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Bioconcentration and endocrine disruption effects of diazepam in channel catfish, *Ictalurus punctatus*

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ABSTRACT

Recently, the detection of pharmaceuticals in surface waters has increased worldwide. Pharmaceuticals are typically found in the environment at concentrations well below therapeutic levels in humans; however, their mechanisms of action may be largely unknown in non-target organisms, such as teleost species. Thus, chronic exposure to these types of compounds warrants further investigation. The goal of this study was to examine the potential for diazepam, a model benzodiazepine drug, to bioconcentrate in tissues of channel catfish and to examine its ability to interact with the endocrine system through modulation of steroid hormones and/or steroidogenic genes. To investigate the bioconcentration potential of diazepam, channel catfish (*Ictalurus punctatus*) were exposed to 1 ng/mL diazepam for seven days, followed by clean water for another seven days, using an abbreviated OECD 305 Fish Bioconcentration Test study design. This concentration of diazepam is well below environmentally relevant concentrations of diazepam (ng/L). To evaluate steroidogenic effects, fish were exposed to 1 ng/mL diazepam for seven days only. Steroid hormone concentrations were analyzed for various tissues, as well as expression of selected steroidogenic genes. Calculated bioconcentration factors for diazepam were well below regulatory threshold values in all tissues analyzed. No changes in steroid hormone concentration were detected in any tissue analyzed; however, the steroidogenic gene cytochrome P450 side chain cleavage (P450scc) was significantly down-regulated at day 5 and 3β-hydroxy steroid dehydrogenase (3β-HSD) was significantly down-regulated at day 7 in the gonad. These results indicate that although diazepam does not significantly bioconcentrate, low-level chronic exposure to diazepam may have the potential to interact with endocrine function by altering gene expression.

1. Introduction

Uptake of pharmaceuticals by fish can occur through various routes, resulting in the accumulation of compounds over time (Corcoran et al., 2010). Accumulation of compounds through gill uptake only (bioconcentration) can be evaluated using the Organization for Economic Cooperation and Development (OECD) 305 Fish Bioconcentration Test (Woodburn et al., 2008). The gill uptake model tends to accurately predict the bioconcentration of moderately non-polar compounds (log $K_{OW} < 5$), such as pharmaceuticals, and works under the assumption that one route of absorption (gill uptake) determines the total accumulation of the compound in fish species using a flow-through test design (Barber, 2008, Erickson et al., 2006, Nichols et al., 2015). The model results in the calculation of the bioconcentration factor (BCF), which is a unit less ratio of the concentration of the contaminant in fish to that in the water (Barber, 2008). However, the significance of the bioconcentration value depends on the country that the test was conducted and the associated regulatory agency, and can range anywhere from 500 to 5000 (Cowan-Ellsberry et al., 2008). Due to the high cost and lengthy time associated with conducting a full OECD 305 study, bioconcentration data is available for only a limited number of pharmaceuticals. Thus, there is a need to acquire BCF data for a large number of chemicals.

A model benzodiazepine pharmaceutical, diazepam, is one of the top 200 drugs dispensed in the U.S. and is also frequently abused; thus, wastewater treatment plants across the United States continually receive diazepam (Lamb, 2009, SAMSA, 2008). Diazepam, as well as its metabolites, has been detected in the environment at concentrations ranging from <1 ng/L in surface waters to 200 ng/L in wastewater (Phillips et al., 2007; Hummel et al., 2006; Baker and Kasprzyk-Hordern, 2011). However, one metabolite of diazepam, oxazepam, has been detected in wastewater at concentrations of up to 482 ng/L (Hummel et al., 2006). Benzodiazepine pharmaceuticals, such as diazepam, act on the central nervous system (CNS) via interaction with the ionotropic GABA receptor, specifically $GABA_A$, which results in modulation of the endogenous inhibitory neurotransmitter gamma-aminobutyric acid (GABA). Activation of the GABA receptor

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results in opening of the ion channel, allowing chloride ions to pass through, which leads to a decreased excitability of neurons. These mechanisms lead to effects such as sedation, hypnosis, decreased anxiety, muscle relaxation, anterograde amnesia and anticonvulsant activity (Charney et al., 2001). Diazepam specifically undergoes cytochrome P450 (CYP450) metabolism in mammals to form the active metabolites nordiazepam, temazepam and oxazepam (Charney et al., 2001). These drugs have similar mechanisms of action in target species (mammals), but unknown actions in non-target species, such as teleost fish. Kwon et al. (2009) examined the concentration of diazepam in livers of wild caught hornyhead turbot (*Pleuronichthys verticalis*) and found that concentrations ranged from 23 to 110 ng/g. These concentrations are higher than the concentrations reported for diazepam in the surface water, indicating that diazepam may exhibit the potential to bioconcentrate in fish tissues. To date, investigations regarding the concentrations of the metabolites of diazepam in fish tissues have not been conducted.

Steroidogenesis represents an important mechanism for controlling many physiological processes in fish, including reproduction. Thus, it is important to understand the mechanism by which environmental contaminants disrupt endocrine function through modulation of steroidogenesis. In mammals, acute changes in steroidogenesis are mediated through non-enzymatic regulation, such as the mobilization of cholesterol via the translocator protein (TSPO) (previously known as the peripheral benzodiazepine receptor (PBR)). In contrast, chronic changes in steroidogenesis, such as the modulation of steroid hormone concentrations, are mediated by changes in gene expression of key steroidogenic enzymes (Kumar et al., 2000). The translocator protein (TSPO), in association with the steroidogenic acute regulatory protein (StAR), functions in the binding and transport of cholesterol into the mitochondria, which is the rate-limiting step in steroidogenesis (Papadopoulos et al., 2005). Once cholesterol reaches the mitochondrial matrix, it is then converted into pregnenolone by cytochrome P450 side chain cleavage (CYP450scc). Pregnenolone is metabolized by various CYP450 enzymes, such as 3β-hydroxy steroid dehydrogenase (3β-HSD), to produce tissue-specific steroid hormones (Papadopoulos et al., 2005).

Numerous benzodiazepine compounds have been shown to exhibit varying degrees of drug specificity for binding to TSPO (Verma and Snyder, 1989; Veenman et al., 2007). Exogenous TSPO ligands, such as benzodiazepine drugs, have been shown to stimulate pregnenolone formation by inducing the transport of cholesterol into the inner mitochondrial membrane, both *in vitro* and *in vivo* (Lacapère and Papadopoulos, 2003). Thus, the TSPO represents a potential target for endocrine disruption in aquatic species including teleost fish. More recently, the gonadal expression of TSPO in largemouth bass (*Micropterus salmoides*) was characterized across reproductive stages in relation to steroid hormone levels and reproductive maturation (Doperalski et al., 2011). The authors found that testicular TSPO was positively correlated with reproductive stage, gonadosomatic index (GSI) and plasma testosterone levels; whereas ovarian TSPO expression was negatively correlated with GSI and plasma levels of testosterone and 17β-estradiol (Doperalski et al., 2011). This study represents the first investigation of the role of TSPO in steroidogenesis in teleost fish and indicates sex differences in mitochondrial cholesterol transport mechanisms. Thus, the potential for TSPO, as well as other steroidogenic enzymes, as a novel target for endocrine disrupting chemicals warrants further investigation.

The aim of this study was to examine the distribution and tissue-specific bioconcentration of diazepam (and metabolites) in channel catfish. Additionally, the endocrine disrupting potential of diazepam was investigated by measuring steroid hormone concentrations as well as the expression of steroidogenic genes/receptors.

2. Materials and methods

2.1. Materials

Diazepam, nordiazepam, temazepam, oxazepam and d_5 diazepam were purchased from Cerilliant Corporation (Round Rock, TX). Pregnenolone, testosterone, estradiol, estrone, progesterone, 17α-hydroxyprogesterone and 11-deoxycortisol (11-DC) were purchased from Sigma-Aldrich (St. Louis, MO). Deuterated internal standards of d_3-estradiol and d_9-progesterone were purchased from Toronto Research Chemicals (Canada). 11-Ketotestosterone and 17α, 20β-dihydroxyprogesterone (17, 20-DP) were purchased from Steraloids (USA). HPLC grade acetonitrile, hexane, ethyl acetate and dimethylformamide (DMF) were purchased from Fisher Scientific (Pittsburgh, PA). TRI reagent, chloroform, isopropanol, ethanol and Tris-EDTA buffer were purchased from Sigma-Aldrich (St. Louis, MO). Forward and reverse primers for 18S, translocator protein (TSPO), P450scc and 3β-HSD were purchased from Invitrogen (Grand Island, NY) (Table 1). The Rotor-Gene SYBR Green RT-PCR kit was purchased from Qiagen (Valencia, CA).

2.2. Research organism

Juvenile channel catfish (*Ictalurus punctatus*) were purchased from Pond King, Inc. (Gainesville, TX) and transported to the University of North Texas Aquatic Toxicology Laboratory. They were maintained in de-chlorinated tap water at 25 ± 2 °C in re-circulating tanks with a 16-h light/8-h dark photoperiod. They were fed ground trout chow (Purina Aquamax Grower 600) *ad libitum* until 24 h prior to any experiments or tissue collections. All research with channel catfish was approved and conducted according to the Institutional Animal Care and Use Committee (IACUC) through the University of North Texas.

2.3. In vivo studies

Juvenile channel catfish (average length = 10.7 ± 0.5 cm) were utilized for all *in vivo* exposures. All exposures were conducted according to OECD 305 guidelines, using an abbreviated study design (Nallani et al., 2011). Conditions for all tests consisted of a 16:8 light/dark photoperiod, daily feeding, and a minimum of weekly monitored water quality measurements. Animals were given approximately one hour for feedings, after which any remaining solid residues were removed from tanks via siphoning. Temperature, pH, dissolved oxygen, alkalinity and hardness in all exposure tanks (n = 20) averaged 18.82 ± 0.36 °C, 7.46 ± 0.18, 8.65 ± 0.24 mg/L, 89.44 ± 6.34 mg/L as $CaCO_3$ and 106.31 ± 10.18 mg/L as $CaCO_3$, respectively.

A continuous flow-through diluter system was used to conduct both tests. A peristaltic pump (Masterflex L/S, Cole-Parmer, Vernon Hill, Il)

Table 1
Primers used in qRT-PCR analysis of steroidogenic enzymes and receptors.

Gene	Forward primer	Reverse primer	R^2	Efficiency (%)
18S (reference gene)[a]	GAGAAACGGCTACCACATCC	GATACGCTCATTCCGATTACAG	0.983	90
TSPO[b]	GGGTCTGACTGCCTTACCAC	GAATTGGGTGGCCTCCATGA	0.997	94
P450scc[a]	GCAAAGATACCTCACTCAAGATGTTGT	TGGTTTGGAGAAGATGCGATAGT	0.989	91
3β-HSD[a]	GCTAAACACACATTTCACCTTCTCTT	CCGACGCTAGCCAATCTGTAGT	0.994	93

[a] Primers originated from Kumar et al. (2000).
[b] Primers developed from GenBank sequence accession number **NM001200736** using Primer3 software (v 0.4.0)