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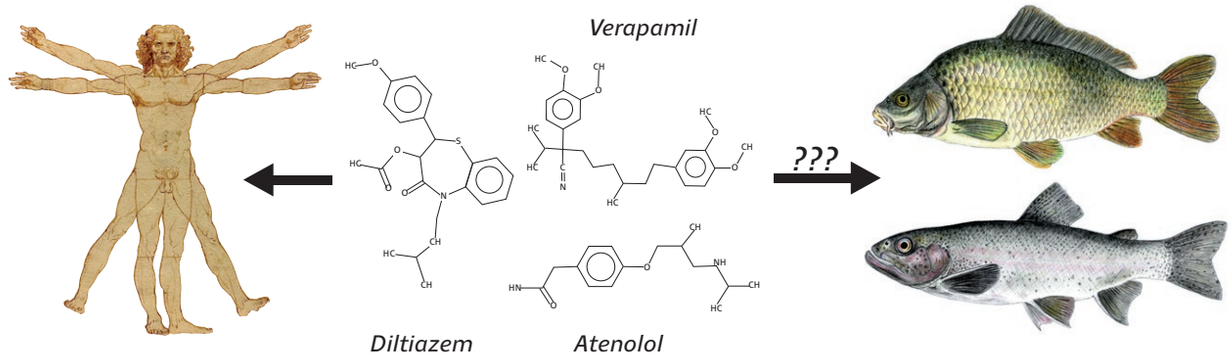
Effect of selected cardiovascular pharmaceuticals found in aquatic environment on fish

Vliv vybraných kardiovaskulárních léčiv nalézaných ve vodním prostředí na ryby



Christoph Antonius Steinbach

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

1.1. Cardiovascular Pharmaceuticals

Pharmaceuticals are a class of emerging environmental contaminants that are extensively and increasingly being used with the consequence of higher levels of their discharge into the environment (Fent et al., 2006; Corcoran et al., 2010). The U.S. Food and Drug Administration (FDA) had approved 1453 small molecule drugs by the end of 2013 (Kinch et al., 2014). Drugs, both prescribed and non-prescribed, are becoming an increasingly complex component of health care (Khetan and Collins, 2007). The increased consumption of human pharmaceuticals can be demonstrated by the pharmaceutical market in the United States where the annual value of sales increased by 62% over 5 years (2000 to 2004) from $153 to $248 billion (Khetan and Collins, 2007).

After the uptake human pharmaceuticals are excreted via urine and/or faeces in their native form or as metabolites and enter the waste water. Due to the fact that waste water treatment plants are not efficient in the elimination of pharmaceuticals, these substances and their metabolites can enter the aquatic environment (Khetan and Collins, 2007; Kümmerer et al., 2008). Therefore, wastewater treatment plants are considered as the main source of this pollution (Huerta et al., 2012). Even though the pharmaceutical concentrations may seem to be relatively low (from ng/l up to low µg/l), they can pose a substantial threat to aquatic organisms because they are designed to be effective at low doses (Gunnarsson et al., 2008). Since the pharmaceuticals are designed to interact with evolutionarily well-conserved drug targets (Gunnarsson et al., 2008; Corcoran et al., 2010), their effects on non-target species are supposed to occur as a result of specific drug target interactions rather than via an unspecific mode of action. Therefore, it is assumed that pharmaceuticals act "therapeutically" rather than toxically (Rand-Weaver et al., 2013). However, the effects of the presence of pharmaceuticals on aquatic organisms, especially after a long-term exposure, are largely unknown (Fent et al., 2006; Crane et al., 2006; Corcoran et al., 2010). Furthermore, the synergistic / antagonistic effects of various pharmaceutical mixtures on aquatic organisms have been scarcely studied (Agerstrand et al., 2015; Backhaus, 2014; Crane et al., 2006; Cleuvers, 2003).

Cardiovascular diseases are responsible for 30% of all deaths worldwide (Kearney et al., 2005). The high prevalence of hypertension worldwide has contributed to the present pandemic of cardiovascular diseases. Globally, the estimated total number of adults with hypertension was 972 million in the year 2000 (Kearney et al., 2005).

In 2012, number of people who died from cardiovascular diseases was estimated at all global deaths. Of these deaths, ca 7.4 million died due to coronary heart disease and ca 6.7 million due to stroke (WHO, 2015). According to the U.S. National Centre for Health Statistics, 17.7% of adults aged 18–64 took at least one cardiovascular agent in the past 30 days in the USA in 2007–2010. And among adults aged 65 and over, 70.2% took at least one cardiovascular agent (U.S. National Institutes of Health, 2014). In 2015, more than one out of three adults worldwide have raised blood pressure – a condition that causes around half of all deaths from stroke and heart disease. It is considered directly responsible for 7.5 million deaths in 2004 – almost 13% of all global deaths (WHO, 2014). In 2015, more than one out of three adults worldwide have raised blood pressure – a condition that causes around half of all deaths from stroke and heart disease. It is considered directly responsible for 7.5 million deaths in 2004 – almost 13% of all global deaths (WHO, 2014). Thus, anti-hypertensive medicines belonging to the group of cardiovascular pharmaceuticals are one of the most commonly prescribed classes of human pharmaceuticals (Jjemba et al., 2008). These pharmaceuticals are with 26–27% of prescription contributing to the most 200 prescribed drugs in the USA (Jjemba et al., 2008). For instance, in the United States 601.4 million prescriptions of cardiovascular and

antihypertensive pharmaceuticals were dispensed in the year 2005 (Jjemba et al., 2008). In the year 2014 the worldwide value of sales of antihypertensive drugs was estimated at 30.5 billion USD. Being at the eights rank of the worldwide drug sales (Hills and Urquhart, 2015). As a result of the high consumption, these pharmaceuticals have been frequently detected in waste waters (at concentrations reaching µg/l levels). The majority of pharmaceuticals enter aquatic environment due to their incomplete removal in sewage treatment works (STWs; Ashton et al., 2004). The removal process of pharmaceuticals in STWs is highly variable. It varies between different substances and is also influenced by the season (Vieno et al., 2007). The cardiovascular drugs are expected to belong to the classes of pharmaceuticals which are hazardous for fish since they are in general well water soluble and because of their chemical properties, they can be easily taken up by fish (Sanderson, et al., 2004a). Moreover, they are supposed to affect similar targets in fish as in humans (Sanderson et al., 2004b).

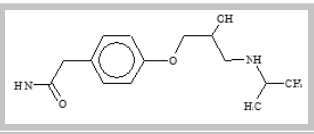
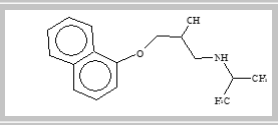
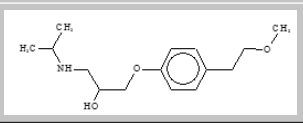
In addition to other groups of pharmaceuticals, arterial hypertension and cardiovascular diseases are treated with β-blockers (blocking β-adrenergic receptors) and calcium channel blockers (inhibiting L-type calcium channels; e.g., Jjemba et al., 2008). β-blockers and calcium channel blockers are widely prescribed and are not totally removed in waste water treatment plants (Jones et al., 2002; Khan and Ongerth, 2004; Hummel et al., 2006; Maurer et al., 2007; Vieno et al., 2007). In accordance to the read across model (Rand-Weaver et al., 2013), these pharmaceuticals are supposed to affect aquatic vertebrates, including fish, since their drug targets, the β-adrenergic receptors and the L-type calcium channels, which are conserved among vertebrates (Lacinova et al., 1995; Nickerson et al., 2001; Rottbauer et al., 2001; Wang et al., 2009).

1.1.1. β-blockers

Chemical structure

β-blockers, also called β-adrenergic receptor antagonists, have a side alkyl chain attached to at least one aromatic ring. The alkyl chain possesses a secondary hydroxyl and amine functional group (Tab. 1). β-blockers are very well soluble in water. Propranolol has the lowest water solubility and is the most lipophilic agent having the highest octanol-water partition coefficients (log K_{ow}) among them (Tab. 1).

Table 1. *Physicochemical properties of selected β-blockers.*

Compound/ CAS number	Molecular formula	Molecular mass (g/mol)	Log K_{ow}	Structure
Atenolol 29122-68-7	$C_{14}H_{22}N_2O_3$	266.34	0.16[a]	
Metoprolol 33286-22-5	$C_{15}H_{25}N_1O_3$	267.37	1.69[a]	
Propranolol 525-66-6	$C_{16}H_{21}N_1O_2$	259.35	3.48[a]	

[a]Alder et al. (2010); CAS-number, molecular formula, molecular mass and structure were gained from EPA's EPIWeb program; K_{ow} = octanol-water partition coefficient.

All β-blockers have one or more chiral centres in their structure; therefore there are, depending on the number of chiral centres, at least two stereoisomers (enantiomers) of each substance. In all cases, at least one of the chiral carbon atoms residing in the side alkyl chain is directly attached to a hydroxyl group (Mehvar and Brocks, 2001).

β-blockers are marketed as racemates (mixture of enantiomers) (Mehvar and Brocks, 2001). The different enantiomers have different activities. Usually, the left (*S*) enantiomer possesses a substantially higher ability to bind to the β-adrenergic receptors compared to right (*R*) enantiomer and is therefore therapeutically more relevant (Mehvar and Brocks 2001).

Mode of action in humans

β-blockers are used in therapy of angina pectoris, glaucoma, heart failure, and arterial hypertensive diseases (Mehvar and Brocks, 2001; Owen et al., 2007). These pharmaceuticals reduce the workload of the heart (Agon et al., 1991) and the heart rate as well (Larsson et al., 2006). This group of substances is divided by the selectivity of the β-adrenergic receptors (ARs; Owen et al., 2007), i.e. the so-called cardio-selectivity group (Cruickshank and John, 1980). Examples of nonselective β-blockers are propranolol, pindolol, nadolol, timolol, and labetalol. All of them bind on both adrenergic receptors (β_1 and β_2). Selective antagonists of the β_1-AR are for instance metoprolol, atenolol, esmolol, and acebutolol (Mehvar and Brocks, 2001). Atenolol was developed as a replacement for propranolol, because propranolol passes through the blood brain barrier and has central nervous system side effects (such as psychological disorders) by binding to the serotonin (5-HT) receptor (Agon et al., 1991; Hampel et al., 2010; Lorenzi et al., 2012). Additional side effects of β-blockers are cardiac dysrhythmia, atrioventricular blockage which could lead to asystole, blood circulation disorders in the skin, hypotension, insomnia, and alteration of glucose and lipid metabolism (Lüllmann et al., 2003). Moreover, treatment with β-blockers is associated with sexual problems in men, namely erectile dysfunction (Lorenzi et al., 2012).

β-blockers are absorbed in the gastrointestinal tract via passive diffusion and are distributed in the circulatory system through binding to albumin and α1-acid glycoprotein in the plasma (Mehvar and Brocks, 2001). Therapeutic, toxic, and comatose-lethal blood plasma concentrations' as well as half-life times of these pharmaceuticals in humans are listed in Tab. 2. Comatose-lethal blood plasma concentrations follow the order of atenolol > metoprolol > propranolol.

Table 2. *Plasma concentrations and half-life time of selected β-blockers (Regenthal et al., 1999).*

	Human blood plasma concentration (mg/l)			Half-life (h)
	Therapeutic	Toxic	Comatose-lethal	
Atenolol	0.1–2	2–3	27	4–14
Metoprolol	0.02–0.5	1	12	3–6
Propranolol	0.02–0.3	0.5–1	4–10	2–6

The metabolic degradation of pharmaceuticals is influenced by their lipophilic characteristics. More hydrophilic β-blockers, like atenolol and sotalol, are excreted in the urine nearly unchanged (Mehvar and Brocks, 2001). On the other hand, lipophilic β-blockers, like propranolol and metoprolol, are metabolized in the liver (Mehvar and Brocks, 2001). Metabolites of these pharmaceuticals, as well as remnants of the parent substance, are then excreted via the urine. For instance approximately 90% of propranolol and metoprolol are metabolized (Walle et al., 1985; Maurer et al., 2007) and only 10% are excreted unchanged. The pathways of propranolol

degradation include glucuronidation, ring hydroxylation, and side-chain oxidation (Mehvar and Brocks, 2001). Metoprolol is metabolized in the same way as propranolol; additionally an *N*-dealkylation pathway is involved (Mehvar and Brocks, 2001). The hydrophilic β-blocker atenolol is metabolised at a low level, about 90% is excreted unchanged. The metabolism comprises hydroxylation and conjugation (Khetan and Collins, 2007).

Sources and concentration in aquatic environment

Nowadays, atenolol and metoprolol represent approximately 80% of the β-blockers consumed in Europe (Vieno et al., 2007). In England, Germany, Austria, Italy and Switzerland, the most-used drugs in this class amount to several tonnes per year (Fent et al., 2006). Accordingly, they are frequently detected in waste water (Alder et al., 2010). In untreated waste water, propranolol, atenolol, and metoprolol were detected at concentration of 50–180 ng/l, 1540 ng/l, and 140–290 ng/l, respectively (Maurer et al., 2007). In U.S. wastewater samples, propranolol and metoprolol were found at concentrations up to 1.9 and 1.2 µg/l, respectively (Huggett et al., 2002). The highest elimination rates of 96, 83, and 61% in STWs were reported for propranolol, metoprolol, and atenolol, respectively. Atenolol, metoprolol, and propranolol are mostly found in surface waters at ng/l concentrations, but can reach even µg/l levels (Ternes, 1998; Zuccato et al., 2000). The β-blocker sotalol was also detected in groundwater up to 560 ng/l (Sacher et al., 2001).

Effects on fish

In general, β-blockers have a moderate lipophilicity (log K_{ow} < 3, Tab. 1), thus they enter the fish's body via the gills, while food seems to be a negligible source. For the same reason β-blockers do not accumulated in fish to a high extent (Owen et al., 2007). Tab. 3 shows the available bioconcentration factors (BCF) of atenolol. As there is no information on BCFs of propranolol and metoprolol, BCFs estimated by the program EPIWeb are presented in Tab. 3. The calculated predicted critical environmental water concentrations of atenolol, metoprolol, and propranolol are 792 µg/l, 15 µg/l, and 2 µg/l, respectively. These environmental concentrations would result in fish blood plasma concentrations equal to human therapeutic blood plasma levels (Fick et al., 2010a); if the concentration in the plasma of a fish were to reach these levels, pharmacological effects would be expected in fish (Rand-Weaver et al., 2013).

Table 3. *Bioconcentration of selected ß-blockers.*

Compound	Species/ Age class	Exposure duration (concentration)	Tissue	BCF
Atenolol	Western mosquitofish *Gambusia affinis*/ Juvenile	96 h (100/1000 µg/l)	homogenate of entire fish	0.13/0.08[a]
Metoprolol				4.5[b]
Propranolol				51[b]

[a]Valdes et al. (2014); [b]Bioconcentration factor (BCF) estimation gained from EPA's EPIWeb program.

β-ARs have been found in vascular tissue, gills, liver, erythrocytes, brain, and muscle of fish (Owen et al., 2007; Corcoran et al., 2010). Since the β-ARs are relatively conserved among vertebrates, β-blockers bind also in non-target organisms like fish (goldfish) and amphibians (salamander; Jozefowski and Plytycz, 1998). For instance, the amino acid sequence of the

transmembrane domain of β_1 and β_2-ARs of rainbow trout (*Oncorhynchus mykiss*) share 57 and 63% identity with the corresponding mammalian β-ARs (Nickerson et al., 2001). Even higher matches for the β_1 and β_2-ARs transmembrane sequence of the β_1 and β_2-ARs are described for zebrafish (*Danio rerio*), where the sequence of the β_1 and β_2 share 77 and 76% identity with their human orthologs (Wang et al., 2009).

Atenolol

Atenolol showed a very low acute toxicity to medaka (*Oryzias latipes*) with a 96hLC_{50} value reaching 1800 mg/l (Yamamoto et al., 2007; Tab. 4). In a short-term (21 d) adult reproductive study on fathead minnow (*Pimephales promelas*), fish were exposed to atenolol at the concentration range of 0.1–10 mg/l; no effects were observed on the gonad weight (GSI) of either sex, egg production or egg hatchability. Atenolol caused an increase in condition index of only male fish (Lowest Observed Effect Concentration (LOEC)$^{\text{condition index}}$ = 3.2 mg/l). Concentration of atenolol reached 0.05 mg/l in the blood plasma of the exposed fish (Winter et al., 2008a). Furthermore, Winter et al. (2008a) carried out a fathead minnow embryo-larval test lasting 28 days at the same concentration range of atenolol as above. Atenolol reduced the growth rate of larvae only at very high concentrations (LOEC$^{\text{growth rate}}$ = 10 mg/l). The other developmental parameters, such as number of hatched fish or occurrence of abnormalities, were not affected by atenolol (Winter et al., 2008a). Therefore, atenolol seems to have low chronic toxicity to early development and reproduction of fathead minnow.

In contrast, a short-term exposure (5 days) to atenolol at the concentration of 11 μg/l changed gene expression patterns in Atlantic salmon (*Salmo salar*). The expression of approximately 30% of 480 candidate genes was altered. Mainly genes involved in metabolic pathways such as adipocytokine signalling pathway, insulin signalling pathway, glycolysis/gluconeogenesis and oxidative phosphorylation were affected. Similarly, in humans, it is known, that the β-blockers atenolol, metoprolol, and propranolol also alter the lipid and carbohydrate metabolism (Jacob et al., 1996; Bakris et al., 2004; Lindholm et al., 2005). Furthermore, changes in the processing of genetic information were observed. The following processes of translation were affected: folding, sorting, and degradation of the synthesized genetic material (Hampel et al., 2010). In the study of Fernandez et al. (2013), rainbow trout gonad cells (RTG-2) were exposed for 24 h to atenolol at a concentration of 500 μg/l. Atenolol did not cause oxidative stress, had no effect on the activity of CYP1A or had no cytotoxic effect on the exposed cells. Therefore, effects of atenolol on fish seems to be more physiological (Hampel et al., 2010) than classically toxicological (Winter et al., 2008a).

In humans, atenolol causes side effects on the central nervous system such as light-headedness, tiredness, fatigue, and lethargy (Wander et al., 2009) even though it hardly crosses the blood brain barrier. Fish are considered to have a relatively leaky blood-brain barrier compared with mammals and the fish brain is rich in β-ARs, a possibly prone target for atenolol. Therefore, changes in the behaviour patterns in atenolol-exposed fish can be expected (Owen et al., 2007).

Propranolol

Due to the nonspecific antagonistic action, propranolol has wide effects on fish, but only at very high concentrations (Fent et al., 2006). The studies of Stanley et al. (2006) and Hugget et al. (2002) reported a 48hLC_{50} of 1.21 and 24.3 mg/l for adult fathead minnows and medaka larvae, respectively (Tab. 4). In addition, Stanley et al. (2006) reported for medaka, a 48hLC_{50} of 1.42 and 1.69 mg/l for the *S*- and *R*-enantiomer of propranolol, indicating no stereospecific effect on acute toxicity of propranolol. Furthermore, Stanley et al. (2006) carried out 7 days lasting toxicity test on medaka larvae; the fish were exposed to the *S*- and *R*-enantiomer and

racemate of propranolol in concentrations ranging from 93.8–15000 μg/l. In the exposed fish, no enantiomer specific effect on survival was observed. The calculated $LOEC^{reduced\ weight}$ for *S*-enantiomer, *R*-enantiomer, and the racemate (*R*, *S*) were 134.4 > 463.6, and 128.2 μg/l, respectively. Similarly, the *S*-enantiomer of propranolol is more potent in the blockage of β-ARs in humans when compared to *R*-enantiomer (Mehvar and Brocks 2001).

In the study of Sun et al. (2014), zebrafish embryos were exposed to propranolol racemate and to the *R*- and *S*-propranolol (at the concentration of 1–16 mg/l). The heart rate and mortality with the hatching rate were assessed after 48 and 96 h, respectively. At very high concentrations, propranolol reduced the heartbeat of the fish ($LOEC^{reduced\ heartbeat}$ = 8 mg/l) and the hatching rate ($LOEC^{hatching\ rate}$ = 8 mg/l). The authors observed no enantiomer specific effects on studied endpoints. In accordance, the heart rate of rainbow trout was not affected by 48-h exposure to high concentrations of propranolol (70.9 μg/l) (Larsson et al., 2006).

Furthermore, Sun et al. (2014) exposed zebrafish larvae for 96 h to propranolol racemate and to the *R*- and *S*-enantiomers of propranolol at concentrations ranging from 0.01 to 1 mg/l. Compared to the controls, neither enantiomers nor the racemate affected transcriptional response of the genes encoding the β_1 and the β_2-AR in exposed fish. Interestingly, *R*-propranolol (the less potent form) affected the expression of β_3-AR (Mehvar and Brocks 2001, Sun et al., 2014). Furthermore, all forms of propranolol at the concentration of 0.01 and 1 mg/l affected in acute exposed zebrafish larvae the expression of genes involved in antioxidant response, detoxification, and apoptosis (Sun et al., 2014).

In the study of Owen et al. (2009) juvenile rainbow trout were exposed for 40 days to propranolol at concentrations ranging from 0.0001 to 10 mg/l. A reduced growth rate was observed in the group of fish exposed for 10 days to the highest propranolol concentration ($LOEC^{reduced\ growth\ rate}$ = 10 mg/l). However, at the end of exposure time, there were no differences in the growth rate among treatment groups and the control ($NOEC^{reduced\ growth\ rate}$ = 10 mg/l), suggesting possible adaptation of fish to propranolol.

Eighty-hour exposure to propranolol at concentrations ranging from 4 to 32 mg/l caused abnormalities in the development of zebrafish (Fraysse et al., 2006). A large pericardial oedema was developed in 40% of the zebrafish larvae exposed to the highest concentration (32 mg/l). When the fish developed an oedema, the heart rate also changed and the blood circulation was reduced in the caudal part. Furthermore, the development of the spine was affected and tail curvatures (mainly lordosis) were observed. Additionally, pigmentation was reduced and the numbers of spontaneous movements increased due to this treatment. Other studied endpoints, e.g., hatching rate and time were not altered (Fraysse et al., 2006).

Giltrow et al. (2009) carried out an adult reproductive study (lasting 21 days) on one-year-old fathead minnow. The fish were exposed to concentrations of propranolol ranging from 0.001 to 10 mg/l. At the concentration of 3.4 mg/l, total mortality was observed. In addition, propranolol decreased the hatching time (number of days to hatch; $LOEC^{hatching\ time}$ = 0.1 mg/l). In female fish, the gonado-somatic index (GSI) increased proportionally to the concentration of propranolol ($LOEC^{female\ GSI}$ = 0.1 mg/l). Also a concentration-related decrease in weight of male fish was observed ($LOEC^{male\ weight}$ = 1.0 mg/l). The concentrations of propranolol in the blood plasma of male fish exposed to nominal concentrations of 0.1 and 1.0 mg/l were 0.34 and 15.0 mg/l, respectively (Giltrow et al., 2009).

In a 2-week medaka reproductive study, where the fish were exposed to propranolol at the concentration range of 0.5–500 μg/l, exposed fish had significant changes in plasma steroid levels; however, no changes in the average number of eggs produced or number of viable eggs which hatched were observed (Huggett et al., 2002). In a 4-week follow-up propranolol exposure, the total number of eggs produced by medaka and the number of viable eggs that hatched were decreased at concentrations as low as 0.5 μg/l (Huggett et al., 2002). Moreover,

Lorenzi et al. (2012) carried out an adult reproductive study (lasting 21 days) on nine-month-old fathead minnows. The fish, were exposed to concentrations of propranolol ranging from 0.05 to 4.11 μg/l. Propranolol did not affect the fecundity, fertilisation rate, hatchability, hatching time, nest rubbing behaviour, time spent near the nests or frequency of males approaching the females. However, males exposed to propranolol in concentration of 0.05 and 0.88 μg/l, visited the nests less often. The exposure to propranolol at the concentration of 4.11 μg/l did not affect the reproductive behaviour or the spawning abilities of fathead minnows, but affected the expression of some genes in the brain of exposed fish, namely myoglobin and calsequestrin and genes involved in calcium ion transport, transcription, proteolysis and apoptosis/anti-apoptosis (Lorenzi et al., 2012). The effect on behaviour and gene expression might be caused by an antagonism of propranolol to the serotonin receptor (5-HT_{1A}; Lorenzi et al., 2012). Therefore, Lorenzi et al. (2012) suggested studying the effect of propranolol on the early development of brain in fish.

The effect of propranolol on the activity of CYP1A in liver and gills was studied by Bartram et al. (2004). In an acute test lasting 98 h, rainbow trout was exposed to propranolol at the concentration of 1 mg/l. Propranolol induced activity of CYP1A in the gills but not in the liver of exposed fish. Furthermore, liver and gill cells were exposed for 72 h *in vitro* to propranolol at concentrations ranging from 0.1 to 400 μg/l. In both gill and liver cell suspensions, propranolol induced the activity of CYP1A at the concentration of 200 μg/l. Similarly, CYP1A2 accounts for 32–50% of the total metabolism of this substance in humans.

Metoprolol

Metoprolol has low acute toxicity for medaka ($48\text{hLC}_{50} \geq 100$ mg/l; Hugget et al., 2002, Tab. 1.). Acute exposure (72 h) to high concentrations of metoprolol affected the embryo-larval development of zebrafish (van den Brandhof and Montforts, 2010). Scoliosis was developed in 35% of the exposed fish at the concentration of 25.3 mg/l and a pericardial oedema was found in 50% of the fish exposed to a higher concentration (50.5 mg/l). When the heart was deformed, the heartbeat also changed. The no-observed-effect concentration (NOEC) for embryo-larval development was estimated at 12.6 mg/l (van den Brandhof and Montforts, 2010). In the study of Sun et al. (2014), zebrafish embryos were exposed for 96 h to metoprolol at concentrations of 1–16 mg/l. The experimental design is described above. Metoprolol caused a reduction of the heartbeat ($\text{LOEC}^{\text{reduced heartbeat}}$ = 1 mg/l) and a reduced hatching rate at very high concentrations ($\text{LOEC}^{\text{reduced hatching rate}}$ = 8 mg/l). Sun et al. (2014) demonstrated the heart beat to be the most sensitive endpoint of the study. As described above, Sun et al. (2014) exposed the fish to the *S*- and *R*-enantiomer and racemate of metoprolol. Metoprolol racemate and the *R*-metoprolol had an effect on the transcription of genes that encode β_2 and β_3-ARs, while the expression of β_1-AR gene was not affected. On the other hand, the *S*-enantiomer of metoprolol affected expression of all β-ARs including the β_1-AR. In addition, fish exposed to *S*-metoprolol exhibited a stronger reduction in the expression of the β–ARs compared to *R*-enantiomer. Consequently, *S*-metoprolol affected the expression of genes involved in antioxidant response, detoxification, and apoptosis, while *R*-enantiomer of metoprolol had smaller effects, and racemate had no effect on the expression of these genes. In line with these findings, *S*-metoprolol is more potent in humans when compared to *R*-metoprolol (Mehvar and Brocks 2001).

Triebskorn et al. (2007) carried out a long-term exposure (28 d) of rainbow trout and common carp (*Cyprinus carpio*) to metoprolol at concentrations of 1, 5, 20, 50, and 100 μg/l. Histopathological changes in the gills ($\text{LOEC}^{\text{mucus cell proliferation in gills}}$ = 20 μg/l; $\text{LOEC}^{\text{chloride cell disorders}}$ = 50 μg/l), trunk kidney ($\text{LOEC}^{\text{trunk kidney}}$ = 1 μg/l), and liver ($\text{LOEC}^{\text{liver}}$ = 1 μg/l) were observed. Additionally, reduced glycogen level was measured in the liver cells ($\text{LOEC}^{\text{reduction of glycogen}}$ =

1 µg/l). The observed histopathological changes (liver, kidney) could have been a result of a reduced blood flow (Triebskorn et al., 2007).

In an *in vitro* experiment, hepatocytes of male Nile tilapia (*Oreochromis niloticus*) were exposed for 24 h to metoprolol at concentrations of 1.07 (environmentally relevant), 10.69, and 106.9 µg/l and the effects of metoprolol on mRNA expression of CYP1A, selected phase-II enzymes, and vitellogenin were studied. At all tested concentrations of metoprolol, the expression of CYP1A was significantly up-regulated compared to the control indicating that metoprolol might be metabolised by CYP1A in fish hepatocytes. At the lowest tested concentration of metoprolol, the expression of GST was induced (1.8 fold) compared to the control, suggesting that GST also is involved in the biotransformation of metoprolol. At the concentration of 10.69 µg/l of metoprolol, the mRNA expression of vitellogenin was increased (Gröner et al., 2014). These findings were interpreted as species-specific endocrine disruption. At present, there is no literature available on the *in vivo* effect of metoprolol on the parameters selected by Gröner et al. (2014).

Conclusion

Acute toxicity of β-blockers is very low up to high (Tab. 4). Atenolol can be classified as non-toxic (96 h LC_{50} > 100 mg/l, OECD, 2001) for larvae. Metoprolol and propranolol can be classified as non-Propranolol can be classifies as a substances of medium toxicity for adult fish (48 h LC_{50} = 10–100 mg/l; Svobodova et al., 1993) and high toxic for the early life stages of fish (48 h LC_{50} = 1–10 mg/l; Svobodova et al., 1993; Tab. 4). Metoprolol and propranolol were more toxic to early life stages of fish compared to adults. Most studies to date have focused on the effects of propranolol, metoprolol, and atenolol. In addition stereospecific effects were studied for propranolol and metoprolol, while there is still limited knowledge on the effects of atenolol enantiomers. An enantiomer-specific effect was reported only for the growth of fish larvae exposed to propranolol. Gene expression patterns were more affected by metoprolol compared to propranolol. In summary, metoprolol and propranolol affects larval development of fish only at very high concentrations (mg/l). Propranolol was found to affect reproductive parameters of fish and had an impact on the parental care of exposed male fish at relatively low concentrations (low µg/l range).

Atenolol affected the growth rate, gene expression patterns, and reproductive parameters of fish in µg/l to mg/l range. Histopathology of gills, kidney, and liver was changed by metoprolol in µg/l range. Chronic effects and bioconcentration of atenolol in juvenile fish were not studied before starting my Ph.D. work.

Table 4. *Lethal concentrations (LC_{50}) of selected β-blockers.*

	Species	Age class	Duration of exposure (h)	LC_{50} (mg/l)	Reference
Atenolol	Medaka (*Oryzias latipes*)	Larvae[a]	96	1800	(Yamamoto et al., 2007)
	Medaka (*Oryzias latipes*)	Larvae	96	> 100	(Kim et al., 2009)
	Rainbow trout (*Oncorhynchus mykiss*)	–[b]	96	> 100	(Winter et al., 2008a)
Metoprolol	Medaka (*Oryzias latipes*)	Adult	48	> 100	(Hugget et al., 2002)
	Zebrafish (*Danio rerio*)	Embryo	96	> 50	(Sun et al., 2014)
Propranolol	Medaka (*Oryzias latipes*)	Larvae	96	11.4	(Kim et al., 2009)
	Zebrafish (*Danio rerio*)	Embryo	96	2.48	(Sun et al., 2014)
	Fathead minnow (*Pimephales promelas*)	Larvae	48	1.21	(Stanley et al., 2006)
	Medaka (*Oryzias latipes*)	Adult	48	24.3	(Hugget et al., 2002)

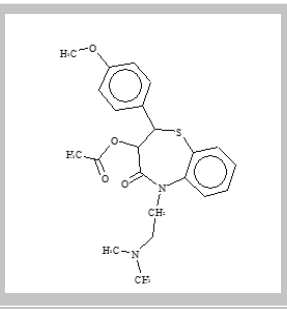
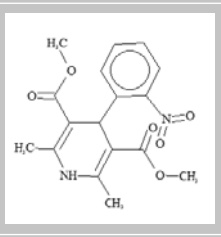
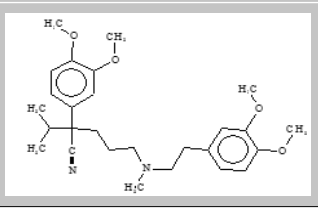
[a]Information on the stage was not provided, but considering that the fish were 10-days old, we suppose that they were in larval stage. [b]Winter et al. (2008a) refer to AstraZeneca (unpublished data), no information on age was provided.

1.1.2. Calcium channel blockers

Chemical structure

The group of calcium channel blockers (also called calcium channel antagonists) is very heterogeneous and consists of three main classes – phenylalkylamines (verapamil), benzothiazepines (diltiazem), and dihydropyridines (nifedipine, isradipine; Budriesi et al., 2007). Selected physicochemical properties of verapamil, diltiazem, and nifedipine are shown in Tab. 5. Calcium channel blockers are chiral substances having two different enantiomers, which differ in activity as well as in bioavailability. For example, verapamil is administered as a racemate; with the *S*-enantiomer being approximately ten times more potent than the *R*-enantiomer (Bhatti and Forster, 1997). As the calcium channel blockers differ in molecular structure, they have also different sites and mode of action and affect various cardiovascular functions (Molden et al., 2002; Lüllmann et al., 2003; Gossman and Messerli; 2004; Bergson et al., 2011). Verapamil and diltiazem are amphipile substances (possessing both hydrophilic and lipophilic properties). Both bind to the calcium channels via the amine functional group (Lüllmann et al., 2003; Budriesi et al., 2007). Nifedipine and isradipine are active due to the dihydropyridin ring (Lüllmann et al., 2003).

Table 5. *Physicochemical properties of selected calcium channel blockers.*

Compound/ CAS number	Molecular formula	Molecular mass (g/mol)	Log K_{ow}	Structure
Diltiazem 33286-22-5	$C_{22}H_{26}N_2O_4S_1$	414.52	2.79	
Nifedipine 21829-25-4	$C_{17}H_{18}N_2O_6$	346.34	2.50	
Verapamil 52-53-9	$C_{27}H_{38}N_2O_4$	454.61	3.79	

K_{ow} = octanol–water partition coefficient; EPA's EPIWeb program [KOWWIN (v1.68)].

Unlike verapamil and diltiazem, nifedipine and isradipine are neither light stable nor well water-soluble (Lüllmann et al., 2003). The water solubility of verapamil is highly dependent on the pH. For instance, solubility at pH 6.3 and pH 6.8 (at 37 °C) is > 100 and 2.71 g/l, respectively (Streubel et al., 2000).

Mode of action in humans

Calcium channel blockers have been used for the treatment of hypertension since the 1980s (Budriesi et al., 2007). They are also used in the therapy of angina pectoris, paroxysmal supraventricular tachycardias, hypertrophic cardiomyopathy, Raynaud phenomenon, and pulmonary hypertension. Calcium channel blockers lower the blood pressure due to a vasodilatation which reduces the peripheral resistance and they also lower the heart rate. In contrast to β-blockers, they do not affect glycogen metabolism (Grossman and Messerli, 2004; Budriesi et al., 2007). Furthermore, calcium channel blockers do not change the lipoprotein profile of the blood plasma (Zaugg, 2000). The typical counter-regulations of body to calcium channel blockers are the release of noradrenaline and rennin (Zaugg, 2000). Different calcium channel blockers attach to different parts of the calcium channel. Calcium channel blockers selectively affect either the cardiac (verapamil) or the arterial (dihydropyridines) calcium channels, whereas diltiazem affects both (Molden et al., 2002). Verapamil and diltiazem are typical antiarrhythmic agents (Lüllmann et al., 2003). The blocking of the voltage-gated

calcium channels reduces the influx of calcium into cells. Mostly, the L-type channels (long-term and height voltage activated) are blocked tonically. Therefore, calcium channel blockers affect mainly the cardiac and smooth muscle cells because these tissues exhibit mostly the L-type of calcium channels (Budriesi, 2007). The T-type calcium channels (transient and owing to small conductance) have well-known functions in the nervous system and are found in the developing heart (Perez-Reyes, 1998; Bergson et al., 2011). Also, in the case of verapamil, the T-type of calcium channels are blocked (Perez-Reyes, 1998; Bergson et al., 2011). Side effects of calcium channel blockers are bradycardia which could lead to asystole and obstipation (Lüllmann et al., 2003). Moreover, the nervous system and skeletal muscles could be affected by calcium channel blockers (Budriesi et al., 2007).

In addition to the cardiovascular effects, verapamil acts as a chemosensitizer in human cancer cell lines (Yusa et al., 1989). The multi-drug resistance (MDR) of tumour cells can reduce the affectivity of chemotherapeutic drugs (Liu et al., 2011). Verapamil can reverse the MDR by a competitive inhibition of the binding sites of P-glycoprotein. This effect can lead to an enhanced retention time of antitumor agents and facilitate the cytotoxic activity of various chemotherapeutics (Yusa et al., 1989; Liu et al., 2011). The calcium channel blockers are taken orally, absorbed via the gastrointestinal tract, and then transported by the blood (Lüllmann et al., 2003). In the Tab. 6, the therapeutic, toxic, and lethal blood plasma concentrations as well as the half-life of these pharmaceuticals in humans are shown.

Table 6. *Plasma concentrations and half-life time of selected calcium channel blockers (Regenthal et al., 1999).*

Compound	Human blood plasma concentration (mg/l)			Half-life (h)
	Therapeutic	Toxic	Comatose-lethal	
Diltiazem	0.1–0.25	0.8	1.3	2–6
Nifedipine	0.025–0.1	0.1	–	2–5
Verapamil	0.05–0.5	1	2.5	6–14

In human verapamil and diltiazem, undergo an extensive first-pass elimination in the liver. Up to 70–95% of verapamil and 50–60% of diltiazem are pre-systemically eliminated (Eichelbaum et al., 1984; Echizen and Eichelbaum. 1986; Pauli-Magnus et al., 2000). Verapamil biotransformation is carried out by the CYP450 enzymes (CYP1A2, CYP2C, CYP2D6, CYP2E1, and CYP3A3/4; Kroemer et al., 1993) and as a result up to 12 different metabolic products can be found in faeces and urine (Sun et al., 2004). *S*-enantiomer of verapamil is metabolized preferentially (Tracy et al., 1999). The main metabolic product of verapamil degradation is norverapamil, which is 80% less effective compared to the parent substance (Mateus et al., 2007). Effects of the other metabolic products are negligible (Sun et al., 2004). In mammals, approximately 75% of diltiazem is metabolized mainly by the CYP3A4 enzyme in the liver (Homsy et al., 1995). In the human body, diltiazem undergoes a complex and intensive biotransformation involving desmethylation and desacetylation (Molden et al., 2003). The main metabolites of diltiazem in humans are monodesmethyl diltiazem, didesmethyl diltiazem and desacetyl diltiazem (Murata et al., 1993; Molden et al., 2003). Interestingly, the diltiazem metabolites *N*-desmethyl diltiazem and *N*-didesmethyl diltiazem have an inhibitory effect on the activity of CYP3A4 and therefore, a long-term diltiazem treatment could lead to a decreased elimination rate of this substance (Sutton et al., 1997; Zhao et al., 2007).

Sources and concentrations in aquatic environment

In Australia, the annual (in the year 1998) volume s of verapamil and diltiazem dispensed were 9786 kg and 8669 kg, respectively (Buser et al., 1999 in Khan and Ongerth, 2004). In Australian wastewater influent, verapamil and diltiazem were found in concentrations of 0.9 and 0.1 μg/l, respectively. The removal rate of diltiazem and verapamil in sewage treatment plants is approximately 35% and 16%, respectively. The sludge sorption of these pharmaceuticals is much lower than the removal rate (Khan and Ongerth, 2004). In the United Kingdom, diltiazem was detected at an average (maximum) concentration of 0.77 (3.20), 0.27 (0.64) and 0.01 (0.20) μg/l in influent, effluent, and surface waters, respectively (Kasprzyk-Hordern et al., 2009). In Germany verapamil was detected in concentration of 3.1 and 0.51 μg/l in influent and effluent waster, respectively (Hummel et al., 2006).

In Australia in the wastewater effluent, verapamil and diltiazem were detected in concentrations of 0.08 and 0.09 μg/l, respectively (Khan and Ongerth, 2004). Additionally, in wastewater effluent in the USA the maximal concentration of verapamil and its metabolite norverapamil were found in concentrations of 0.190 and 0.07 μg/l, respectively (Batt et al., 2008). In the USA, highest detected concentration of diltiazem in waste water was 0.106 μg/l (Kolpin et al., 2004). The calculated predicted critical environmental water concentrations of diltiazem and verapamil are 27884 ng/l and 24 ng/l, respectively. These environmental concentrations would cause fish blood plasma concentrations equal to human therapeutic blood plasma levels (Fick et al., 2010a).

Effects on fish

The uptake of calcium channel blockers in fish is supposed to be similar to the uptake of β-blockers since their lipophilicity (log K_{ow} < 3, Tab. 5) and the molecular weights are comparable (Owen et al., 2007). Therefore, calcium channel blockers will be taken up mainly via the gills. In general, substances having molecular masses lower than 1000 g/mol can be taken up by the gills of fish (Sanderson et al., 2004a). Diltiazem accumulates to a certain extent in liver and muscle of fish. The mean concentrations found in wild fish were 0.15 and 0.7 μg/kg in fillets and liver, respectively (Ramirez et al., 2009). Fick et al. (2010b) reported that the blood plasma of rainbow trout exposed to waste water was containing verapamil. Tab. 7. shows the BCFs of diltiazem and verapamil.

The drug target of the calcium channel blockers, the calcium channels are relatively conserved among vertebrates. For instance, polypeptide sequences of human α-subunit of the L-type calcium channel, which is one of the targets of verapamil, has a 78% match with the sequence of zebrafish (Lacinova et al., 1995; Rottbauer et al., 2001). The zebrafish heart possesses both L- and T-type calcium channels. Interestingly, in most mammals T-type channels are only expressed in the developing heart or under pathophysiological conditions (Nemtsas et al., 2010). The calcium channels are also essential for the muscle activity in both fish and man. In zebrafish, the L-type calcium channels trigger the skeletal muscle excitation-contraction and also the coupling of the membrane depolarization (Schredelseker et al., 2010).

As stated, calcium channel blockers are chiral substances (Bhatti and Forster 1997). However, to the best of our knowledge no research addresses the effect of different enantiomers of calcium channel blockers on fish.

Table 7. *Bioconcentration of calcium channel blockers.*

Compound	Species / Age class	Exposure time / Medium	Tissue	BCF
Diltiazem[a]	Eastern mosquitofish (*Gambusia holbrooki*) / –	7 days / Waste water	Whole fish homogenate	16
Verapamil[b]	Rainbow trout (*Oncorhynchus mykiss*) / Juvenile	14 days / Waste water	Blood plasma	> 33 to 175

[a]Wang and Gardianli (2013) – authors provided only information on the length of the fish (2.5–5.2 cm); [b]Fick et al. (2010b); BCF = bioconcentration factor.

Verapamil

Li et al. (2010a) carried out an acute exposure study (lasting 96 h) on juvenile rainbow trout with verapamil at the concentration range of 0.5–10.0 mg/l. The 96hLC_{50} value was estimated at 2.72 mg/l.

Li et al. (2010b) also carried out chronic toxicity test on juvenile rainbow trout lasting 42 days. The fish were exposed to verapamil at sub-lethal concentrations ranging from 0.5 to 270 μg/l. After the 21-day exposure, the body length, liver weight, and condition factor significantly decreased at the highest tested concentration (270 μg/l). Haematological changes as well as changes in biochemistry of the blood plasma appeared at the concentrations of 27 and 270 μg/l. After further exposure, verapamil caused changes in haematological and biochemical parameters of blood even at lower concentrations. Verapamil induced also antioxidant responses and caused stress in exposed fish. Stress index (integrated biomarker response) increased with increasing concentration of verapamil and time of exposure (Li et al., 2010b). Moreover, Li et al. (2011) carried out a short-term toxicity test on juvenile rainbow trout lasting 7 days. The fish were simultaneously exposed to verapamil at sub-lethal concentrations ranging from 50 to 100 μg/l and to cadmium at concentration of 50 μg/l. Levels of oxidative stress biomarkers (thiobarbituric acid-reactive substances, carbonyl protein) was increased and the antioxidant response was decreased in the group exposed only to cadmium. Li et al. (2011) suggested that verapamil might reduce the cadmium uptake through calcium channels. Therefore, it could decrease the cadmium-induced physiological stress in rainbow trout (Li et al., 2011). In the study of Overturf et al. (2012), long-term exposure (28 days) of fathead minnow larvae to verapamil at the concentration of 600 μg/l caused a significant decrease in growth rate.

In an acute toxicity test lasting 3 h zebrafish embryos were exposed to verapamil at concentrations ranging from 0.45 to 456 mg/l (Berghmans et al., 2008). At a very high level of verapamil (hundreds of mg/l), the atrial and ventricular rates and the number of gut contractions were reduced, similar to the effect and side effects observed in humans.

Winter et al. (2008b) who carried out an acute toxicity test on zebrafish, observed abnormal swimming patterns ($NOEC^{swimming}$ = 41 μg/l) after a 1-hour exposure to verapamil (Winter et al., 2008b). Furthermore, Rihel et al. (2010) carried out a rest/wake behavioural assay where zebrafish larvae were exposed for three days to a high concentration of verapamil (4.51 mg/l). Verapamil-exposed fish showed longer rest time compared with the control.

In rainbow trout hepatocyte cell culture, 7-day lasting exposure to verapamil but not diltiazem (both at the concentration of 0.45 mg/l) reduced vitellogenin concentration (Yeo and Mugiya, 1997).

In many aquatic species belonging to various taxonomic classes the presence of transmembrane ATP-dependent P-glycoprotein (P-gp) and/or the function of MXR proteins have been demonstrated (Smital and Sauerborn, 2002). The pharmaceutical verapamil has a high affinity for P-gp binding sites and therefore can act as its competitive inhibitor, preventing binding and active transport of other xenobiotics (Smital et al., 2004). Additionally, verapamil acts as chemosensitizer in fish as demonstrated e.g., for common carp (Smital and Sauerborn, 2002) and therefore, could increase the intracellular accumulation and toxic effects of xenobiotics (Kurelec, 1997).

Diltiazem

Unfortunately, to the best of our knowledge only three studies dealing with the effect of diltiazem on fish have been published. In an acute toxicity test carried out by Kim et al. (2007) it was found that diltiazem has a very low toxicity to medaka (48hLC_{50} = 26.5 mg/l). In zebrafish injected with diltiazem at a dose of 1.3 mg, an increase of the nucleotide and ATP levels was observed in the blood plasma (Klein et al., 2009).

Wang and Gardinali, (2013) exposed eastern mosquitofish (*Gambusia holbrooki*) to treated (reclaimed) waste water containing diltiazem at the concentrations of 144 ng/l. The 7- day exposure period was followed by a 14-day long depuration phase. Diltiazem reached a BCF of 16 after the 7-day exposure and its half-life was estimated at 117 h. Half-life time of diltiazem in fish is much longer compared to that of humans (2–6 h; Regenthal et al., 1999) suggesting a slower depuration rate in fish.

Conclusion

Hitherto, most studies have been focused on the effects of verapamil and diltiazem while no data is available for nifedipine or other calcium channel blockers. The acute toxicity of diltiazem and verapamil is in the range of mg/l. Verapamil can be classified as toxic (> 1 < 10 mg/l; OECD, 2001) while diltiazem as harmful or toxic to fish (> 10 < 100 mg/l; OECD, 2001; Tab. 8). Diltiazem has a low level of bioconcentration in fish (BCF = 16). In addition, fish have a longer depuration time of diltiazem when compared with humans. Chronic effects of diltiazem on fish had not been studied before starting my Ph.D. work. Also, no experimental data were available on BCF or on half-life of verapamil and diltiazem in fish. The predicted environmental concentration that would result in concentration of these pharmaceuticals in the blood plasma of fish reaching the human therapeutic levels is for diltiazem 1161 times higher than for verapamil. Besides the effect on the cardiovascular system, verapamil is also supposed to act as chemosensitizer. In summary from the literature, effects on fish were found only at environmentally not relevant concentrations.

Table 8. *Lethal concentration (96hLC_{50}) of selected calcium channel blockers.*

	Species	Age class	LC_{50} (mg/l)	Reference
Verapamil	Rainbow trout (*Oncorhynchus mykiss*)	Juvenile	2.72	(Li et al., 2010a)
Diltiazem	Medaka (*Oryzias latipes*)	Juvenile-adult[a]	15.0	(Kim et al., 2007)

[a]Information on the age class of the fish was not provided. Based on given length (2.0 ± 1.0 cm), fish could be either juvenile or adult.

1.1.3. General conclusions

In general, only a few articles describing the effects of cardiovascular drugs on fish were published before starting my Ph.D. work, and even fewer focused on the effects of a long-term exposure to low concentrations. Most of the effects of the cardiovascular drugs on fishes have been at very high concentrations. However, there have been only few attempts to search for some specific targets of their action in fishes. Most studies were focused on nonspecific endpoints, such as mortality, growth rate, biomarkers of oxidative stress etc. Moreover, at present there is limited information on the bioconcentration and the half-life time of cardiovascular drugs in fish. Therefore, there is an urgent need for further research.

1.1.4. The outline and aims of the thesis

This thesis was focused on effects of selected cardiovascular pharmaceuticals on fish. The compounds of interest were namely the β-blocker atenolol and the calcium channel blockers verapamil and diltiazem. These pharmaceuticals are used for the treatment of arterial hypertension and angina pectoris (Lüllmann et al., 2003). They are highly prescribed and not fully degraded in wastewater treatment plants. As a result relatively high concentrations can be traced in the aquatic environment (Jones et al., 2002; Hummel et al., 2003; Kolpin et al., 2004; Khan and Ongerth, 2004; Maurer et al., 2007; Vieno et al. 2007; Kasprzyk-Hordern et al., 2009). However, there was little information available on their long-term effects, bioconcentration, metabolism, and half-life in fish.

The specific objectives of this thesis were to:

- Study the bioconcentration and half-life of verapamil, atenolol, and diltiazem in fish.
- Study the sub-chronic effects of verapamil, atenolol, and diltiazem on fish.
- Study metabolism (mainly the phase I) of diltiazem in rainbow trout.
- Develop a protocol for the unified assessment and evaluation of the histopathological alterations in the heart of fish.

Sub-chronic effects, bioconcentration, and depuration of verapamil and atenolol in the early-life stages of common carp and juvenile rainbow trout are described in Chapter 2 and Chapter 3, respectively. Chapter 4 reports on the effect of long-term exposure of diltiazem on rainbow trout including diltiazem tissue-specific bioconcentration, metabolism and depuration. A method of determination of the phase I metabolites isoforms of diltiazem in fish is described in Chapter 5. In Chapter 6, a unified method for the evaluation of histopathological alterations of the heart in fish is presented.

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CHAPTER 2

TOXIC EFFECTS, BIOCONCENTRATION AND DEPURATION OF VERAPAMIL IN THE EARLY LIFE STAGES OF COMMON CARP (*CYPRINUS CARPIO* L.)

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Toxic effects, bioconcentration and depuration of verapamil in the early life stages of common carp (*Cyprinus carpio* L.)

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HIGHLIGHTS

- Study of the acute and sub-chronic toxicity of verapamil on early-life stages of common carp.
- Acute exposure to verapamil reduced the heart rate in early-life stages of common carp.
- The bioconcentration factor of verapamil ranged from 6.6 to 16.6.
- The half-life of verapamil in fish was estimated to be 10.2±1.6days.

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ABSTRACT

Verapamil is a pharmaceutical that belongs to a group of calcium channel blockers and is mainly used as a treatment of angina pectoris and arterial hypertension. Verapamil has been detected in aquatic environments in concentrations ranging from ng L^{-1} to µg L^{-1}. In the present study, a series of acute toxicity tests of verapamil on various developmental stages of common carp (*Cyprinus carpio*) were conducted. As a result, 96hLC_{50} values of verapamil were estimated at 16.4 ± 9.2, 7.3 ± 1.5 and 4.8 ± 0.2 mg L^{-1} for embryos (E5–E9) and common carp larvae L2 and L5, respectively. Lethal concentrations of verapamil decreased with an increase in the age of the fish. Acute exposure to verapamil significantly reduced the heart rate in the embryos and larvae. In an embryo-larval toxicity test (sub-chronic exposure), the bioconcentration, depuration, and toxic effects of verapamil were assessed in common carp. The fish were exposed to verapamil in a concentration of 0.463 (environmentally relevant), 4.63, 46.3 and 463 µg L^{-1}. Verapamil had no effect on the accumulated mortality, hatching, condition factor, growth or ontogeny of the fish in any of the tested concentrations. In carp exposed to 463 and 46.3 µg L^{-1} of verapamil, significantly higher occurrences of malformations and edemas were observed compared to the control. The bioconcentration factor of verapamil in whole fish homogenates ranged between 6.6 and 16.6 and was therefore below the critical value for hazard substances (BCF > 500). The half-life and the 95% depuration time for the tested compound were estimated to be 10.2 ± 1.6 days and 44.2 ± 8.6 days, respectively. No effects of verapamil on the studied endpoints were observed at environmentally relevant concentrations.

1. Introduction

Typically, human pharmaceuticals are excreted after application and enter aquatic ecosystems via wastewater because they are not completely removed in sewage treatment plants (Khetan and Collins, 2007). Even if the concentrations of most pharmaceuticals in aquatic environments are relatively low (from ng L^{-1} up to low µg L^{-1}), the pharmaceuticals might pose a substantial threat to aquatic organisms (Corcoran et al., 2010; Gunnarsson et al., 2008) because they are designed to be effective in low doses. In general, effects on non-target species are supposed to occur as a result of specific drug target interactions rather than via unspecific modes of action, whereas pharmaceuticals are designed to interact with evolutionarily well-conserved drug targets (Corcoran et al., 2010; Gunnarsson et al., 2008). However, there is only limited knowledge available on the long-term influence of pharmaceuticals on aquatic organisms (Fent et al., 2006).

Verapamil, a phenylalkylamine, is a pharmaceutical belonging to a group of calcium channel blockers. This highly prescribed drug is an active antianginal, antiarrhythmic and antihypertensive that is used in the treatment of cardiovascular diseases (Johnston et al., 1981; Lüllmann et al., 2003). The reported concentrations of verapamil in

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treated waste waters and surface waters lie in the range of μg L^{-1} and ng L^{-1}, respectively (Batt et al., 2008; Hummel et al., 2006; Khan and Ongerth, 2004).

Acute toxicity of verapamil is relatively low, as demonstrated by an acute toxicity test on rainbow trout (*Oncorhynchus mykiss*) by Li et al. (2010a). In their study, the 96hLC_{50} value for verapamil was estimated at 2.7 mg L^{-1}. The acute exposure of zebrafish larvae (*Danio rerio*) to verapamil at a concentration of 455 mg L^{-1} affected their heart rate and gut activity (Berghmans et al., 2008). However, verapamil has also caused effects in fish at lower concentrations. A short-term exposure (96 h) of rainbow trout (*O. mykiss*) to verapamil at a concentration of 270 μg L^{-1} caused oxidative stress (Li et al., 2010a) and an increase in the stress index (integrated biomarker response; Li et al., 2010b) after long-term exposure (42 days).

Fish and amphibians are vertebrates that undergo their metamorphosis, a very sensitive life period, in surface waters. Consequently, their embryo-larval stages are supposed to be highly sensitive to chemicals polluting the aquatic environment in which they live (Richard et al., 2008). In toxicological risk assessments, the fish embryo-larval test (Machova et al., 2009; OECD, 1992, 1996) and amphibian metamorphosis assay (OECD, 2009) are established test procedures. To date, few studies have focused on the effects of verapamil on early-life stages of fish and amphibians (Burgess and Vere, 1989; Overturf et al., 2011). A study by Burgess and Vere (1989) revealed that verapamil at a concentration of 72 μg L^{-1} caused abnormalities in the development of the African clawed frog (*Xenopus laevis*). In a recent study by Overturf et al. (2011), long-term exposure (28 days) of verapamil to the fathead minnow (*Pimephales promelas*) larvae at a concentration of 600 μg L^{-1} caused a significant decrease in growth rate. Verapamil is a cationic amphiphile substance that has a bioconcentration potential because it is slightly lipophilic (log P = 4.8 and 3.4 for its uncharged and charged form, resp.; Fick et al., 2010a,b; Lüllmann et al., 2003; OECD, 1996). However, only limited data on the bioconcentration of verapamil in fish are available (Fick et al., 2010a,b). Bioconcentration properties of a substance can be described by the bioconcentration factor (BCF), which is defined as the ratio of the concentration of the substance in the fish to the concentration in the water (Mackay and Fraser, 2000; OECD, 1996; Springer et al., 2008). The elimination rate, presented as the half-life, is another important pharmacokinetic parameter. The half-life is defined as the time it takes for the concentration of a substance in an organism to halve its steady-state (Lüllmann et al., 2003; OECD, 1996). It reflects the capacity for biotransformation and elimination of a substance in the exposed organisms. However, the bioconcentration factor and half-life are important endpoints for environmental hazard assessment (Franke, 1996; Mackay and Fraser, 2000; Nordberg and Rudén, 2007; OECD, 1996, 2001; Springer et al., 2008). In fact, only a limited number of studies have described them for waterborne human pharmaceuticals in fish (Berghmans et al., 2008; Alderton et al., 2010; Fick et al., 2010a, 2010b; Gomez et al., 2010; Nallani et al., 2011; Mehinto et al., 2010; Paterson and Metcalfe, 2008).

The aim of the present study was to determine the toxic effects of acute and long-term exposure of verapamil on the early-life stages of common carp, a well-established model species used to test the adverse effects of chemicals on the early development of fish (OECD, 1992, 1996). To our knowledge, this is the first study describing the effects of verapamil on the occurrence of malformations during early development of fish. Moreover, this study is among the first to investigate both the bioconcentration factor and half-life of verapamil in fish.

2. Material and methods

2.1. Experimental animals

Fertilized eggs and larvae of the common carp (*Cyprinus carpio* L.) were obtained from the breeding station of the Research Institute of Fish Culture and Hydrobiology in Vodnany (Czech Republic). Eggs were produced according to standard methods of artificial reproduction (Kocour et al., 2005). Twenty four hours post-fertilization, all eggs were treated with malachite green solution (0.25 mg L^{-1}) and formaldehyde (0.125 μg L^{-1}) for two hours to prevent possible fungal infection (Sudova et al., 2007). White unfertilized eggs were removed.

2.2. Experimental conditions

The fish were kept in dechlorinated tap water. Throughout the experiment, all basins were gently aerated. The water quality parameters were the following: dissolved oxygen >5.8 mg L^{-1}, temperature 20.1 ± 1.7 °C, pH 7.9 ± 0.2, $ANC_{4.5}$ (acid neutralization capacity) 1.1 mmol L^{-1}, COD_{Mn} (chemical oxygen demand) 1.10 mg L^{-1}, NH_4^+ 0.02 mg L^{-1}, NO_3^- 9.2 mg L^{-1}, PO_4^{3-} 0.02 mg L^{-1}, Cl^- 10.65 mg L^{-1}. The basins were placed in a laboratory (open-air conditions) with natural light exposure (16:8 h light: dark).

2.3. Chemicals

Verapamil hydrochloride (CAS number: 152-11-4) and Diltiazem hydrochloride (CAS number: 33286-22-5) were obtained from Sigma-Aldrich Corporation (USA). LC–MS grade acetonitrile (LiChrosolv Hypergrade) was purchased from Merck (Darmstadt, Germany). Verapamil concentrations are expressed as concentrations of the active substance verapamil throughout the study.

2.4. Acute toxicity tests

The 96 h acute toxicity tests were conducted using the OECD guideline No. 212 (OECD, 1998), with modifications as described below. Three early-life stages of common carp were used: embryos – stage E5 and larvae – stages L2 and L5. The larvae of the stages L2 and L5 were 6 and 20 days post hatched, respectively. At the conclusion of the tests, embryos reached stage E9 and larvae remained at stages L2 and L5, respectively. Developmental stages were classified according to Penaz et al. (1983). At each experimental replicate, 20 embryos or 15 larvae were randomly distributed to crystallization basins containing 190 mL of a solution corresponding to the following treatments: 1) eggs – control (verapamil-free water), 1, 2, 5, 10, and 20 mg L^{-1} verapamil and 2) larvae – control (verapamil-free water), 1, 2, 5, 10, 15, and 20 mg L^{-1} verapamil. Each treatment and the control were replicated three times. The test solutions were exchanged daily by light draining each basin and adding new solution. Mortalities were recorded and dead fish were removed on a daily basis. Once the larvae were able to take up food, they were fed daily *ad libitum* with freshly hatched brine shrimp (*Artemia salina*) nauplii after water was exchanged.

At the end of the exposure, the heart rate (beats per minute) of the fish (stages E9 and L2) was recorded from five fish per replicate in the following treatments: control, 1, 2, 5, and 10 mg L^{-1} verapamil. The embryos and larvae exposed to higher verapamil concentrations died before the end of the tests. The heart was not visible in the third stage (L5); therefore, the heart rate could not be measured in this case. The heart rate was determined by counting the number of visual beats under a stereomicroscope (Olympus SZXZ; magnification ×10) over a period of 30 s. At the end of the experiments, all surviving fish were fixed in a 4% formalin solution. Morphological anomalies were evaluated according to Jezierska et al. (2000).

2.5. Sub-chronic toxicity test

The embryo-larval toxicity test was conducted using the modified test design of the OECD guideline No. 210 (OECD, 1992). The test started one day after egg fertilization. At each experimental replicate, 100 eggs were randomly distributed to each of the crystallization basins containing 300 mL of the solution corresponding to the following

treatments: control (verapamil-free water); 0.463 μg L^{-1} verapamil (group 1); 4.63 μg L^{-1} verapamil (group 2); 46.3 μg L^{-1} verapamil (group 3); 463 μg L^{-1} verapamil (group 4). Each treatment and the control were replicated three times. The solutions from each treatment were renewed daily by light draining each basin and adding new solution. Hatching and mortality were recorded daily; dead eggs, embryos and larvae were removed. Once the larvae were able to take up food, they were fed daily *ad libitum* with freshly hatched brine shrimp (*A. salina*) nauplii after the water was exchanged. The experiment was terminated after 31 days.

To assess development, ten fish per replicate were sampled after 10, 15, 20, 24 and 31 days. The fish were stored in a 4% formalin solution. Determination of developmental periods and stages followed Penaz et al. (1983). Morphological anomalies were evaluated according to Jezierska et al. (2000). The total length, relative mass after fixation, developmental stages and occurrence of morphological anomalies were checked under a stereomicroscope (Olympus SZXZ). The total length was measured with an accuracy of 0.01 mm by a fine digital caliper. The relative weight after fixation, to a resolution of 0.1 mg, was measured by a Mettler Toledo AB204-S balance. The weight of fish was taken at the time when the weight was already stabilized, i.e. after 3 months of formalin preservation (Kristoffersen and Salvanes, 1998). The Fulton's condition factor (FCF; Nash et al., 2004) and the mean specific growth rate (SGR; Machova et al., 2009; Cook et al., 2000) for the fish in each of the experimental groups were calculated. After the termination of the embryo-larval test, the replicates of each treatment were put together and kept in five aquaria (40 L) in verapamil-free water. The fish were fed daily with dry food (Tetra). Fifty percent of the water in the tanks was exchanged at least every third day. These fish were kept in the aquaria five months post-termination of the exposure to verapamil to evaluate the half-life of verapamil in fish. The fish mortality was also recorded during this period.

2.6. Analysis of verapamil in water and fish

In the acute toxicity tests, water samples were taken from each basin immediately after the water was exchanged and 24 h post exchange during each test. In the sub-chronic toxicity test, water samples were taken from each basin after 0, 2, 6 and 24 h on days 10, 20 and 31. Additionally, tap water samples were taken. All water samples were taken using separate syringes. At the end of the exposure, seven fish from each replicate (105 in total) were sampled. Additionally, seven fish from each experimental group (35 in total in every sampling term) were sampled after two and six weeks post-exposure. All sampled fish were dried with filter paper and stored in 2 mL Eppendorf tubes at 20 °C until analysis. The concentration of verapamil was determined in whole fish homogenates. The homogenization and extraction of the samples followed modified Ramirez et al. (2009). The sample extraction was conducted with acetonitrile in the high-speed homogeniser with stainless-steel balls. Diltiazem was used as an internal standard.

Quality assurance/quality control (QA/QC) of the analytical method included blank samples (to be sure that target compound is not introduced from the sample handling), duplicates (every tenth sample was duplicated) and fortified samples (every tenth sample of fish from control group was spiked with the target compound at the concentration 100 ng g^{-1} to check the method accuracy). Matrix-matched standards ("clean" fish extracts spiked with both target compound and internal standard) were used to correct response factor of the calibration curve prepared in methanol.

The water samples were analyzed using line SPE liquid chromatography with tandem mass spectrometry (LC–MS/MS; Kvarnryd et al., 2011). Samples of two highest concentrations were diluted prior to analysis. Fish homogenate samples were analyzed using LC–MS/MS. These analyses were performed with a TSQ Ultra MS/MS (Thermo Fisher Scientific, San Jose, CA, USA) coupled with Accela 1250 and Accela 600 HPLC pumps (Thermo Fisher Scientific, San Jose, CA, USA) and a HTS-XT autosampler (CTC Analytics AG, Zwingen, Switzerland).

The bioconcentration factor (BCF) was calculated in accordance with OECD Guideline No. 305 (OECD, 1996) for each treatment group as follows: the mean concentration of verapamil in the fish sampled at the termination of the experiment was divided by the mean concentration of all analyzed water samples in the respective group.

First-order kinetics was assumed to determine the depuration rate. The half-life (50% depuration, t_{50}) and 95% loss in the depuration phase (t_{95}) of verapamil in fish were calculated using linear regression of the natural logarithm (ln) of the detected concentrations (group 2, group 3, group 4) and the value of the slope (k) of the graph: (t_{50}) = 0.693/k and (t_{95}) = 3.0/k, respectively (OECD, 1996). The first group was excluded from half-life calculations because the detected concentrations were too close or below the LOQ.

2.7. Statistical analysis

Statistical analysis was performed using Statistica version 9 (StatSoft, Czech Republic). The differences in the accumulated mortality, hatching time and occurrence of macroscopic morphological anomalies between the test groups were measured using the χ^2 test. Data for the total length, relative mass after fixation, and FCF were tested for normality using the Kolmogorov–Smirnov test and for homogeneity using Levene's test. If the statistical criteria for normality and homogeneity were fulfilled, a one-way analysis of variance (ANOVA) was implemented with subsequent Dunnett's multiple-range post hoc test. If these criteria were not satisfied, the nonparametric test (Kruskal–Wallis test) was applied. The results are presented as the mean ± standard deviation. Statistical differences between the experimental groups were considered significant when $p < 0.05$.

The 96hLC_{50} values were calculated by probit analysis using EKOTOX 5.1 software (Ingeo Liberec, Czech Republic).

3. Results

3.1. Determination of verapamil in water and fish

3.1.1. Analytical method performance

Performance of the analytical method was assessed including its linearity, repeatability, sensitivity and recovery. Six point calibration curves were prepared for water and fish analysis in the range 10–5000 ng L^{-1} and 1–500 ng g^{-1} respectively. Good linearity was observed with R^2 coefficient 0.998. Method repeatability was tested for ten replicates; relative standard deviation (RSD) of replicates was 10%. The recovery of verapamil from fish tissue was studied by spiking "clean" fish sample with the target compound before the extraction procedure. Average recovery of verapamil was 110% with the RSD of ten replicates 5%. The limits of quantification (LOQ) of verapamil in fish and water were 0.006 μg g^{-1} and 0.07 μg L^{-1}, respectively.

3.1.2. Concentration of verapamil in water

Concentrations of verapamil in all treatment baths measured immediately (0 h) and 24 h post-exchange in acute toxicity tests as well as sub-chronic test are shown in Table 1. A comparison of the determined verapamil water concentration with nominal values for all test groups of the sub-chronic test is listed in Table 2. Concentrations of verapamil in the controls and the tap water were below the limit of quantification.

3.1.3. Concentration of verapamil in fish

The concentrations of verapamil in fish homogenates at the conclusion of the sub-chronic test are summarized in Table 3. Verapamil concentrations in the fish were linearly correlated to the concentrations in

Table 1
Concentration of verapamil in water in the 96-h acute toxicity tests and in the 31-day embryo-larval toxicity test (concentrations measured immediately (0 h) and (24 h) after water exchange). Values are expressed as the mean ± SD. Acute toxicity tests: n = 9, concentrations in mg L^{-1}; 31-day embryo-larval toxicity test: n = 3, concentrations in μg L^{-1}.

Test	Treatment/sampling time	Concentration of verapamil	
		0 h	24 h
Acute tests	Control	<LOQ	<LOQ
	Nominal conc. 1 mg L^{-1}	0.7 ± 0.1	0.8 ± 0.1
	Nominal conc. 2 mg L^{-1}	1.2 ± 0.1	2.0 ± 0.1
	Nominal conc. 5 mg L^{-1}	4.2 ± 1.6	3.7 ± 1.8
	Nominal conc.10 mg L^{-1}	8.1 ± 1.8	8.5 ± 1.8
	Nominal conc. 15 mg L^{-1}	13.9 ± 4.4	16.6 ± 4.6
	Nominal conc. 20 mg L^{-1}	16.2 ± 3.3	21.6 ± 4.6
Sub-chronic test	Control	<LOQ	<LOQ
	Nominal conc. 0.463 μg L^{-1}	1.1 ± 0.5	0.7 ± 0.6
	Nominal conc. 4.63 μg L^{-1}	4.0 ± 0.9	5.5 ± 1.4
	Nominal conc. 46.30 μg L^{-1}	42.5 ± 13.8	26.1 ± 13.3
	Nominal conc. 463 μg L^{-1}	363 ± 129	222 ± 89

water ($y = 3.9151x + 67.147$, $R^2 = 0.9947$; x and y represent the concentration of verapamil in water and fish homogenates). The BCF calculated for groups 1 to 4 was as follows: 16.6, 14.0, 10.1, and 6.6. The half-life and 95% loss period in the depuration phase of verapamil in fish were estimated at 10.2 ± 1.6 and 44 ± 8.6 days, respectively. Hence, verapamil was still detectable in the fish six weeks after time of exposure.

3.2. Acute toxicity tests

3.2.1. Lethal concentrations

The 96hLC_{50} values of verapamil were estimated at 16.4 ± 9.2, 7.3 ± 1.5, and 4.8 ± 0.2 mg L^{-1} for embryos (E5–E9) and larvae L2 and L5, respectively. Lethal concentrations of verapamil decreased with increasing age of fish.

3.2.2. Heart rate

Verapamil exposure was found to significantly reduce heart rate. The exponential regression analysis of the heart rate (y) on verapamil concentration in the water (x) for embryos (E9) and larvae (L2) of common carp yielded values as follows: $y = 94.96e^{-0.1543x}$, $R^2 = 0.87$ and $y = 104.85e^{-0.1333x}$, $R^2 = 0.85$, respectively (Fig. 1). The heart rate could not be measured in stage L5 larvae, because their hearts were not visible.

3.2.3. Macroscopic morphological anomalies

Macroscopic morphological anomalies observed at the conclusion of the acute toxicity tests are given in Table 4. These included the axial and lateral curvature of the spine (kyphosis, lordosis, and scoliosis) and skull malformation, as well as yolk sac, visceral, and heart edemas (Fig. 2). A higher incidence of edemas compared to the controls was observed in most verapamil-treated groups of all stages (Table 4). Furthermore, at stage L2, all verapamil-treated groups had a significantly higher occurrence of malformations (Table 4) compared to the controls. Occurrence of malformations could not be assessed in groups treated with higher verapamil concentrations (higher than 10 or 5 mg L^{-1} in the case of E9 and L2, and L5, respectively), as all the fish in these treatments died before the termination of the test.

Table 2
Concentration of verapamil in water of all treatments and differences of determined concentrations to the nominal concentrations during the 31-day embryo-larval toxicity test.

	Control	Group 1	Group 2	Group 3	Group 4
		0.463 μg L^{-1}	4.63 μg L^{-1}	46.3 μg L^{-1}	463 μg L^{-1}
c (μg L^{-1})					
Mean ± SD	<LOQ	0.9 ± 0.6	4.0 ± 1.2	33.7 ± 15.5	298.8 ± 117.7
Min–max		0.1–2.3	1.8–6.5	9.3–64.2	88.3–612.8
Difference to nominal conc. (%)	–	196 ± 124	87 ± 27	72 ± 35	64 ± 25

Table 3
Concentration of verapamil in fish homogenates at the conclusion of the 31-day embryo-larval toxicity test (0 day) and after 14 and 42 days post-exposure. Values are expressed as the mean ± SD.

Group/sampling time	Concentration of verapamil (μg kg^{-1})		
	0 day	14 days	42 days
Control	<LOQ	<LOQ	<LOQ
Group 1: 0.463 μg L^{-1}	15.1 ± 6.6	<LOQ	<LOQ
Group 2: 4.63 μg L^{-1}	56.1 ± 22.0	17.3 ± 9.6	4.5 ± 7.0
Group 3: 46.3 μg L^{-1}	340 ± 91.2	66.2 ± 22.4	19.2 ± 5.9
Group 4: 463 μg L^{-1}	1870 ± 274	282 ± 57.1	42.8 ± 15.6

3.3. Sub-chronic toxicity test

3.3.1. Hatching

Hatching began 5 days after the initial time of exposure, and the majority of eggs in all treatment groups hatched by day 7. Verapamil had no significant effect on the hatching time (χ^2 test, $p > 0.05$).

3.3.2. Accumulated mortality

The accumulated mortality did not differ statistically among treatments (χ^2 test, $p > 0.05$). No mortality was observed five months after the termination of the experiment in all the groups, including in the control.

3.3.3. Length, weight, FCF and SGR

Exposure to verapamil had no significant effect on the length, relative mass after fixation or FCF of the tested groups (ANOVA, $p > 0.05$, Table 5). The specific growth rate in the control, group 1, group 2, group 3 and group 4 were 14.0, 13.4, 15.4, 13.1, and 15.7, respectively.

3.3.4. Macroscopic morphological anomalies

Verapamil caused the following macroscopic morphological anomalies in the exposed larvae: deformation of the intestine, axial and lateral curvature of the spine (kyphosis, lordosis, and scoliosis), skull malformation, fin malformations, underdeveloped eyes and visceral edema with hematocele (the number of checked fish in each treatment ranged between 24 and 31). The most predominant malformations were axial curvature of the spine and visceral edema with hematocele (Fig. 3, Table 6).

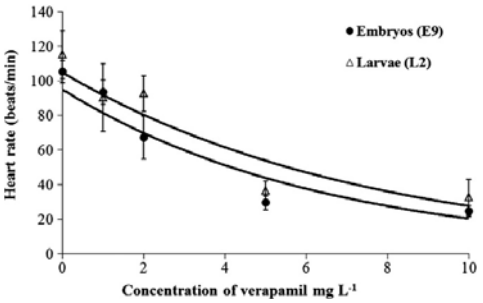

Fig. 1. Exponential regression of heart rate (y) on the verapamil concentration in water (x) for embryos (E9) and larvae (L2) of common carp, respectively; $y = 94.96e^{-0.1543x}$, $R^2 = 0.87$; $y = 104.85e^{-0.1333x}$, $R^2 = 0.85$.

Table 4
Occurrence of macroscopic morphological anomalies in embryos (E9) and larvae (L2, L5) of common carp at the conclusion of the 96-h acute toxicity tests with verapamil. MA — sum of all malformations, Sp — axial and lateral curvature of the spine, SM — skull malformation, E — sum of all edemas, VE — visceral edema, YE — yolk sac edema and CE — cardiac edema. Asterisks indicate a significant difference compared to the control: * $p < 0.05$, ** $p < 0.01$ (χ^2 test). N.E. — not evaluated.

Stage	c (mg L^{-1})	MA (%)	Sp (%)	SM (%)	E (%)	YE (%)	VE (%)	CE (%)
	Control	4.7	4.7	0	2.3	2.3	0	0
	1	15.4	15.4	5.1	20.5**	17.9*	0	15.4*
E9	2	15.0	15.0	7.5	30.0**	12.5	0	22.5*
	5	17.0	17.0	10.6*	59.6**	36.2**	0	34.0**
	10	66.7**	59.0**	28.2**	82.1**	79.5**	0	69.2**
	Control	3.0	3.0	0	0	N.E.	0	0
	1	33.3**	25.9**	7.4	14.8*	N.E.	14.8*	3.7
L2	2	27.8**	27.8**	16.7*	11.1	N.E.	11.1*	5.6
	5	57.1**	57.1**	35.7**	75.0**	N.E.	53.6**	67.9**
	10	77.8**	66.6**	55.6**	55.6**	N.E.	44.4**	33.3**
	Control	0	0	0	2.4	N.E.	2.4	N.E.
	1	0	0	0	4.4	N.E.	4.4	N.E.
L5	2	4.5	4.5	0	15.9*	N.E.	15.9*	N.E.
	5	6.5	6.5	0	25.8**	N.E.	25.8**	N.E.

The highest malformation rates in verapamil-treated fish were observed on days 20 and 24 (Table 6). No malformations were observed at the last sampling time (day 31). In groups 3 and 4 (treated with the two highest concentrations), a significantly higher occurrence of malformations and edema with hematocele was observed compared to the control.

3.3.5. Early ontogeny

Verapamil did not cause a delay in the development of the exposed fish. The differences in the early ontogeny among the tested groups were not significant (Wilcoxon–Mann–Whitney-test, $p > 0.05$, Table 6).

4. Discussion

4.1. Acute toxicity tests

4.1.1. Lethal concentrations of verapamil

In the present study the 96hLC_{50} values of verapamil for common carp embryos and larvae ranged from 16.6 to 4.8 mg L^{-1}; therefore, verapamil can be classified as a harmful ($>10 < 100$ mg L^{-1}) or toxic ($>1 < 10$ mg L^{-1}; OECD, 2001) substance. This finding is in accordance with the studies by Li et al. (2010a) and Villegas-Navarro et al. (2003), who estimated the lethal concentration of verapamil for juvenile rainbow trout and *Daphnia magna* to be 2.7 mg L^{-1} (96hLC_{50}) and 7.0 mg L^{-1} (48hLC_{50}). Interestingly, acute lethal concentrations (96hLC_{50}) of verapamil determined in the present study were close to the comatose-lethal blood plasma concentration for humans (2.5 mg L^{-1}; Regenthal et al., 1999).

The toxicity of verapamil seemed to increase slightly with the age of the fish, with the oldest stage (L5) being the most sensitive. The tested developmental stages of common carp differed in food intake as well as in advancement of physiological development (Penaz et al., 1983). However, the differences in sensitivity might be explained by the advancement of gill development, as the fish were only exposed to waterborne verapamil in the study. Fish of the L5 stage had fully developed gills compared to the other tested stages (E5-9 and L2) (Penaz et al., 1983); therefore, verapamil could enter their body much easier. In general, larvae appear to be more sensitive to most toxic substances compared to embryos (Kroupova et al., 2010; Machova et al., 2009).

4.1.2. Effect of verapamil on heart rate

In the present study, verapamil reduced the heart rate of carp embryos and larvae in acute toxicity tests in a concentration-dependent manner, which is well in line with the finding of Berghmans et al. (2008), who exposed zebrafish larvae to verapamil for 3 h in the concentration range of 136–227 mg L^{-1}. Furthermore, Shin et al. (2010) found a reduced heart rate in zebrafish embryos exposed to verapamil at the concentration of 1 mg L^{-1} for 12 h compared to the controls. The present study, together with the results of Berghmans et al. (2008) and Shin et al. (2010), reveal that verapamil has a similar pharmacological effect on fish as it does on humans, where it is used therapeutically to reduce the heart rate (Grossman and Messerli, 2004). The similarity in the mode of action in fish and humans is not surprising, whereas most drug targets are supposed to be highly conservative among vertebrate species (Gunnarsson et al., 2008). This finding is further supported by the fact that comparing the polypeptide sequences for the human and zebrafish orthologs of the α-subunit of the L-type calcium channel, which is one of the targets of the calcium channel blocker verapamil, revealed a 78% match (Lacinova et al., 1995; Rottbauer et al., 2001). Sequence of carp α-subunit of the L-type calcium channel is not available, but it is highly probable that carp and zebrafish polypeptide sequences are very similar since both fish species belong to the same family, family of cyprinid fishes.

4.2. Macroscopic morphological anomalies

Carp embryos and larvae exposed acutely to high verapamil concentrations (1–10 mg L^{-1}) had a higher occurrence of macroscopic morphological anomalies than fish exposed sub-chronically to lower concentrations (0.463–463 μg L^{-1}). However, in both cases, verapamil exposure was associated with significantly higher occurrence of spine malformations and edemas. Occurrence of edemas was also observed in frogs treated with verapamil during their early development (Burgess and Vere, 1989). Similarly, peripheral edema is a non-cardiovascular side effect of verapamil treatment in humans (Drenth et al., 1992). Burgess and Vere (1989) attributed this effect of verapamil to disturbances in the ionic equilibrium. Moreover, long-term exposure of carp larvae to verapamil resulted in a higher incidence of hematocele in the intestine compared to the control, whereas gastrointestinal hemorrhage is another side effect of verapamil treatment in humans (Pahor et al., 1996). Gastrointestinal hemorrhage is caused by inhibition of platelet aggregation as well as the inhibition of the vasoconstrictive response to bleeding (Pahor et al., 1996). These effects might also be responsible for hematocele in fish.

4.3. Sub-chronic toxicity test

4.3.1. Sub-chronic toxicity

Long-term exposure to verapamil at concentrations of 0.463–463 μg L^{-1} had no effect on the survival and morphometric parameters (length, relative weight after fixation, and FCF) of the carp embryos and larvae in the present study. Similarly in a study by Overturf et al. (2011) that exposed fathead minnow embryos and larvae (*P. promelas*) to verapamil (600 μg L^{-1}) for 28 days, no effect on mortality was observed; however, verapamil exposure caused a significant reduction in dry weight. In the present study, the occurrence of macroscopic morphological anomalies appeared to be the most sensitive endpoint that was analyzed. Verapamil exposure resulted in a significantly higher occurrence of malformations, and edema with hematocele, in fish exposed to the two highest verapamil concentrations (46.3 and 463 μg L^{-1}) compared to the control. However, neither of the studied endpoints showed any effect of verapamil at environmentally relevant concentrations on carp larvae and embryos.

4.3.2. Bioconcentration factor

In the present study, the bioconcentration factor (BCF) of verapamil in fish homogenates ranged between 6.6 and 16.6. To the best of our knowledge, there are no other experimental data available on BCF in fish tissues or fish homogenates. Predicted BCF for whole fish is 3.2

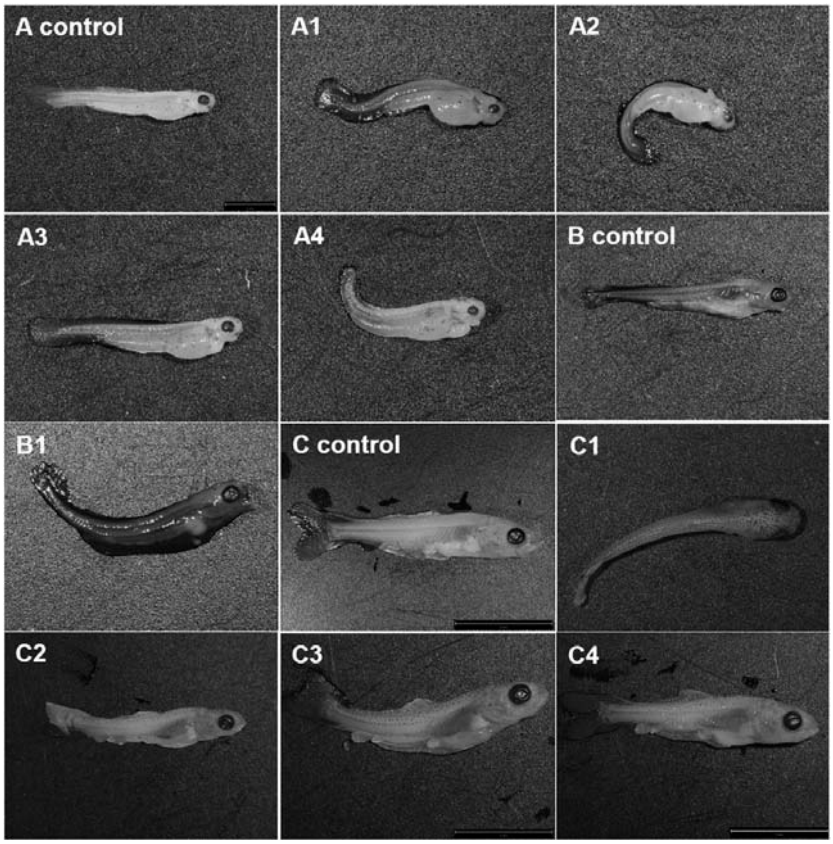

Fig. 2. Types of deformed embryos and larvae of common carp at the conclusion of the 96-h acute toxicity tests with verapamil; A — normal embryo (control, E9), A1 — axial curvature, kyphosis (5 mg L^{-1}, E9), A2 — deformed skull, axial curvature; kyphosis lateral spinal curvature; scoliosis, yolk sac edema (10 mg/l, E9), A3 — yolk sac edema, cardiac edema (1 mg L^{-1}, E9), A4 — cardiac edema, yolk sac edema, spinal curvature; c – shape (10 mg L^{-1}, E9), B — normal larva (control, L2), B1 — cranial malformation, cardiac edema, visceral edema, spinal curvature (5 mg L^{-1}, L2), C — normal larva (control, L5), C1 — lateral spinal curvature, scoliosis (2 mg L^{-1}, L5), C2 — visceral edema, spinal curvature; lordosis (5 mg L^{-1}, L5), C3 — visceral edema, axial spinal curvature; c – shape (5 mg L^{-1}, L5), C4 — visceral edema (5 mg L^{-1}, L5).

(based on log P = 3.4, calculated by EPI Suite™ v4.11 – US EPA, 2012) which is in agreement with our experimental values. Moreover, we can compare our values only with those found in fish blood plasma. Namely, Fick et al. (2010b) reported that the plasma of rainbow trout exposed to waste water containing verapamil had a BCF ranging from <33 to 175, whereas the predicted BCF value of the plasma of fish was 40 (based on log P = 3.4; Fick et al., 2010b). The BCF determined in the present study is lower, but follows the same order of magnitude. A BCF of <500 in fish is considered to be indicative of a low level of bioconcentration in the given substance (OECD, 2001).

Although the plasma BCF seems to be low, verapamil could reach dangerous levels in fish plasma. In accordance with "fish plasma model" proposed by Huggett et al. (2003), Fick et al. (2010a,b) calculated critical environmental concentrations (CECs) of verapamil causing fish plasma concentration equal to the therapeutic human plasma level (10 ng mL^{-1}; Schulz and Schmoldt, 2003). Based on predicted and measured blood BCFs, they estimated CECs at 230 and 53 ng L^{-1}, respectively (Fick et al., 2010a,b). Therefore, both similar pharmacological effect of verapamil in man and fish and relatively low critical environmental concentrations indicate a high risk of verapamil to fish.

4.3.3. Depuration of verapamil

The half-life of verapamil in carp larvae was estimated to be 10.2 days, which is much longer than the half-life in humans (4.5–12 h; Wood et al., 1999). Similar results were obtained for fluoxetine (Paterson and Metcalfe, 2008), which had a half-life three times greater when exposed in medaka compared to mammalian species. The longer half-life of verapamil in fish compared to humans implies the existence of differences in the biotransformation and/or excretion of the substance between these species. Verapamil undergoes extensive first-pass elimination in the liver of humans; therefore, only 20–30% of orally administrated verapamil is bioavailable (Eichelbaum et al., 1984). Similarly, Luurtsema et al. (2005)) who systemically administrated (by injection) verapamil to rats observed an intensive metabolism of this substance in liver (88.9% 60 h post-injection). Verapamil undergoes extensive oxidative metabolism in both humans and rats mediated by

Table 5
Total length (TL), relative mass after fixation (w), and Fulton's condition factor (FCF) of the common carp larvae during the 31-day embryo-larval toxicity test with verapamil. Values are expressed as the mean ± SD.

Sampling time		Control	Group 1	Group 2	Group 3	Group 4
			0.463 μg L^{-1}	4.63 μg L^{-1}	46.3 μg L^{-1}	463 μg L^{-1}
Day 10	TL (mm)	7.7 ± 0.3	7.5 ± 0.5	7.3 ± 0.4	7.5 ± 0.5	7.2 ± 0.3
	w (mg)	3.0 ± 0.4	2.7 ± 0.4	2.1 ± 0.5	2.8 ± 0.5	2.0 ± 0.4
	FCF	0.7 ± 0.1	0.7 ± 0.2	0.5 ± 0.1	0.9 ± 0.1	0.5 ± 0.1
Day 15	TL (mm)	8.6 ± 0.4	8.8 ± 0.5	8.8 ± 0.6	8.8 ± 0.5	8.8 ± 0.6
	w (mg)	4.2 ± 1.1	4.7 ± 0.8	4.7 ± 1.1	4.67 ± 1.2	5.0 ± 1.1
	FCF	0.7 ± 0.2	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Day 20	TL (mm)	10.1 ± 0.7	10.3 ± 0.6	10.0 ± 0.6	10.3 ± 1.0	10.2 ± 0.9
	w (mg)	8.6 ± 2.5	9.3 ± 2.2	8.7 ± 1.8	9.5 ± 2.7	8.9 ± 2.2
	FCF	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.2
Day 24	TL (mm)	11.8 ± 0.7	12.0 ± 0.8	12.4 ± 0.9	12.3 ± 0.8	12.35 ± 0.9
	w (mg)	20.0 ± 5.5	16.8 ± 4.9	20.7 ± 5.2	16.7 ± 4.4	19.1 ± 5.4
	FCF	1.2 ± 0.2	1.0 ± 0.3	1.1 ± 0.11	0.9 ± 0.10	1.0 ± 0.2
Day 29	TL (mm)	14.8 ± 1.47	14.5 ± 0.91	14.8 ± 1.0	14.5 ± 1.5	14.7 ± 1.5
	w (mg)	35.0 ± 12.0	39.4 ± 2.9	38.5 ± 9.3	38.7 ± 12.4	41.7 ± 12.5
	FCF	1.5 ± 0.1	1.4 ± 1.6	1.2 ± 0.2	1.2 ± 0.1	1.3 ± 0.2
Day 31	TL (mm)	16.7 ± 1.0	15.9 ± 1.1	16.2 ± 1.1	16.3 ± 1.7	15.9 ± 1.3
	w (mg)	56.7 ± 12.4	45.5 ± 10.6	53.4 ± 11.9	56.1 ± 21.5	54.0 ± 15.7
	FCF	1.2 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1

several cytochrome P450 isoenzymes whereas the main pathways are N-dealkylation, N-demethylation and O-demethylation (Reder-Hilz et al., 2004). However, the metabolism of verapamil in fish is not yet fully understood. On the one hand, in a study by Alderton et al. (2010), verapamil produced similar metabolic products in zebrafish larvae and humans, indicating similarities in their metabolism. On the other hand, Burkina et al. (2012) reported that in contrast to humans, long-term exposure of verapamil to rainbow trout had no significant effect on the activity of the selected CYP450 enzymes that are involved in the metabolism of verapamil and other pharmaceuticals in humans. It is possible that other biotransformation enzymes might be involved in fish; however, the biotransformation and/or elimination of verapamil were far slower in fish than in humans.

5. Conclusion

In summary, the effects caused by verapamil in fish can be considered similar to the therapeutic effects and side effects that are found in humans (reduction of heart rate, bradycardia, peripheral edema and gastrointestinal hemorrhage). These similarities in the mode of action can be explained by the high structural similarities of L-type calcium channels in fish and humans (Rottbauer et al., 2001).

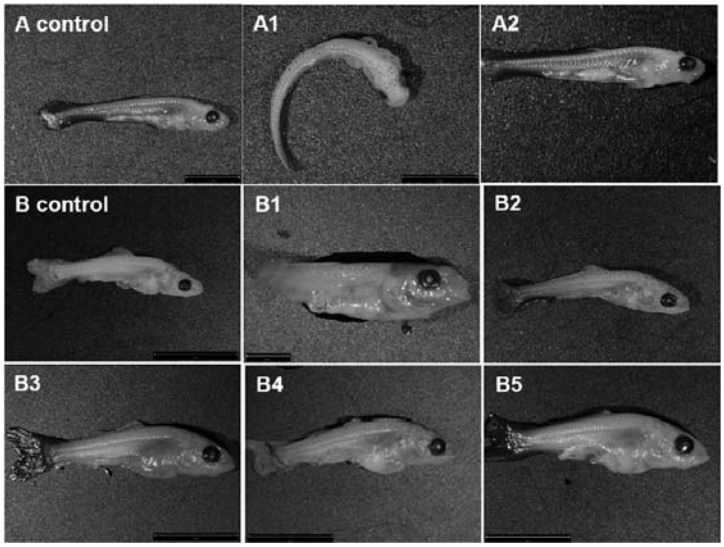

Fig. 3. Types of deformed larvae of common carp during the 31-day embryo-larval toxicity test with verapamil; A — normal larva (control, L4), A1 — axial curvature; kyphosis, underdeveloped eye (46.3 μg L^{-1}, L4), A2 — intestine edema (46.3 μg L^{-1}, L4), B — normal larva (control, L5), B1 — deformed skull (4.36 μg L^{-1}, L5), B2 — axial curvature; kyphosis (46.3 μg L^{-1}, L5), B3 — visceral edema with haematocele (46.3 μg L^{-1}, L5), B4 — coelom edema (463 μg L^{-1}, L5), B5 — intestine deformation; strangulation and dilatation (463 μg L^{-1}, L5).

Table 6
Developmental stages (steps, DS) of the larval period and occurrence of macroscopic morphological anomalies during the 31-day embryo-larval toxicity test on common carp. MA — sum of all malformations, EH — edema and hematocele. Asterisks indicate a significant difference compared to the control: * $p < 0.05$, ** $p < 0.01$ (χ^2 test).

Groups/Sampling Time		Day 10	Day 15	Day 20	Day 24	Day 29	Day 31	Σ
Control	DS	L3-L4	L4	L5	L5	L6	L6	–
	MA (%)	0	3.6	0	0	0	0	0.6
	EH (%)	0	0	0	7.7	0	0	1.3
Group 1	DS	L3-L4	L4	L5	L5	L6	L6	–
0.463 μg L^{-1}	MA (%)	7.1	0	3.4	3.2	0	0	2.4
	EH (%)	0	0	0	6.5	0	0	1.2
Group 2	DS	L3-L4	L4	L5	L5-L6	L6	L6	–
4.63 μg L^{-1}	MA (%)	0	0	0	0	3.5	0	0.6
	EH (%)	0	0	0	30.8*	0	0	4.7
Group 3	DS	L3-L4	L4	L2-L5	L5	L6	L6-J1	–
46.3 μg L^{-1}	MA (%)	7.4	11.1	3.7	3.7	0	0	4.2*
	EH (%)	3.7	0	0	48.1**	3.6	0	9.0*
Group 4	DS	L3-L4	L4	L4-L5	L4-L6	L5-L6	L6	–
463 μg L^{-1}	MA (%)	8	3.4	16.0*	4.2	3.3	0	5.6*
	EH (%)	0	3.4	0	37.5*	0	0	6.2*

Fish exposed sub-chronically to verapamil at a concentration of 4.63–463 μg L^{-1} appeared to bioconcentrate the pharmaceutical at a low level (BCF ranging between 6.6 and 16.6), but the half-life was relatively long (10.2 days) compared to that in humans, indicating the slow rate of biotransformation and/or elimination of verapamil in fish. Although we did not observe any effects of verapamil on studied endpoints at environmentally relevant concentration in the present study, we cannot exclude the possibility that it might affect fish after a longer exposure.

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CHAPTER 3

THE SUB-LETHAL EFFECTS AND TISSUE CONCENTRATION OF THE HUMAN PHARMACEUTICAL ATENOLOL IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

Steinbach, C., Burkina, V., Fedorova, G., Grabicova, K., Stara, A., Velisek, J., Zlabek, V., Schmidt-Posthaus, H., Grabic, R., Kocour Kroupova, H., 2014. The sub-lethal effects and tissue concentration of the human pharmaceutical atenolol in rainbow trout (*Oncorhynchus mykiss*). Science of the Total Environment 497–498, 209–218.

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The sub-lethal effects and tissue concentration of the human pharmaceutical atenolol in rainbow trout (*Oncorhynchus mykiss*)

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HIGHLIGHTS

- Atenolol affects the haematological and biochemical profile of trout.
- Atenolol has an effect on the vascular system of trout.
- Atenolol doesn't affect the activities of the studied CYP450 isoforms in the liver.
- Atenolol was found to have a very low bioconcentration factor (reaching 0.27).

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ABSTRACT

Atenolol is a highly prescribed anti-hypertensive pharmaceutical and a member of the group of β-blockers. It has been detected at concentrations ranging from ng L^{-1} to low μg L^{-1} in waste and surface waters. The present study aimed to assess the sub-lethal effects of atenolol on rainbow trout (*Oncorhynchus mykiss*) and to determine its tissue-specific bioconcentration. Juvenile rainbow trout were exposed for 21 and 42 days to three concentration levels of atenolol (1 μg L^{-1} — environmentally relevant concentration, 10 μg L^{-1}, and 1000 μg L^{-1}). The fish exposed to 1 μg L^{-1} atenolol exhibited a higher lactate content in the blood plasma and a reduced haemoglobin content compared with the control. The results show that exposure to atenolol at concentrations greater than or equal to 10 μg L^{-1} significantly reduces both the haematocrit value and the glucose concentration in the blood plasma. The activities of the studied antioxidant enzymes (catalase and superoxide dismutase) were not significantly affected by atenolol exposure, and only the highest tested concentration of atenolol significantly reduced the activity of glutathione reductase. The activities of selected CYP450 enzymes were not affected by atenolol exposure. The histological changes indicate that atenolol has an effect on the vascular system, as evidenced by the observed liver congestion and changes in the pericardium and myocardium. Atenolol was found to have a very low bioconcentration factor (the highest value found was 0.27). The bioconcentration levels followed the order liver > kidney > muscle. The concentration of atenolol in the blood plasma was below the limit of quantification (2.0 ng g^{-1}). The bioconcentration factors and the activities of selected CYP450 enzymes suggest that atenolol is not metabolised in the liver and may be excreted unchanged.

1. Introduction

Over the last few years, various pharmaceuticals have been widely detected in aquatic environments at concentrations ranging from ng L^{-1} to low μg L^{-1} (Fent et al., 2006). However, because pharmaceuticals are designed to be effective even at low doses, these low concentrations may affect aquatic organisms (Gunnarsson et al., 2008). In general, pharmaceuticals have the potential to affect non-target species because many drug targets are evolutionarily conserved across vertebrate species (Gunnarsson et al., 2008; Corcoran et al., 2010). This fact raises a concern regarding the long-term influence of pharmaceuticals on aquatic organisms (Fent et al., 2006).

Arterial hypertension is a highly prevalent chronic disease in humans. The estimated total number of adults with hypertension worldwide was 972 million in the year 2000 (Kearney et al., 2005). Therefore, it is not surprising that anti-hypertensive and cardiovascular drugs are one of the most commonly prescribed classes of human pharmaceuticals (Jjemba, 2008; Jones et al., 2002). For instance, in the United States, 601 million prescriptions of antihypertensive and

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cardiovascular pharmaceuticals were dispensed in the year 2005 (Jjemba, 2008). As far as atenolol is concerned, Jones et al. (2002) reported the prescription of 29 tons of this drug in England in the year 2000. In the Czech Republic, atenolol prescription for treatment of cardiovascular diseases was 2.7 tons in the year 2011 (The State Institute for Drug Control (Czech Republic); http://www.sukl.eu).

β-blockers are one of the classes of cardiovascular drugs that are used not only to treat arterial hypertonic diseases but also in the treatment of angina pectoris, heart failure, and glaucoma (Owen et al., 2007). As their name suggests, β-blockers block β-adrenergic receptors (β-ARs), which are mainly found in the heart, blood vessels, and the lungs in the human body and can be stimulated by binding with catecholamines (e.g., adrenaline and noradrenaline) (Frishman, 2003; Owen et al., 2007). For instance, β-AR stimulation leads to an increase in the heart rate, heart muscle contraction, blood pressure, and relaxation of smooth muscle in the bronchial tubes in the lungs (Frishman, 2003). The administration of β-blockers to patients blocks the access of catecholamines to their receptors, which results in reductions in the heart rate and blood pressure (Frishman, 2003; Prichard et al., 2001). Atenolol, which belongs to the β-blocker class of pharmaceuticals, is a selective antagonist of the β_1-AR that exhibits a greater binding affinity for the β_1 than for the β_2-AR (Mehvar and Brocks, 2001; Owen et al., 2007).

β-ARs are relatively conserved among vertebrates (Owen et al., 2007). For example, the transmembrane sequence of the rainbow trout β_1 and β_2-ARs share 57 and 63% identity with the corresponding mammalian β-ARs (Nickerson et al., 2001). β-ARs can be found in several fish organs (heart, gills, liver, brain, spleen, head kidney, and muscles) and cells (erythrocytes; Owen et al., 2007). Thus, the physiological processes regulated by β-ARs in fish, such as blood flow, blood pressure, homeostasis, and immunocompetence, may be affected by β-blockers (Nickerson et al., 2002; Owen et al., 2007, 2009; Massarsky et al., 2011).

Atenolol has been widely detected in concentrations ranging from ng L^{-1} up to μg L^{-1} in waste and surface waters. It can be traced in surface water since it is not fully eliminated in STPs, e.g. Vieno et al. (2007) and Golovko et al. (2014) reported for atenolol an elimination rate of 10–61% depending on season. For example, Maurer et al. (2007) reported an atenolol concentration of 1.54 μg L^{-1} in untreated wastewater. In effluent from SPT in Ceske Budejovice (Czech Republic), atenolol concentration was ranging between 0.39 and 1.69 μg L^{-1} during one year monitoring study (Golovko et al., 2014). Zuccato et al. (2006) found 0.17 μg L^{-1} atenolol in surface water. Vieno et al. (2006) and Daneshvar et al. (2010) reported atenolol in concentration of 0.01 to 0.11 μg L^{-1} in river water in Sweden and Finland, respectively. Moreover, Jones et al. (2002) predicted an atenolol concentration of 0.89 μg L^{-1} in English rivers. In addition, atenolol is expected to be present in aquatic environment because it is relatively stable to photolysis by sunlight (half-life = 77 to 730 h), its microbial biodegradation is slow (half-life = 340 to 2900 h), and it has a low affinity for absorption to sediments (Jones et al., 2002; Yamamoto et al., 2009; Küster et al., 2010).

At present, there is limited information available on the effect of atenolol on fish. Atenolol has been found to have low acute toxicity (96hLC_{50} > 100 mg L^{-1}) to medaka (*Oryzias latipes*; Kim et al., 2009). Hampel et al. (2010) reported that a short-term exposure (5 days) to atenolol (11 μg L^{-1}) alters the gene expression patterns in the brain of Atlantic salmon (*Salmo salar*). Winter et al. (2008), who conducted a short-term reproduction study (21 days) and early life stage test (28 days) on fathead minnow exposed to atenolol, found that atenolol affected the studied endpoints at high concentrations. At the concentration of 3.2 mg L^{-1} atenolol caused an increase in condition index of male fish only and it reduced the growth rate of fathead minnow larvae at 10 mg L^{-1} (Winter et al., 2008). In addition, a few in vitro studies have reported an effect of atenolol on fish hepatocytes and gonad cells (Ings et al., 2012; Fernandez et al., 2013). However, long-term exposure studies with environmentally relevant concentrations are lacking.

In mammals, pharmaceuticals are mainly metabolised by the cytochrome P450 (CYP450) superfamily, particularly the members of the CYP1–CYP4 families, which are predominantly expressed in the liver (Hasler et al., 1999). The hepatic CYP3A4, which contributes to the metabolism of 28% of the drugs in human, is one of the most important isoforms associated with this process (Lewis, 2004). CYP1A (EROD) is the most studied isoform in connection with the metabolism of various substances, mainly xenobiotics, in fish and is therefore widely used as a biomarker of environmental contamination (Uno et al., 2012). For example, propranolol, a member of the β-blocker group, induces the activity of CYP1A in rainbow trout primary hepatocytes (Laville et al., 2004). Furthermore, Corcoran et al. (2012) demonstrated that propranolol and other pharmaceuticals are able to induce the expression of CYP450 enzymes in carp primary hepatocytes through their interaction with pregnane X receptors (PXR). In contrast, atenolol does not induce the activity of CYP1A in rainbow trout gonad cells (Fernandez et al., 2013). *In vivo* studies on CYP450 enzyme activities in fish exposed to atenolol are missing.

During the insertion of oxygen into a substrate, a reaction that is catalysed by the CYP450 enzyme system, the uncoupling of the catalytic turnover from substrate oxidation can result in the production of superoxide or H_2O_2, which may lead to oxidative stress (Richard et al., 2008; Lawrence and Hemingway, 2003). Moreover, oxidative stress can be a response to general stress conditions (Lushchak, 2011). Exposure to pollutants is known to enhance the production of reactive oxygen species (ROS), leading to a deficiency in the antioxidant system and thereby resulting in an imbalance of the antioxidant enzyme activities (Morel and Barouki, 1999; van der Oost et al., 2003; Sieroslawska et al., 2012). The activity of antioxidant enzymes has also been used as a biomarker of oxidative stress due to pharmaceuticals, namely diazepam, clofibric acid, and clofibrate (Nunes et al., 2008). At present, only one *in vitro* study on rainbow trout gonad cells exposed to atenolol is available, and the results showed no effect of this substance on ROS production (Fernandez et al., 2013). Information on the antioxidant response of fish exposed to atenolol *in vivo* is missing.

Owen et al. (2007) suggested to study the histological changes in fish exposed to β-blockers to determine an organ-specific response; however, the effect of atenolol on the histological features of fish tissues, particularly the heart and liver, had not been assessed prior to the present study.

The objective of the present study was to assess the sub-chronic effects of the β-blocker atenolol at sub-lethal concentrations on juvenile rainbow trout (*Oncorhynchus mykiss*). Specifically, the present study aimed to 1) investigate the impact of atenolol exposure on the haematological and biochemical profiles of the blood plasma and the histology of the liver and heart, 2) measure the enzymatic activity of the enzymes involved in the detoxification and oxidative stress response in the liver and gills, and 3) analyse the tissue-specific bioconcentration of atenolol.

2. Materials and methods

2.1. Chemicals

Atenolol ((+)-4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy] benzeneacetamide, CAS No.: 56715-13-0, purity 99%), resorufin, 7-ethoxyresorufin (ER), 7-methoxyresorufin (MR), 7-pentoxyresorufin (PR), 7-benzyloxy-4-trifluoromethylcoumarin (BFC), nicotinamide adenine dinucleotide phosphate (NADPH), glutathione disulphide (GSSG), ethylenediaminetetraacetic acid (EDTA), reduced dipotassium salt of β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitrotetrazolium blue chloride (NBT), and 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIS) were obtained from Sigma-Aldrich (Steinheim, Germany). The slides used to determine the biochemical parameters of blood plasma were obtained from IDEXX Laboratories Inc. (Westbrook, ME, USA). LC–MS-grade methanol and acetonitrile (LiChrosolv Hypergrade) were purchased from Merck (Darmstadt,

Germany). Formic acid, which was used to acidify the mobile phases, was purchased from Labicom (Olomouc, Czech Republic). The Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea) was used for the preparation of ultrapure water. The internal standard trimethoprim (2,4-diamino-5-(3,4,5-trimethoxybenzyl) pyrimidine; CAS No.: 738-70-5, purity 99%) was obtained from Sigma-Aldrich (Steinheim, Germany).

2.2. Experimental design

Juvenile rainbow trout (*O. mykiss*; mean body length: 23.4 ± 1.3 cm, mean body weight: 145 ± 22 g) were obtained from a local commercial hatchery (Husinec, Czech Republic). Before the start of the experiment, the fish were acclimated for 14 days to the laboratory conditions. The animals were maintained in aquaria with 200 L of fresh water, and 20 fish were randomly distributed in each of eight aquaria. The aquaria were set up with continuous aeration, a constant temperature of 15.8 ± 1.2 °C, and a 12-h light/12-h dark photoperiod. The dissolved oxygen concentration and pH were measured before bath exchange on a daily basis. The dissolved oxygen concentration and pH were 8.3 ± 0.8 mg L^{-1} and 7.43 ± 0.31, respectively. During the acclimation and experimental periods, the fish were fed commercial fish food (Bio Mar, Denmark) at a ratio of 1% of body weight on a daily basis.

The experimental set up followed the OECD guideline 305 (OECD, 2012) with some modifications, as follows. The fish were exposed to atenolol at three different concentration levels under semi-static conditions for 42 days: 1 μg L^{-1} (environmentally relevant concentration), 10 μg L^{-1}, and 1000 μg L^{-1} (sub-lethal concentrations). In addition, a control group of fish maintained in atenolol-free water was included. All of the experimental treatments were conducted in duplicate. One hundred and fifty litres of the solution used for the atenolol treatments and control were refreshed daily with new atenolol-fortified or untreated water, respectively. The mortality during the experimental and acclimation periods was recorded. This study was performed in accordance with the principals of the EU-harmonised Animal Welfare Act of the Czech Republic.

2.3. Fish sampling

At the start of the experiment and after 21 and 42 days of exposure, eight fish from each group (including the replicate groups) were individually sampled. First, blood samples were collected from the *vena caudalis* and stabilised with 40 IU of sodium heparin per 1 mL of blood. Subsequently, the fish were euthanised in buffered ethyl 3-aminobenzoate methanesulfonic acid (MS 222®; Argent Chemical Laboratories, Redmont, WA, USA), and the spinal cord of the animals was severed. The total length and the body, liver, heart, and gonad weights were recorded. The condition factor (FCF), mean specific growth rate (SGR), hepatosomatic index (HSI), cardiosomatic index (CSI), and gonadosomatic index (GSI) were calculated as described by Machova et al. (2009), Velisek et al. (2010), and Powell et al. (2011). Furthermore, to determine the concentration of atenolol, samples of the blood plasma, kidney, liver, and muscle were collected and stored at −20 °C. To determine the enzymatic activity, the liver and gill tissues were sampled and stored at −80 °C. In addition, on the last sampling day (after 42 days of exposure), samples of the liver and heart from the fish exposed to the highest concentration (1000 μg L^{-1}) and the control group were collected and fixed in 10% buffered formalin for histopathological examination.

2.4. Analysis of atenolol in water and fish tissues

Water samples were collected from each aquarium immediately after the water was exchanged and 24 h after the exchange and stored at −18 °C. The extraction of the tissues was conducted in acetonitrile according to the method described by Grabicova et al. (2013) with some modifications. Briefly, samples of 0.5 g of tissue and 200 μL of plasma were used for the determination of the atenolol concentration and were spiked with 20 ng and 50 ng of the internal standard (trimethoprim), respectively. Matrix-matched standards were used for each matrix (tissues or water) to correct possible matrix effects, which can cause the differences from response factor derived from the calibration curve. The QA/QC samples were prepared and analysed to ensure the quality of the results. Blank samples were prepared with each series of processed samples. The accuracy and precision of the method were tested using duplicates and fortified samples: every tenth sample was duplicated, and every tenth sample of fish from the control group was spiked with the target compound at a concentration of 100 ng g^{-1}. The LOQs were based on peak area corresponding to the lowest calibration point fitting the criterion of response factor linearity. This value of peak area was set to the calculation formula for each sample taken into consideration IS peak area, sample amount, and response factor.

The atenolol in the water samples was analysed using on-line solid-phase extraction (SPE) liquid chromatography with tandem mass spectrometry for lower concentrations and direct injection for the highest concentration. The details of the on-line SPE of the MS/MS are described by Grabic et al. (2012) and Khan et al. (2012). The tissue extracts were analysed using the LC–MS/MS method (Grabic et al., 2012; Grabicova et al., 2013). The analyses were performed using TSQ Ultra MS/MS (Thermo Fisher Scientific, San Jose, CA, USA) coupled with Accela 1250 and Accela 600 HPLC pumps (Thermo Fisher Scientific) and a HTS-XT autosampler (CTC Analytics AG, Zwingen, Switzerland). A Cogent Bidentate column (50-mm length, 2.1-mm ID, and 4-μm particle size; MicroSolv Technology Corporation, NJ, USA) was used as the analytical column. A Hypersil Gold column (20 mm × 2.1-mm ID, 12-μm particles; Thermo Fisher Scientific) was used as the pre-concentration column for the on-line SPE. The LC–LC and LC gradients and MS/MS transitions are reported in Supplements 1, 2, and 3, respectively.

The bioconcentration factor (BCF) was calculated according to the OECD Guideline No. 305 (OECD, 2012). For each experimental group (and each tissue), the mean concentration of atenolol at each sampling time was divided by the mean concentration of atenolol in the water of the respective group.

2.5. Haematological and biochemical profile of the blood

The haematological profile of the blood, i.e., the erythrocyte count (RBC), haematocrit (Hc), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), leukocyte count (Leuko), and differential leukocyte count, was determined as described by Svobodova et al. (1991). For the biochemical analysis, the plasma was separated from the blood by centrifugation (10 min at 12,000 g) at 4 °C. Until analysis, the plasma samples were stored at −80 °C. The biochemical analysis of the blood plasma was conducted using a VETTEST 8008 analyser (IDEXX Laboratories Inc., USA). The following indices were measured: glucose (GLU), total protein (TP), triacylglycerols (TRIG), alanine aminotransferase (AMT), creatine kinase (CK), and lactate (LAC).

2.6. Analysis of enzyme activities in the liver and gills

2.6.1. Preparation of post-mitochondrial supernatant and microsomal fraction

The microsome fraction was prepared by differential centrifugation (Burkina et al., 2013) and used to measure the CYP450-mediated reactions. The analysis of the antioxidant parameters was performed using the post-mitochondrial supernatant of the liver tissue, as described by Stara et al. (2012).

2.6.2. Protein analysis

The protein levels were estimated spectrophotometrically through the method described by Smith et al. (1985) using bovine serum albumin as the standard. The microsomes were diluted to obtain a protein

concentration of 4 mg mL^{-1}, and the post-mitochondrial supernatant was diluted to obtain a protein concentration of 10 mg mL^{-1}.

2.6.3. Total CYP450 content

The total CYP450 content in the microsomes was determined from the sodium dithionite-reduced carbon monoxide difference spectrum with an extinction coefficient of 91 mM^{-1} cm^{-1} (Omura and Sato, 1964).

2.6.4. Microsomal enzyme activities

The ethoxyresorufin-O-deethylase (EROD), methoxyresorufin-O-demethylase (MROD), and pentoxyresorufin-O-depethylase (PROD) assays were performed based on the protocol described by Kennedy and Jones (1994). The 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylation (BFCOD) assay was conducted according to the method described by Renwick et al. (2000). The reaction incubations were performed as described by Grabicova et al. (2013).

The alkyl-resorufin reactions were assessed based on the rates of the transformations of ethoxyresorufin (ER), methoxyresorufin (MR), and pentoxyresorufin (PR) to resorufin, and the activity of BFCOD was estimated based on the rate of the transformation of 7-benzyloxy-4-trifluoromethylcoumarin (BFC) to 7-hydroxy-4-trifluoromethylcoumarin (HFC). An Infinite 200 spectrophotometer (TECAN) was used for the analysis of the concentrations of resorufin (excitation/emission: 544/590 nm) and HFC (excitation/emission: 410/538 nm). The enzymatic activities were expressed as pmol of resorufin or HFC produced per min and mg of microsomal proteins (the detection limits for resorufin and HFC were 2 and 1 pmol, respectively).

2.6.5. Activity of antioxidant enzymes

The total superoxide dismutase (SOD) activity was determined using the method developed by Ewing and Janero (1995) with slight modifications. The reaction mixtures in a final volume of 250 μL were added to each well of a 96-well plate. The activity determinations were made for the complete reaction system, which included 25 μL of post-mitochondrial supernatant, 200 μL of reaction solution (48 μM NBT and 80 μM NADPH), and 25 μL of PMS (3.5 μM) in phosphate buffer (50 mM with 0.1 mM EDTA, pH 7.4). This assay was conducted in triplicate for each separate sample. The SOD activity was assessed spectrophotometrically at 560 nm using NBT as the substrate and is expressed as the amount of enzyme per milligramme of protein.

The catalase (CAT) activity was determined using the method developed by Aebi (1984) with slight modifications. The CAT activity was determined by measuring the decrease in hydrogen peroxide in cuvettes. The post-mitochondrial supernatant was diluted 1:50 in phosphate buffer (50 mM with 1 mM EDTA, pH 7.0). Hydrogen peroxide was diluted to a concentration of 0.09% in TRIS buffer (50 mM with 0.1 mM EDTA, pH 7.2). To measure the enzymatic activity, 100 μL of the diluted post-mitochondrial supernatant was mixed with 500 μL of the diluted hydrogen peroxide. The decomposition of the substrate (H_2O_2) was recorded at 240 nm. The calculations were made using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

The glutathione reductase (GR) activity was determined using the method developed by Carlberg and Mannervik (1975) with some modifications. The reaction mixtures in a final volume of 200 μL were added to each well of a 96-well plate. The activity determinations were made for the complete reaction system, which consisted of 50 μL of the post-mitochondrial supernatant, 20 μL of GSSG (1 mM), 20 μL of NADPH (0.1 mM), 30 μL of cold distilled water, and 80 μL of phosphate buffer (0.1 mM with 0.5 mM EDTA, pH 7.0). The assay was performed in triplicate for each sample. The GR activity was assayed spectrophotometrically at 340 nm to measure the level of NADPH oxidation.

An Infinite 200 spectrophotometer (TECAN) was used to measure the absorbances in 96-well plates, and a Specord 210 spectrophotometer (Analytic Jena) was used for the measurement of the absorbance of solutions in cuvettes. The enzymatic activities were determined for three replicates and are expressed as nanomoles or micromoles of substrate hydrolysed per minute per mg of protein.

2.7. Histopathological analysis

Fixed samples of the liver and heart were paraffin-embedded and routinely processed for histological examination. Sections with a thickness of 3 μm were cut, stained with haematoxylin-eosin (H&E), and examined using a light microscope. The histopathological changes were graded as 0 (no), 1 (scattered), 2 (mild), 3 (mild to moderate), 4 (moderate), 5 (moderate to severe), or 6 (severe) (Schmidt-Posthaus et al., 2013; Bettge et al., 2009; Grabicova et al., 2013). The liver tissue was additionally stained with periodic acid-Schiff (PAS) to determine the glycogen content in hepatocytes.

2.8. Statistical analysis

The statistical software STATISTICA (version 8.0 for Windows, StatSoft, Czech Republic) was used to compare the differences between the tested groups, including the control group. The normality and homoscedasticity of the variance were checked by the Kolmogorov–Smirnov test and Bartlett's test, respectively. If the conditions of normal distribution and homogeneity were satisfied, one-way analysis of variance (ANOVA) was employed to determine whether there were significant differences in the measured variables between the experimental groups. Subsequently, Dunnett's multiple range test was applied. If the conditions for ANOVA were not satisfied, a nonparametric test (Kruskal–Wallis) was utilised. A p value of less than 0.05 was chosen as a valid significance level.

3. Results

3.1. Concentration of atenolol in water and fish tissues

For the on line SPE method and direct injection LC–MS/MS the limits of quantification (LOQs) of atenolol in water samples ranged from 0.0014 to 0.0021 μg L^{-1} and from 0.9 to 1.1 μg L^{-1}, respectively (footnotes of Table 1). On average the LOQs of atenolol in water were for on line SPE and direct injection LC–MS/MS 0.0017 μg L^{-1} and 0.98 μg L^{-1}, respectively. The limits of quantification (LOQs) of atenolol in fish tissues ranged from 0.34 to 4.3 ng g^{-1} with the average of 1.3 ng g^{-1}. The tissue specific LOQs are listed as footnotes of Table 2. The differences in the LOQs of atenolol in water and tissues were a result of the different methods of detection as well as different weights of the samples. The average recoveries (trueness of the method) of atenolol from fortified matrices were 95% (relative standard deviation (RSD) of ten replicates: 6%), 107% (RSD of four replicates: 13%) and 102% (RSD of ten replicates: 4%) in the fortified samples of fish tissues, blood plasma, and the water samples,

Table 1
Concentrations of atenolol in the water used in the sub-chronic toxicity test. The concentrations were measured immediately after the water exchange (0 h) and 24 h after the exchange. The values are expressed as the means ± S.D. (n = 6). LOQ = limit of quantification.

Group	Sample time (h)	Water concentration (μg L^{-1})	Difference to the nominal concentration (%)	Min–Max
Control	0	<LOQ		
	24	<LOQ		
1 μg L^{-1}	0	1.6 ± 0.3	60	1.3–2.1
	24	1.6 ± 0.4	60	1.2–2.3
10 μg L^{-1}	0	12.2 ± 1.9	22	8.7–14.2
	24	12.0 ± 1.6	20	9.5–13.4
1000 μg L^{-1}	0	1120 ± 93	12	1040–1240
	24	1070 ± 103	7	975–1218

On-line SPE LC–MS/MS, LOQ (1–10 μg L^{-1}) = 0.0014–0.0021 μg L^{-1}; Direct injection LC–MS/MS LOQ (1000 μg L^{-1}) = 0.9–1.1 μg L^{-1}.

Table 2
Concentrations of atenolol, number of atenolol positive samples and bioconcentration factors (BCFs) of atenolol in the blood plasma, liver, kidney, and muscle of rainbow trout sub-chronically exposed to atenolol. The values are expressed as the means ± S.D. (n = 8). LOQ = limit of quantification.

Tissue	Exposure time (days)	Control	1 μg L^{-1}	10 μg L^{-1}	1000 μg L^{-1}
Plasma	21	<LOQ (0/8)	<LOQ (0/8)	<LOQ (0/8)	<LOQ (0/8)
	42	<LOQ (0/8)	<LOQ (0/8)	<LOQ (0/8)	<LOQ (0/8)
Liver	21	<LOQ (0/8)	<LOQ (0/8)	<LOQ (0/8)	300 ± 260; (8/8); BCF = 0.27
	42	<LOQ (0/8)	<LOQ (0/8)	2.40 ± 0.39; (2/8); BCF = 0.20	230 ± 210; (8/8); BCF = 0.21
Kidney	21	<LOQ (0/8)	<LOQ (0/8)	<LOQ (0/8)	161 ± 220; (8/8); BCF = 0.15
	42	<LOQ (0/8)	<LOQ (0/8)	<LOQ (0/8)	110 ± 24; (8/8); BCF = 0.10
Muscle	21	<LOQ (0/8)	<LOQ (0/8)	<LOQ (0/8)	3.6 ± 1.4; (8/8); BCF = 0.003
	42	<LOQ (0/8)	<LOQ (0/8)	<LOQ (0/8)	2.3 ± 1.4; (8/8); BCF = 0.002

Tissue specific LOQ (average and range) of on-line SPE LC–MS/MS: plasma = 2.00 ng g^{-1}; (1.0–4.3 ng g^{-1}), liver = 1.2 ng g^{-1} (0.68–4.0 ng g^{-1}), kidney = 1.2 ng g^{-1} (0.70–2.0 ng g^{-1}), muscle = 0.89 ng g^{-1} (0.34–2.0 ng g^{-1}).

respectively. It was not possible to prepare fortified plasma samples due to the insufficient amount of these samples. Therefore, the average recovery of atenolol in blood plasma was assessed using samples collected from fish not involved in the experiment.

The concentration of atenolol in the water samples from the control group was below the LOQ. The starting atenolol concentration and the concentration of atenolol 24 h after exposure in the testing aquaria are reported as an average of three samplings within the experimental period (1st, 12th, and 25th days). The measured atenolol water concentrations with nominal values for all of the treatment groups are shown in Table 1.

The concentrations of atenolol in the control fish during the experimental period and in the fish before the start of the exposure were below the LOQ (Table 2). After 21 days of exposure, atenolol was detected only in the fish that were exposed to the highest tested concentration. After 42 days of exposure, atenolol was detected in the fish exposed to the intermediate and the highest tested concentrations. The bioconcentration factor (BCF) of atenolol in the different tissues ranged from 0.002 to 0.27 and is given in Table 2. The bioconcentration of atenolol followed the order liver > kidney > muscle. The atenolol concentrations in the liver and kidney were significantly higher compared to those in muscle. In the plasma, the detected concentrations were below the LOQ.

3.2. Survival and morphological parameters

During the acclimation and experimental periods, no mortality was detected in any of the treatment groups, including the control.

The atenolol-exposed fish were not significantly different in their total length, body weight, CF, HSI, GSI, and CSI compared with the control (Supplement 4). The SGRs calculated at the end of the exposure were 9.52, 8.39, 9.45, and 9.31 in the control group and in the fish exposed to 1, 10, and 1000 μg L^{-1} atenolol, respectively.

3.3. Haematological and biochemical parameters of the blood

The haemoglobin concentration was significantly reduced in the fish exposed to atenolol for 21 days compared with the control. However, the haemoglobin concentration was not additionally affected by continued exposure to atenolol. The haematocrit value was significantly reduced in fish exposed to atenolol at concentrations of 10 and 1000 μg L^{-1} for 21 days compared with the control (Table 3). After 42 days, the haematocrit value was significant reduced in fish exposed to 10 μg L^{-1} atenolol compared with the control (Table 4). The remaining haematological indices (erythrocyte and leucocyte counts, MCV, MCHC, and differential leucocyte count) did not differ significantly between the groups (Table 3 and Supplement 5).

Table 3
Haematological parameters in rainbow trout sub-chronically exposed to atenolol. Hc — haematocrit, Hb — haemoglobin, WBC — white blood cell (leukocyte) count, MCV — mean corpuscular volume, MHC — mean corpuscular haemoglobin, MCHC — mean corpuscular haemoglobin concentration. The values are expressed as the means ± S.D. (n = 8). The asterisks indicate a significant difference compared with the control: *p < 0.05 (ANOVA).

Indices	Exposure time (d)	Control	1 μg L^{-1}	10 μg L^{-1}	1000 μg L^{-1}
Hc (%)	21	44.4 ± 7.3	39.3 ± 4.2	33.3 ± 4.5*	35.9 ± 7.9*
	42	43.8 ± 6.3	40.4 ± 6.7	33.3 ± 5.9*	40.3 ± 4.3
Hb (g L^{-1})	21	82.1 ± 14.6	63.5 ± 5.9*	55.8 ± 5.8*	66.5 ± 13.1*
	42	84.9 ± 15.1	82.8 ± 11.9	72.8 ± 15.1	81.5 ± 8.8
Erythrocytes (T L^{-1})	21	1.2 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.2
	42	1.2 ± 0.2	1.1 ± 0.2	1.0 ± 0.1	1.2 ± 0.2
WBC (g L^{-1})	21	51.5 ± 12.1	68.5 ± 27.6	65.8 ± 38.4	59.5 ± 9.2
	42	53.7 ± 17.7	53.7 ± 9.9	64.4 ± 22.71	59.8 ± 29.2
MCV (fl)	21	378 ± 57	388 ± 58	371 ± 71	369 ± 54
	42	381 ± 79	390 ± 68	370 ± 95	428 ± 105
MCH (pg)	21	69.5 ± 18.0	62.6 ± 8.9	62.1 ± 12.0	68.4 ± 9.0
	42	71.3 ± 6.4	74.4 ± 11.3	71.9 ± 9.5	71.5 ± 7.7
MCHC (g L^{-1})	21	188 ± 29	162 ± 8	168 ± 13	186 ± 10
	42	198 ± 41	206 ± 20	218 ± 14	203 ± 19

After 21 days, all of the atenolol-exposed fish exhibited a significantly enhanced lactate concentration in the blood plasma compared with the control. After 42 days, only the fish exposed to the highest tested concentration of atenolol (1000 μg L^{-1}) presented a significantly enhanced lactate concentration compared with the control (Table 4). The concentration of glucose was significantly lower in fish exposed to 10 μg L^{-1} atenolol for 21 days compared with the control (Table 4). The concentrations of total protein and triacylglycerols and the activities of aminotransferase and creatine kinase were not affected by atenolol exposure, regardless of the duration (Table 4).

3.4. Activity of selected CYP450 enzymes in the liver

The catalytic activity of EROD (CYP1A1/2), MROD (CYP1A1/2), PROD (CYP2B), and BFCOD (CYP3A) and the total CYP450 content were not significantly affected by atenolol exposure (Supplement 6).

Table 4
Biochemical parameters of the blood plasma of rainbow trout sub-chronically exposed to atenolol. GLU — glucose, TP — total protein, TRIG — triacylglycerols, AMT — aminotransferase, CK — creatine kinase, LAC — lactate. The values are expressed as the means ± S.D. (n = 8). The asterisks indicate a significant difference compared with the control: *p < 0.05 (ANOVA).

Indices	Exposure time (d)	Control	1 μg L^{-1}	10 μg L^{-1}	1000 μg L^{-1}
GLU	21	4.0 ± 0.3	4.2 ± 0.5	3.4 ± 1.1*	4.5 ± 0.5
(mmol L^{-1})	42	4.1 ± 0.9	3.9 ± 0.7	3.7 ± 0.7	3.4 ± 0.8
LAC	21	0.4 ± 0.3	0.6 ± 0.2*	0.7 ± 0.1*	0.7 ± 0.3*
(mmol L^{-1})	42	0.61 ± 0.1	0.7 ± 0.1	0.9 ± 0.4	1.6 ± 0.6*
TP	21	35.4 ± 4.94	36.0 ± 3.6	37.7 ± 6.3	28.9 ± 13.3
(g L^{-1})	42	44.6 ± 6.1	43.0 ± 5.0	37.1 ± 5.6	40.9 ± 3.6
TRIG	21	0.9 ± 0.6	0.7 ± 0.2	0.7 ± 0.1	0.7 ± 0.3
(mol L^{-1})	42	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.7 ± 0.2
AMT	21	4.9 ± 3.0	5.3 ± 3.2	3.8 ± 2.5	2.5 ± 2.1
(μkat L^{-1})	42	11.8 ± 4.0	10.3 ± 7.4	7.7 ± 3.9	10.0 ± 5.6
CK	21	831 ± 524	379 ± 408	555 ± 557	487 ± 360
(μkat L^{-1})	42	1113 ± 604	1537 ± 313	1060 ± 305	1815 ± 1219

Table 5
Activity of antioxidant enzymes in the liver of rainbow trout sub-chronically exposed to atenolol. SOD — superoxide dismutase, CAT — catalase, GR — glutathione reductase. The values are expressed as the means ± S.D. (n = 8). The asterisks indicate a significant difference compared with the control: *p < 0.05 (ANOVA).

Indices	Exposure time (d)	Control	1 μg L^{-1}	10 μg L^{-1}	1000 μg L^{-1}
CAT	21	0.04 ± 0.02	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
μmol (min^{-1} mg^{-1})	42	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	0.03 ± 0.01
GR	21	0.57 ± 0.21	0.63 ± 0.20	0.59 ± 0.14	0.47 ± 0.10
nmol (min^{-1} mg^{-1})	42	0.60 ± 0.09	0.45 ± 0.09	0.49 ± 0.16	0.29 ± 0.19*
SOD	21	0.24 ± 0.18	0.24 ± 0.13	0.28 ± 0.21	0.28 ± 0.07
nmol (min^{-1} mg^{-1})	42	0.09 ± 0.05	0.10 ± 0.05	0.13 ± 0.08	0.09 ± 0.03

3.5. Activity of antioxidant enzymes

In the liver, the activities of CAT and SOD in the atenolol-exposed fish at any of the sampling times were not significantly different compared with those of the control group (Table 5). The activity of GR was significantly reduced only with the highest tested concentration of atenolol after 42 days of exposure compared with the control (Table 5).

In the gills, the activities of CAT, SOD, and GR were not significantly changed in fish exposed to atenolol compared with the control (Supplement 7).

3.6. Histological changes

In the liver, the control and atenolol-exposed fish showed varying amounts of fat vacuoles in hepatocytes, and the cytoplasm showed mild to moderate granulation. The glycogen content in hepatocytes was not affected by treatment with atenolol (data not shown). In contrast to the controls, the fish exposed to the highest tested concentration of atenolol (1000 μg L^{-1}) displayed mild to severe sinusoidal congestion (Fig. 1 and Supplement 8). The analysis of the heart of atenolol-exposed fish showed that their pericardium exhibited more pronounced signs of inflammation compared with the control (Fig. 2 and Supplement 8). In addition, ranges of scattered to moderate histopathological alterations were observed in the heart of atenolol-exposed fish, e.g., edematous pericardial fibrous tissue, infiltration in the sub-endocardial space, and myocardial edema (Fig. 2 and Supplement 8). One of the atenolol-exposed trout presented multiple vessel walls that were thickened and edematous, and its endothelial cells were occasionally disrupted. The same animal exhibited obvious sub-endocardial infiltration of inflammatory cells in the atrium. The histopathological changes are summarised in Supplement 8. Most of the histopathological alterations caused by atenolol ranged from mild to moderate. Hence, no severe histopathological alterations in the heart and liver were expected in response to exposure to the lower tested concentrations of atenolol. Therefore, the fish exposed to the lower tested concentrations were not examined.

4. Discussion

4.1. Mortality and morphological parameters

The present study showed that long-term exposure to atenolol at a concentration in the range of 1 to 1000 μg L^{-1} did not cause mortality in rainbow trout. This finding is in line with the very low acute toxicity of atenolol (96hLC_{50} > 100 mg L^{-1}) that was found for medaka (*Oryzias latipes*, Kim et al., 2009). The present study revealed that atenolol has no effect on the studied morphological parameters (length, weight, FCF, HSI, and CSI) and no effect on fish fitness and growth. In an embryo–larval study (28 day) on fathead minnow (*Pimephales promelas*), atenolol reduced the growth rate of the exposed larvae; however, the concentration of atenolol used was 10-fold higher than the highest concentration used in the present study (LOEC growth = 10 mg L^{-1}, Winter et al., 2008).

4.2. Bioconcentration in fish

Due to the low lipophilicity (log P = 0.16; EPI Suite™ v4.11 — US EPA, 2012) atenolol is expected to be present mainly in the water phase. Consequently the uptake via the gills in fish can be hypothesised with only negligible contribution of uptake via food (Owen et al., 2007). For the same reason, atenolol is not expected to accumulate to a high extent in fish bodies. In accordance with this assumption, the bioconcentration factors (BCFs) of atenolol in different rainbow trout tissues calculated in the present study were very low, ranging from 0 to 0.27. To the best of our knowledge, this study provides the first demonstration of the tissue-specific bioconcentration of atenolol in fish. In western mosquitofish (*Gambusia affinis*) that were exposed for 96 h to atenolol at concentrations of 100 and 1000 μg L^{-1}, the BCFs in the fish homogenates were estimated to be 0.13 and 0.08, respectively (Valdes

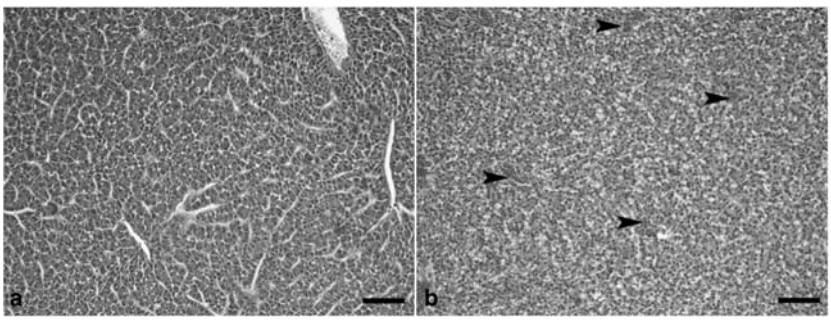

Fig. 1. Histopathological findings in the liver of rainbow trout: a — control fish with normal liver structure; b — fish exposed to 1000 μg L^{-1} atenolol. The exposed fish present small blood vessels and congested sinusoids (arrowheads), as determined through H&E staining; scale bar = 50 μm.

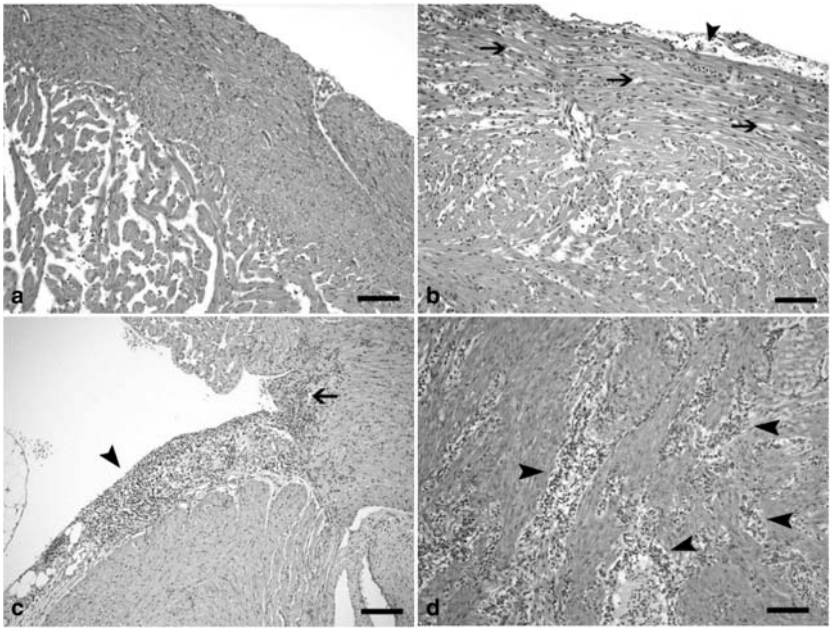

Fig. 2. Histopathological findings in the heart of rainbow trout: a — control fish with normal heart structure; b to d — fish exposed to 1000 μg L^{-1} atenolol. b — pericardial edema (arrowhead) and myocardial edema (arrows), c — subepicardial infiltration with lymphocytes, plasma cells, and macrophages (arrowhead), which multifocally infiltrate into the myocardial tissue (arrow), d — subendocardial infiltration with lymphocytes, plasma cells, and macrophages (arrowheads). The findings are shown through H&E staining; scale bars = 50 μm (a–c) and 25 μm (d).

et al., 2014). These values are comparable to those calculated for rainbow trout tissues in the present study. Furthermore, the atenolol concentration did not reach the LOQ (1.3 ng mL^{-1}) even in the blood plasma of rainbow trout exposed to an atenolol concentration as high as 1000 μg L^{-1}. Winter et al. (2008) detected an atenolol concentration of 48.6 μg L^{-1} in a pooled plasma sample of adult male fathead minnow exposed to atenolol at a concentration of 1000 μg L^{-1}. This value is close to the predicted value presented by Fick et al. (2010), who calculated that atenolol would reach the human therapeutic blood plasma level of 100 μg L^{-1} (Schulz and Schmoldt, 2003) in fish if the animals were exposed to an atenolol concentration of 792.3 μg L^{-1}. However, similarly to the juvenile rainbow trout analysed in the present study, the plasma concentration in adult females of fathead minnow exposed to the same concentration of atenolol did not reach the LOQ (0.5 ng mL^{-1}) (Winter et al. (2008). The differences in the atenolol concentration in the blood plasma among fish of different sexes and ages may be a result of their different elimination rates of atenolol. For instance, Parks and LeBlanc (1998), who exposed fathead minnow to testosterone, discovered that adult females eliminate some testosterone metabolites at a significantly greater rate than males and that juvenile fish eliminate nearly all testosterone metabolites at greater weight-normalized rates than the adults. Similarly, Wiegand et al. (2000) observed that the activity of enzymes involved in detoxification and excretion had higher activity in juvenile compared to adult fish.

In addition, at the pH level used in our experimental protocol (pH 8.3), atenolol can be expected to be present mainly in its protonated positively charged form (pKa = 9.6). Because charged substances are much less membrane-permeable than uncharged ones (Owen et al., 2009; http://pubchem.ncbi.nlm, 2013), this finding may explain why the blood plasma concentration of atenolol in rainbow trout was below the LOQ and why the BCFs in the tissues were lower than the predicted value for the whole fish.

4.3. Haematological and biochemical profiles of the blood plasma

In response to pollutants, the haematological and biochemical profiles of the blood of fish can provide valuable information regarding their internal environment (Velisek et al., 2010). In the present study, atenolol (at the environmentally relevant concentration) induced haematological and biochemical changes in the blood of rainbow trout. The present study verified that changes within the haeme pathway are sensitive biomarkers of pollutants at their environmental concentrations, as was also demonstrated for, e.g., cadmium (van der Oost et al., 2003).

The changes in the haematocrit value and haemoglobin concentration of rainbow trout in response to atenolol observed in the present study may indicate that this substance affects the β-ARs in the organs associated with erythropoiesis, i.e., head kidney and spleen. The presence of β_1-ARs in these organs has been documented in salmonid fish (Owen et al., 2007). However, we cannot rule out the possibility that these changes are part of a general stress response. In the present study, as a consequence of the reduction in the haemoglobin concentration caused by atenolol, the oxygen supply may have been reduced, which may have led to the observed increase in the lactate concentration.

Furthermore, in the present study, a decrease was observed in the glucose concentration in fish exposed to atenolol, indicating an effect of this substance on the carbohydrate and energy metabolisms. Similarly, in the brain of Atlantic salmon (*Salmo salar*) exposed to 11 μg L^{-1} atenolol for five days, Hampel et al. (2010) observed changes in the mRNA expression of genes related to the carbohydrate and energy metabolisms. For example, phosphoenolpyruvate carboxykinase, which is a key

enzyme in gluconeogenesis, presented the most reduced mRNA level. In rainbow trout hepatocytes, Ings et al. (2012) reported that atenolol (2.7 ng L^{-1}) abolishes the epinephrine-induced glucose production. Additionally, van Den Thillart et al. (2001) reported that atenolol reduces the glucose content in the blood plasma of isoproterenol (non-selective β-agonist)-treated common carp. The reduction in the glucose concentration in fish exposed to β-blockers may be a consequence of the blockage of β-ARs, which are then not accessible to catecholamines (e.g., noradrenaline and adrenaline), because catecholamines lead to increases in the blood plasma glucose content (Evans, 1997).

4.4. Activity of enzymes in the liver and gills

Atenolol did not cause any changes in the total CYP450 content and the activities of the selected isoforms of CYP450 enzymes in the liver, which indicates that it is not detoxified/metabolised but most likely directly eliminated in rainbow trout. Similarly, in the human body, atenolol is only partly metabolised (10%) and is mainly excreted unchanged via the kidney (Reeves et al., 1978; Wander et al., 2009). Although atenolol is not significantly metabolised in the human body, pharmaceuticals with log Kow values as low as that of atenolol are substrates for CYP450 enzymes (Lewis, 2000). Therefore, to accurately describe the metabolism of atenolol in fish, further studies on the presence of atenolol metabolites in tissues and excretes are required.

The activities of the antioxidant enzymes SOD and CAT were also not affected by atenolol exposure. In contrast, the activity of another antioxidant enzyme, GR, was decreased in the liver of fish exposed to the highest tested concentration of atenolol for 42 days. GR maintains the redox balance of glutathione, an antioxidant that prevents damage to cells caused by reactive oxygen species, by catalysing the reduction of glutathione disulphide to glutathione (van der Oost et al., 2003; Richard et al., 2008). Therefore, the inhibition of GR activity can cause a decrease in the content of glutathione (Lushchak, 2011; Srikanth et al., 2013). In accordance, atenolol reduces the glutathione content in human blood plasma (Komala et al., 2013). Oxidative stress occurs when the redox homoeostasis within cells is altered by a deficiency of the antioxidant system (Komala et al., 2013). Therefore, fish exposed to atenolol at a concentration of 1000 μg L^{-1} may be at higher risk of oxidative stress.

4.5. Histological changes

The results of the present study demonstrate an absence of structural changes in hepatocytes and no effect on the glycogen level in atenolol-exposed fish. This finding demonstrates that atenolol does not appear to be hepatotoxic for rainbow trout. Similarly, atenolol was not found to have a cytotoxic effect on rat primary hepatocytes (Bandyopadhyay et al., 1990). In humans, β-blockers are also not expected to be hepatotoxic, and in fact, only a very few cases of hepatotoxicity caused by atenolol have been reported (Boillot and Scoazec, 2009). These results further support the hypothesis that, similarly to humans (Flockhart and Tanus-Santos, 2002), atenolol is mainly excreted by the kidney with no obvious metabolism in the liver in fish.

However, the results of the present study show an effect of atenolol on the cardiovascular system of exposed fish. Namely, atenolol causes degenerative changes in the blood vessels of the pericardium and an inflammatory reaction in the pericardium and subendocardium. In line with our findings, an increased incidence of atrial degeneration of the heart has been described in rats fed a very high dosage of atenolol (300 mg atenolol/kg/day) (RxList, 2013). Fish exposed to atenolol also show congestion of the sinusoids (sinusoidal blood vessels), indicating circulatory changes (Henrion, 2012). Because the liver of rainbow trout is rich in β_2-ARs (Owen et al., 2007), it can be assumed that atenolol blocks the β_2-ARs and thereby causes vasodilation. The congestion of the sinusoids may be a consequence of this effect. The congestion may also be related to a reduced oxygen supply due to the observed reduction in the haemoglobin concentration. In this case, the congestion may be regarded as a compensational reaction due to hypoxia in the tissues (Henrion, 2012).

5. Conclusions

In conclusion, the bioconcentration of atenolol in rainbow trout exposed to sub-lethal levels (1–1000 μg L^{-1}) of atenolol is very low: the blood plasma concentration was found to be below the LOQ (2.0 ng g^{-1}), and the maximum BCF in the tissues was found to be 0.27. Furthermore, the absence of structural changes in the hepatocytes, the lack of an effect on the glycogen level, and the unchanged activity of the CYP450 enzymes in the liver indicate that atenolol is not metabolised but rather directly excreted by fish. Similarly, in humans, atenolol is only insignificantly metabolised in the liver and is excreted mainly unchanged by the kidney (Flockhart and Tanus-Santos, 2002).

Contrary to the read-across concept (Huggett et al., 2003; Rand-Weaver et al., 2013), which is based on the hypothesis that a pharmaceutical affects fish if the plasma concentration in the fish is similar to the human therapeutic plasma concentration, atenolol did affect the exposed fish in the present study. At the environmentally relevant concentration, atenolol exposure leads to a decrease in the haemoglobin concentration and an increase in the lactate content of blood plasma, suggesting a reduction in the oxygen supply. Some effects caused by atenolol, mainly at the higher concentrations tested, in fish can be considered to be similar to those observed in humans, particularly the effects on the vascular system of the liver and heart. These changes may be mediated by β-ARs because β-ARs in fish and humans are supposed to have high structural similarities (as demonstrated, e.g., for the β_2-AR, Nickerson et al., 2001; Owen et al., 2009). Taken together, the data demonstrate that atenolol exhibits potential for the induction of sublethal effects on non-target organisms, such as fish, in aquatic environments.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2014.07.111.

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CHAPTER 4

BIOCONCENTRATION, METABOLISM, AND SUB-LETHAL EFFECTS OF THE HUMAN THERAPEUTIC DRUG DILTIAZEM ON RAINBOW TROUT *ONCORHYNCHUS MYKISS*

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BIOCONCENTRATION, METABOLISM, AND SUB-LETHAL EFFECTS OF THE HUMAN THERAPEUTIC DRUG DILTIAZEM ON RAINBOW TROUT *ONCORHYNCHUS MYKISS*

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ABSTRACT

The objective of the present study was to assess the effect of sub-chronic exposure to the human therapeutic drug diltiazem on rainbow trout Oncorhynchus mykiss. Juvenile trout were exposed for 21 and 42 days to three nominal concentrations of diltiazem: 0.03 μg L^{-1} (environmentally relevant concentration), 3 μg L^{-1}, and 30 μg L^{-1} (sub-lethal concentrations). At the highest tested concentration, the haematocrit value was significantly elevated. The number of mature neutrophilic granulocytes was significantly increased in fish exposed to 3 μg L^{-1} and 30 μg L^{-1} diltiazem. Antioxidant enzyme activity was affected in liver and gill of fish exposed to all tested concentrations of diltiazem. Diltiazem exposure at 30 μg L^{-1} inhibited hepatic CYP3A activity. The highest concentration was associated with histological changes in heart, liver, and kidney. These alterations can be attributed to the effects of diltiazem on the cardiovascular system, similar to those observed in the human body, as well as to its metabolism. The bioconcentration factor (BCF) of diltiazem was relatively low (0.5–194) in analysed tissues, following the order kidney > liver > muscle > blood plasma. The half-life of diltiazem in liver, kidney, and muscle was 1.5 h, 6.2 h, and 49 h, respectively. The metabolization rate of diltiazem in liver, kidney, muscle, and blood plasma was estimated to be 85 ± 9%, 64 ± 14%, 46 ± 6%, and 41 ± 8%, respectively. Eight diltiazem metabolites were detected. The presence of desmethyl diltiazem (M1), desacetyl diltiazem (M2), and desacetyl desmethyl diltiazem (M3) suggests that rainbow trout metabolize diltiazem via desmethylation, desacetylation, similar to mammals. In addition, diltiazem undergoes hydroxylation in fish. At the environmentally relevant concentration, diltiazem was found to induce alterations in the blood, gill, and liver of fish, indicating its potential for adverse effects on non-target organisms in the aquatic environment.

Keywords: *Calcium channel blocker; CYP3A; haematology; histopathological changes; metabolites; half-life; bioconcentration factor*

Highlights

- Diltiazem at concentrations of 3 and 30 μg L^{-1} affected the haematological and blood biochemical profile of rainbow trout.
- Diltiazem affected antioxidant enzyme activity in liver and gill of rainbow trout at an environmentally relevant concentration (0.03 μg L^{-1}).
- Histological changes observed at the highest concentration of diltiazem indicated effects on the trout vascular system.
- Exposure to diltiazem at 30 μg L^{-1} inhibited CYP3A activity in liver.
- Diltiazem was detected in kidney of fish exposed to the highest concentration (30 μg L^{-1}), with bioconcentration factor reaching 194.
- The half-life of diltiazem followed the order muscle > kidney > liver.
- Diltiazem metabolites detected in trout were similar to those reported in mammals.

1. Introduction

Many pharmaceuticals are not fully degraded in sewage treatment plants (STP), thus they are being continually released into the aquatic environment where have been increasingly detected in recent years. This is alarming since pharmaceuticals are designed to have a biological effect and to be relatively persistent in order to avoid inactivation before accomplishing the intended therapeutic effect (Corcoran et al., 2010). Moreover, as with other harmful xenobiotics, they are able to cross the cell membrane and interact with evolutionarily-conserved targets (Corcoran et al., 2010; Gunnarsson et al., 2008; Sanderson et al., 2004a; Sanderson et al., 2004b).

Calcium channel blockers (CCB) inhibit the movement of calcium ions across the cell membrane by blocking the L-type calcium channel (Lüllmann et al., 2002). In humans, L-type calcium channels are found chiefly in the heart, aorta, and viscera. The CCB binding to calcium channels leads to relaxation in the affected tissue. Calcium channel blockers are prescribed to reduce heart rate and force of contraction. They also cause vasodilatation, leading to reduction in blood pressure (Grossman and Messerli, 2004; Lüllmann et al., 2002).

Diltiazem is a CCB with widespread use in the treatment of angina pectoris, hypertension, and prevention of migraine (Acheson et al., 2002, Chaffman, 1985). Its major therapeutic effect is lowering of blood pressure, primarily through vasodilatation leading to a reduction in peripheral blood vessel resistance (Grossman and Messerli, 2004; Gunnarsson et al., 2008). The metabolism of diltiazem in mammals is well documented (Homsy et al., 1995; Molden et al., 2000; Molden et al., 2002a). Approximately 75% of the drug is metabolized in the mammalian liver (Homsy et al., 1995) by enzymes of the cytochrome p 450 (CYP) family, with CYP3A4 being the most important metabolizing enzyme, and CYP2D6, CYP2C8, and CYP2C9 contributing to a lesser extent (Molden et al., 2000; Molden et al., 2002a; Molden et al., 2002b; Fradette and du Souich, 2004; Connors et al., 2013). Briefly, the major pathways of diltiazem metabolism are desmethylization and deacetylization (Homsy et al., 1995; Molden et al., 2000a). The desmethylation of diltiazem is carried out by CYP450 enzymes, whereas the deacetylation is suggested to be the action of esterases (Molden et al., 2000; Molden et al., 2000a; Zhao et al., 2007; Molina et al., 2007). The main metabolites of diltiazem in mammals are desmethyl diltiazem and deacetyl diltiazem (Murata et al. 1993).

In 2011, diltiazem was one of the most prescribed CCBs in the Czech Republic (2.1 tonnes; The State Institute for Drug Control (Czech Republic); http://www.sukl.eu). Diltiazem is not fully degraded in STPs. Golovko et al. (2014) and Khan and Ongerth (2004) reported elimination rates of 47–62%. In effluent of the STP in Ceske Budejovice (Czech Republic), diltiazem was

reported at concentrations ranging from 0.007–0.030 μg L^{-1} (Golovko et al., 2014). In surface waters, diltiazem has been detected at concentrations of 0.02–0.106 μg L^{-1} (Kasprzyk-Hordern et al., 2009; Kolpin et al., 2004). Jones et al. (2002) predicted a concentration of 0.67 μg L^{-1} in English rivers. Since diltiazem is a moderately hydrophobic compound (K_{ow} =2.7), it is bioconcentrated to a certain extent in fish tissue. Maximum concentrations in muscle and liver of wild fish have been reported as 0.2 μg Kg^{-1} and 0.9 μg Kg^{-1}, respectively (Ramirez et al., 2009; Wang and Gardinali, 2013). Wang and Gardinali (2013) demonstrated a half-life of 117 h and a bioconcentration factor (BCF) of 16 in whole homogenates of the eastern mosquito fish *Gambusia holbrooki* exposed to diltiazem at 0.14 μg L^{-1}.

At present there is limited available information with respect to the biological effects of diltiazem in fish. Diltiazem has very low acute toxicity. The 48 h LC50 values for medaka *Oryzias latipes* have been reported as high as 25.6 mg L^{-1} (Kim et al., 2007). Klein et al. (2009) reported an increase of nucleotide and ATP levels in blood plasma of zebrafish *Danio rerio* injected with 1.3 mg diltiazem. Connors et al. (2013), in an *in vitro* study of biotransformation of diltiazem at 414.5 μg L^{-1} by the microsomal S9 fraction in hepatocytes of rainbow trout *Oncorhynchus mykiss*, found that it was not metabolized by the enzymes CYP1A, uridine diphosphate–glucuronosyltransferase, or glutathione S-transferase (GST). No information on metabolites of diltiazem in fish is currently available.

Exposure to xenobiotics often leads to oxidative stress (Lushchak, 2011), which can result in the inhibition of antioxidant enzymes, depletion of radical scavengers, lipid peroxidation, and, eventually, in damage to cell membranes (Kelly et al., 1998). Information on calcium channel blockers as a cause of oxidative stress in fish is limited to that of Li et al. (2010), who reported on oxidative stress in rainbow trout exposed to the CCB verapamil for 42 days.

The goal of the present study was to assess effects of sub-lethal diltiazem concentrations in juvenile rainbow trout. Objectives were to (1) investigate the impact of diltiazem exposure on the haematological and biochemical profile of blood plasma and the histology of liver, kidney, brain, and heart; (2) measure activity of enzymes involved in the oxidative stress response in liver and gill; (3) analyse the tissue-specific bioconcentration and half-life of diltiazem; and (4) evaluate activity of CYP enzymes and concentration of diltiazem metabolites in blood plasma and other tissues.

2. Materials and methods

2.1. Chemicals

Diltiazem [(2S,3S)-5-(2-dimethylaminoethyl)-2-(4-methoxyphenyl)-4-oxo-2,3-dihydro-1,5-benzothiazepin-3-yl acetate, CAS no.: 42399-41-7, purity 98 %]; resorufin; 7-ethoxyresorufin; 7-benzyloxy-4-trifluoromethylcoumarin (BFC); nicotinamide adenine dinucleotide (NADH); nicotinamide adenine dinucleotide phosphate (NADPH); glutathione disulfide (GSSG); tris(hydroxymethyl)aminomethane (TRIS); diethylenetriaminepentaacetic acid (DTPA); 1-chloro-2,4-dinitrobenzene (CDNB); reduced glutathione (GSH); and phenazine methosulfate were obtained from Sigma-Aldrich (Steinheim, Germany). The LC-MS grade acetonitrile and methanol (LiChrosolv Hypergrade) were obtained from Merck (Darmstadt, Germany). The internal standard verapamil hydrochloride [(±)-2-isopropyl-2,8-bis(3,4-dimethoxyphenyl)-6-methyl-6-azaoctannitril hydrochloride, CAS no.: 52-53-9] was obtained from Sigma Aldrich (Steinheim, Germany). The slides used to determine the biochemical blood plasma parameters were purchased from IDEXX Laboratories (Westbrook Inc., USA). Formic acid used to acidify the mobile phases was purchased from Labicom (Olomouc, Czech Republic). The Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea) was used for ultrapure water preparation.

2.2. Experimental design

Juvenile rainbow trout (mean body length: 23.1 ± 1.5 cm, mean weight: 164 ± 28 g) were obtained from a local commercial hatchery (Husinec, Czech Republic). The fish were acclimated to laboratory conditions for 14 days before beginning the experiment. Twenty fish were randomly placed into each of eight aquaria containing 200 L of fresh water with continuous aeration. The temperature was constant at 15.4 ± 1.4 °C. Photoperiod was 12:12 h light:dark. Dissolved oxygen concentration and pH were 8.5 ± 0.5 mg L^{-1} and 7.7 ± 0.5, respectively. During the acclimation and experimental periods, the fish were fed a commercial preparation (Bio Mar, Denmark) at 1% of body weight per day.

The experimental design followed the OECD guideline 305 (OECD, 2012) with some modifications. The fish were exposed to diltiazem at three concentration levels under semi-static conditions for 42 days: 0.03 μg L^{-1} (environmentally relevant concentration), 3 μg L^{-1}, and 30 μg L^{-1} (sub-lethal concentrations). A control group of fish was maintained in diltiazem-free water. All experimental and control trials were duplicated and carried out with de-chlorinated tap water. One-hundred-fifty L of the diltiazem solution and the water in control tanks were renewed daily. Mortality was recorded during the acclimation and experimental periods. This study was performed in accordance with the principals of the EU-harmonized Animal Welfare Act of the Czech Republic. After 42 days exposure, 8 fish exposed to 30 μg L^{-1} diltiazem were transferred into diltiazem-free water for depuration. At 7 and 14 days of depuration, 4 fish were sampled to assess levels of diltiazem and its metabolites.

2.3. Fish sampling

Prior to the start of the experiment (at the end of the acclimatisation period), and at 21 and 42 days exposure, eight fish from each replicate of each group were sampled. Blood samples were collected from the *vena caudalis* and stabilized with 40 IU sodium heparin per 1 mL blood. Subsequently, the fish were euthanized in 2-phenoxyethanol (Sigma-Aldrich, Czech Republic), and the spinal cord was severed. The total length and body, liver, heart, and gonad weights were recorded. Fulton's condition factor (FCF), mean specific growth rate (SGR), hepatosomatic index (HSI), cardiosomatic index (CSI), and gonadosomatic index (GSI) were calculated as described by Máchová et al. (2009), Velíšek et al. (2012), and Nash et al. (2004). Samples of blood plasma, kidney, liver, and muscle were collected and stored at -20 °C for determination of diltiazem concentration. To determine enzyme activity, liver and gill samples were collected and stored at -80 °C. After 42 days of exposure, samples of liver, brain, heart, and kidney of the fish exposed to the highest concentration (30 μg L^{-1}), and of the control group, were fixed in 10% buffered formalin for histological examination (2.7). For determining levels of diltiazem and its metabolites (2.4) four fish were sampled at 7 and 14 days depuration.

2.4. Determination of diltiazem and its metabolites

2.4.1. Determination of diltiazem concentration in water, tissues, and blood plasma

Water samples were collected from each aquarium immediately after, and 24 h after, water exchange. In addition, water samples from de-chlorinated tap water were taken. Concentration of diltiazem was determined by in-line-SPE-liquid chromatography with tandem mass spectrometry (LC/LC/MS/MS, Fedorova et al., 2014a). The analyses were performed using a TSQ Ultra MS/MS (Thermo Fisher Scientific, San Jose, CA, USA) coupled to Accela

1250 and Accela 600 HPLC pumps (Thermo Fisher Scientific, San Jose, CA, USA) and an HTS-XT autosampler (CTC Analytics AG, Zwingen, Switzerland).

The concentration of diltiazem was determined in kidney, liver, muscle, and blood plasma. Extraction was conducted in acetonitrile according to a modified method of Fedorova et al. (2014b). In addition, a blank sample and matrix control was conducted for method verification and detection of sample contamination. Samples of tissue (0.5 g) and plasma (200 μL) were used to determine diltiazem levels and were spiked with the internal standard verapamil at 20 ng and 45 ng, respectively.

To detect the formation of metabolites as an indication of photo-degradation and oxidation products, two aquaria were filled with 200 L water at the highest concentration of diltiazem (30 $\mu g\ L^{-1}$). Testing was conducted under the same conditions as described for the experiment, at the beginning of the trial and after 24 h. No fish were involved the stability test.

The BCF of diltiazem in liver, muscle, and blood plasma was calculated in accordance with OECD Guideline No. 305 (OECD, 2012). At each sampling time, the mean concentration of diltiazem in each tissue type from each experimental group, was divided by the mean concentration in water from which the fish was taken. First-order kinetics was assumed to determine the depuration rate of diltiazem in liver, muscle, and kidney. For exposed fish, the half-life (50% depuration, t50) and 95% loss in the depuration phase (t95) of diltiazem were calculated using linear regression of the natural logarithm (ln) of the detected concentrations in liver, kidney, and muscle and the value of the slope (k) of the graph: (t50) = 0.693/k and (t95) = 3.0/k (OECD, 2012).

2.4.2. Determination of diltiazem metabolites in tissues and blood plasma

The rate of metabolism of diltiazem was calculated based on its concentration expressed as μmol. Metabolites of diltiazem in fish tissue and blood plasma were analysed using a hybrid quadrupole-orbital trap mass spectrometer Q-Exactive (Thermo Fisher Scientific) coupled to an Accela 1250 LC pump (Thermo Fisher Scientific) and HTS XT-CTC autosampler (CTC Analytics AG) using direct injection of the samples. Parent compounds and metabolites were chromatographically separated using a Hypersil GOLD C18 column (50 mm x 2.1 mm ID x 3 μm particles, Thermo Fisher Scientific). All compounds matched in the 5 ppm range from exact mass were confirmed in High Resolution Product Scan mode, in which the parent ion was isolated and fragmented in the collision cell, and product spectrum was collected at 17500 FWHM resolution. Some metabolites are not commercially available, so semi-quantification was based on assumption that all metabolites have the same response at ESI+ ionization mode as diltiazem.

2.5. Haematological and biochemical blood profiles

The haematological profile, comprising erythrocyte count (RBC), haematocrit (Hc), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), leukocyte count (Leuko), and differential leukocyte count was determined as described in Svobodova et al. (1991). For biochemical analysis, the plasma was obtained by centrifugation for 10 min at 12000×g at 4 °C. Plasma samples were stored at -80 °C until analysis. The biochemical analysis of plasma was carried out with a VETTEST 8008 analyser (IDEXX Laboratories Inc., USA). The following indices were measured: glucose (GLU), triacylglycerol (TAG), γ-glutamyltranspeptidase (GGT), creatine kinase (CK), lactate (LAC), and calcium (Ca). The total protein (TP) was spectrophotometrically measured according to Smith et al. (1985).

2.6. Analysis of enzyme activity in liver and gill

2.6.1. Preparation of post-mitochondrial supernatant and microsome fraction

The protocol used to obtain the post-mitochondrial supernatant (PMS) was described by Howcroft et al. (2009). Briefly, samples were homogenized in 1.5 mL potassium-phosphate buffer (0.1 M, pH 7.4) using an ultrasonic homogenizer (Bandelin electronic D-12207; Berlin, Germany). From the homogenate, 100 μL were separated for determination of lipid peroxidation. The remaining tissue homogenate (1400 μL) was centrifuged at 10000×g for 20 min at 4 °C to isolate the PMS. The microsome fraction was prepared using differential centrifugation as described by Burkina et al. (2013).

2.6.2. Protein analysis

The protein concentration in the PMS was determined according to Smith et al. (1985) at 562 nm using bovine serum albumin as a standard. The samples of liver and gill tissue were diluted to a protein content of 6 mg mL^{-1} and 4 mg mL^{-1}, respectively.

2.6.3. Microsome enzyme activity of selected isoforms of CYP_{450}

The ethoxyresorufin-*O*-deethylase (EROD) assay was performed as described in Kennedy and Jones (1994). The 7-benzyloxy-4-trifluoromethylcoumarin *O*-debenzylation (BFCOD) assay was conducted according to the method of Renwick et al. (2000). The reaction incubations were described in Grabicova et al. (2013).

The rates of the transformation of ethoxyresorufin to resorufin and the activity of BFCOD were estimated from the rate of transformation of 7-benzyloxy-4-trifluoromethylcoumarin (BFC) to 7-hydroxy-4-trifluoromethylcoumarin (HFC). An Infinite 200 spectrophotometer (TECAN) was used for determination of the concentrations of resorufin (excitation/emission: 544/590 nm) and 7-benzyloxyresorufin (excitation/emission: 410/538 nm). The enzyme activity was expressed as pmol of resorufin or HFC formed per min and mg of microsomal proteins. The detection limits of resorufin and HFC were 2 and 1 pmol, respectively.

2.6.4. Antioxidant enzyme activity and lipid peroxidation

The catalase (CAT) activity was measured by at 240 nm by monitoring the decrease of absorbance due to degradation of hydrogen peroxide (H_2O_2) to $2H_2O$ and O_2, as described by as described by Clairborne (1985). The measurement was carried out by TECAN in a 96-well flat-bottom UV-transparent microtitre plate.

We mixed 15 μL PMS with protein content diluted 1:10 with 185 μL potassium phosphate (0.05 M, pH 7.0) and 100 μL H_2O_2 (0.03 M). The decomposition of the substrate H_2O_2 was recorded at 240 nm. Calculations were made using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

Glutathione reductase (GR) activity was determined using the method of Cribb et al. (1989) with some modifications. Approximately 0.2 mg mL^{-1} PMS was combined with 150 μL of reaction solution. This reaction solution consisted of NADPH (0.4 mM), GSSG (2 mM), and DTPA (1 mM) in 50 mL potassium phosphate buffer (0.05 M, pH 7.0). The enzyme activity was quantified by measuring the disappearance of NADPH at 340 nm. Calculations were made using the molar extinction coefficient of 6220 $M^{-1}cm^{-1}$.

The glutathione peroxidase (GPx) activity was measured by the method of Mohandas et al. (1984). The incubation mixture (0.3 mL) contained PMS (approximately 0.06 mg mL^{-1}), potassium phosphate buffer (0.05 M, pH 7.0), EDTA (1 mM), sodium azide (1 mM) and GR (7.5 mL from stock with 1 U/ml). GSH (4 mM) and NADPH (0.8 mM) were added as substrate to the solution. The reaction was initiated by the addition of 0.5 mM of H_2O_2. Oxidation of NADPH was recorded at 340 nm. Calculations were made using the molar extinction coefficient of 6220 M^{-1} cm^{-1}.

The GST activity was determined using 1-chloro-2,4-dinitrobenzene as substrate according to the method of Habig et al. (1974) adapted to the microplate reader by Frasco and Guilhermino (2002). The incubation mixture (0.3 mL) contained PMS with protein content diluted 1:10 with a reaction solution containing 2.55 mL of CDNB (10 mM), 15.30 mL of GSH (10 mM), and 84.5 mL of potassium phosphate buffer (0.1 M, pH 6.5). The CDNB conjugate was measured as increase in absorbance at 340 nm. Calculations were made using the molar extinction coefficient of 9.6 mM^{-1} cm^{-1}.

The total superoxide dismutase (SOD) activity was determined using the method developed by Ewing and Janero (1995) with slight modifications. The reaction mixtures at a final volume of 250 μL were added to each well of a 96-well plate. The activity was determined for the complete reaction system, which included 25 μL of PMS, 200 μL of reaction solution (48 μM nitro blue tetrazolium and 80 μM NADPH), and 25 μL of phenazine methosulphate (3.5 μM) in phosphate buffer (50 mM with 0.1 mM EDTA, pH 7.4). All assays were conducted in triplicate. The SOD activity was assessed spectrophotometrically at 560 nm using NBT as the substrate and is expressed as the amount of enzyme per mg of protein.

An Infinite M200 spectrophotometer (TECAN, Mannedorf, Switzerland) was used for reading absorbance of the incubation reactions for all assays. For each sample all assays were conducted in triplicate. Samples were processed on ice, with analysis made at 25 ºC. The relationship between absorbance and time in each reaction well was linear (R2 > 0.8). Enzyme activity was expressed as units of substrate hydrolyzed per minute, per mg of protein.

In gill and liver, the oxidative damage was assessed by determining the level of lipid peroxidation following the methodology of Ohkawa et al. (1979) with slight modifications. Briefly, 100 μL of homogenate was mixed with 333 μL of cold trichloroacetic acid (12%), 200 μL of 60 mM Tris-HCl with 0.1 mM DTPA, and 333 μL 2-thiobarbituric acid (0.73%). Tubes were heated for 1 h at 99 ºC. Following centrifugation at 3000×g for 5 min at 25 ºC, the absorbance of the supernatant was determined at 535 nm. Calculations were made using the molar extinction coefficient: ε = 156000 M^{-1} cm^{-1}. Oxidative damage was expressed as micromols of TBARS per g wet weight of tissue.

2.7. Histological analysis

Fixed samples of liver, heart, kidney, and brain were paraffin-embedded and routinely processed for histological examination. Sections (4 μm) were stained with haematoxylin-eosin (H&E) and examined by light microscopy. Pathological changes were graded as 0 (none), 1 (scattered), 2 (mild), 3 (mild to moderate), 4 (moderate), 5 (moderate to severe), or 6 (severe) (Bettge et al., 2009; Schmidt-Posthaus et al., 2013). In addition, liver tissue was stained with periodic acid-Schiff (PAS) in order to detect glycogen droplets in hepatocytes.

2.8. Statistical analysis

Data were analyzed using STATISTICA (version 8.0 for Windows, StatSoft, Czech Republic). Data were checked for normality and homoscedasticity by the Kolmogorov–Smirnov test and Bartlett's test, respectively. If these criteria were fulfilled, a one-way analysis of variance (ANOVA) was employed to detect significant differences among the experimental groups in the measured variables. Subsequently, Dunnett's multiple range test was applied. If the conditions for ANOVA were not satisfied, the nonparametric test (Kruskal–Wallis) was used. As significance level $p < 0.05$ was applied. The data were presented as mean ± standard deviation (SD).

Table 1. *Concentration of diltiazem in water in the sub-chronic toxicity test on rainbow trout. The concentrations were measured immediately after water exchange (0 h) and 24 h post-exchange. The values are expressed as mean ± S.D. (n = 4). LOQ = limit of quantification.*

Group	Sample time (h)	Water concentration (μg L^{-1})	Difference to the nominal concentration (%)	Min – Max
Tap water		< LOQ		
Control	0	< LOQ		
	24	< LOQ		
0.03 μg L^{-1}	0	0.06 ± 0.02	188	0.01–0.22
	24	0.04 ± 0.02	145	0.02–0.08
3 μg L^{-1}	0	2.8 ± 1.3	92	2.2–3.1
	24	3.1 ± 1.9	103	2.0–5.2
30 μg L^{-1}	0	34 ± 34	115	5.6–98
	24	21 ± 12	67	4.1–37

On-line SPE LC–MS/MS, LOQ = 0.009–0.017 μg L^{-1}.

Table 2. *Concentration and bioconcentration factor (BCF) of diltiazem in blood plasma, liver, kidney, and muscle of rainbow trout sub-chronically exposed to diltiazem (0.03, 3, and 30 μg L^{-1}) for 21 and 42 days. Diltiazem concentrations are given as μg kg^{-1} (mean ± S.D., n = 8), and BCF is in parenthesis. LOQ = limit of quantification.*

Tissue	Exposure time (d)	Control	0.03 μg L^{-1}	3 μg L^{-1}	30 μg L^{-1}
Plasma	0	≤ LOQ			
	21	≤ LOQ	≤ LOQ	≤ LOQ	12.2 ± 4.1; (0.46)
	42	≤ LOQ	≤ LOQ	≤ LOQ	26.3 ± 5.2; (0.98)
Liver	0	≤ LOQ			
	21	≤ LOQ	3.4; (69)	110 ± 59; (37)	809 ± 228; (30)
	42	≤ LOQ	≤ LOQ	71 ± 34; (23)	564 ± 539; (21)
Kidney	0	≤ LOQ			
	21	≤ LOQ	6.4 ± 2.2; (128)	290 ± 174; (89)	2388 ± 1387; (84)
	42	≤ LOQ	9.7 ± 4.2; (194)	261 ± 87; (88)	2397 ± 1854; (90)
Muscle	0	≤ LOQ			
	21	≤ LOQ	≤ LOQ	4.8 ± 1.6; (1.6)	43.1 ± 12.2; (1.6)
	42	≤ LOQ	≤ LOQ	1.8 ± 0.3; (0.7)	15.8 ± 8.6; (0.6)

3. Results

3.1. Diltiazem content in water and fish tissues

3.1.1. Analytical method performance

In the water samples, the limit of quantification (LOQ) of diltiazem in the ranged 0.010–0.017 μg L^{-1}. The LOQ in tissue was in the range 0.04–33 ng g^{-1} (Supplement 1). The recovery rates in plasma, muscle, liver, and kidney as well as in experimental solutions were obtained from clean water samples with 126 ± 29%, 93 ± 6%, 110 ± 19%, 144 ± 6% and 95 ± 10%, respectively.

The concentration of diltiazem in the de-chlorinated tap water, in control fish during the trial, and in fish prior to exposure, was below the LOQ (Tab. 1, 2). The concentration of diltiazem in water over the course of 24 h as average of the four sampling dates compared to the nominal value for all test groups is presented in Tab. 1. The average water concentration in treatments with 0.03, 3.0, and 30 μg L^{-1} diltiazem were 0.05, 2.95, and 26.72 μg L^{-1}, respectively. Calculation of BCF was based on these concentrations.

3.1.2. Concentration of diltiazem in tissues

Diltiazem was detected in the blood plasma of fish exposed to 30 μg L^{-1} diltiazem for 42 days and found to exhibit a low BCF, reaching 0.98. Diltiazem was detected in muscle of fish exposed to 3 and 30 μg L^{-1} diltiazem for 21 and 42 days. In muscle, BCF ranged from 0.59 to 1.9. After 21 and 42 days of exposure diltiazem was present in liver of fish exposed to 3 and 30 μg L^{-1} diltiazem, with a BCF ranging from 21 to 69. In kidney of all fish exposed to diltiazem, BCFs in ranging from 46 to 194 were calculated. In general, the bioconcentration levels of diltiazem followed the order kidney > liver > muscle > plasma (Tab. 2). The half-life of diltiazem in liver, kidney, and muscle was 1.5 h, 6.2 h, and 49 h, respectively. The 95% depuration time in liver, kidney, and muscle was estimated to be 6.5 h, 27.5 h, and 214 h, respectively. The depuration time of diltiazem followed the order muscle > kidney > liver. Diltiazem was present in the blood plasma at higher concentrations during the depuration phase than seen during exposure; hence, in plasma, no depuration of diltiazem was observed.

3.1.3. Diltiazem metabolites

Nine diltiazem related compounds (including diltiazem) were detected. The positions of biotransformation (*O* or *N*–metabolites) is difficult to identify using LC-HRMS/HRPS as there is one highly prevailing fragmentation path always leading to the same product; therefore the specific isoforms of the diltiazem metabolites are not presented in this manuscript. No metabolite was found during 24 h in the spiked water without fish. The biotransformation of diltiazem in fish includes desmethylation, desacetylation, and hydroxylation (Fig. 1), with the first two pathways being the most important. The major metabolite detected was desmethyl diltiazem (M1), followed by desacetyl diltiazem (M2), and desacetyl, desmethyl diltiazem (M3). Lesser amounts of desacetyl, didesmethyl diltiazem (M4) and didesmethyl diltiazem (M5) were detected. Hydroxylation represents only a minor pathway in diltiazem metabolization. The following metabolites were detected at lower levels: hydroxy diltiazem (M6); desmethyl, hydroxy diltiazem (M7) and desacetyl, hydroxy diltiazem (M8). The combined concentration

level of all detected metabolites followed the order kidney > liver > muscle > plasma (Supplement 2–5). In fish exposed to 0.03 μg L^{-1} diltiazem, the major metabolites M1, M2, and M3 were detected only in kidney. At this concentration, no diltiazem metabolites were found in other tissue. The low or very low level of metabolites (M9, M5, M11, M4) were found in fish exposed to 3 and 30 μg L^{-1} diltiazem. Higher levels of metabolites were found after 42 days exposure than after 21 days of exposure.

In fish exposed to diltiazem at 30 μg L^{-1}, the uptake phase was followed by a depuration phase. Compared to the uptake phase, during the depuration phase (7 and 14 days post-exposure) higher levels of minor metabolites were detected in all tissues with the exception of muscle (Supplement 2–5).

The estimated metabolism rate of diltiazem followed the order liver > kidney > muscle > plasma at 85 ± 9%, 64 ± 14%, 46 ± 6% and 41 ± 8%, respectively.

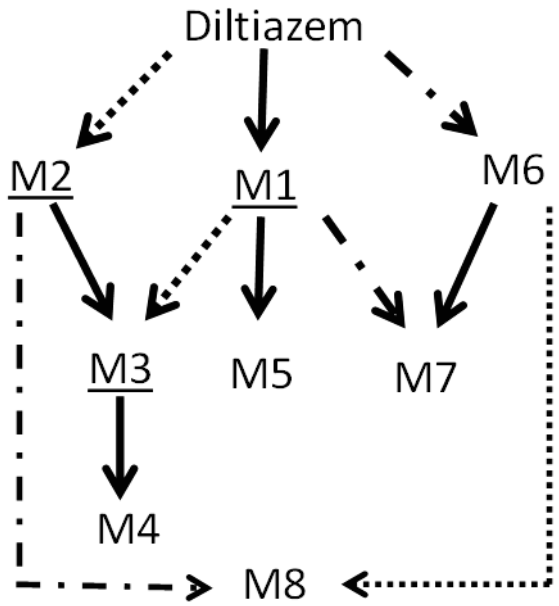

Figure 1. *Metabolic pattern of diltiazem in rainbow trout. Solid line – desmethylation; dotted line – desacetylation; dash dotted line – hydroxylation. Major metabolites are underlined: M1 – desmethyl diltiazem; M2 – desacetyl diltiazem; M3 – desacetyl, desmethyl diltiazem; M4 – desacetyl, didesmethyl diltiazem; M5 – didesmethyl diltiazem; M6 – hydroxy diltiazem; M7 – desmethyl, hydroxy diltiazem; M8 – desacetyl, hydroxy diltiazem (modified according to Molden et al., 2000).*

3.2. Survival and morphological parameters

No mortality occurred during the acclimation and experimental periods in either control or exposed fish. The diltiazem exposed fish did not show significant differences from controls in total length, body weight, CF, HSI, or CSI (Supplement 6). During 42 days exposure, the SGRs of the control group and the fish exposed to 0.03, 3, and 30 μg L^{-1} diltiazem were 9.82, 9.57, 9.84, and 9.40, respectively.

3.3. Haematological and blood biochemical parameters

The haematocrit value was significantly enhanced in fish exposed to 30 μg L^{-1} diltiazem for 21 days compared to the control group (ANOVA, $p > 0.05$, Tab. 3). The HC, RBC, WBC, MCV, MCHC, and MHC, as well as the differential leukocyte count (all indices, except mature neutrophil granulocytes) were not significantly affected by diltiazem exposure in any other treatment group compared to controls (Supplement 7 and Supplement 8). Fish exposed to diltiazem at 3 and 30 μg L^{-1} for 42 days showed significantly increased numbers of mature neutrophil granulocytes compared with the control group (ANOVA, $p > 0.05$, Supplement 8).

At all sampling times, GLU, GGT, TRIG, and Ca concentration was not significantly different in the exposed fish compared to controls (Tab. 3). The LAC concentration was significantly reduced in fish exposed to 3 and 30 μg L^{-1} diltiazem for 21 days than in the control group. However, after 42 days exposure, no significant difference was found in fish exposed to 3 and 30 μg L^{-1} diltiazem compared to the control. The TP content was significantly reduced in fish exposed at 3 and 30 μg L^{-1} for 21 days compared with the control group (ANOVA, $p < 0.05$). After 42 days, a significantly enhanced concentration of CK was detected in all diltiazem exposed fish compared with the control.

Table 3. *Biochemical parameters of blood plasma of rainbow trout sub-chronically exposed to diltiazem. GLU – glucose; GGT – gamma-glutamyltranspeptidase; TP – total protein; TRIG – triacylglycerols; CK – creatine kinase; Ca – calcium; LAC – lactate. Values are expressed as mean ± S.D. (n = 8). Asterisks indicate significant difference compared to the control: * p < 0.05 (ANOVA).*

Indices	Exposure time (d)	Control	0.03 μg L^{-1}	3 μg L^{-1}	30 μg L^{-1}
GLU	0	6.1 ± 0.7			
(mmol L^{-1})	21	5.8 ± 0.8	5.6 ± 0.9	4.8 ± 0.8	5.2 ± 1.3
	42	5.9 ± 0.7	6.1 ± 1.0	6.4 ± 1.5	5.4 ± 0.8
TP	0	22.7 ± 4.2			
(g L^{-1})	21	23.3 ± 2.4	20.7 ± 5.8	18.3 ± 4.9*	16.9 ± 2.2*
	42	20.8 ± 3.9	20.1 ± 5.0	24.3 ± 8.0	19.8 ± 7.0
TRIG	0	1.8 ± 0.4			
(mol L^{-1})	21	1.7 ± 0.4	1.7 ± 0.5	1.7 ± 0.3	1.8 ± 0.7
	42	2.0 ± 0.3	1.8 ± 0.5	1.9 ± 0.7	2.0 ± 0.5
GGT	0	0.4 ± 0.1			
(μkat L^{-1})	21	0.31 ± 0.1	0.30 ± 0.1	0.38 ± 0.1	0.39 ± 0.1
	42	0.2 ± 0.1	0.1 ± 0.04	0.1 ± 0.03	0.1 ± 0.01
Ca	0	2.4 ± 0.2			
(mmol L^{-1})	21	2.4 ± 0.1	2.4 ± 0.1	2.2 ± 0.2	2.2 ± 0.1
	42	2.3 ± 0.1	2.1 ± 0.2	2.5 ± 0.2	2.4 ± 0.1
CK	0	768 ± 49			
(μkat L^{-1})	21	767 ± 46	747 ± 41	757 ± 55	762 ± 57
	42	863 ±168	2167 ± 1420*	3055 ± 873*	2224 ± 1053*
LAC	0	1.4 ± 0.8			
(mmol L^{-1})	21	2.9 ± 1.9	3.3 ± 1.4	1.1 ± 0.9*	0.9 ± 0.2*
	42	1.5 ± 0.5	1.6 ± 1.0	1.4 ± 0.4	2.6 ± 1.4

3.4. Activity of selected CYP450s in liver

The catalytic activity of CYP1A1 in the fish hepatic microsome was determined by measuring EROD activity. Constitutive EROD activity in fish exposed to diltiazem was not significantly different from that of the control (Supplement 9).

Significant differences were observed in CYP3A activity, showing a reduction in fish exposed to 30 μg L^{-1} diltiazem for 21 days compared to control (ANOVA, $p < 0.05$, Supplement 9). At 42-days exposure, no significant difference in CYP3A activity among groups was found.

3.5. Antioxidant enzyme activity and lipid peroxidation

3.5.1. Liver

The activity of all measured antioxidant enzymes, except GR and GST, in liver was affected by diltiazem exposure (Fig. 2). The CAT activity was significantly induced in fish exposed to 0.03 and 3 μg L^{-1} diltiazem for 21 days compared with the control (ANOVA, $p < 0.05$). However, CAT activity was not significantly different from the control in those groups after 42-days exposure (ANOVA, $P > 0.05$, Fig. 2). After 21 days, SOD activity was significantly reduced in fish exposed to 0.03 and 3 μg L^{-1} diltiazem compared with the control group (ANOVA, $p < 0.05$). Fish exposed to these concentrations for 42 days exhibited significantly induced SOD activity compared to the control. After 21 days, the activity of GPx was significantly higher in all diltiazem-exposed fish than in controls, but, after 42 days exposure, GPx activity was significantly higher only in fish exposed to 0.03 and 3 μgL^{-1} diltiazem. After 42 days, fish exposed to 3 and 30 μg L^{-1} diltiazem exhibited significantly lower TBARS concentration than observed in control (ANOVA, $p > 0.05$).

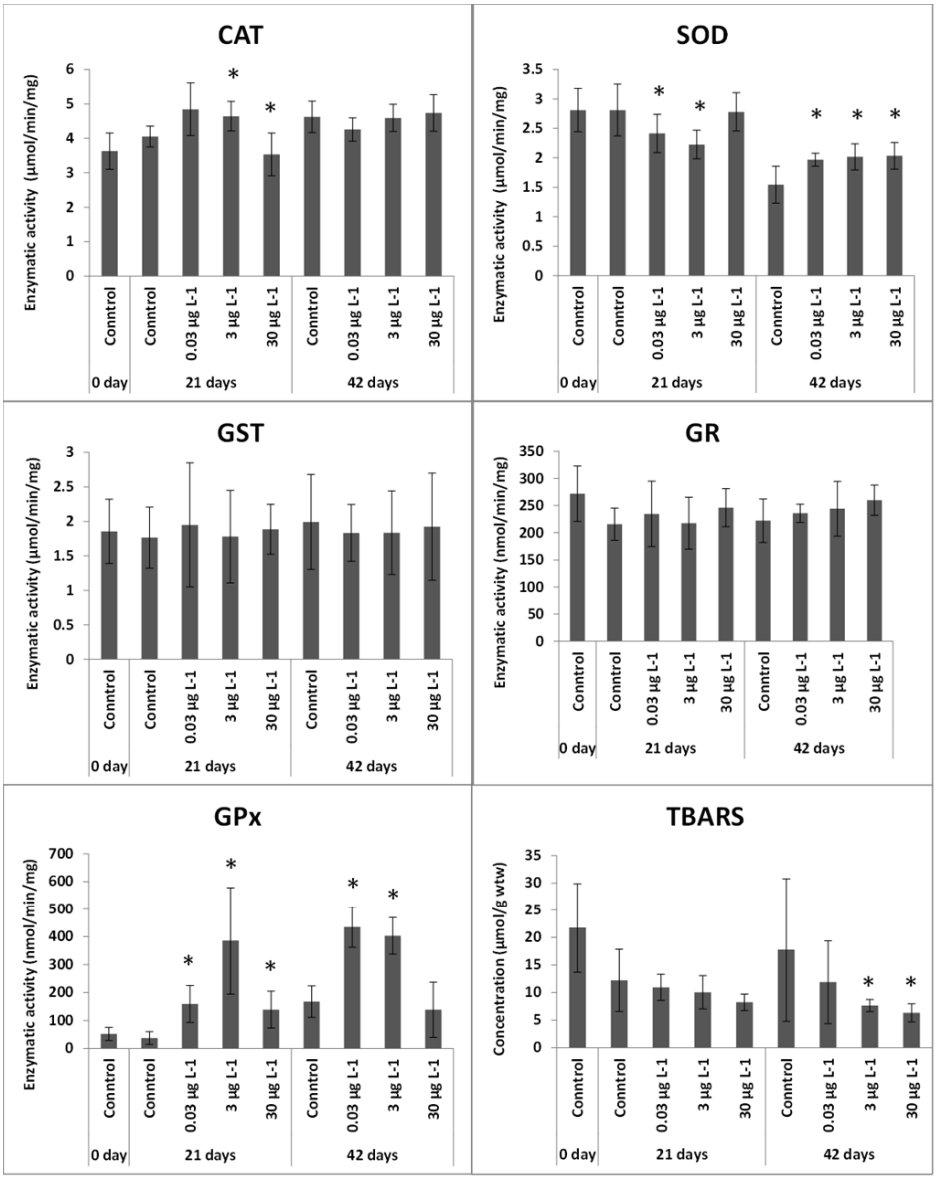

Figure 2. *Antioxidant enzyme activity in liver of rainbow trout sub-chronically exposed to diltiazem. SOD – superoxide dismutase; CAT – catalase, GR – glutathione reductase. Values are expressed as mean ± S.D. (n = 8). Asterisks indicate significant difference compared to the control. *: p < 0.05 (ANOVA).*

3.5.2. Gill

The activity of CAT and GR in gill of fish exposed to diltiazem did not differ significantly from the control (Fig. 3). After 21 days exposure, SOD activity at all tested concentrations of diltiazem was not significantly different from control. The SOD activity was significantly reduced in fish exposed for 42 days to 3 µg L^{-1} diltiazem, but not to 0.03 and 30 µg L^{-1} (ANOVA, $p > 0.05$, Fig. 3). The GPx activity in gill was significantly induced in fish exposed to 0.03 and 30 µg L^{-1} diltiazem for 21 days, but not in fish exposed to 3 µg L^{-1} diltiazem. After 21 days

exposure, GST activity was not significantly different in fish exposed to diltiazem from that in controls. GST activity after 42 days exposure was significantly reduced in fish exposed to 0.03 and 30 μg L^{-1} diltiazem compared with the control (ANOVA, $P < 0.05$), but not in those exposed to 3 μg L^{-1}. After 21 days exposure, the concentration of TBARS was significantly enhanced only in fish exposed to 0.03 μg L^{-1} diltiazem compared with the control (ANOVA, $p < 0.05$).

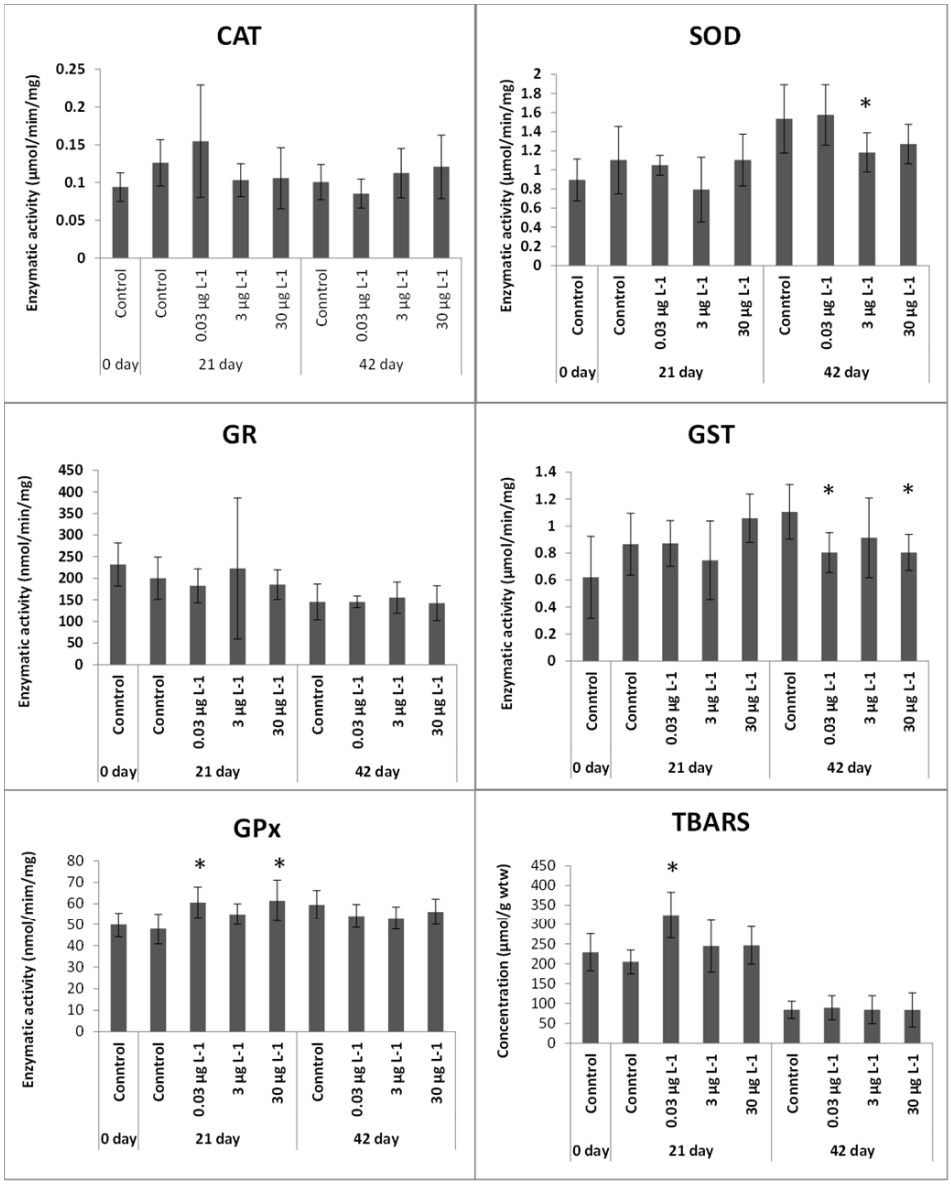

Figure 3. *Antioxidant enzyme activity in gill of rainbow trout sub-chronically exposed to diltiazem. SOD – superoxide dismutase; CAT – catalase, GR – glutathione reductase. Values are expressed as mean ± S.D. (n = 8). Asterisks indicate significant difference compared to the control. * p < 0.05 (ANOVA).*

3.6. Histological changes

Liver of control and diltiazem-exposed fish showed moderate numbers of fat vacuoles in hepatocytes. In fish exposed to 30 μg L^{-1} diltiazem, the cytoplasm of the hepatocytes was characterized by mild granulation with enhanced glycogen content (PAS positive) (Supplement 10). Liver of fish exposed to diltiazem showed scattered to mild sinusoidal congestion. Two diltiazem treated fish displayed mild to moderate rupture of cell membranes in highly vacuolated hepatocytes, and one of the diltiazem exposed fish presented single cell necrosis characterized by karyopycnosis in hepatocytes (Fig. 4, Supplement 10).

Heart of control animals showed no histological changes. Alterations in fish exposed to 30 μg L^{-1} diltiazem ranged from scattered to moderate. Signs of inflammation with lymphocytes, plasma cells, and macrophages in the epicardium were present, along with degeneration and rupture of pericardial vessel walls (Fig. 5). In a single fish exposed to 30 μg L^{-1} diltiazem, degeneration of muscle fibres in the ventricle and focal calcification was observed (Supplement 10).

Kidney of diltiazem-exposed fish was characterized by scattered to moderate tubulonephrosis and tubular neogenesis (Fig. 6, Supplement 11). Scattered degenerated tubular epithelial cells were also observed in control fish.

No histological differences were found in brain of control and diltiazem exposed fish; all groups exhibited scattered to mild signs of inflammation, primarily lymphocytes in the meninges.

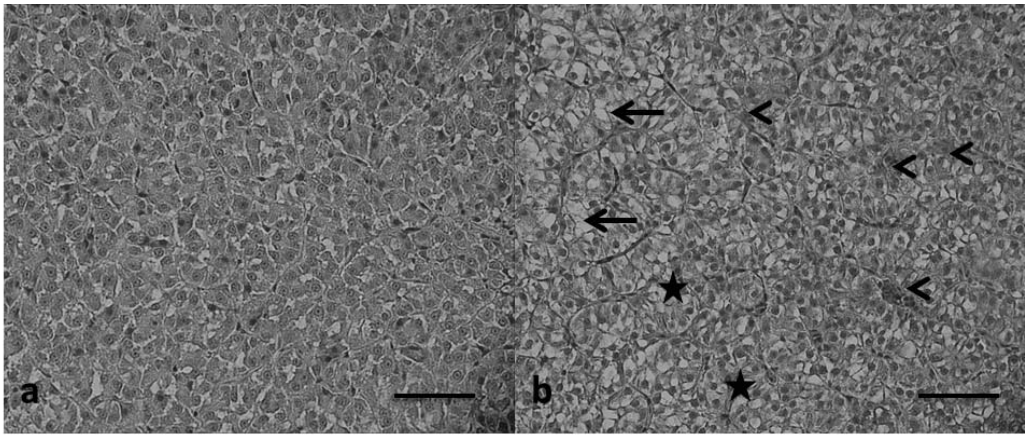

Figure 4. *Histological findings in liver of rainbow trout. a – control fish with normal liver structure; b – fish exposed to 30 μg L^{-1} diltiazem. Arrowheads – small blood vessels and congested sinusoids; stars – granulation of the cytoplasm; arrows – rupture of the cell membrane of the hepatocytes. H&E staining; scale bar = 25 μm.*

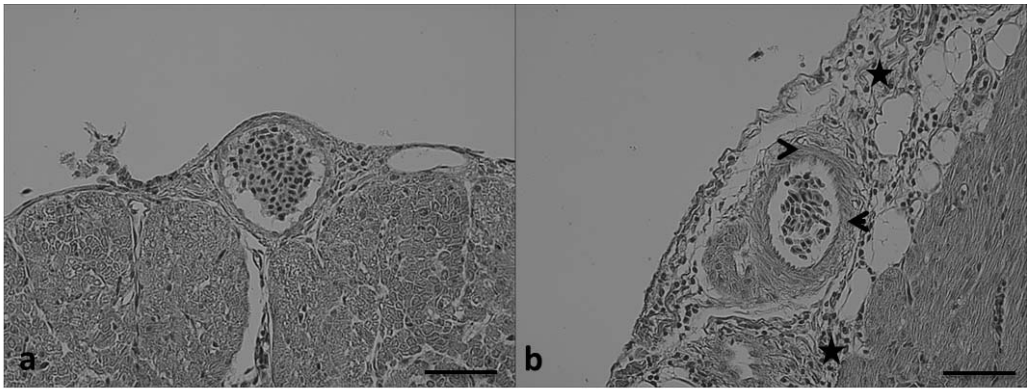

Figure 5. *Histological findings in heart of rainbow trout. a – control fish with normal heart structure; b – fish exposed to 30 µg L^{-1} diltiazem. Stars – thickening of the pericardium by infiltration with lymphocytes, plasma cells, and macrophages and oedema. H&E staining; scale bar = 25 µm.*

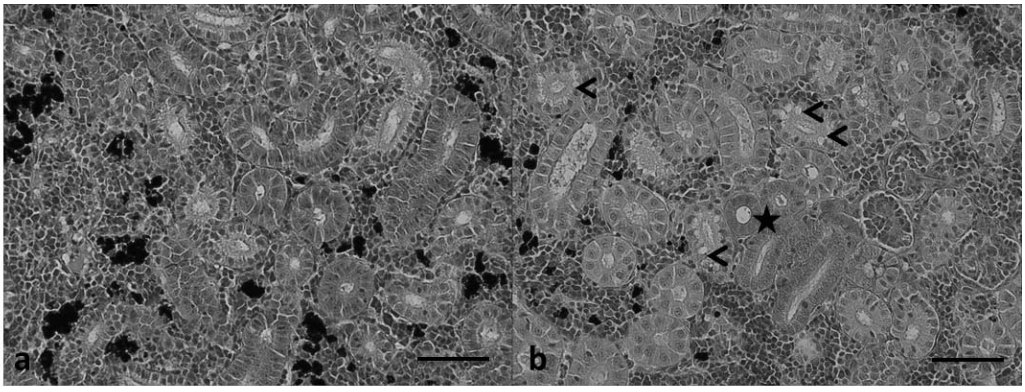

Figure 6. *Histological findings in kidney of rainbow trout. a – control fish with normal kidney structure; b – fish exposed to 30 µg L^{-1} diltiazem. Arrowhead – tubular necrosis; star – tubuloneogenesis. H&E staining; scale bar = 25 µm.*

4. Discussion

4.1. Bioconcentration and half-life of diltiazem in fish

Bioconcentration of substances in aquatic organisms can give rise to toxic effects over a long period of time even when water concentrations are low (OECD, 2001). In the present study, the BCFs of diltiazem in kidney, liver, muscle, and blood plasma ranged from 0.5 to 194. The OECD (2001) classifies diltiazem as a substance with a low level of bioconcentration in fish (BCF < 500). The predicted BCF of diltiazem in whole fish homogenates calculated by EPI Suite™ (v. 4.11, US EPA, 2012), based on its log K_{ow} (2.7), was 28. In agreement with the modelled BCF, Wang and Gardinali (2013) reported a BCF of 16 for diltiazem in mosquito fish homogenates. Considering the range of BCF values calculated for different tissues in the present study, BCF in our fish homogenates ought to have differed by approximately an order of magnitude from the predicted value and that calculated for eastern mosquito fish by Wang and Gardinali (2013).

In the present study, the BCF values followed the order kidney > liver > muscle > plasma. This is in agreement with Ramirez et al. (2009) who reported higher diltiazem concentration in liver of wild fish compared to that in muscle. To the best of our knowledge, no data is available on the level of diltiazem in kidney of fish for comparison. In plasma, diltiazem was detected only in fish exposed to the highest tested concentration (30 $\mu g\ L^{-1}$), with the BCF ranging from 0.5 to 0.98. However, Fick et al. (2010) reported a BCF of 24 in the blood plasma of rainbow trout exposed for 2 weeks to treated waste water with diltiazem concentration as low as 0.037 $\mu g\ L^{-1}$. The BCF of 14 calculated by Fick et al. (2010) according to Hugget's blood plasma model was also higher than the BCF found in the present study. This could be attributed to the rapid metabolic turnover of diltiazem in liver. In the study of Fick et al. (2010), fish were not fed during the experimental period of 14 days, and nutritional deficiencies might have led to lower rates of xenobiotic metabolism (Yang et al., 1992) compared to that in our study. Moreover, the fish were exposed to sewage effluent containing a cocktail of pharmaceuticals, including a chemosensitizer, verapamil (Kurelec, 1997). The Hugget's blood plasma model does not take into account metabolism of the target substance, an important factor affecting final concentration of the substance in plasma.

The half-life calculated for diltiazem in various tissues of rainbow trout ranged from 1.5 to 49 h in the present study. Tissue-specific differences in lipid content are not considered to affect the bioconcentration and half-life of diltiazem, since it is an ionisable (pK_{as}= 8.94) and moderately hydrophobic compound (K_{OW} =2.7; Ramirez et al., 2009, EPI Suite™ v4.11, US EPA, 2012). The relatively high concentration and short half-life of diltiazem in trout liver indicate high metabolic turnover. Compared to the other analyzed tissue types, liver exhibited the highest content of diltiazem metabolites during the exposure phase. The high BCF and longer half-life of diltiazem in the kidney could be attributed to glomerular filtration, renal tubular secretion, and/or reabsorption. A longer half-life associated with reduced metabolic turnover of diltiazem was determined in muscle when compared to liver and kidney, suggesting muscle tissue as a diltiazem deposit in fish.

4.2. Diltiazem metabolites

The present study demonstrated that diltiazem is transformed into 8 metabolites in rainbow trout. It is metabolized preferentially into desmethyl diltiazem (M1), desacetyl diltiazem (M2), and desacetyl, desmethyl diltiazem (M3), which suggests desmethylation and desacetylation as major metabolic pathways. Similarly, M1, M2, and M3 metabolites are found in human blood plasma (Christensen et al., 1999; Dasandi et al., 2009). As in this study, Alderton et al. (2010), in a study of the metabolic profile of verapamil, as a representative calcium channel blocker, found considerable overlap between fish and humans. In the present study, hydroxyl diltiazem was determined as a metabolite of diltiazem. To the best of our knowledge, there is no information available in the literature describing the presence of hydroxyl diltiazem in humans or other mammals, indicating that this metabolite is fish-specific.

4.3. Histological changes

Diltiazem was found to be associated with pathologic alterations in liver, heart, and kidney, while the brain showed no differences between control and exposed animals. In mammals, diltiazem is metabolized chiefly in the liver (Homsy et al., 1995). The granulation of hepatocytes associated with enhanced glycogen content that was observed in trout liver in the present study indicated increased metabolic activity related to diltiazem detoxification. In humans, the metabolism of drugs is associated with pathological changes in the liver (Chang and

Schiano, 2007; Tredger and Davis, 1991). Sarachek et al. (1985) also reported pathological changes in the liver characterized by granulation of hepatocytes with diltiazem exposure. Only single cell necrosis was observed in few exposed trout in the present study.

Among the studied tissues, kidney displayed the highest rate of pathological alterations as well as the highest diltiazem concentration. Necrotic changes in the tubuli resulted in increased tubuloneogenesis, indicative of regeneration. *De novo* development of tubuli is a common response to nephrotoxic injury in fish (Reimschuessel, 2001). The alterations in kidney can be attributed to the metabolic demands of enhanced excretion and reabsorption and/or a toxic effect of diltiazem and its metabolites.

The histological changes in heart and the blood vessels of the liver in exposed trout suggest vasodilatation similar to the pharmacological effect of diltiazem in the human body (Lüllmann, 2003). Since the L-type calcium channel, the target of diltiazem in the human body, has a high degree of similarity in human and fish (Rottbauer et al., 2001), we can hypothesize a similar mode of action.

4.4. Haematological and biochemical profiles of blood plasma

In the present study, diltiazem induced haematological and biochemical changes in the blood plasma of rainbow trout. Fish exposed 42 days to diltiazem in concentrations of 3 and 30 μg L^{-1} presented increased numbers of mature granulocytes in the blood, which could be associated with inflammation in liver and heart.

The reduced TP content in plasma of fish exposed for 21 days to diltiazem 3 and 30 μg L^{-1} indicated liver and kidney damage (Kolarova and Velisek, 2012), which was confirmed by histology. Lower TP might also reflect the increased metabolic demands of detoxification of diltiazem. Lactate in plasma was also reduced in diltiazem exposed fish. In rainbow trout, lactate can be rapidly metabolized and is an important source of energy for the heart (Battiprolu et al., 2007). Therefore, the reduced lactate content could reflect increased metabolic demand related to diltiazem exposure. Similarly, an effect on energy balance represented by reduced ATP level in plasma was described by Klein et al. (2009) for zebrafish injected with a high dose of diltiazem (1.3 mg).

At all tested diltiazem concentrations, the CK content was significantly increased in plasma, suggesting damage of the cardiac muscle. Increased CK levels are also found in the human body as a consequence of myocardial injury (Clarkson et al., 2006; Adams et al., 1993). The present study found degeneration of cardiac muscle fibre in a single exposed fish.

4.5. Activity of CYPs and antioxidant enzymes in the liver and gills

Activity of CYP1A, which carries out the *O*-deethylase reaction, is a widely used biomarker of xenobiotic exposure in fish (Whyte et al., 2000). In the present study, diltiazem was not shown to affect CYP1A activity, nor did we detect metabolites of this enzyme in exposed fish. Therefore, it is probably not involved in diltiazem metabolism in trout. Similarly, Connors et al. (2013) did not observe metabolism of diltiazem by CYP1A in liver S9 fraction of rainbow trout *in vitro*. In mammals, CYP1A1 activity has not been shown to be affected by diltiazem, and this isoform is not supposed to be involved in its metabolism (Connors et al., 2013).

CYP3A4 is the principal enzyme in human drug metabolism, involved in 28% of metabolism of 50% of currently available drugs (Lewis, 2000; Lewis, 2004). The key step in the metabolism of diltiazem, desmethylation, is extensively catalysed by CYP3A4 (Molden et al., 2002a). In the present study, reduced activity of the CYP3A isoform was found in fish exposed to the highest tested concentration of diltiazem. Excess diltiazem and its major metabolite *N*-desmethyl

diltiazem, which was also detected in the present study, are known to inhibit the activity of CYP3A4 in humans. In mammals, the desmethylation of diltiazem is also carried out by CYP2D6, CYP2C8, and CYP2C9, and, as a result, *O*-desmethyl diltiazem is formed in addition to *N*-desmethyl diltiazem (Molden et al., 2000; Sutton et al., 1997). The subfamily of CYP2C and CYP2D is not present in fish, and therefore diltiazem may not undergo *O*-desmethylation in fish (Kirischian et al., 2011). The specific isoforms of diltiazem metabolites could not be identified by our analytical method; hence, we could not distinguish *O*-desmethyl diltiazem from *N*-desmethyl diltiazem.

As in mammals (Molden et al., 2002a), desacetylation was the second major step observed in diltiazem metabolism in rainbow trout in the present study. The desacetylation of diltiazem in mammals is carried out by esterases, mainly in the intestines, and CYPs are not involved (Molina et al., 2007; Molden et al., 2002a; Homsy et al., 1995). Therefore, desacetyl metabolites of diltiazem indicated extra-hepatic metabolism of diltiazem and the presence of these esterases in fish.

Diltiazem undergoes a hydroxylation reaction in fish that is not seen in mammals. However, the corresponding metabolic pathway remains unknown. It is possible that CYPs in addition to those we analyzed are involved in biotransformation of diltiazem in fish.

In the present study, increased activity of SOD, CAT, and GPx compared to the control was observed in liver of trout exposed to diltiazem for 42 days, indicating an antioxidant response to diltiazem exposure. These enzymatic reactions probably led to a reduction in the concentration of reactive oxidative species (ROS) O_2^- and H_2O_2 (van der Oost et al., 2003; Cazenave et al., 2006), as indicated by a concurrent reduced concentration of TBARS. On the other hand, in gill, TBARS levels were transiently (after 21 days) elevated in fish exposed to the lowest tested diltiazem concentration when compared to the control. In general, activity of nearly all antioxidant enzymes was lower in gill compared to liver, indicating that gill may be more vulnerable to xenobiotic exposure than is liver.

5. Conclusions

The bioconcentration factor of diltiazem in rainbow trout exposed to sub-lethal concentrations of diltiazem (0.03–30 μg L^{-1}) was relatively low, ranging from 0.5 to 194. Therefore, diltiazem can be classified as having a low level of bioconcentration (OECD, 2001). The highest BCF was calculated for the kidney, then following the order liver > muscle > blood plasma. The half-life calculated for diltiazem in different tissues of rainbow trout ranged from 1.5 to 49 h, following the order liver, kidney, and muscle. The highest content of diltiazem metabolites was detected in liver and then followed the order kidney > muscle > blood plasma. The tissue-specific half-life corresponded to the rate of diltiazem metabolism. The detected metabolites demonstrated desacetylation and desmethylation as major metabolic pathways of diltiazem, indicating similarities of diltiazem metabolism in fish and humans. We determined the hydroxylation of diltiazem as a fish specific pathway. In accordance, the histopathological alterations in the liver and kidney can be attributed to the metabolism and excretion of diltiazem. At the highest tested concentrations, some effects observed in fish can be considered to be similar to those described for mammals: specifically the inhibition of CYP3A activity and the effects on the vascular system of the heart and liver. The effect on the cardiovascular system could have been mediated by the L-type calcium channel, a suggestion supported by structural conservation of the protein structure of the L-type calcium channel in fish and human (Rottbauer et al., 2001). At the environmentally relevant tested concentration, diltiazem exposure led to altered activity of antioxidant enzymes in the liver and gill as well as to transient elevation of TBARS level in the gills. In addition, at this concentration, the CK

content in blood plasma was increased, indicating damage to cardiac muscle. The present study indicates that diltiazem, when present in the aquatic environment, could be a source of sub-lethal detrimental effects in fish.

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Supplement 1. *Minimum and maximum limit of quantification (LOQs) of diltiazem in acetonitrile extract of the blood plasma, liver, muscle, and kidney.*

Tissue	Tested groups/ minimal and maximal LOQ of diltiazem (μg Kg^{-1})				
	Control	0.03 μg L^{-1}	3 μg L^{-1}	30 μg L^{-1}	Depuration phase
Plasma	3.1–8.9	3.4–15	3.3–9.8	5.2–15	8.7–23
Liver	0.99–29.6	2.8–25	0.62–4.5	0.7–4.6	1.1–2.6
Kidney	4.0–33	15–32	7.0–11.8	2.6–18	16–21
Muscle	0.13–6.0	0.94–2.6	1.0–4.3	2.2–30	0.6–1.2

Supplement 2. *Diltiazem and metabolite levels: concentration (μM), mean (%), and prevalence in kidney of rainbow trout exposed to diltiazem at 0.03, 3, and 30 μgL^{-1} for 21 and 42 days and in fish during the depuration phase (7 and 14 d post-exposure). M1- desmethyl diltiazem; M2- desacetyl diltiazem; M3- desacetyl, desmethyl diltiazem; M4- desacetyl, didesmethyl diltiazem; M5- didesmethyl diltiazem; M6- hydroxy diltiazem; M7- desmethyl, hydroxy diltiazem; M8- desacetyl, hydroxy diltiazem. LOQ = limit of quantification.*

Group	Substance	21 (d) exposure	42 (d) exposure	7 (d) post exposure	14 (d) post exposure
0.0 3μg L^{-1}	Diltiazem	15; 66%; 8/8	24; 42%; 6/8		
	M1	8; 34%; 4/8	16; 29%; 8/8		
	M3	≤ LOQ	7; 12 %; 1/8		
	M2	≤ LOQ	9; 17%; 2/8		
	M8	≤ LOQ	≤ LOQ		
	M4	≤ LOQ	≤ LOQ		
	M6	≤ LOQ	≤ LOQ		
	M7	≤ LOQ	≤ LOQ		
	M5	≤ LOQ	≤ LOQ		
3 μg L^{-1}	Diltiazem	699; 45 %; 8/8	631; 33%; 8/8		
	M1	526; 34 %; 8/8	952; 50%; 8/8		
	M3	129; 8%; 7/8	176; 9%; 8/8		
	M2	210; 11%; 7/8	159; 20%; 8/8		
	M8	≤ LOQ	≤ LOQ		
	M4	≤ LOQ	≤ LOQ		
	M6	≤ LOQ	≤ LOQ		
	M7	≤ LOQ	≤ LOQ		
	M5	≤ LOQ	21; 1 %; 8/8		
30 μg L^{-1}	Diltiazem	5768; 38% 8/8	5790; 24%;8/8	317; 2%; 4/4	232; 2%; 4/4
	M1	6267; 41%; 8/8	11033; 46%; 8/8	15446; 71%; 4/4	9727; 73%; 4/4
	M3	1337;9%; 8/8	2603;11%; 8/8	5153; 24%; 4/4	2658; 21%; 4/4
	M2	1732; 11% ;8/8	1718; 7%; 8/8	480; 2.2%; 4/4	293; 2%; 4/4
	M8	7; < 1%; 5/8	5; < 1%; 2/8	6; < 1%;1/4	≤ LOQ
	M4	9; < 1%; 4/8	20; < 1%; 8/8	313; < 1%;4/4	182; < 1%;2/4
	M6	10; < 1%; 8/8	14.4; < 1%; 8/8	11; < 1%; 4/4	6; < 1%; 4/4
	M7	6; < 1%; 2/8	15; < 1%; 8/8	7; < 1%; 2/4	≤ LOQ
	M5	38; < 1%; 8/8	201; < 1%; 8/8	215; 1%; 4/4	153; 1%; 4/4

Supplement 3. *Diltiazem and metabolite levels: concentration (µM), mean (%), and prevalence in liver of rainbow trout exposed to diltiazem at 0.03, 3, and 30 µg L^{-1} for 21 and 42 days and in fish during the depuration phase (7 and 14 d post-exposure). M1 – desmethyl diltiazem; M2 – desacetyl diltiazem; M3 – desacetyl, desmethyl diltiazem; M4 – desacetyl, didesmethyl diltiazem; M5 – didesmethyl diltiazem; M6 – hydroxy diltiazem; M7 – desmethyl, hydroxy diltiazem; M8 – desacetyl, hydroxy diltiazem. LOQ = limit of quantification.*

Group	Substance	21 (d)exposure	42 (d) exposure	7 (d) post exposure	14 (d) post exposure
	Diltiazem	8; 24; 2/8	≤ LOQ		
	M1	26; 76%; 8/8	30; 100%; 8/8		
	M3	≤ LOQ	≤ LOQ		
0.03 µg L^{-1}	M2	≤ LOQ	≤ LOQ		
	M8	≤ LOQ	≤ LOQ		
	M4	≤ LOQ	≤ LOQ		
	M6	≤ LOQ	≤ LOQ		
	M7	≤ LOQ	≤ LOQ		
	M5	≤ LOQ	≤ LOQ		
	Diltiazem	228; 16%; 5/8	163; 12%; 8/8		
	M1	1245; 73%; 7/8	1057; 78%; 8/8		
	M3	92; 5%; 7/8	36; 3 %; 8/8		
	M2	18; 1%; 7/8	10; 1%; 7/8		
3 µg L^{-1}	M8	≤ LOQ	≤ LOQ		
	M4	≤ LOQ	≤ LOQ		
	M6	≤ LOQ	≤ LOQ		
	M7	≤ LOQ	≤ LOQ		
	M5	32; 11%; 3/8	53; 4%; 3/8		
	Diltiazem	1954; 12%; 8/8	1364; 9%;8/8	37; 4%; 4/4	55; 8%; 4/4
	M1	10630; 75%; 8/8	11587; 72%; 8/8	617; 72% 8; 4/4	489; 74%; 4/4
	M3	808; 6%; 8/8	611; 4%; 8/8	151; 17%; 4/4	89; 14%; 4/4/
	M2	189; 1%; 8/8	140; 1%; 7/8	14; 2%;8/8	13; 2%; 3/4
30 µg L^{-1}	M8	≤ LOQ	≤ LOQ	≤ LOQ	≤ LOQ
	M4	34; < 1%; 7/8	19; < 1%;8/8	31; 4%; 2/4	13; 2%; 2/4
	M6	8; < 1%; 4/8	15; < 1%; 2/8	≤ LOQ	≤ LOQ
	M7	≤ LOQ	≤ LOQ	1; < 1%; 1/4	≤ LOQ
	M5	490; 4%; 8/8	763; 5%; 7/8	25; 3%; 4/4	27; 4%; 4/4

Supplement 4. *Diltiazem and metabolite levels: concentration (µM), mean (%), and prevalence in muscle of rainbow trout exposed to diltiazem at 0.03, 3, and 30 µg L^{-1} for 21 and 42 days and in fish during depuration phase (7 and 14 d post-exposure). M1 – desmethyl diltiazem; M2 – desacetyl diltiazem; M3 – desacetyl, desmethyl diltiazem; M4 – desacetyl, didesmethyl diltiazem; M5 – didesmethyl diltiazem; M6 – hydroxy diltiazem; M7 – desmethyl, hydroxy diltiazem; M8 – desacetyl, hydroxy diltiazem. LOQ = limit of quantification.*

Group	Substance	21-d exposure	42-d exposure	7-d post exposure	14-d post exposure
0.03 µg L^{-1}	Diltiazem	≤ LOQ	≤ LOQ		
	M1	≤ LOQ	≤ LOQ		
	M3	≤ LOQ	≤ LOQ		
	M2	≤ LOQ	≤ LOQ		
	M8	≤ LOQ	≤ LOQ		
	M4	≤ LOQ	≤ LOQ		
	M6	≤ LOQ	≤ LOQ		
	M7	≤ LOQ	≤ LOQ		
	M5	≤ LOQ	≤ LOQ		
3 µg L^{-1}	Diltiazem	10; 46%; 6/8	5; 62%;5/8		
	M1	12; 54%; 2/8	3; 38%;4/8		
	M3	≤ LOQ	≤ LOQ		
	M2	≤ LOQ	≤ LOQ		
	M8	≤ LOQ	≤ LOQ		
	M4	≤ LOQ	≤ LOQ		
	M6	≤ LOQ	≤ LOQ		
	M7	≤ LOQ	≤ LOQ		
	M5	≤ LOQ	≤ LOQ		
30 µg L^{-1}	Diltiazem	104; 42%; 5/8	38; 53%: 8/8	23; 31%;4/4	23; 47%; 4/4
	M1	94; 38%; 8/8	42; 47%;8/8	37; 49%;4/4	16; 34%;4/4
	M3	≤ LOQ	≤ LOQ	11; 14%;4/4	5; 10%;4/4
	M2	15; 6%; 2/8	≤ LOQ	4; 6%; 4/4	4; 8%; 4/4
	M8	≤ LOQ	≤ LOQ	≤ LOQ	≤ LOQ
	M4	≤ LOQ	≤ LOQ	≤ LOQ	≤ LOQ
	M6	22; 9%; 1/8	≤ LOQ	≤ LOQ	≤ LOQ
	M5	≤ LOQ	≤ LOQ	≤ LOQ	≤ LOQ
	M7	≤ LOQ	≤ LOQ	≤ LOQ	≤ LOQ

Supplement 5. *Diltiazem and metabolite levels: concentration (µM), mean (%), and prevalence in plasma of rainbow trout exposed to diltiazem at 0.03, 3, and 30 µg L^{-1} for 21 and 42 days and in fish during depuration phase (7 and 14 d post-exposure). M1 – desmethyl diltiazem; M2 – desacetyl diltiazem; M3 – desacetyl, desmethyl diltiazem; M4 – desacetyl, didesmethyl diltiazem; M5 – didesmethyl diltiazem; M6 – hydroxy diltiazem; M7 – desmethyl, hydroxy diltiazem; M8 – desacetyl, hydroxy diltiazem. LOQ = limit of quantification.*

Group	Substance	21-d exposure	42-d exposure	7-d post exposure	14-d post exposure
0.03µg L^{-1}	Diltiazem	≤ LOQ	≤ LOQ		
	M1	≤ LOQ	≤ LOQ		
	M3	≤ LOQ	≤ LOQ		
	M2	≤ LOQ	≤ LOQ		
	M8	≤ LOQ	≤ LOQ		
	M4	≤ LOQ	≤ LOQ		
	M6	≤ LOQ	≤ LOQ		
	M7	≤ LOQ	≤ LOQ		
	M5	≤ LOQ	≤ LOQ		
3 µg L^{-1}	Diltiazem	≤ LOQ	≤ LOQ		
	M1	≤ LOQ	≤ LOQ		
	M3	≤ LOQ	≤ LOQ		
	M2	≤ LOQ	≤ LOQ		
	M8	≤ LOQ	≤ LOQ		
	M4	≤ LOQ	≤ LOQ		
	M6	≤ LOQ	≤ LOQ		
	M7	≤ LOQ	≤ LOQ		
	M5	≤ LOQ	≤ LOQ		
30 µg L^{-1}	Diltiazem	29; 52 %; 1/8;	64; 67%; 4/8;	96; 77%; 4/4	138; 3/4; 76%
	M1	31; 48 %; 1/8	31; 33 %; 4/8	≤ LOQ	≤ LOQ
	M3	≤ LOQ	≤ LOQ	≤ LOQ	≤ LOQ
	M2	≤ LOQ	≤ LOQ	≤ LOQ	≤ LOQ
	M8	≤ LOQ	≤ LOQ	≤ LOQ	≤ LOQ
	M4	≤ LOQ	≤ LOQ	≤ LOQ	≤ LOQ
	M6	≤ LOQ	≤ LOQ	≤ LOQ	≤ LOQ
	M7	≤ LOQ	≤ LOQ	29; 23%; 1/8	45; 24%; 3/4
	M5	≤ LOQ	≤ LOQ	≤ LOQ	≤ LOQ

Supplement 6. *Morphological parameters of rainbow trout sub-chronically exposed to diltiazem. FCF – Fulton's condition factor, HSI – hepatosomatic index, CSI – cardiac- somatic index. Values are expressed as mean ± S.D. (n = 8).*

Indices	Exposure time (d)	Control	0.03 µg L^{-1}	3 µg L^{-1}	30 µg L^{-1}
Length	21	241 ± 13	241 ± 21	248 ± 14	248 ± 13
(mm)	42	245 ± 14	258 ± 16	257 ± 10	255 ± 16
Weight	21	174 ± 31	175 ± 40	196 ± 36	197 ± 21
(g)	42	225 ± 37	219 ± 30	226 ± 26	215 ± 34
FCF	21	1.32 ± 0.07	1.24 ± 0.13	1.26 ± 0.10	1.30 ± 0.11
	42	1.23 ± 0.14	1.22 ± 0.08	1.32 ± 0.056	1.29 ± 0.08
HIS	21	1.27 ± 0.15	1.12 ± 0.96	1.10 ± 0.18	1.02 ± 0.38
	42	1.08 ± 0.19	1.13 ± 0.10	1.26 ± 0.12	1.20 ± 0.18
CSI	21	0.15 ± 0.01	0.15 ± 0.03	0.14 ± 0.02	0.27 ± 0.31
	42	0.16 ± 0.02	0.15 ± 0.02	0.14 ± 0.02	0.15 ± 0.02

Supplement 7. *Haematological parameters of rainbow trout sub-chronically exposed to diltiazem. Hc – haematocrit, Hb – haemoglobin, WBC – white blood cell (leukocyte) count, MCV – mean corpuscular volume, MHC – mean corpuscular haemoglobin, MCHC – mean corpuscular haemoglobin concentration. Values are expressed as mean ± S.D. (n = 8). Asterisks indicate significant difference from control. * : P <0.05 (ANOVA).*

Indices	Exposure time (d)	Control	0.03 µg L^{-1}	3 µg L^{-1}	30 µg L^{-1}
Hc (%)	0	46 ± 8			
	21	50 ± 5	46 ±4.	49 ± 5	57 ± 3*
	42	38 ± 2	44 ± 7	39 ± 8	38 ± 8
Hb (g L^{-1})	0	83 ± 21			
	21	81 ± 8	77 ± 4	77 ± 8	85 ± 3
	85 ± 10		84 ± 10	65 ± 10	75 ± 13
Erythrocytes (T L^{-1})	0	1.2 ± 0.2			
	21	1.1 ± 0.3	1.0 ± 0.1	0.9 ± 0.2	1.0 ± 0.2
	42	1.1 ± 0.3	1.0 ± 0.2	0.9 ± 0.2	1.0 ± 0.1
WBC (g L^{-1})	0	86 ± 41			
	21	107 ± 35.9	81 ± 26	83 ± 36	90 ± 23
	42	119 ± 47	109 ± 37	101 ± 27	118 ± 71
MCV (fl)	0	375 ± 83			
	21	474 ± 111	446 ± 26	472 ± 27	524 ± 40
	42	315 ± 47	297 ± 93	336 ±74	288 ± 32
MCH (pg)	0	68 ± 25			
	21	76 ± 11	61 ± 8	55 ± 10	62 ± 16
	42	71 ± 16	55 ± 15	62 ± 12	64 ± 13
MCHC (g L^{-1})	0	0.18 ± 0.03			
	21	0.18 ± 0.04	0.16 ± 0.03	0.14 ± 0.02	0.12 ± 0.02
	42	0.23 ± 0.1	0.19 ± 0.04	0.19 ± 0.05	0.23 ± 0.06

Supplement 8. *Differential leukocyte count (leukogram) of rainbow trout sub-chronically exposed to diltiazem. Values are expressed as mean ± S.D. (n = 8). Asterisks indicate significant difference from control. * P < 0.05 (ANOVA).*

Indices	Exposure time (d)	Control	0.03 µg L^{-1}	3 µg L^{-1}	30 µg L^{-1}
Small lymphocytes (%)	0	96.0 ± 3.4			
	21	94.8 ± 2.0	91.7 ± 6.0	94.9 ± 3.1	94.8 ± 2.6
	42	94.6 ± 5.8	96.5 ± 3.0	91.6 ± 11.2	95.6 ± 3.1
Large lymphocytes (%)	0	0			
	21	0.5 ± 0.4	0.5 ± 0.9	0	0
	42	0	0	0	0
Monocytes (%)	0	1.6 ± 1.5			
	21	1.5 ± 1.3	2.7 ± 1.0	3.3 ± 2.0	2.3 ± 1.9
	42	1.9 ± 1.5	2.3 ± 3.4	2.2 ± 2.7	1.9 ± 2.1
Young neutrophil granulocytes (%)	0	2.2 ± 1.8			
	21	1.8 ± 1.4	4.4 ± 5.1	2.5 ± 2.1	2.5 ± 2.3
	42	2.7 ± 3.6	2.1 ± 2.7	0.3 ± 0.5	1.2 ± 1.4
Mature neutrophil granulocytes (%)	0	0.2 ± 0.2			
	21	0.2 ± 0.3	0.2 ± 0.5	0.3 ± 0.2	0.9 ± 0.8
	42	0.4 ± 0.5	0.3 ± 0.4	1.8 ± 1.4*	1.6 ± 2.2*

Supplement 9. *Activity of CYP450 enzymes in liver of rainbow trout sub-chronically exposed to diltiazem. CYP1A1 (EROD) and CYP3A (BFCOD) in liver of rainbow trout sub-chronically exposed to diltiazem. EROD – ethoxyresorufin-O-deethylase, BFCOD – 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylation. Values are expressed as mean ± S.D. (n = 8). Asterisks indicate significant difference from control. * P < 0.05 (ANOVA).*

Indices	Exposure time (d)	Control	0.03 µg L^{-1}	3 µg L^{-1}	30 µg L^{-1}
EROD pmol $min^{-1}mg^{1}mg^{-1}$	0	28.6 ± 15.0			
	21	48.1 ± 20.4	33.1 ±7.2	32.0 ± 15.6	31.6 ± 16.5
	42	37.4 ±13.6	33.0 ± 18.1	25.7 ± 15.6	29.8 ± 16.2
BFCOD pmol $min^{-1}mg^{1}mg^{-1}$	0	16.4 ± 8.1			
	21	29.4 ± 8.3	23.7 ±9.2	21.3 ±8.8	18.3 ± 8.4*
	42	15.8 ± 2.6	21.4 ± 8.4	20.0 ± 9.9	17.1 ± 8.7

Supplement 10. *Histological findings in liver and heart of rainbow trout exposed to 30 µg L^{-1} diltiazem for 42 days (n = 8) and control fish. 0 = none, 1 = scattered, 2 = mild, 3 = mild to moderate, 4 = moderate, 5 = moderate, and 6 = severe pathological changes. The PAS-stain, pos. = positive, neg. = negative.*

	Liver								**Heart**				
Animal number	Sinusoidal congestion	Inflammatory cells	Rupture of cell membrane	Areas with macrophage infiltration	Fat vacuoles	Glycogen content (PAS – reaction)	Hepatocytes with Karyopygnosis	Granulated cytoplasm	Pericardial infiltration with lymphocytes, plasma and macrophages	Subendocardial infiltration	Degeneration of vessel wall	Muscle fibre degeneration in ventricle associated with plasma cells	Focal calcification of the myocardia
Control animals													
1	0	0	0	0	4	neg.	0	0	0	0	0	0	0
2	0	0	0	0	4	neg.	0	0	0	0	0	0	0
3	0	0	0	3	4	neg.	0	0	0	0	0	1	0
4	0	0	0	0	4	neg.	0	0	0	0	0	0	0
5	0	0	0	0	2	neg.	0	0	0	0	0	0	0
6	0	0	0	0	1	neg.	0	0	1	0	0	0	0
7	0	0	0	0	2	neg.	0	0	0	0	0	0	0
8	1	0	0	0	4	neg.	0	0	2	0	0	0	0
Exposed animals													
1	0	0	0	0	3	pos.	0	2	0	0	0	0	0
2	0	0	0	0	4	neg.	0	0	3	0	2	0	0
3	0	0	0	0	3	pos.	0	2	1	0	2	0	0
4	0	0	0	0	4	pos.	0	2	0	0	0	0	0
5	1	0	0	0	5	neg.	0	1	0	2	0	0	2
6	2	2	2	0	4	neg.	0	1	1	0	0	1	0
7	2	2	0	0	4	neg.	2	1	1	0	0	0	0
8	1	2	3	0	4	neg.	0	1	1	0	0	0	0

Supplement 11. *Histological findings in kidney and brain of rainbow trout exposed to 30 µg L^{-1} diltiazem for 42 days (n = 8) and control fish (n = 8). 0 = none, 1 = scattered, 2 = mild, 3 = mild to moderate, 4 = moderate, 5 = moderate to severe, and 6 = severe pathological changes. NE = not evaluated.*

	Kidney				Brain	
Animal number	Tubulus necrosis; characterised by vascularisation and karyopygnosis	Tubulus with hyaline like degeneration	Tubular neogenesis	Deposition of eosinophylic epithel in the mesenchymal loops	Meningeal infiltration with lymphocytes	Gliosis in dience2phalon and myelencephalon
Control animals						
1	1	0	0	0	0	0
2	1	1	0	0	2	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	1	0	0	0	1	0
6	0	0	0	0	2	0
7			NE		2	0
8	0	1	0	0		NE
Exposed animals						
1	1	0	2	0	0	0
2	1	1	0	0	2	0
3	1	0	0	0	2	0
4	1	0	3	0	2	0
5	1	0	1	1	1	0
6	3	0	3	0	0	0
7	2	0	1	0	1	1
8	2	0	4	0	1	0

CHAPTER 5

INVESTIGATION OF DILTIAZEM METABOLIC PATHWAY IN FISH USING HYBRID QUADRUPOLE/ORBITAL TRAP MASS SPECTROMETER QEXACTIVE

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INVESTIGATION OF DILTIAZEM METABOLIC PATHWAY IN FISH USING HYBRID QUADRUPOLE/ORBITAL TRAP MASS SPECTROMETER QEXACTIVE

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ABSTRACT

A rapid and sensitive LC-HRMS method has been developed in present study. New approach based on accurate mass measurement using hybrid quadrupole-orbital trap mass spectrometer Q-Exactive was applied to diltiazem metabolites determination in fish tissues in order to extend lack of knowledge about diltiazem metabolization in aquatic organisms (fish). Blood plasma, muscle, liver and kidney of rainbow trout (Oncorhynchus mykiss) exposed for 42 days to 30 μg L^{-1} were used for the method development. In the present method, no standards of metabolites were required for identification of the diltiazem biotransformation products in fish. Finally, 17 diltiazem metabolites (including isomeric forms) were detected and identified using MassFrontier spectral interpretation software. A semi-quantification approach has been applied for organ dependent comparison of metabolite concentrations. The data suggest complex metabolic pathways of diltiazem in fish with the involvement of desmethylation, desacetylation and hydroxylation, which was firstly investigated in the present study, and their combinations.

1. Introduction

The calcium channel blocker diltiazem is used mainly in the treatment of cardiovascular diseases like angina pectoris and hypertension (Budriesi et al., 2007). In addition, it is prescribed for the prevention of the migraine and in the treatment of chronic anal fissure as ointment (Batt et al., 2008; Silberstein, 2009). Due to its wide use and the incomplete elimination in the sewage treatment plants (STPs) diltiazem has been frequently detected in STPs effluents and in surface waters (Golovko et al., 2014; Kasprzyk-Hordern et al., 2009; Khan and Ongerth, 2004; Kolpin et al., 2004).

Diltiazem is bioconcentrated to a certain extent in fish tissues (Ramirez et al., 2009; Wang and Gardinali, 2013). For instance, diltiazem was traced in the liver and muscle of wild fish, white sucker (*Catostomus commersonii*) and jonny darter (*Etheostoma nigrum*), downstream of a local STP in the USA (Ramirez et al., 2009). Under laboratory conditions, (Wang and Gardinali, 2013) demonstrated a quick uptake (uptake rate = 0.033 mL g^{-1} h^{-1}) and relatively long half-life time (117 h) of diltiazem in whole homogenates of the eastern mosquito fish (*Gambusia holbrooki*). Unfortunately, at present, information about presence and behavior of diltiazem metabolites in fish is missing. However, this could improve our understanding of chronic effects of diltiazem on fish and provide the information on its metabolic pathways.

In mammals, metabolism of diltiazem is well documented (Homsy, 1995; Molden et al., 2000; Molden et al., 2002a; Molden et al., 2002b; Nakamura et al., 1987; Sutton et al., 1997; Yan et al., 2013; Zhao et al., 2007). In humans, it is metabolised into three major products, i.e. *O*-desacetyl diltazem, *N*-desmethyl diltiazem, and *N*-desmethyl-*O*-desacethyl

diltiazem (Christen et al., 2010; Dasandi et al., 2009) which can be found in the blood plasma. Interestingly, *O*-desacethyl and *N*,*O*-didesmethyl diltiazem are pharmacologically active and their effects differ from the parent compound. For instance, these metabolites are more potent inhibitors of the blood cell aggredation and adenosine transport by erythrocytes than diltiazem (Yeung et al., 1996). Similarly, *N*-desmethyl and *N*,*N*-didesmethyl diltiazem were stroger inhibitors of the activity of CYP450 3A compared to diltiazem (Sutton et al., 1997; Zhao et al., 2007).

Most of the studies focused on diltiazem metabolites generally deal with tissues of mammals and humans. Recent studies have presented analytical methods for limited number of diltiazem metabolites, ussually only two major ones (*N*-desmethyl diltiazem and desacetyl diltiazem) (Chankvetadze et al., 2002; Dasandi et al., 2009) were studied. To the best of our knowledge, just one study has showed method for determination of 11 phase I metabolites of diltiazem in human plasma (Molden et al., 2003).

Due to limited information on diltiazem metabolites in fish tissues, new approach based on accurate mass measurement using hybrid quadrupole-orbital trap mass spectrometer Q-Exactive was developed. This method, which does not require employing standards of metabolites, was used for identification of all possible metabolites of phase I biotransformation of diltiazem. Consequently, tandem mass spectrometry was used for confirmation of metabolites identity. Finally, the method based on liquid chromatography with high resolution product scan (HRPS) mass spectrometry was developed and optimized for determination and semi-quantification of diltiazem and its phase I metabolites in fish tissues.

2. Material and method

Chemicals:

Diltiazem [(2S,3S)-5-[2-(dimethylamino)ethyl]-2-(4-methoxyphenyl)-4-oxo-2,3-dihydro-1,5-benzothiazepin-3-yl] acetate (CAS No.: 42399-41-7) and the internal standard Verapamil hydrochloride (CAS No. 152-11-4) were purchased from Sigma-Aldrich (Steinheim, Czech Republic). LC–MS grade acetonitrile (LiChrosolv Hypergrade) was obtained from Merck (Darmstadt, Germany). Formic acid, which was used to acidify the mobile phases, was purchased from Labicom (Olomouc, Czech Republic). The ultrapure water was prepared by the Aqua-MAX-Ultra System (Younglin, Kyounggi-do, Korea).

Instrumentation:

Homogenizer TissueLyser II (Quiagen, Germany) and Micro 200R centrifuge (Hettich Zentrifugen, Germany) were used for tissue extraction. An Accela 1250 LC pump (Thermo Fisher Scientific, San Jose, CA, USA) coupled with a hybride quadrupole/orbital trap Q-Exactive mass spectrometer (Thermo Fisher Scientific) and a HTS XT-CTC autosampler (CTC Analytics AG, Zwingen, Switzerland) were used for separation and detection of target analytes. An analytical Hypersil Gold column (50 mm length, 2.1 mm i.d, 3 μm particles; Thermo Fisher Scientific) preceded with the same phase pre column (10 mm length, 2.1 mm i.d., 3 μm particles) was used for chromatographic separation of target analytes.

Fish exposure setup:

Juvenile rainbow trout (*Oncorhynchus mykiss*; mean body length 23.1 ± 1.5 cm, mean body weight 163.8 ± 27.5 g) were obtained from a local commercial hatchery (Husinec, Czech Republic). The fish were kept in aquaria with 200 L of fresh water in a semi-static system. The aquaria were set up with continuous aeration and the temperature was kept constant at 15.4 ± 1.4 °C with a photoperiod of 12:12 h (light–dark). During the acclimation time and the experimental period, the fish were fed with a commercial fish food (Bio Mar, Denmark)

at a ratio of 1% of body weight on a daily basis. 150 L of experimental bath were daily refreshed with new diltiazem fortified water. The fish used in the present study were exposed to diltiazem at concentration level of 30 μg L^{-1} for 42 days. Samples of kidney, liver, muscle and blood plasma of fish were taken and stored at -20 °C until further analysis. All samples were labeled to be traceable to corresponding individuals.

Extraction:

About 0.5 g of tissue samples were weighted to the Eppendorf vial, 20 ng of IS standard and 1 ml of acetonitrile acidified with 1% of formic acid was added. The samples were homogenized (TissueLyser II, Qiagen,Germany) and extracted using stainless steel ball at 1800 oscillations/ min for 10 min. The extracts were centrifuged at 9500 x g for 10 min. The supernatant was frozen overnight at -20 °C and then filtered through the syringe filter (regenerated cellulose, 0.45 μm pores). An aliquot of 100 μL was taken for LC-MS analysis.

Five ng of verapamil as internal standard was added to 200 μL of plasma in Eppendorf vial. The samples were shaken together with 200 μL of acetonitrile acidified with 1% of formic acid. The extract were handled the same way as tissue samples.

LC-HRMS/HRPS analysis

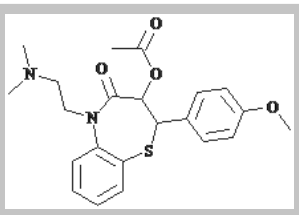
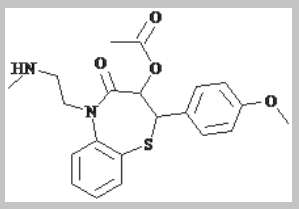
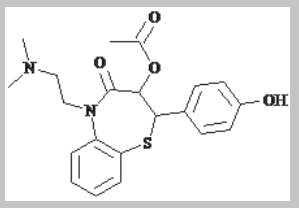
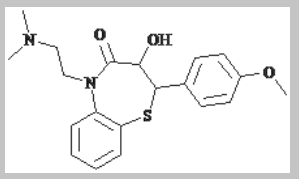
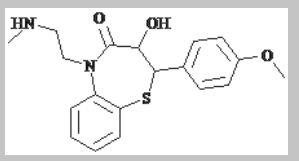
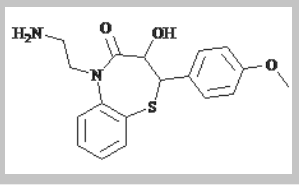
In order to ionize target compounds a heated electrospray ionization (HESI-II) interface was used for both HRMS full scan and HRPS mode. Spray voltage was set up to 3.5 kV. Nitrogen (purity > 99.999%) was used as sheath gas (40 arbitrary units), auxiliary gas (10 arbitrary units) and collision gas. Vaporizer was heated to 250 °C and capillary to 325 °C. There were two methods, full scan HRMS and HRPS (High Resolution Product Scan), applied in current study. General properties of full scan MS were set as follows: resolution 70000 FWHM, AGC target 1e6, maximum filling time 100 ms and scan range from 50 to 550 m/z. MS^2 experiment was performed using the mass inclusion list. Key parameters of HRPS were optimized and used as follows: isolation window at the quadrupole 1 m/z, resolution of orbital trap 17500 FWHM, AGC target 1e6, maximum filling time 30 ms.

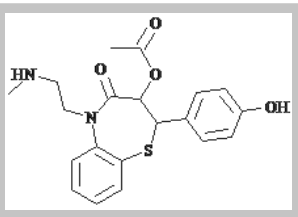
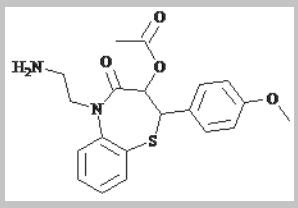
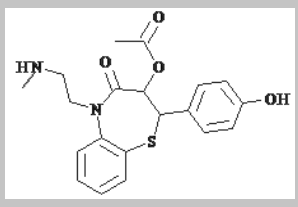
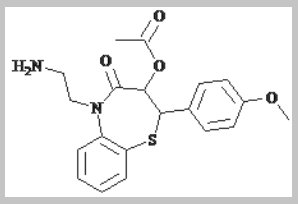
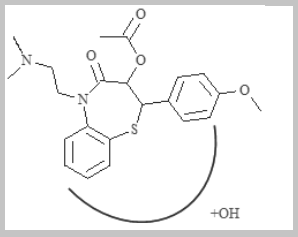
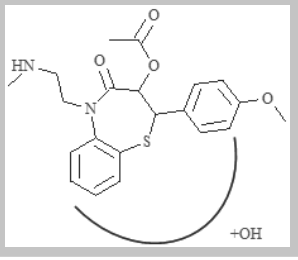
3. Results and discussion

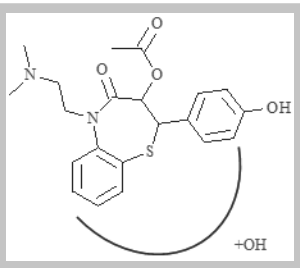
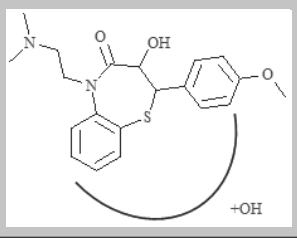
Metabolites detection

Diltiazem was measured as a protonated molecule ($[M+H]^+$) at m/z 415.1686 with retention time (RT) 7.30 min. corresponding to chromatographic gradient presented in the Tab. S1. Based on to the literature research and general principles of drugs metabolism in different living tissues (Zhang et al., 2008; Dasandi et al., 2009; Molden et al., 2003) a list of all prospective metabolites of diltiazem was prepared for further investigation. Generally, products of different kind of drugs biotransformation and decomposition are assumed to be more hydrophilic and they should appear before RT of parent compound under given chromatographic conditions. Targeted screening in the full scan mode has showed all possible metabolites present in the blood plasma, muscle, kidney, and liver of fish. Due to complexity of matrices, full scan data was not satisfactory for further analysis especially of minor metabolites (Fig. S1). Hence, peaks in extracted chromatograms (XIC) corresponding to exact masses of those metabolites (calculated from their element composition), which matched 5 ppm window from exact mass, were selected for the next step of analysis (Tab. 1). Then HRPS mode was applied for further identity confirmation of selected structures. Finally, eight groups of metabolic transformation products were detected (Tab. 1): desmethyl diltiazem (M1), desacetyl diltiazem (M2), desacetyl desmethyl diltiazem (M3), desacetyl didesmethyl diltiazems (M4), didesmethyl diltiazems (M5), hydroxy diltiazems (M6), desmethyl hydroxy diltiazems (M7) and desacetyl hydroxy diltiazems (M8).

Table 1. *Diltiazem and its possible selected metabolites.*

Compound, (metabolite name used in current study)	Formula	Structure	Exact mass (Da)	Measured mass (Da)*	Mass error (mDa)	Mass error (ppm)
Diltiazem	$C_{22}H_{26}N_2O_4S$		415.1686	415.1679	-0.7	1.69
N-desmethyl diltiazem (M1)	$C_{21}H_{24}N_2O_4S$		401.1530	401.1523	-0.7	1.74
O-desmethyl diltiazem	**		**	**	**	**
O-desacetyl diltiazem (M2)	$C_{20}H_{24}N_2O_3S$		373.1580	373.1574	-0.6	1.61
N-desmethyl, *O*-desacetyl diltiazem (M3)	$C_{19}H_{22}N_2O_3S$		359.1424	359.1418	-0.6	1.67
O-desmethyl, *O*-desacetyl diltiazem	**		**	**	**	**

N,*O*-didesmethyl, *O*-desacetyl diltiazem (M4a)	$C_{18}H_{20}N_2O_3S$		345.1267	345.1336	-0.6	1.74
N,*N*-didesmethyl, *O*-desacetyl diltiazem (M4b)	**		**	**	**	**
N,*O*-didesmethyl diltiazem (M5a)	$C_{20}H_{22}N_2O_4S$		387.1373	387.1365	-0.8	2.07
N,*N*-didesmethyl diltiazem (M5b)	**		**	**	**	**
Hydroxy diltiazem (M6a,b,c,d)	$C_{22}H_{26}N_2O_5S$		431.1635	431.1336	0.1	0.23
N-desmethyl, hydroxy Diltiazem (M7a,b,c)	$C_{21}H_{24}N_2O_5S$		417.1479	417.1477	-0.2	0.48

O-desmethyl, hydroxy diltiazem	**		**	**	**	**
O-desacetyl, hydroxy diltiazem (M8a,b,c)	$C_{20}H_{24}N_2O_4S$		389.1530	389.1525	-0.5	1.28

* Diltiazem and its transformation products were measured as $[M + H]^+$ ions
** Isomeric form (all the parameters are written in the previous line)

Identification of metabolites and their isomers

MassFrontier software was used to predict all possible MS^2 products from proposed structures. An approach employing HRPS and results obtained from software allowed to confirm or disprove findings based only on accurate mass measurements.

Due to the same fragmentation pathway of diltiazem (Fig. S2) and its metabolites in collision cell, the major product ion at m/z 178.0320 was present in all compounds of our interest. Less intensive product ions were observed almost in all metabolites (Fig. S2.) also with appearance of minor fragments.

The fragment ions 137.0595, 150.0370, 178.0320, 223.0898, 310.0892, 312.1049, 328.0994, and 370.1104 of diltiazem were identified by the accurate mass measurements with errors of -1.46, 1.33, -0.56, -0.9, -1.29, -1.28, -2.44, and -1.08 ppm (Tab. S2), respectively. This helped us to exclude possibility of interferences and to make their identification easier.

According to the measured data, all metabolites can be divided into three groups: 1) metabolites M1, M2, and M3 appeared as a single peak despite possible existence of isomers for some of them (Tab. 1), 2) only two isomeric forms were found for M4 and M5 because of different mechanism of the second desmethylation, 3) M6, M7, and M8 might have higher number of isomers due to the hydroxylation process which can take place at different sites of diltiazem molecule (Tab. 1).

The M1 and M2 have been most frequently identified and measured in wide range of matrices (Christensen et al., 1999; Dasandi et al., 2009). Metabolites present as single isomers (M1, M2 and M3) were eluted at 7.19, 6.51 and 6.46 min, respectively. All metabolites of this group have the pathway of fragmentation similar to diltiazem (Fig. 1). In this study, according to spectra and proposed fragment structures, M1 and M3 were identified as products of desmethylation of methyl group located at N atom (Fig. 1). Using software and theoretical knowledge based on precise mass shifts of fragments, structures of specific fragments were examined. Specific fragments which helped us to distinguish where desmethylation took place were measured as 209.0739, 219.0647, 326.0840 with errors -1.91, -2.28, -1.53, respectively for M1 and 209.0747, 284.0736, 341.1297 with errors -1.91, -1.41,-6.16, respectively for M3 (Tab. S2). On the other hand, M2 was identified much easier due to matching of measured spectra to acetyl diltiazem spectra available at m/z Cloud database [www.mzcloud.org].

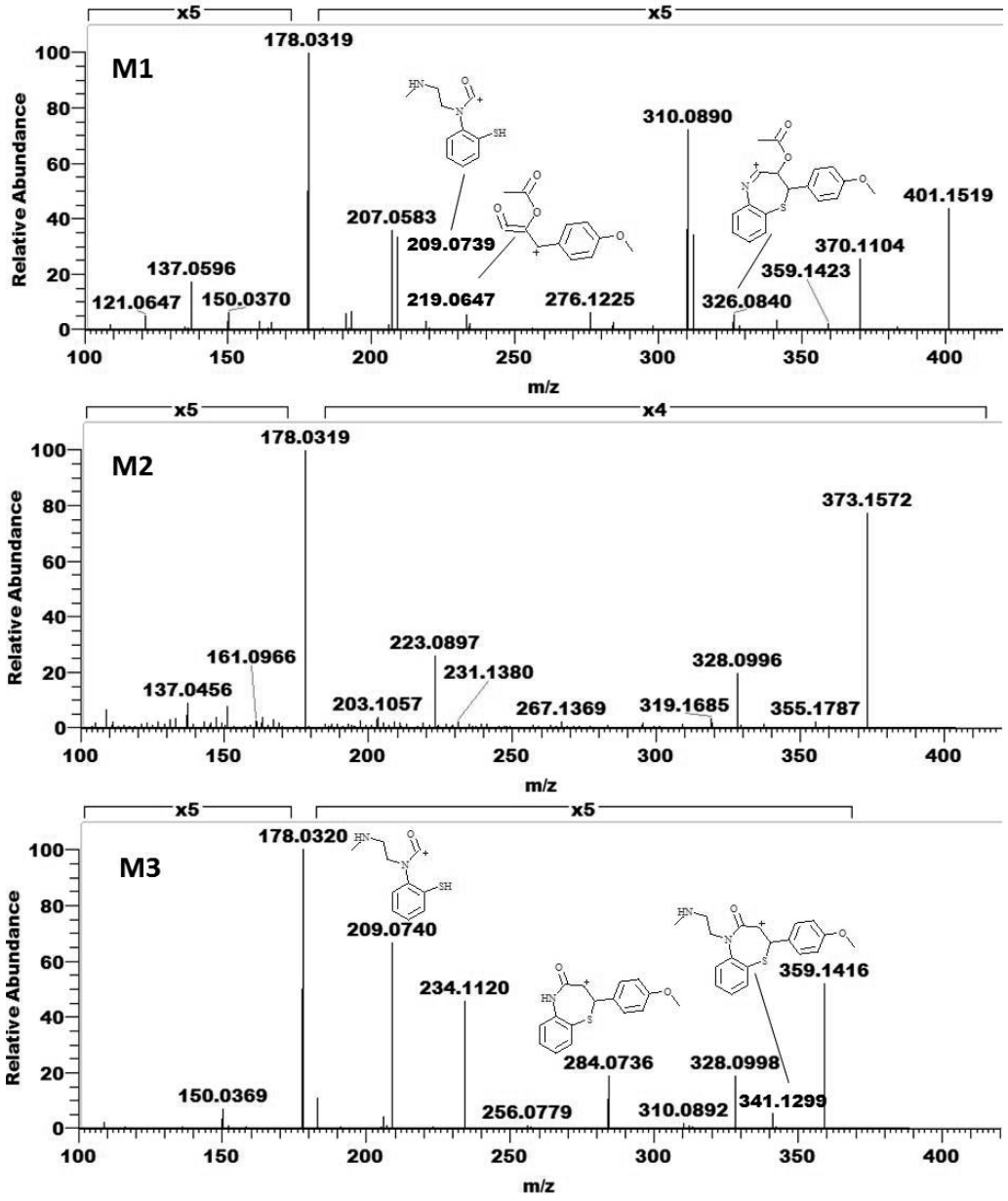


Figure 1. *HRPS spectra of M1–M3: mass range 100–177 was amplified 5 times and 179–410 was amplified four times to improve readiness of the figure.*

M4 and M5 (didesmethyl desacetyl diltiazem and didesmethyl diltiazem) were present in two isomeric forms. Taking into account the structure of diltiazem, second desmethylation might be located at two different sites of the molecule – *N* or *O* atom. Based on possible reaction mechanisms and fragmentation of mentioned compounds, *N*,*O*-didesmethyl and N,N-didesmethyl diltiazem were identified. MS^2 spectra and specific fragments used for identification of both metabolites are given in the Fig. 2–3. Few particular fragments which helped to confirm identity of metabolites were found as follows 209.0747 (1.91 ppm),

314.0838 (-2.23 ppm) for M4a; 195.0589 (1.03 ppm) for M4b; 209.0739 (-1.9 ppm), 296.0735 (-1.69 ppm), 298.0889 (-2.35 ppm), 356.0942 (-2.53 ppm) for M5a and 161.0597 (0 ppm), 195.0587(0 ppm), 220.0966 (-0.91 ppm), 327.1153 (-2.75ppm) for M5b (error values are given in the brackets after each fragment) (Tab. S2).

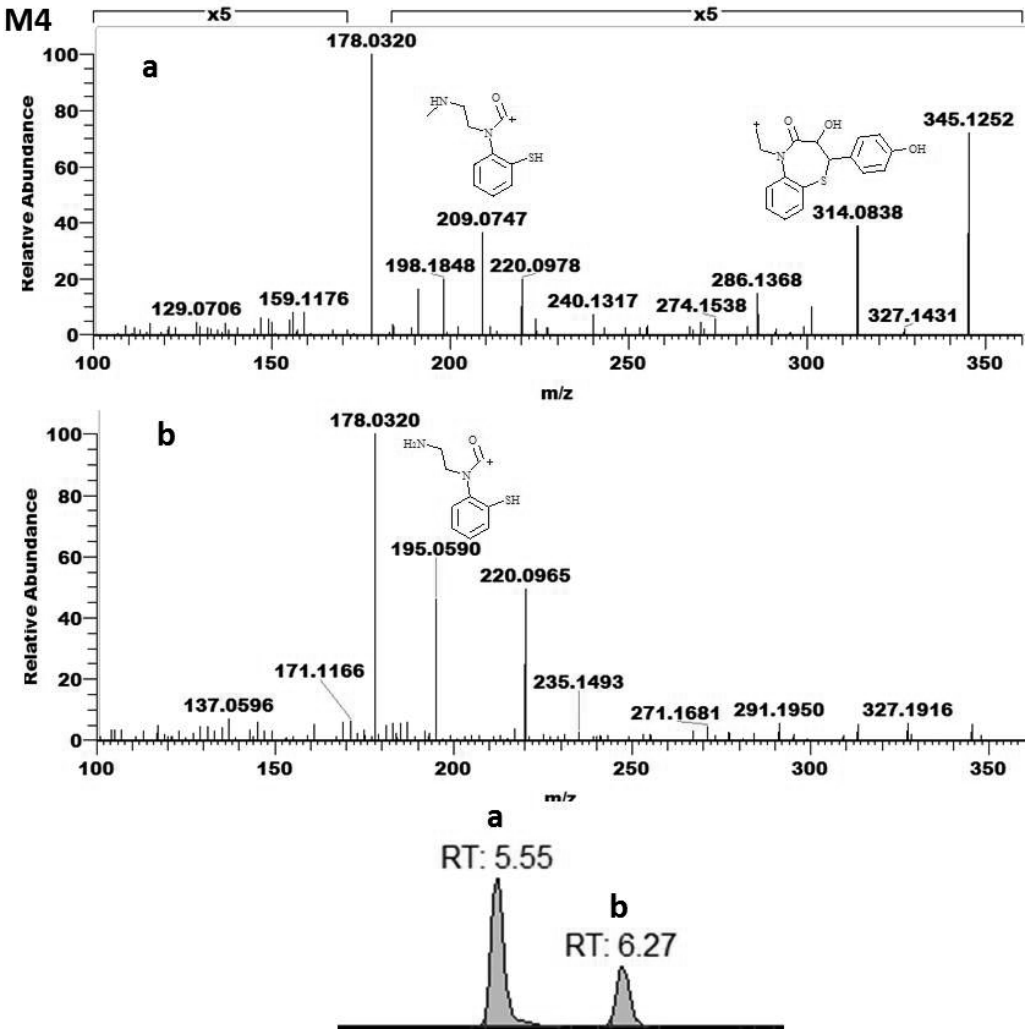


Figure 2. *HRPS spectra of M4 in fish liver (a – N,O-didesmethyl O-desacetyl diltiazem, b – N,N-didesmethyl O-desacetyl diltiazem) mass range 100–177 was amplified 5 times and 179–410 was amplified 5 or two times respectively to improve readiness of the figure.*

Astonishingly, LC behavior of isomers of M4 and M5 under mentioned conditions was identical. Namely, *N*,*O*-isomer (M4a and M5a) was eluted earlier than *N*,*N*-isomer (M4b and M5b).

The importance of correct identification of corresponding isomers can be demonstrated on extreme differences observed among tissue samples of different individuals. The most interesting trends were found in kidney and liver tissue. For example, M4a,b and M5a,b were present in both tissues but amount of M5a was lower than M5b in both mentioned tissues (in

case of kidney, the difference was higher), while amount of M4a in the kidney was much lower than content of M4b contrary to opposite isomer ratio in the liver.

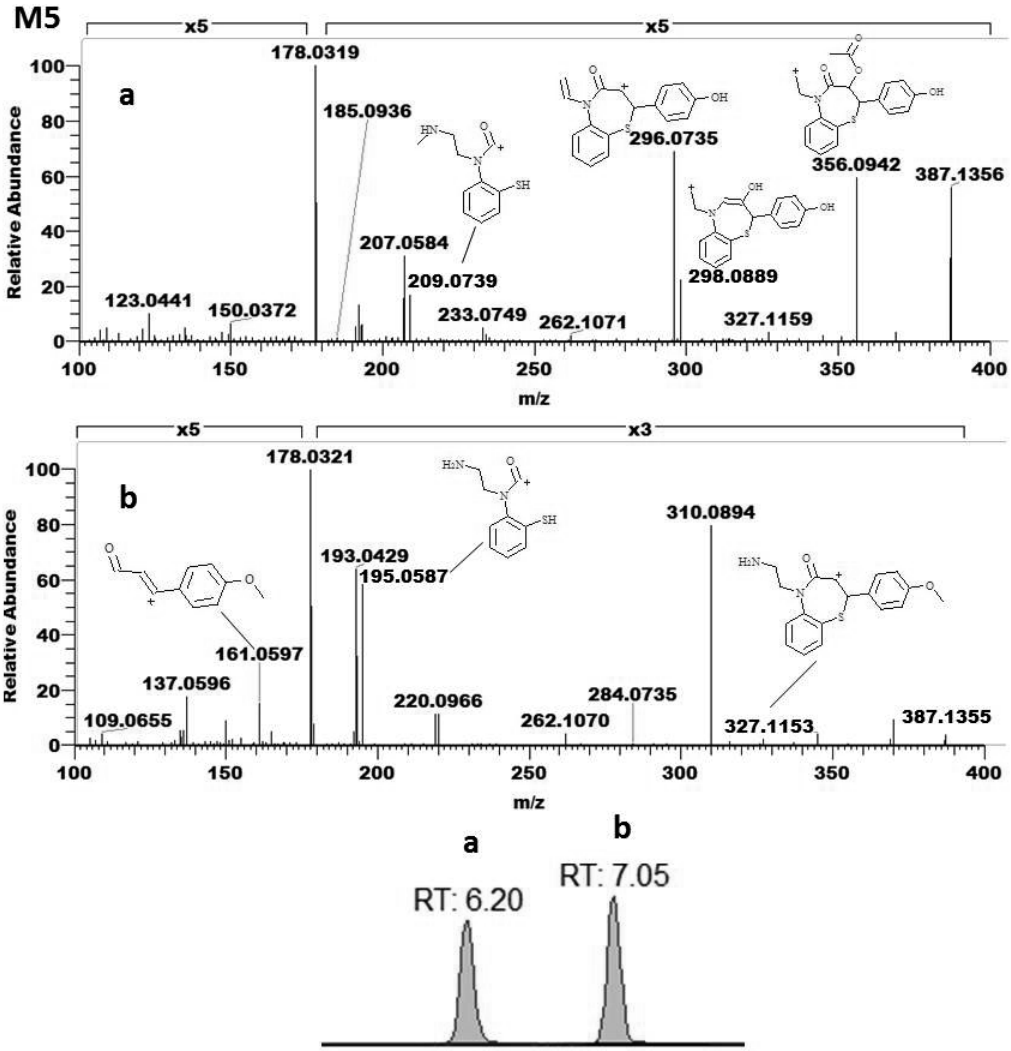


Figure 3. *HRPS spectra of M5 in fish liver (a – N,O-didesmethyl diltiazem, b – N,N-didesmethyl diltiazem) mass range 100–177 was amplified 5 times and 179–410 was amplified 5 or 3 times respectively to improve readiness of the figure.*

Identification of the third group of isomers was the most complex and the most complicated issue. To the best of our knowledge, hydroxy metabolites M6-M8 have never been detected in any kind of biota samples before.

According to position, where hydroxylation takes place (Tab. 1), different specific fragments appear during MS^2 fragmentation. When the hydroxyl group was situated at methoxyphenyl ring (further in article given as first ring), the same HRPS prevailing fragment as for diltiazem, i.e. 178.0320 m/z, was found. Presence of hydroxyl group at core diltiazem structure (further in article given as second ring) leads to generation of new specific fragments 194.0272 (1.03

ppm), 177.0546 (0 ppm), 328.0996 (-2.33), 166.0321 (0 ppm), 219.0653 (0.46 ppm) and 344.0968 (4.94 ppm) (order with intensity decrease; error values are given in the brackets after each fragment) (Tab. S2) as core structure forming 178.0320 is substituted.

Hydroxy diltiazems M6 were found mainly in the liver. Earlier eluted metabolites with RT 5.62 (a) and 5.89 (b) correspond to hydroxylation at the second ring, the rest ones with RT 6.52 (c) and 7.46 (d) are related to hydroxylation at first ring. M6a and M6b were not detected in kidney samples; it might be explained with fast Phase II metabolic biotransformation with subsequent excretion via bile. One more potential isomer (M6e) eluted in HRPS at RT 8.07 min was found just in the liver but it was identified as interference because of completely different fragmentation pattern.

The same depiction was obtained for M7 group. M7 metabolite family was identified as hydroxylation and N-desmethylation products (N-form because of specific fragmentation). There were three isomers (a, b, c) of M7 in the liver tissue eluted at 5.57, 5.76 and 6.38 min, respectively. Specific MS^2 products at 161.0596 (-0.62 ppm), 177.0546 (0 ppm) and 194.0272 (1.03 ppm) (Tab. S2) m/z indicated hydroxylation at second ring for M7a and M7b. M7c had the same fragmentation as diltiazem. It means that hydroxylation of M7c in the liver took place in the first ring. The same isomer pattern as for M6, was found for M7 in the liver, too. Only one metabolite (M7c) was present in the kidney. Another possible isomeric form M7d appeared in all studied tissues but fragmentation pathway was the same as for M6e and it could not be matched with proposed fragmentation mechanisms. Only three of four M7 metabolites found in HRPS were identified as diltiazem products.

Identification of desacetyl hydroxy metabolites (M8) was the most interesting and difficult. Firstly, the highest number (six) of possible isomers M8a, M8b, M8c, M8d, M8e, M8f eluted at 4.17, 4.71, 5.78, 6.11, 6.64 and 7.04 min respectively was detected in the kidney, only five were present in the liver. M8f had the same fragmentation pattern as M6e and M7d and it was immediately excluded from the list of isomers. M8c, M8d and M8e in the kidney had the specific fragment for diltiazem, i.e. 178.0320 m/z, but after checking mass error (ranged from 89 to 254 ppm for different isomers) for precursor ion 389.1530 m/z, M8d and M8e were also excluded as obvious interferences. A different situation was observed for the same isomeric forms in the liver, where M8c was not present, and M8d was matched to hormones spectra according to m/z Cloud. Another prospective metabolite M8e was not possible to identify as compound related to diltiazem at all. M8a and M8b were present in both studied tissues but corresponding HRPS spectra contained both 178.0320 and 194.0272 m/z fragments. According to information given above, this fact means that hydroxyl groups were present in both diltiazem rings simultaneously, what is impossible. Further investigation using predicting software brought an explanation of this observation. M8a and M8b were identified like hydroxylation products at first ring. In case of M8 metabolite group, desacetylation preceding hydroxylation step enabled appearance of the 194.0272 m/z fragment. The fragment, which was further fragmented to 178.0320 m/z, had a specific structure (Fig.4a) and the same element composition as for other cases. A fragment with the same mass 194.0272 m/z was also formed for M6 and M7 metabolites (Fig.6b) which were hydroxylated at the second ring, further fragmentation pathway was different and it did not include 178.0320 m/z.

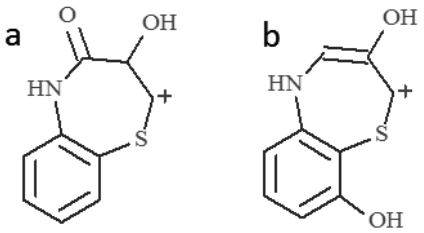


Figure 4. *Mass Frontier predicted structure of 194.0272 m/z fragment for M8 (a), M6, and M7 (b) metabolites families.*

Semi-quantification of metabolites in the fish tissues

As just a few reference standards are commercially available for diltiazem metabolites, we used semi-quantification approach for characterization of differences among tissues. Full scan data did not show high enough selectivity so we based the method on assumption that the signals at transition of protonated molecular ion of metabolites $[M(1-8)-H]^+$ compare to prevailing fragment of 178.0320 m/z have equal intensity. Consequently we used peak area corresponding to above mentioned mass for calculation of metabolites concentration and determination of metabolic rate in the different fish tissues (Fig. 5). Using HRPS allowed us to semi-quantify diltiazem and its metabolites at ng g^{-1} levels.

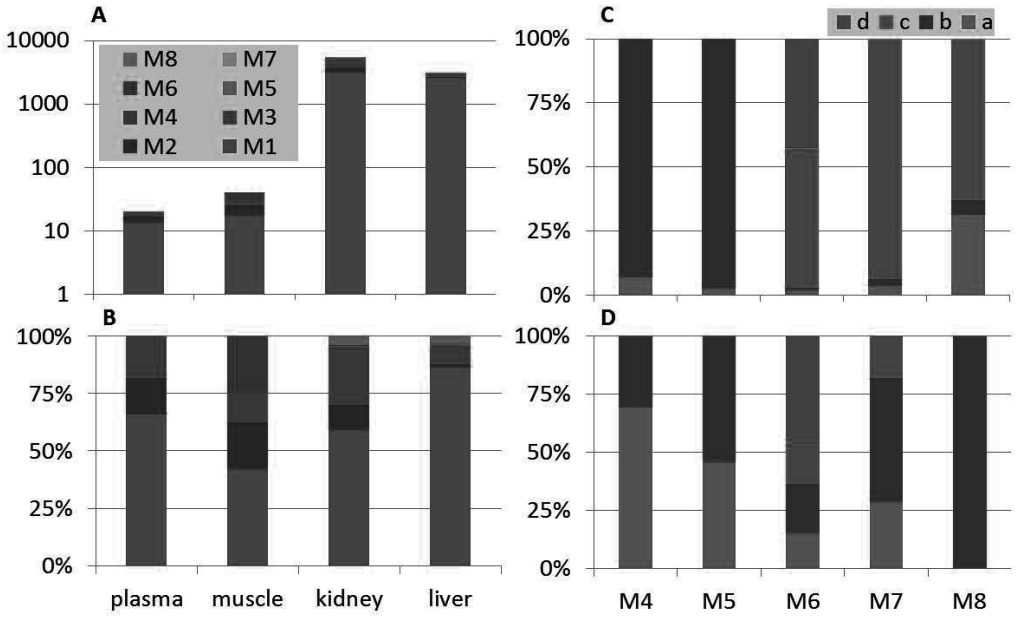


Figure 5. *(A): Metabolites concentrations (in ng g^{-1}, logarithmic scale with base 10); (B): distribution of total amount of metabolites in all studied tissues; (C): isomers composition of diltiazem metabolites in the kidney and (D): isomers composition of diltiazem metabolites in the liver (a, b, c, d are different isomeric forms).*

Based on data showed in the Fig. 5 A, B, plasma and muscle did not contain all possible metabolites. These tissues did not show such a high variability of metabolite isomeric forms (Fig. S1) as kidney and liver did. According to the present study, the highest total concentration of metabolites was present in the kidney but the biggest number of isomeric forms was observed in the liver (Fig. 5).

Biotransformation of diltiazem in fish

Seventeen diltiazem metabolites and their isomeric forms were detected and identified in the present study. In mammals diltiazem undergoes extensive metabolism in hepatic and extrahepatic tissues. *N*-desmethyl diltiazem (M1) and desacetyl diltiazem (M2) are two of the main metabolites of diltiazem that retain pharmacological activity (Fraile et al., 2001). M1 and M2 were also the main metabolites of diltiazem which were detected in fish tissues in the present study.

According to (Molden et al., 2000) CYP2D6 carries out the *O*-desmethylation of diltiazem in mammals. The subfamily of CYP2D is not present in fish (Kirischian et al., 2011). This fact explains why M1 and M3 metabolites were identified as products of desmethylation at *N* atom only.

M4 and M5 are products of double desmethylation and desacetylation which might be explained by the fact that other enzymes than CYP2D6 could be involved in the formation of *N*,*O*-didesmethyl diltiazem in fish. In agreement, Molden et al. (2000) supposed that in addition to CYP2D6 also other enzymes can be involved in the formation of *N*,*O*-didesmethyl diltiazem in human. (Molden et al., 2002a).

M6, M7 and M8, the hydroxylated diltiazem metabolites, were identified in this study for the first time indicating that hydroxylation of diltiazem is fish-specific. Due to the usage of hybrid quadrupole-orbital trap high resolution mass spectrometer QExactive (Thermo Scientific) it was possible to distinguish among metabolite isomers which were products of desmethylation and hydroxylation. Namely, it was possible to determine if the desmethylation or hydroxylation took place at the first or the second ring of diltiazem molecule. To our knowledge, there are no studies available which describe usage of the same advanced instrumentation for diltiazem metabolite detection and identification. The method presented in this study helped to extend knowledge on diltiazem biotransformation processes in fish and might be applied to mammal tissues in the future as well.

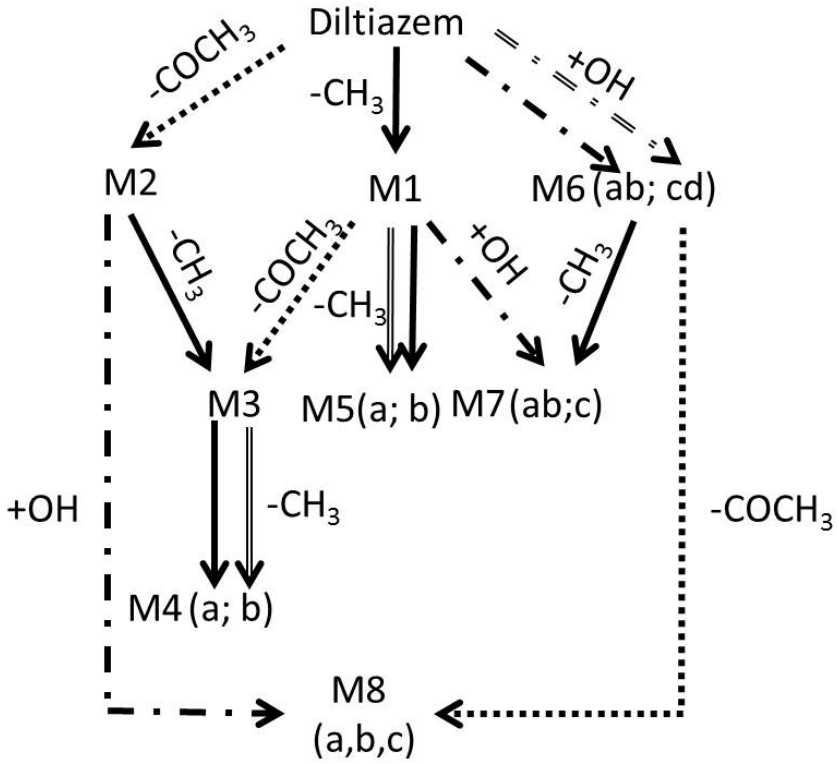


Figure 6. *Isomeric forms of diltiazem metabolites in rainbow trout. Solid line– N-desmethylation; solid line double– O-desmethylation; dotted line– desacetylation; dash dotted line – hydroxylation at the core diltiazem structure, double dash dotted line – hydroxylation at the methoxyphenyl-ring). M1 – N-desmethyl diltiazem; M2 – desacetyl diltiazem; M3 – desacetyl, N-desmethyl diltiazem; M4 – desacetyl, (a – N,O; b – N,N) – didesmethyl diltiazem; M5- (a – N,O; b – N,N)- didesmethyl diltiazem M6 – (ab – core diltiazem structure; cd – methoxyphenyl-ring) – hydroxy diltiazem; M7 – N-desmethyl (ab-core diltiazem structure; c-methoxyphenyl-ring)- hydroxy diltiazem; M8 – desacetyl, hydroxy (a, b, c – hydroxylation products at methoxyphenyl-ring) diltiazem.*

Conclusions

The present study demonstrated a successful application of hybrid quadrupole-orbital trap mass spectrometer Q Exactive and modern spectral interpretation software MassFrontier for the determination of diltiazem metabolites in fish, showing complex metabolic pathways of studied compound. To our knowledge, presented LC-HRPS method is the most sensitive (it was possible to detect metabolites in amount less than 1 ng g^{-1}) and might be used for detection of the high number of metabolites when compared with methods which already exist. The study of organ dependent formation of diltiazem metabolites can be conducted with this method using semi-quantification approach when reference standards are not available. The method was established for fish tissues but further application for mammal tissues is also possible.

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SUPPLEMENT MATERIALS

Table S1. *Gradient for the elution of target compounds*

Time, min.	Mobile phase composition/vol. %		Flow rate, μL min^{-1}
	Water*	ACN*	
0.00	95	5	300
1.00	95	5	300
7.00	60	40	350
11.00	0	100	400
12.00	0	100	400
12.01	95	5	350
15.00	95	5	300

* Constituents acidified with 0.1 vol. % of FA.

Table S2. *Measured fragments of diltiazem metabolites with mass error values.*

Metabolite name	Exact mass (Da)	Measured m/z (Da)	Mass error (mDa)	Mass error (ppm)
Diltiazem	137.0597	137.0595	-0.2	-1.46
	150.0372	150.0370	-0.2	-1.33
	178.0321	178.0320	-0.1	-0.56
	223.0900	223.0898	-0.2	-0.90
	310.0896	310.0892	-0.4	-1.29
	312.1053	312.1049	-0.4	-1.28
	328.1002	328.0994	-0.8	-2.44
	370.1108	370.1104	-0.4	-1.08
M1	209.0743	209.0739	-0.4	-1.91
	219.0652	219.0647	-0.5	-2.28
	326.0845	326.084	-0.5	-1.53
M3	209.0743	209.0740	-0.3	-1.43
	284.0740	284.0736	-0.4	-1.41
	341.1318	341.1299	-1.9	-5.57
M4a	209.0743	209.0747	0.4	1.91
	314.0845	314.0838	-0.7	-2.23
M4b	195.0587	195.0590	0.3	1.54
M5a	209.0743	209.0739	-0.4	-1.91
	296.0740	296.0735	-0.5	-1.69
	298.0896	298.0889	-0.7	-2.35
	356.0951	356.0942	-0.9	-2.53
M5b	161.0597	161.0597	0	0
	195.0587	195.0587	0	0
	220.0968	220.0966	-0.2	-0.91
	327.1162	327.1153	-0.9	-2.75
M6a,b	166.0321	166.0321	0	0
	177.0546	177.0546	0	0
	194.0270	194.0272	0.2	1.03
	219.0652	219.0653	0.1	0.46
	344.0951	344.0968	1.7	4.94
	386.1057	386.1048	-0.9	-2.33
M7a,b	161.0597	161.0596	-0.1	-0.62
	177.0546	177.0546	0	0
	194.0270	194.0272	0.2	1.03

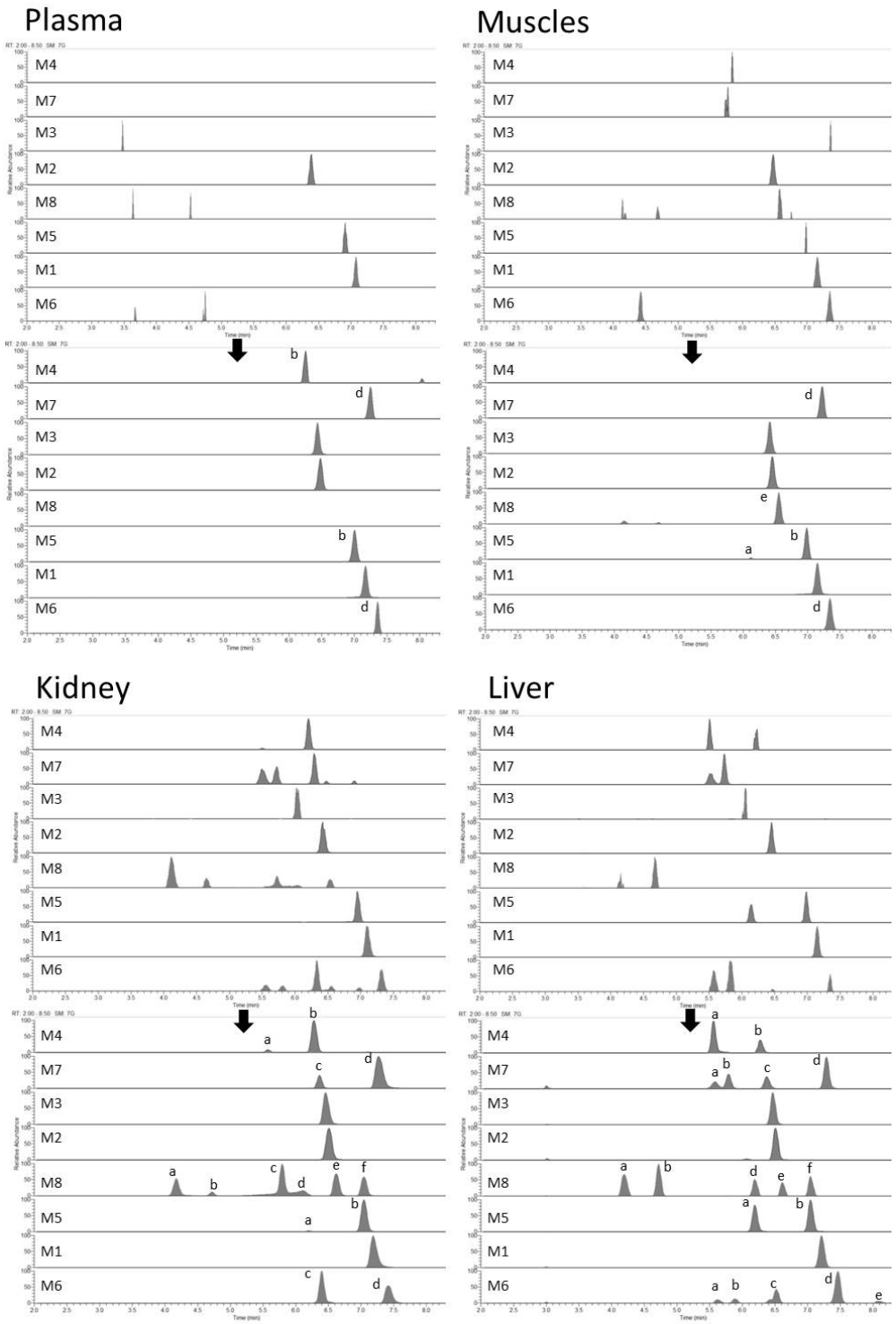

Figure S1. *Comparison between XIC of HRMS measurement, picture above, (5 ppm window) and HRPS data at extracted fragment 178.0320 (10 ppm window) for all studied tissues.*

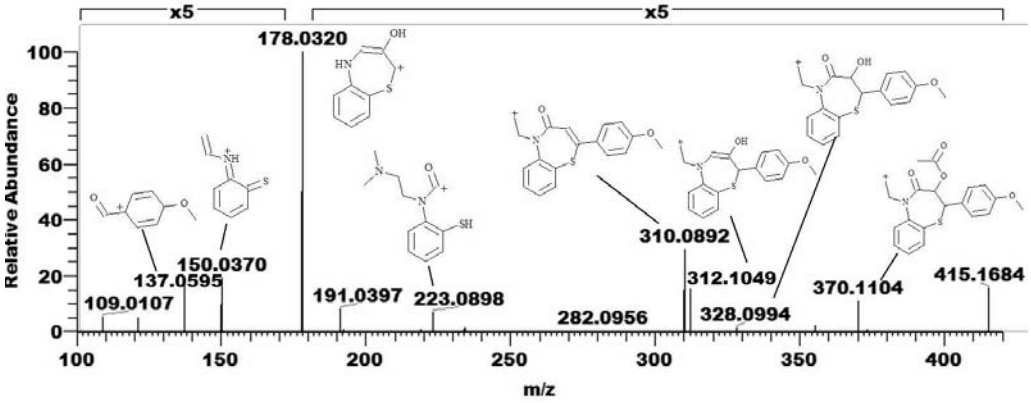

Figure S2. *HRPS spectra of diltiazem with fragment structures (17500 FWHM resolution): intensities of mass range 100–177 was amplified 5 times and 179–410 was amplified 5 or 3 times respectively to improve readiness of the figure.*

CHAPTER 6

HISTOPATHOLOGICAL ALTERATIONS OF THE CARDIOVASCULAR SYSTEM IN FISH – A PROPOSAL FOR THE ASSESSMENT

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HISTOPATHOLOGICAL ALTERATIONS OF THE CARDIOVASCULAR SYSTEM IN FISH – A PROPOSAL FOR THE ASSESSMENT

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ABSTRACT

Histopathological alterations in the heart are reported in fish as a result of exposure to a variety of chemical compounds. However, at present a method for the evaluation of histopathological alterations in the cardiovascular system of fish and the calculation of the organ index is missing. Therefore, we designed a method for a standardised assessment and evaluation of histopathological alterations in the heart of fish. As a model species, rainbow trout (Onchorynchus mykiss) was used, but the protocol was also successfully applied on other fish species belonging to different systematic orders. To test the protocol, we re-evaluated sections of atenolol exposed and unexposed rainbow trout obtained in our previous study. The results were in accordance with those previously published demonstrating the applicability of the protocol. The protocol provides an universal method of the comparative evaluation of histopathological changes in the cardiovascular system of fish.

Keywords: *Cardiovascular system; heart – index; myocard; coronary blood vessels; histopathology*

1. Introduction

Histopathological alterations, defined as structural and functional changes in cells, tissues, and organs have been used for diagnosis of fish diseases and as biomarkers of e.g. stress and exposure to pollution and/or toxins (Feist and Segner, 2013; Haschek et al., 2010; Raskovic et al., 2013). For instance, the histopathological alterations of the fish gonads are used as criteria in the regulatory risk assessment of chemicals (Feist and Segner, 2013).

Mostly, histopathological alterations are presented as semi-quantitative values. However, such data do not allow statistical evaluation of the results. Bernet et al. (1999) developed a protocol for the standardised assessment of histopathological changes in the liver, gill, kidney and skin of fish which employs the "organ-index". This protocol provides a mechanism to compare in more unbiased and objective way the same tissue from different individuals subjected to different treatments. Bernet et al. (1999) selected gills and skin for evaluation because they come into direct contact with pollutants, and liver and kidney due to their function in metabolism and excretion of pollutants. However, structural changes in other organs are widely used to judge effects in toxicological studies as well (Feist and Segner,

2013; Haschek et al., 2010, Raskovic et al., 2013, Marchand et al., 2012, Schmidt et al., 1999, Steinbach 2014). In these other organs, statistical evaluations of histological changes were not possible until now as a standardized scheme for these organs was missing.

The heart is one of the tissues of interest. For example, the study of Steinbach et al. (2014) described histopathological alterations in the heart of rainbow trout (*Onchorynchus mykiss*) that were sub-chronically exposed to atenolol. Another study by Marchand et al. (2012) described pathologic effects of polluted river water in various tissues including the heart of sharptooth catfish (*Clarias gariepinus*) and tilapia (*Oreochromis mossambicus*). With a standardized protocol for the assessment of histological alterations, statistical evaluation would be possible and studies using this scheme could complement each other e.g. to give insights in the cardio-toxicity of selected pollutants.

Therefore, the aim of the present study was to develop and test a unified protocol for the assessment and evaluation of histopathological alterations in the heart ventricle and coronary blood vessels of fish. To test this protocol, samples of the heart of unexposed rainbow trout and those exposed to the β – blocker atenolol (Steinbach et al., 2014) were re-evaluated and assessed according to the protocol. In addition, heart tissues of fish belonging to different families (*Salmonidae, Cyprinidae, Acipenseridae* and *Percidae*) were used to test the adaptability of this protocol.

2. Material and methods

Development of the histological scheme

Based on the histological atlases of Takashima and Hibiya (1995) and Genten et al. (2009), categories describing the anatomy and the histology of the heart ventricle and the coronary blood vessels were defined. The statistical principles are based on the protocol of Bernet et al. (1999).

Application of the histological scheme

The developed protocol, as given in Tab. 1, was applied on heart sections of rainbow trout originating from a previous study (Steinbach et al., 2014). Briefly, fish had been exposed to atenolol at a concentration of 1000 µg/l for 42 days. From these fish and an untreated control group, hearts had been removed and processed for histology. The experimental conditions are described in detail in Steinbach et al. (2014). In order to validate the histological protocol, five sections from each experimental group were re-evaluated. Then the heart index (Equation 1) for the different experimental groups (exposed and unexposed) was calculated.

In addition, the developed protocol was applied on slides obtained from routine diagnostic work provided by the Centre for Fish and Wildlife Health (CFWH, Bern, Switzerland). The sections originated from five species belonging to three different families as follows: Cyprinidae: Zebrafish (*Danio rerio*), and Koi (*Cyprinus carpio* koi); Acipenseridae: Russian sturgeon (*Acipenser gueldenstaedtii*) and Percidae: European perch (*Perca fluviatilis*). From each of the different species one slide was evaluated according to the scheme described.

Statistical analysis

Differences within the organ indices of the exposed fish and the control fish were tested by the statistic programme STATISTICA (version 8 Windows. StarSoft, Czech Republic). The normality and homoscedasticity of the variance of the organ indices were checked by the Kolmogorov-Smirnov test and Bartlett's test, respectively. If the conditions of normal distribution and homogeneity were satisfied, the student T-test was used to determine significant differences between the experimental groups. As a valid significance level, a p value of less than 0.05 was chosen.

3. Development of histological scheme

Histological scheme for the assessment of pathological alterations and calculation of the heart index

The pathological alterations were classified into five reaction patterns (circulatory disturbance, regressive and progressive changes, inflammation and tumour). To each alteration an importance factor was assigned depending on the impact of the alteration on the health condition. These importance factors ranged from 1 - minimal impact, over 2 - moderate pathological importance – to 3 – severe pathological importance (Tab. 1). Then changes in the heart were scored from very mild (1) up to severe (6). For each of the determined alterations, the score value was multiplied with the importance factor resulting in an alteration index. The tissue index was calculated as the sum of the alteration indices of the respective functional tissue units (Equation 1; Tab. 1). The following functional tissue units were evaluated: the coronary blood vessels and capillaries, the epicardium, the endocardium and the myocardium (Fig. 1). Afterwards the heart index was calculated as the sum of the tissue indices (Equation 2). In the supplement we present a detailed calculation of the heart and the tissue index of the atenolol exposed fish and the respective control (Supplement 1).

Equation 1: Tissue index = Σ (score value * importance factor)

Equation 2: Heart index = Σ (Tissue indices)

Table 1. *Protocol for the assessment of histopathological alteration in the heart of fish. The importance factor (I) is ranging from 1 to 3.*

Functional unit of the tissue		Reaction pattern	Alteration	I
Coronary vessels	**Endothelial cells**	Circulatory changes	Hyperaemia	1
			Aneurysm	1
			Thrombus	2
			Intracellular edema	1
		Regressive changes	Architectural and structural alterations	1
			Plasma alterations	1
			Nuclear alterations	1
			Atrophy	2
			Necrosis	3
			Rupture if the endothelial cells	1
		Progressive changes	Hypertrophy	1
			Hyperplasia	2
		Tumour	Benign tumour	2
			Malignant tumour	3
	Basement membrane	Circulatory changes	Haemorrhage	1
			Intracellular edema	1
		Regressive changes	Architectural and structural alterations	1

Functional unit of the tissue		Reaction pattern	Alteration	I
			Plasma alterations	1
			Deposits	1
			Nuclear alterations	2
			Atrophy	2
			Necrosis	3
			Rupture of the basement membrane	1
		Progressive changes	Hypertrophy	1
			Hyperplasia	2
		Inflammation	Exudate	1
			Infiltration	2
	Connective tissue (*Tunica adventitia*)	Circulatory changes	Haemorrhage	1
			Intracellular edema	1
		Regressive changes	Architectural and structural alterations	1
			Plasma alterations	1
			Deposits	1
			Nuclear alterations	2
			Atrophy	2
			Necrosis	3
		Progressive changes	Hypertrophy	1
			Hyperplasia	2
		Inflammation	Exudate	1
			Infiltration	2
		Tumour	Benign tumour	2
			Malignant tumour	3
Coronary	**Endothelia cells**	Circulatory changes	Hyperaemia	1
capillary			Thrombus	2
			Intracellular edema	1
		Regressive changes	Architectural and structural alterations	1
			Plasma alterations	1
			Nuclear alterations	2
			Atrophy	2
			Necrosis	3
			Rupture of the endothelia cells	1
		Progressive changes	Hypertrophy	1
			Hyperplasia	2

Functional unit of the tissue		Reaction pattern	Alteration	I
		Tumour	Benign tumour	2
			Malignant tumour	3
	Basement membrane	Circulatory changes	Intracellular edema	1
		Regressive changes	Architectural and structural alterations	1
			Plasma alterations	1
			Nuclear alterations	2
			Atrophy	2
			Necrosis	3
		Progressive changes	Hypertrophy	1
			Hyperplasia	2
		Tumour	Benign tumour	2
			Malignant tumour	3
			Rupture of the basement membrane	1
	Pericytes	Circulatory changes	Intracellular edema	1
		Regressive changes	Architectural and structural alterations	1
			Plasma alterations	1
			Nuclear alterations	2
			Atrophy	2
			Necrosis	3
		Progressive changes	Hypertrophy	1
			Hyperplasia	2
		Tumour	Benign tumour	2
			Malignant tumour	3
Epicardium	**Epithelial cells**	Circulatory changes	Haemorrhage	1
			Intracellular edema	1
		Regressive changes	Architectural and structural alterations	1
			Plasma alterations	1
			Deposits	1
			Nuclear alterations	2
			Atrophy	2
			Necrosis	3
		Progressive changes	Hypertrophy	1
			Hyperplasia	2
		Inflammation	Exudate	1
			Infiltration	2

Functional unit of the tissue		Reaction pattern	Alteration	I
		Tumour	Benign tumour	2
			Malignant tumour	3
	Mesothelium	Circulatory changes	Haemorrhage	1
			Intracellular edema	1
		Regressive changes	Architectural and structural alterations	1
			Plasma alterations	1
			Deposits	1
			Nuclear alterations	2
			Atrophy	2
			Necrosis	3
		Progressive changes	Hypertrophy	1
			Hyperplasia	2
		Inflammation	Exudate	1
			Infiltration	2
		Tumour	Benign tumour	2
			Malignant tumour	3
	Connective tissue	Circulatory changes		1
			Intracellular edema	1
		Regressive changes	Architectural and structural alterations	1
			Plasma alterations	1
			Deposits	1
			Nuclear alterations	2
			Atrophy	2
			Necrosis	3
		Progressive changes	Hypertrophy	1
			Hyperplasia	2
		Inflammation	Exudate	1
			Infiltration	2
		Tumour	Benign tumour	2
			Malignant tumour	3
Myocardium	**Cardiomyocytes**	Circulatory changes	Haemorrhage	1
			Intracellular edema	1
		Regressive changes	Architectural and structural alterations	1
			Plasma alterations	1
			Deposits	1
			Nuclear alterations	2
			Atrophy	2

Functional unit of the tissue		Reaction pattern	Alteration	I
			Necrosis	3
		Progressive changes	Hypertrophy	1
			Hyperplasia	2
		Inflammation	Exudate	1
			Infiltration	2
		Tumour	Benign tumour	2
			Malignant tumour	3
	Fibroelastic tissue	Circulatory changes	Haemorrhage	1
		Regressive changes	Deposits	1
		Progressive changes	Hypertrophy	1
			Hyperplasia	2
		Inflammation	Exudate	1
			Infiltration	2
		Tumour	Benign tumour	2
			Malignant tumour	3
Endocardium	**Endothelia cells**	Circulatory changes	Congestion	1
			Intracellular edema	1
		Regressive changes	Architectural and structural alterations	1
			Plasma alterations	1
			Deposits	1
			Nuclear alterations	2
			Atrophy	2
			Necrosis	3
		Progressive changes	Hypertrophy	1
			Hyperplasia	2
		Tumour	Benign tumour	2
			Malignant tumour	3

4. Evalution of the protocol

To test the applicability, the scheme was applied on heart sections of the study of Steinbach et al. (2014) (Fig. 1). The alterations within the functional units in fish exposed to atenolol and control fish are listed below. The respective groups the tissue indices were calculated based on the heart scheme for each of the functional units. Then, based on the heart scheme, the heart indices for both groups were calculated. The calculations of the tissue indices and the heart index are given in detail in Supplement 1.

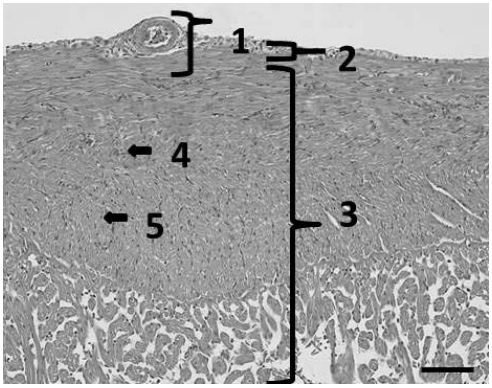

Figure 1. *Anatomic structure of the heart ventricle of rainbow trout (Oncorhynchus myciss). 1 = coronary blood vessel, 2 = epicardium, 3 = myocardium, 4 = capillary and 5 = endocardium. H&E staining; scale bar = 50 µm.*

Determined histopathological alterations in the heart in rainbow trout

The coronary blood vessels and capillaries

The coronary blood vessels in the control and exposed fish had a tissue index of 0.8 ± 1.4 and 8.6 ± 2.7, respectively (Supplement 1). In both groups, the membranes of endothelial cells as well as the basement membrane (*Tunica media*) displayed ruptures. In addition, in control and exposed fish intercellular edema were found as well as signs of an inflammatory reaction; this was defined as an infiltration with lymphocytes and macrophages (Fig. 2a). The connective tissue (*Tunica adventitia*) in both groups showed intercellular edema and infiltration with the same inflammatory cells (Fig. 2a). The capillaries were not altered and showed no alterations of the endothelial cells, the basement membrane or the pericytes.

Epicardium (Lamina visceralis pericardii)

The tissue index for the epicardium was estimated in control and exposed fish at 1.3 ± 1.6 and 8.6 ± 4.7, respectively (Supplement 1). The epithelial cells and the mesothelium of the epicardium in exposed animals were haemorrhagic and in the control and exposed fish, the epithelial cells and the mesothelium were inflamed by lymphocytes and macrophages (Fig. 2a). The connective tissue of exposed animals also showed signs of an inflammatory reaction and was found to be oedematous.

Myocardium

The tissue index in the myocardium in exposed fish was 1.6 ± 0.6 but the control fish presented no alterations (tissue index 0; Supplement 1). The cardiomyocytes of exposed animals were oedematous (Fig. 2b) and presented signs of an inflammatory reaction (Fig. 2b). No alterations were found in the fibroelastic tissue.

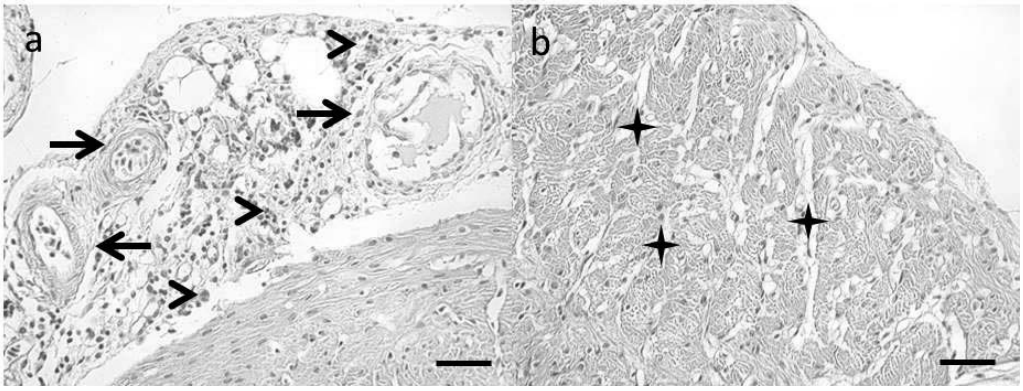

Figure 2. *Histopathological findings in the heart of rainbow trout (Oncorhynchus mykiss) exposed to 1000 µg/l atenolol. a. The exposed fish showed an epicardial infiltration with lymphocytes, plasma cells, and macrophages (arrowheads) and degeneration of the coronary blood vessel (arrows). b. The exposed fish presented an intracellular vacuolization of the myocardium (oedematous) (star). H&E staining; scale bar = 50 µm.*

Endocardium

The endothelia cells did not present any signs of histopathological alterations in any group (Supplement 1).

Heart index

The histopathological alterations found in fish exposed to atenolol and control fish, as well as the calculations of the heart – indices, are presented in Supplement 1. The fish exposed to atenolol had a significantly higher organ index when compared to the control group ($p < 0.05$) (Fig. 3).

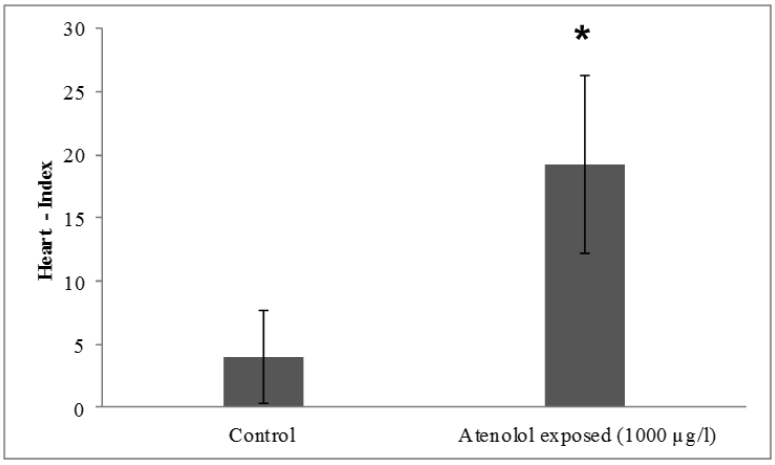

Figure 3. *Heart index of rainbow trout (Oncorhynchus mykiss) from control group and group exposed to 1000 µg/l atenolol. The asterisks indicate significant difference compared to the control: * $p < 0.05$ (t-test).*

The heart protocol applied on fish belonging to the orders of Cyprinidae, Acipenseriformes and Percidae

The scheme was tested on *sturgeon, percid* and *cyprinid* fishes using slides from a previous diagnostic study; these were re-evaluated according to the present protocol. All functional tissue units in heart of those fish species could be assessed and a heart index could be calculated. This heart index was in agreement with the qualitative assessment done by the CFWH. Therefore, this protocol was found to be applicable to species other than rainbow trout. Differences from the rainbow trout model were increased blood content in coronary vessels in small fish species, like zebrafish (Fig. 4a) or perch (Fig. 4b), high amounts of epicardial lipid tissue in koi carp (Fig. 4c) and subepicardial haematopoietic tissue in sturgeon (Fig. 4d). These species-specific features were not included into the heart protocol so as to keep it more widely applicable.

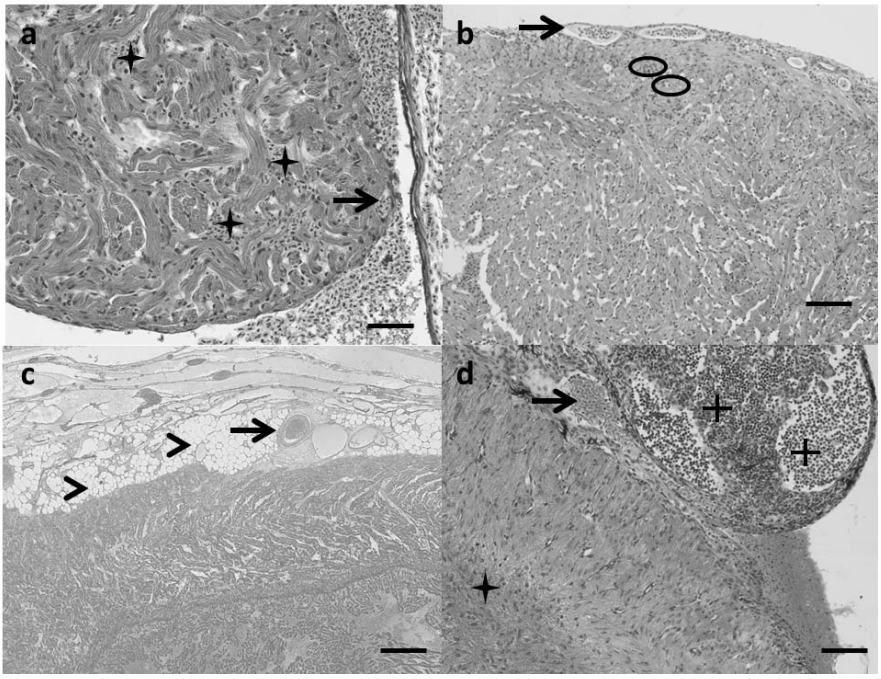

Figure 4. *Anatomic structure of the hearts of representatives of cyprinid, percid and sturgeon fish. a – In the juvenile zebrafish (Danio rerio) the ventricle is filled by blood (star), the coronary blood vessel is indicated by arrow. b – In the European perch (Perca fluviatilis) the ventricle is party filled by remaining blood (star), with well pronounced coronary blood vessels (arrows) and the capillaries in the myocardia (circles). c – In koi carp (Cyprinus carpio koi) the lipid tissue in the epicardium (arrow head) is partly embedding the coronary blood vessel (arrow). d – The heart of the Russian sturgeon (Acipenser gueldenstaedtii) is characterised by the subepicardial haematopoietic tissue (cross) close to the coronary blood vessels (arrow). H&E staining; scale bars = 50 μm (a, d) and 100 μm (b, c).*

5. Discussion

Histopathological alterations in the heart of fish can be caused by various compounds like heavy metals, pharmaceuticals and pesticides (Mohamed, 2008; Steinbach et al., 2014, Magar and Dube, 2013). Because of the paucity of a standardised evaluation method, these alterations had to be presented in a qualitative way without possibility of statistical evaluation (Mohamed, 2008; Steinbach et al., 2014; Magar and Dube, 2013).

In the present work a protocol was developed for the quantitative standardised assessment of histopathological changes in the heart ventricle and coronary blood vessels in fish employing a heart index. Histopathological alterations in the heart of atenolol-exposed rainbow trout were assessed and quantified. Heart indices calculated in the present study for atenolol-exposed and the control fish were significantly different which was well in line with the results of the previous qualitative assessment (Steinbach et al., 2014). Thus, our protocol was found to be well applicable for assessing histopathological alterations in the heart of rainbow trout. In the present study the calculation of the heart – index was based on the calculation of the indices of each functional unit. In addition, these indices can be used as indicators of damage at the level of the functional unit and allow an easy localisation of the alterations within the tissue.

In addition to rainbow trout which is used in toxicological research as a model species (OECD, 2012), our protocol was successfully applied on zebrafish, another model species (OECD, 2012), as well as on several other fish species belonging to different systematic families. However, special anatomic features, such as the haematopoietic tissue of sturgeons, were not included in the protocol as we wanted to avoid generating a species-specific index that would not be comparable to those of other species. Species-specific patterns should be reported separately and not included into the heart index.

We also did not include histopathological alterations of the bulbus arteriosus and the valves into the protocol as we wanted to determine mainly alterations in the myocardial and vascular tissue. Not including the bulbus arteriosus and the valves also makes the heart protocol easier to use, because in case of small fish, heart valves are often not visible, or only partly included on the histological sections and the bulbus arteriosus shows high species-specific differences in anatomy (Poppe and Ferguson, 2006). Thus, inclusion of these structures would prevent comparing data from different fish species.

Moreover, alterations in the lipid content of the epicardium were not included in the protocol, because it is an effect of nutrition rather than a "classic" histopathological alteration, thus it also has to be reported separately. In case of a tumour, we recommend that it should be classified according to the WHO classification developed for mammals (Slayter et al., 1994) and to determine the cells of origin. For example, the haemangioma is found as one typical neoplasm of the vascular system and can be induced by aniline compounds, vinyl toluene and 1,3-butadiene in mammals (Haschek et al., 2010).

Unlike the heart in mammals, the heart of adult fish possesses a capability of regeneration (Itou et al., 2014). This regeneration is similar to the skeletal muscle regeneration pattern in mammals (McGavin et al., 2001) and is indicated by an increased number of nuclei per myocyte and a hyperplasia of cardiomyocytes (Takashima and Hibiya, 1995). Therefore, a qualitative description of the changes is still necessary to distinguish e.g. between degenerative or regenerative changes possibly showing similar indices. To overcome this dilemma, the indices for example, regenerative and progressive changes, are reported independently, allowing a further classification of the determined processes.

As gross pathological alterations of the heart are important indicators (Roberts, 2012), we recommend reporting them in addition to the heart index.

6. Conclusion

In conclusion, our unified protocol for the quantitative assessment of histopathological alterations on the heart ventricle and coronary blood vessels employing heart index calculation was found to be applicable for several fish species. In addition, this protocol enables a quantifiable evaluation of the alterations within the functional units of the ventricle and coronary blood vessels (coronary blood vessels and capillaries, the epicardium, the endocardium and the myocardium). Furthermore, to enhance the power of this model we recommend reporting gross morphological changes, alterations in the bulbus arteriosus and the valves, alterations in lipid content, signs of regeneration, tumour classification and species specific patterns like alterations in haematopoietic tissue separately. For the future, our protocol offers the possibility of a comparison between different studies and allows a better quantification of dose dependencies in the assessment of toxic effects.

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CHAPTER 7

7.1. GENERAL DISCUSSION

Bioconcentration

Bioconcentration properties of a substance can be described by the bioconcentration factor (BCF), which is defined as the ratio of the concentration of the substance in the fish to the concentration in the water (OECD, 1996; Mackay and Fraser, 2000; Springer et al., 2008).

The BCF of verapamil in whole fish homogenates ranged between 6.6 and 16.6. To the best of our knowledge, there are, except for blood plasma, no other experimental data available on BCF of verapamil in fish tissues or fish homogenates. Predicted BCF for whole fish is 3.2 which is in agreement with the experimental values of the present work. Fick et al. (2010a) reported that the plasma of rainbow trout exposed to waste water containing verapamil had a BCF ranging from < 33 to 175, whereas the predicted BCF value for the plasma of fish was 40 (based on log Kow = 3.4; Fick et al., 2010b). The BCF determined in the present work is lower, but follows the same order of magnitude. Although the plasma BCF seems to be low, verapamil could reach dangerous levels in fish plasma. In accordance with "fish plasma model" proposed by Huggett et al. (2003), Fick et al. (2010b) calculated critical environmental concentrations (CECs) of verapamil causing fish plasma concentration equal to the therapeutic human plasma level (10 ng/ml; Schulz and Schmoldt, 2003). Based on predicted and measured blood BCFs, they estimated CECs at 230 and 53 ng/l, respectively (Fick et al., 2010b). Therefore, both similar pharmacological effect of verapamil in man and fish and relatively low critical environmental concentrations indicate a high risk of verapamil to fish.

The BCFs of diltiazem in kidney, liver, muscle, and blood plasma of rainbow trout ranged from 0.5 to 194. The predicted BCF of diltiazem in whole fish homogenates calculated by EPI Suite™ (v. 4.11, US EPA, 2012), based on its log K_{ow} (2.7), was 28. In agreement with the modelled BCF, Wang and Gardinali (2013) reported a BCF of 16 for diltiazem in mosquito fish homogenates. Considering the range of BCF values calculated for different tissues in the present work, BCF in fish homogenates ought to have differed by approximately an order of magnitude from the predicted value and that calculated for eastern mosquito fish by Wang and Gardinali (2013). In the present work, the BCF values of diltiazem in rainbow trout followed the order kidney > liver > muscle > plasma. This is in agreement with Ramirez et al. (2009) who reported higher diltiazem concentration in liver of wild fish compared to that in muscle. To the best of our knowledge, no data is available on the level of diltiazem in kidney of fish for comparison. In plasma, diltiazem was detected only in fish exposed to the highest tested concentration (30 μg/l), with the BCF ranging from 0.5 to 0.98. However, Fick et al. (2010a) reported a BCF of 24 in the blood plasma of rainbow trout exposed for 2 weeks to treated waste water with diltiazem concentration as low as 0.037 μg/l. The theoretical plasma BCF of 14 calculated by Fick et al. (2010a) according to Hugget's blood plasma model was also higher than the BCF found in the present work. This could be attributed to the rapid metabolic turnover of diltiazem in liver. In the study of Fick et al. (2010a), fish were not fed during the experimental period of 14 days, and nutritional deficiencies might have led to lower rates of xenobiotic metabolism (Yang et al., 1992) compared to that in the present work. Moreover, the fish were exposed to sewage effluent containing a cocktail of pharmaceuticals, including a chemosensitizer, verapamil (Kurelec, 1997). Regarding the Hugget's blood plasma model, it does not take into account metabolism of the target substance, an important factor affecting final concentration of the substance in plasma.

Due to the low lipophilicity (log K_{ow} = 0.16; EPI Suite™ v4.11 – US EPA, 2012) atenolol is expected to be present mainly in the water phase. Consequently the uptake via the gills in fish

can be hypothesised with only negligible contribution of uptake via food (Owen et al., 2007). For the same reason, atenolol is not expected to accumulate to a high extent in fish bodies. In accordance with this assumption, the BCFs of atenolol in different rainbow trout tissues calculated in the present work were very low, ranging from 0 to 0.27. To the best of our knowledge, this work provides the first demonstration of the tissue-specific bioconcentration of atenolol in fish after a long-term exposure. In western mosquitofish that were exposed for 96 h to atenolol at concentrations of 100 and 1000 µg/l, the BCFs in the whole fish homogenates were estimated to be 0.13 and 0.08, respectively (Valdes et al., 2014). These values are comparable to those calculated for rainbow trout tissues in the present work.

In summary none of the tested calcium channel blockers or the tested β-blocker can be classified, based on their BCFs, as hazardous or bioaccumulative compound (BCF ≥ 2000; Brooks et al., 2012).

Half-life time

The biological half-life (t_{50}) is the time taken for an organism to eliminate half of the toxicant content from its body or tissues. This time displays the persistence of a substance in the organism or tissue (Wright et al., 2002). In addition, the half–life of pharmaceuticals in the human blood plasma is well known and can be compared to those estimated for fish (Regenthal et al., 1999). Unfortunately, there is a limited number of studies describing the half-life of β-blockers and calcium channel blockers in fish (Wang and Gardianli 2013).

The half-life of verapamil in carp larvae was estimated to be 10.2 days, which is much longer than the half-life in humans (4.5–12 h; Wood et al., 1999). Similar results were obtained e.g., for antidepressant fluoxetine (Paterson and Metcalfe, 2008), which had a half-life three times greater when exposed in medaka compared to mammalian species. The longer half-life of verapamil in fish compared to humans implies the existence of differences in the biotransformation and/or excretion of the substance between these species.

The half-life calculated for diltiazem in various tissues of rainbow trout after a long-term exposure ranged from 1.5 to 49 h in the present work. Tissue-specific differences in lipid content are not considered to affect the bioconcentration and half-life of diltiazem, since it is an ionisable (pKa= 8.94) and moderately hydrophobic compound (K_{OW} =2.7; Ramirez et al., 2009, EPI SuiteTM v4.11, US EPA, 2012). The relatively high concentration and short half-life of diltiazem in trout liver indicates a high metabolic turnover. Compared to the other analysed tissue types, liver exhibited the highest content of diltiazem metabolites during the exposure phase. The high BCF and longer half-life of diltiazem in the kidney could be attributed to glomerular filtration, renal tubular secretion, and/or reabsorption. A longer half-life associated with reduced metabolic turnover of diltiazem was determined in muscle when compared to liver and kidney, suggesting muscle tissue as a diltiazem deposit in fish.

The half-life of atenolol was not studied in the present work, because of its very low bioconcentration.

Metabolites of diltiazem

At present, metabolites of cardiovascular pharmaceuticals in the aquatic environment and their effect as well as the biotransformation in fish is quite a "new" field in aquatic toxicology. The reason for this is a paucity of adequate methods for the determination of these compounds and their metabolites. Therefore, only few studies have described metabolites of cardiovascular pharmaceuticals in fish (Alderton et al., 2010; Lahti et al., 2011). However, studying these metabolites in fish can provide valuable information on the toxicological profile

of the compound of interest, since the metabolism or biotransformation of a xenobiotic can dramatically alter its distribution and action. This can subsequently lead to detoxification and excretion, or to bioactivation and toxicity (Haschek et al., 2010). In addition, the metabolites of a compound can indicate the pathways of its biotransformation (Molden et al., 2002). Furthermore, metabolites of pharmaceuticals are mostly well studied in mammals, allowing an easy comparative approach if studied in fish.

The present work described a method for the determination of diltiazem phase-I metabolites and their isoforms in rainbow trout. These compounds were identified by their specific fragmentation patterns using the hybrid quadrupole-orbital trap mass spectrometer. Diltiazem was transformed into 8 groups of metabolites with 17 different isoforms in rainbow trout. In fish the biotransformation of diltiazem involved desmethylation, desacetylation and hydroxylation and combinations of these pathways. Diltiazem was metabolized preferentially into *N*-desmethyl diltiazem, desacetyl diltiazem and *N*-desmethyl desacetyl diltiazem, which suggests desmethylation and desacetylation as major metabolic pathways. Similarly, *N*-desmethyl diltiazem, desacetyl diltiazem and *N*-desmethyl desacetyl diltiazem are found in human blood plasma (Dasandi et al., 2009; Christensen et al., 2010). In fish, *N*-desmethyl diltiazem was exclusively found while *O*-desmethyl diltiazem was not revealed. Consequently, both isoforms were found for didesmethyl diltiazem, indicating that the *N*-desmethylation is followed by the *O*-desmethylation. *N*,*N*-didesmethyl and *N*,*O*- didesmethyl diltiazem are formed in fish as well as in the human body (Molden et al., 2002). In mammals, the *N*-desmethylation of diltiazem is carried out by CYP3A4 (Molden et al., 2002), while its *O*-desmethylation is carried out by CYP2D6 (Sutton et al., 1997; Molden et al., 2000; Molden et al., 2002). The subfamily of CYP2D is not present in fish (Kirischian et al., 2011), indicating that other enzymes than CYP2D were involved in the *O*-desmethylation of diltiazem in fish. In agreement, Molden et al. (2000) supposed that in addition to CYP2D6 other enzymes can be involved in the formation of *N*,*O*-didesmethyl diltiazem in human.

As in mammals (Molden et al., 2000), desacetylation was the second major step observed in diltiazem metabolism in rainbow trout in the present work. The desacetylation of diltiazem in mammals is carried out by esterases mainly in the intestines, and CYPs are not involved (Homsy et al., 1995; Molden et al., 2002a; Molina et al., 2007). Therefore, desacetyl metabolites of diltiazem indicated extra-hepatic metabolism of diltiazem and the presence of these esterases in fish.

In fish, hydroxylation was involved, even if to a minor extent in diltiazem metabolism. To the best of our knowledge, there is no information available in the literature describing the presence of hydroxyl diltiazem in humans or other mammals, indicating that this metabolite is fish-specific.

As in this study, Alderton et al. (2010), when studying the metabolic profile of verapamil, a representative of calcium channel blockers, found considerable overlap between fish and humans.

In the present work, a reduced activity of the CYP3A isoform was found in fish exposed to the highest tested concentration of diltiazem (30 µg/l). Excess of diltiazem and its metabolites *N*-desmethyl and *N,N*-didesmethyl diltiazem, which were also detected in the present work, are known to inhibit the activity of CYP3A4 in humans (Sutton et al., 1997; Zhao et al., 2007).

Effects in fish similar to mode of action in human body

Human pharmaceuticals can affect fish by interacting with evolutionarily well conserved drug targets (Gunnarsson et al., 2008; Corcoran et al., 2010). Therefore pharmacologically similar effects in fish compared to those found in the human bodies could be expected and

pharmaceuticals are supposed to act "therapeutically" rather than toxically on non-target organisms (Rand-Weaver et al., 2013).

Verapamil reduced the heart rate of carp embryos and larvae in acute toxicity tests in concentration dependent manner, which is well in line with the finding of Berghmans et al. (2009), who exposed zebrafish larvae for 3 h to verapamil in the concentration range of 136–227 mg/l. Furthermore, Shin et al. (2010) found reduced heart rate in zebrafish embryos exposed to verapamil at the concentration of 1 mg/l for 12 h compared to control. The present work together with the results of Berghmans et al. (2008) and Shin et al. (2010) revealed that verapamil has a similar pharmacological effect on fish and human where it is used therapeutically to reduce the heart rate (Grossman and Messrli, 2004). The similarity in the mode of action in fish and human is not surprising considering that most drug targets are supposed to be highly conserved among vertebrate species (Gunnarsson et al., 2008). This is further supported by the fact that the polypeptide sequences for the human and zebrafish orthologs of the α-subunit of the L-type calcium channel which is one of the targets of the calcium channel blocker verapamil revealed 78% identity (Rottbauer et al., 2001).

Moreover, carp embryos exposed acutely to high concentrations (1–10 mg/l) and sub-chronically to lower concentrations (0.463–463 μg/l) of verapamil had higher occurrence of spine malformations and edemas. Occurrence of edemas was also observed in frogs treated with verapamil during their early development (Burgess and Vere, 1989). Similarly, peripheral edema is a non-cardiovascular side effect of verapamil treatment in human (Drenth et al., 1992). Burgess and Vere (1989) attribute this effect of verapamil to the disturbances in the ionic equilibrium. Moreover, long-term exposure of carp larvae to verapamil resulted in higher incidence of haematocele in the intestine compared to the control whereas gastrointestinal haemorrhage is another side-effect of verapamil treatment in human (Pahor et al., 1996). Gastrointestinal haemorrhage is caused by inhibition of platelet aggregation as well as the inhibition of the vasoconstrictive response to bleeding (Pahor et al., 1996). These effects might be also responsible for the haematocele in fish.

Diltiazem caused histopathological alterations in the heart (inflammation, degeneration and rupture of pericardial blood vessels) and liver (sinusoidal congestion and vacuolisation in hepatocytes) of rainbow trout exposed sub-chronically to the highest tested level (30 μg/l) in the present work. The histological changes in heart and the blood vessels of the liver in exposed trout suggested vasodilation similar to the pharmacological effect of diltiazem in the human body (Lüllmann, 2003). Since the L-type calcium channel, the target of diltiazem in the human body, has a high degree of similarity in human and fish (Rottbauer et al., 2001), a similar mode of action can be hypothesized.

The data showed that atenolol, diltiazem and verapamil have a similar pharmacological effect on fish as described for humans. This proves the read-across concept stipulating that a drug will have an effect in non-target organisms only if the molecular targets have been conserved (Gunnarsson et al., 2008; Rand-Weaver et al., 2013).

Atenolol caused histopathological changes in the heart of rainbow trout. Namely, it caused degenerative changes in the blood vessels of the pericardium and an inflammatory reaction in the pericardium and subendocardium. In line with these findings, an increased incidence of atrial degeneration of the heart has been described in rats fed a very high dosage of atenolol (300 mg atenolol/kg/day) (RxList, 2013). Fish exposed to atenolol also showed congestion of the sinusoids (sinusoidal blood vessels) indicating circulatory changes (Henrion, 2012). Because the liver of rainbow trout is rich in β_2-ARs (Owen et al., 2007), it can be assumed that atenolol blocked the β_2-ARs and thereby caused vasodilation. The congestion of the sinusoids may be a consequence of this effect. The congestion may also be related to a reduced oxygen supply (Henrion, 2012) which was indicated by reduction in the haemoglobin concentration

in atenolol exposed fish. For other representatives of β-blockers similar effects in fish to the therapeutic effects in humans were described as well. Sun et al. (2014) demonstrated a reduced heart rate in metoprolol exposed zebrafish. In addition, the studies of Sun et al. (2014) and Stanley et al. (2006) showed that metoprolol and propranolol affected the expression of the β_1- and the β_2-ARs in exposed zebrafish. The similarities in the mode of action of the β-blockers atenolol, metoprolol and propranolol might be caused by a conservation of the drug targets (β_1 and β_2-ARs) among fish and mammals (Nickerson et al., 2001).

The heart index in fish

The present work introduced a method for the standardized evaluation of histopathological alterations in the heart ventricle and the coronary blood vessels of fish. Based on the alterations of the functional units of the heart, namely the coronary blood vessels and capillaries, the epicardium, the endocardium and the myocardium, the heart index can be determined. In addition, these indices can be used as indicators of damage at the level of the functional unit and allow an easy localisation of the alterations within the tissue.

This protocol was developed using the heart of rainbow trout, but it was also successfully applied on fish belonging to different systematic orders like *cyprinid, sturgeon* and *percid* fish. The protocol has also some limitations. It was not possible to include gross morphological changes, alterations in the *bulbus arteriosus* and the valves, alterations in lipid content, signs of regeneration, tumour classification and species specific patterns into the calculation of the heart index and therefore these features have to be reported separately.

Taken together, the heart–index enables a quantitative evaluation of histopathological changes in the cardiovascular system in fish. Therefore, it can help to compare the effects of xenobiotics including cardiovascular pharmaceuticals on the cardiovascular system of fish.

Biomarker response at environmentally relevant concentrations

In general, biomarker response can be defined as a biological response to exposure or as toxic effects of environmental chemicals, which can be used to assess the health status of organisms and to obtain early-warning signals of environmental risks (van der Oost et al., 2003).

Verapamil at environmentally relevant concentration (0.463 µg/l) did not affect hatching, development, or morphological parameters nor did it have an effect on the mortality of exposed fish. Similarly, in the study of Li et al. (2010b) such verapamil concentrations had no effect on rainbow trout exposed for 42 days. Although the present work and the study of Li et al. (2010b) could not demonstrate any effects of verapamil on studied endpoints at environmentally relevant concentrations, we cannot exclude the possibility that it might affect fish after a longer exposure. Moreover, this pharmaceutical inhibits multixenobiotic resistance in fish (Smital and Sauerborn, 2002) and therefore it could enhance the sensitivity of fish to other compounds (Kurelec, 1997). This special characteristic of verapamil would deserve further attention.

Diltiazem at the environmentally relevant concentration (0.03 µg/l) caused changes in activity of antioxidant enzymes in the liver and gills as well as elevation of TBARS level in the gills. In addition, at this concentration, the CK content in the blood plasma was increased, indicating damage to cardiac muscle (Adams et al., 1993; Clarkson et al., 2006). Indeed, degeneration of cardiac muscle fibre was observed in a single exposed fish. Other studied endpoints, namely parameters of the haematological and biochemical profile of the blood plasma and the activity of CYP1A and CYP3A4 were not affected by diltiazem at environmental concentration.

Atenolol at environmentally relevant concentration (1 μg/l) affected the biochemical and haematological profile of the blood, namely by increasing lactate concentration and reducing the haemoglobin content in the blood. This indicated that atenolol caused a reduction in oxygen supply. The present study verified that changes within the haem pathway are sensitive biomarkers of exposure to pollutants at their environmental concentrations, as was also demonstrated for e.g., cadmium (van der Oost et al., 2003). The changes in the haematocrit value and haemoglobin concentration of rainbow trout in response to atenolol observed in the present study may indicate that this substance affects the β-ARs in the organs associated with erythropoiesis, i.e. in the head kidney and spleen. The presence of β_1-ARs in these organs has been documented in salmonid fish (Owen et al., 2007). However, we cannot rule out the possibility that these changes are part of a general stress response. Atenolol had no effect on other biomarkers, such as activity of antioxidant enzymes and activity of selected CYP450 isoforms (CYP1A; CYP2B; and CYP3A4) at environmentally relevant concentration.

In summary, atenolol and diltiazem affected the exposed fish while verapamil had no effect on the studied endpoints at environmental levels in the present work.

Conclusions

In conclusion, atenolol, diltiazem and verapamil cannot be classified as hazardous or bioaccumulative compounds (BCF ≥ 2000) due to their relatively low BCFs in fish. At high levels, all tested pharmaceuticals had effects on fish that were similar to the effects and side effects described for the human body. This indicates a similar mode of action of these pharmaceuticals in fish and human. In addition, the data showed also similarities in the metabolism of diltiazem and its half-life time between fish and human. At environmentally relevant concentration verapamil had no effect on studied endpoints while atenolol and diltiazem caused some physiological changes in exposed fish. Therefore, atenolol and diltiazem, when present in the aquatic environment, could be a source of sub-lethal detrimental effects in fish.

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7.2. ENGLISH SUMMARY

Effect of selected cardiovascular pharmaceuticals found in aquatic environment on fish

Christoph Antonius Steinbach

Cardiovascular pharmaceuticals are among the most prescribed drugs. As a result of the high consumption, these pharmaceuticals have been frequently detected in waste and surface waters. Verapamil, diltiazem and atenolol are very important representatives of cardiovascular pharmaceuticals; therefore, the present research focused on their acute and sub-chronic effects, bioconcentration, half-life time and metabolism in fish. Moreover, unified protocol for the quantitative assessment of histopathological alterations on the heart ventricle and coronary blood vessels employing heart index calculation was developed with the aim to better assess histopathological changes in fish heart which is one of the targets of cardiovascular pharmaceuticals and other chemicals.

The effects caused by high concentrations of the studied substances, verapamil, diltiazem and atenolol, in fish can be considered similar to the therapeutic effects and side effects that are found in humans. The acute exposure to verapamil at the human therapeutic plasma level reduced the heart rate in common carp embryos and larvae. In addition, the acute and chronic exposure to this substance caused peripheral edema and gastrointestinal haemorrhage in carp. Similarly, the histological changes in heart and the blood vessels of the liver in diltiazem exposed rainbow trout suggested vasodilatation similar to the pharmacological effect of diltiazem in the human body. In rainbow trout sub-chronically exposed to atenolol at a human therapeutic blood plasma concentration, histopathological changes in the cardiovascular system were found.

The bioconcentration of verapamil, diltiazem and atenolol in fish can be classified as low. Bioconcentration factor (BCF) of verapamil in whole body homogenates of common carp ranged between 6.6 and 16.6. The BCF of diltiazem was also relatively low (0.5–194) in analysed tissues of trout, following the order kidney > liver > muscle > blood plasma. BCF of atenolol in rainbow trout tissues was the lowest among the tested substances (BCF = 0.002–0.27), following the order of liver > kidney > muscle. In the blood plasma, the concentration of atenolol was below the limit of quantification. Verapamil showed a longer half-life time (10.6 days) in fish compared to the human body, indicating the slow rate of biotransformation and/or elimination of verapamil in fish. Estimated half-life times of diltiazem in liver (1.5 h) and kidney (6.2 h) were in the same order of magnitudes as those determined for the human blood plasma. The half-life time of atenolol in trout was not studied, because of its very low bioconcentration.

In diltiazem exposed rainbow trout, 8 groups of metabolites of diltiazem with 17 different isoforms were identified using liquid chromatography/high resolution mass spectrometry method. Diltiazem was found to undergo a biotransformation involving desmethylation, desacethylation and hydroxylation in fish. These results showed that diltiazem was metabolised in fish in a similar way like in the human body by desmethylation and desacethylation. On the other hand, hydroxylation, which was involved to a minor extent, seemed to be species specific.

Verapamil had no effect on early life stages of common carp at the environmentally relevant concentration after one month lasting exposure. On the other hand, atenolol and diltiazem in environmentally realistic concentrations caused after 42-day exposure some physiological changes in rainbow trout. Namely, atenolol affected haematological and biochemical parameters of the blood in exposed rainbow trout and diltiazem caused changes in the

activity of antioxidant enzymes in trout liver and gills. These data indicated that atenolol and diltiazem, when present in the aquatic environment, could be a source of sub-lethal detrimental effects in fish.

7.3. CZECH SUMMARY

Vliv vybraných kardiovaskulárních léčiv nalézaných ve vodním prostředí na ryby

Christoph Antonius Steinbach

Kardiovaskulární léčiva patří v České republice k nejvíce předepisovaným. V důsledku jejich vysoké spotřeby jsou jejich rezidua často detekována v odpadních a povrchových vodách. Verapamil, diltiazem a atenolol jsou významnými zástupci kardiovaskulárních léčiv, proto jsem se ve své dizertační práci zaměřil na studium jejich akutních i subchronických účinků na ryby, především na schopnost jejich kumulace v těle ryb, metabolizmus a biologický poločas. S cílem zlepšit metodiku posuzování histopatologických změn rybího srdce jsem vypracoval jednotný protokol pro kvantitativní hodnocení výsledků histologického vyšetření srdeční komory a koronárních cév, včetně výpočtu srdečního indexu. Kvalitní a systematická vyšetření histopatologických změn srdce jsou velmi důležitá, neboť srdce přestavuje jeden z cílů působení kardiovaskulárních léčiv a dalších chemických látek.

Verapamil, diltiazem a atenolol vyvolaly při expozici ryb vysokým koncentracím těchto látek změny, které jsou známy jako terapeutické a vedlejší účinky u lidí. Akutní expozice embryí a larev kapra obecného verapamilu v koncentraci, v níž se vyskytuje v krevní plazmě lidí léčených touto látkou, vyvolala u pokusných organizmů snížení tepové frekvence srdce. Akutní i chronická expozice kaprů této látce u nich navíc způsobila periferní otok a tvorbu krvácenin v trávicím traktu. U pstruhů duhových exponovaných diltiazemu bylo při histopatologickém vyšetření zjištěno rozšíření srdečních a jaterních cév. To znamená, že i tento prostředek má obdobný farmakologický účinek na ryby jako na lidi. Také subchronická expozice pstruha duhového atenololu v koncentraci, v níž se vyskytuje v krevní plazmě lidí léčených touto látkou, vyvolala histopatologické změny kardiovaskulárního systému.

Verapamil, diltiazem a atenolol můžeme klasifikovat jako látky s malou schopností biokoncentrace v rybách. Biokoncentrační faktor (BCF) verapamilu v homogenátech celého kapřího těla se pohyboval v rozmezí od 6,6 do 16,6. BCF diltiazemu v tkáních pstruha byl také relativně nízký (0,5–194), přičemž nejvyšší hodnota BCF byla prokázána u ledvin a v dalších tkáních klesala v následujícím pořadí: játra > svalovina > krevní plazma. BCF atenololu v tkáních pstruha duhového byl z testovaných látek nejnižší a pohyboval se v rozmezí 0,002–0,27. Nevyšší hodnoty BFC byly zjištěny v játrech, následovaly ledviny a nejnižší ve svalovině. V plazmě byla koncentrace atenololu pod mezí kvantifikace. Verapamil vykazoval delší biologický poločas u ryb (10,6 dne) než u lidí, což indikuje nízkou rychlost biotransformace anebo eliminace verapamilu u ryb. Odhadovaný biologický poločas diltiazemu v játrech (1,5 hodiny) a v ledvinách (6,2 hodiny) byl řádově shodný s hodnotou zjištěnou v krevní plazmě lidí. Biologický poločas atenololu u pstruha studován nebyl, neboť u této látky byla prokázána velmi nízká schopnost biokoncentrace.

U ryb exponovaných diltiazemu bylo pomocí kapalinové chromatografie s hmotnostní detekcí s vysokým rozlišením (LC/HRMS) identifikováno osm metabolitů této látky se 17 různými izoformami. Bylo zjištěno, že diltiazem v rybách podléhá biotransformaci zahrnující demethylaci, deacetylaci a hydroxylaci, což potvrzuje, že diltiazem byl v rybách metabolizován podobným způsobem jako v lidském těle, tedy převážně demethylací a deacetylací. Na druhou stranu, hydroxylace, která se na biotransformaci podílela v nejmenší míře, se jeví jako druhově specifická.

Při studiu vlivu verapamilu na raná vývojová stádia kapra obecného, která byla vystavena environmentálně relevantním koncentracím tohoto léčiva po dobu jednoho měsíce, nebyly u pokusných organizmů prokázány žádné negativní účinky. Ale u pstruha duhového, který

byl vystaven po dobu 42 dnů atenololu a diltiazemu v koncentracích, které jsou detekovány v životním prostředí, byly zjištěny některé fyziologické změny. V případě atenololu se jednalo o změny hematologických a biochemických ukazatelů v krvi a u diltiazemu o změny v aktivitě antioxidačních enzymů v játrech a žábrách. Tato data naznačují, že výskyt atenololu a diltiazemu ve vodním prostředí může negativně ovlivňovat zdravotní stav ryb.

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- GAJU/125/2013/Z: Grant Agency of the University of South Bohemia (2012, responsible leader Dipl.-Biol. Christoph Steinbach).

7.5. LIST OF PUBLICATIONS

Peer-reviewed journals with IF

Steinbach, C., Burkina, V., Fedorova, G., Grabicova, K., Stara, A., Velisek, J., Žlábek, V., Schmidt-Posthaus, H., Grabic, R., Kocour Kroupova, H., 2014. The sub-lethal effects and bioconcentration of the human pharmaceutical atenolol in rainbow trout (*Oncorhynchus mykiss*). Science of the Total Environment 497–498, 209–218.

Grabicova, K., Fedorova,G., Burkina,V., **Steinbach, C.,** Schmidt-Posthaus, H., Zlabek, V., Kocour Kroupova, H., Grabic, R., Randak, T., 2013. Presence of UV filters in surface water and the effects of phenylbenzimidazole sulfonic acid on rainbow trout (*Oncorhynchus mykiss*) following a chronic toxicity test. Ecotoxicology and Environmental Safety 96, 41–47.

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Steinbach, C., Fedorova, G., Prokes, M., Grabicova, K., Machova, J., Grabic, R., Valentova, O., Kocour Kroupova, H., 2013. Toxic effects, bioconcentration and depuration of verapamil in the early life stages of common carp (*Cyprinus carpio* L.). Science of the Total Environment. 461–462, 198–206.

Švinger, V.W., Policar, T., **Steinbach, C.,** Poláková, S., Jankovych, A., Kouřil, J., 2013. Synchronization of ovulation in brook char (*Salvelinus fontinalis*, Mitchill 1814) using emulsified D-Arg6Pro9NEt sGnRHa. Aquaculture International 21, 783–799.

Submitted manuscripts to peer-reviewed journals with IF

Lebeda, I., **Steinbach, C.,** Bytyutskyy, D., Flajšhans, M., 2015. Use of flow cytometry to assess success rate of gynogenesis induction and separate nongynogenetic progeny of sturgeon. Aquaculture Research. (submitted)

Manuscripts

Koba, O., **Steinbach, C.**, Kocour Kroupova, H., Grabicova, K., Randak T., Grabic, R., 2015. Investigation of diltiazem metabolic pathway in fish using hybrid quadrupole/orbital trap mass spectrometer QExactive

Steinbach, C., Burkina, V., Grabic, R., Fedorova, G., Schmidt-Posthaus, H., Zlabek, V., Stara, A., Koba, O., Golovko O., Grabicova, K., Kolarova, J., Velisek, J., Randak, T., Kocour Kroupova, H., 2015. Bioconcentration, metabolism, and sub-lethal effects of the human therapeutic drug diltiazem on rainbow trout *Oncorhynchus mykiss*.

Steinbach, C., Kocour Kroupova, H., Wahli, T., Schmidt-Posthaus, H., 2015. Histopathological alterations of the cardiovascular system in fish – a proposal for the assessment.

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Steinbach, C., Burkina, V., Fedorova, G., Zlabek, V, Randak, T., Schmidt-Posthaus, H., Grabic, R., Kocour Kroupova, H., 2012. The sub-lethal toxic effects of the human pharmaceutical diltiazem on rainbow trout (*Oncorhynchus mykiss*). DIFA II, Vodnany, Czech Republic. Books of abstracts. p. 99.

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Zlabek, V., Burkina, V., Kroupova, H., **Steinbach, C.,** Grabic, R., Fedorova, G., Velisek, J., Randak, T., 2012. Chronic effect of atenolol on physiological indices in rainbow trout (*Oncorhynchus mykiss*). ABSTRACT BOOK 6th SETAC World Congress/SETAC Europe, Berlin, Germany (TU075) 309.

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Svinger, V.W., Policar, T., Polakova, S., **Steinbach, C.,** Jankovych, A., Kouril, J., 2011., Induction and advancement of ovulation in brook char (*Salvelinus fontinalis* Mitchill) using administration of emulsified D-Arg6Pro9NEt-sGnRHa. Diversification in Inland Finfish Aquaculture Conference, 16–18 May, 2011, Písek, Czech Republic, p. 56.

7.6. TRAINING AND SUPERVISION PLAN DURING STUDY

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Period	1st October 2010 until 30th September 2015

Ph.D. courses	**Year**
Aquatic toxicology	2010
Pond aquaculture	2010
Applied hydrobiology	2011
Ichthyology and systematics of fish	2011
Basics of scientific communication	2011
English language	2012

Scientific seminars	**Year**
Seminar days of RIFCH and FFPW	2011 2012 2013 2013 2015

International conferences	**Year**
Diversification in Inland Finfish Aquaculture Conference, Písek, Czech Republic (*Poster presentation*)	2011
27th Ichthyoparasitological symposium in Austria; Neusiedl, Austria (*Oral presentation*)	2011
Diversification in Inland Finfish Aquaculture Conference, Vodňany, Czech Republic (*Poster presentation*)	2012
Diversification in Inland Finfish Aquaculture Conference, Vodňany, Czech Republic (*Poster presentation*)	2012
49th Congress of the European Societies of Toxicology (EUROTOX), Interlaken, Switzerland (*Poster presentation*)	2012
15th Bi-annual meeting of the German-speaking branches of the Association of Fish Pathologists (EAFP), Starnberg, Germany (*Poster presentation*)	2014

Foreign stays during Ph.D. study at RIFCH and FFPW	**Year**
Prof. Teresa Własow, Department of Ichthyology, Faculty of Environmental Science and Fisheries, University of Warmia and Mazury in Olsztyn, Poland (1 month, fish histology and acute toxicity of pesticides)	2012
Dr. Heike Schmidt-Posthaus, Centre for Fish and Wildlife health, Institute of Animal Pathology, University of Bern, Switzerland (4 months, fish diseases and fish histopathology)	2013 2014

Pedagogical activity		Year
Teaching	127 h	2010–2015
Tutor of bachelor students Miroslav Vaněček	Reduction of the toxicity of nitrites and ammonia to fish by humid substances	2012–2013
Petra Beranová	The occurrence of β-blockers and calcium channel blockers in the aquatic environment and their effects on fish	2014–2015
Summer school student supervision Marek Pípal Razmara Parastoo	Analysis of gene expression in environmental toxicological research, a case study of rainbow trout (*Oncorhynchus mykiss)* exposed to a human cardiovascular pharmaceutical	2013
Xènia Pérez Sitjà Stanislav Valenta	Analysis of gene expression in toxicological research	2014
Project leader Grant agency of the University of South Bohemia in České Budějovice	Toxic effects and bioconcentration of human pharmaceutical, atenolol, in rainbow trout (*Oncorhynchus mykiss*)	2013

7.7. CURRICULUM VITAE

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Histopathological seminar (University of Bern), Histological seminar (FFPW),
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