TISSUE-SPECIFIC BIOCONCENTRATION FACTOR OF THE SYNTHETIC STEROID
HORMONE MEDROXYPROGESTERONE ACETATE (MPA) IN
THE COMMON CARP, Cyprinus carpio

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Thesis Prepared for the Degree of
MASTER OF SCIENCE

UNIVERSITY OF NORTH TEXAS
August 2013

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Due to the wide spread occurrence of medroxyprogesterone acetate (MPA), a pharmaceutical compound, in wastewater effluent and surface waters, the objectives of this work were to determine the tissue specific uptake and bioconcentration factor (BCF) for MPA in common carp. BCFs were experimentally determined for MPA in fish using a 14-day laboratory test whereby carp where exposed to 100 μg/L of MPA for a 7-day period followed by a depuration phase in which fish were maintained in dechlorinated tap water for an additional 7 days. MPA concentrations in muscle, brain, liver and plasma were determined by liquid chromatography/mass spectrometry (LC/MS).

The results from the experiment indicate that MPA can accumulate in fish, however, MPA is not considered to be bioaccumulative based on regulatory standards (BCF ≥ 1000). Although MPA has a low BCF value in common carp, this compound may cause reproductive effects in fish at environmentally relevant concentrations.
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ACKNOWLEDGMENTS

I would like to thank my graduate advisor, Dr. La Point, for his much appreciated guidance and patience throughout my graduate research and coursework. I want to thank Dr. Huggett for all the time he generously gave to my research. His advice was crucial in the design and implementation of my experiment as well as the interpretation of my experimental results. I thank Dr. Venables for the countless hours he spent helping me operate the analytical machinery. If it were not for his help, I would not have obtained the data necessary to complete my research.

I appreciate all the help from my fellow graduate students. I am particularly thankful to Santos Garcia, David Hala, Sid Barnes, and David Baxter, who significantly contributed to the successful completion of my experiment. I thank the biology department for the financial assistance received in the form of a Beth Baird Scholarship and a TA.

I am grateful for the constant support and encouragement from my family. Special thanks to my parents, Margaret and Buddy, and my brother, Hamilton.
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CHAPTER 1

INTRODUCTION

The purpose of this chapter is to introduce the research objective and hypotheses as well as explain the overall importance of this study. The chapter is divided into seven sections. Section 1 states the research objectives, and the importance of these objectives is rationalized in sections 2-6. The issue of pharmaceutical compounds as emerging contaminants is explained in sections 2 and 3. Section 4 introduces concerns with the presence of pharmaceutical steroid hormones in surface waters. The 5th section details the risks posed by synthetic progestins, a particular class of synthetic hormones that, for the most part, have gone overlooked in ecotoxicology. Section 6 defines and explains the importance of bioconcentration factors (BCFs) in the risk evaluation of contaminants in the aquatic environment. Lastly, the hypotheses of the study are stated in section 7.

1.1 Research Objectives

The objectives of this research are to determine the extent to which MPA accumulates in various fish tissues (muscle, brain, liver, and plasma). To accomplish these goals, bioconcentration factors (BCFs) were experimentally determined for MPA in fish. The laboratory BCF experiment followed OECD 305 guidelines (OECD 1996) and involved a reduced sampling design (explained in chapter 2, section 4) (Springer et al. 2008). In addition to wet weight BCFs, lipid normalized BCFs were determined using tissue sample lipid weights. A freshwater species, the common carp (Cyprinus carpio), was the model organism used for the study. This species was chosen because it is common in lentic and lotic habitats throughout
North America (Parkos Iii et al. 2003), widely used in aquatic toxicology studies, and tolerable of handling and varying water quality conditions (USEPA 1996). Because there are currently no data on the accumulation potential of MPA in fish tissue, results from this study should offer insight into the effects of MPA on fish while furthering current understanding of the risks synthetic progestins pose to freshwater ecosystems.

1.2 Emerging Contaminants in the Aquatic Environment

Over 72,000 chemicals are in commercial use within the United States, yet only about 10% of them have been screened for toxicity (Dodds and Whiles 2010). Whether from point sources such as sewage treatment plants or non-point sources including agricultural and urban run-off, freshwater ecosystems receive a wide variety of synthetic compounds, and many of these ecosystems have been diminished as a result. A Report on national water quality by the US Environmental Protection Agency indicates that 44% of assessed river and stream miles and 64% of assessed lake acres are impaired, and among the leading causes of such impairment is exposure to inorganic and organic contaminants (USEPA 2004).

Much of the attention on chemical pollution has focused on “priority contaminants” which pertain to chemicals that are acutely toxic or carcinogenic and display persistence in the environment (Daughton and Ternes 1999). With the passage of the Clean Water Act in 1972, considerable progress has been made in reducing the amount of these contaminants in many freshwaters throughout the United States (Lettenmaier et al. 1991, Brown and Froemke 2012). However, many chemicals present in surface waters are unregulated either because they are
relatively new or they have not been detected. Many of these new and emerging contaminants could pose considerable threats to aquatic organisms and ecosystems.

1.3 Pharmaceutical Compounds: Emerging Contaminants in Surface Waters

1.3.1 Sources and Occurrence

Pharmaceuticals are emerging contaminants that have received increasing concern over their widespread occurrence and potential environmental effects in freshwaters throughout the world (Daughton and Ternes 1999, Kolpin et al. 2002, Fent et al. 2006, Brooks et al. 2009, Ramirez et al. 2009, Fick et al. 2010, Schultz et al. 2010b). This diverse class of compounds includes human and veterinary drugs as well as their respective metabolites and transformation products (Kummerer 2010). The occurrence of pharmaceuticals in U.S. freshwaters was first reported in 1976, when clofibric acid, an anti-inflammatory drug, was detected in treated wastewater at a range of 0.8 – 2 µg/L (Garrison et al. 1976). During a survey of 139 U.S. streams, Kolpin et al. (2002) detected human pharmaceuticals and personal care products in 80% of sampled streams. With recent advances in trace contaminant analysis technology, several pharmaceutical compounds, including beta blockers, anti-inflammatory drugs, Neuroactive compounds and synthetic hormones have been detected in streams and wastewater effluent at concentrations ranging from the ng/L to the low µg/L range (Ternes 1998, Carballa et al. 2004, Clara et al. 2004, Zhou et al. 2012). Most pharmaceuticals enter wastewater treatment plants (WWTPs) after they are dumped or excreted into sewage systems by humans. WWTPs are not designed to remove pharmaceuticals and their metabolites, so these compounds are subsequently discharged into aquatic habitats (Daughton and Ternes
Hormones and antibiotics used in livestock farms enter aquatic habitats as non-point source pollutants through run-off during times of rainfall and snow melt (Daughton and Ternes 1999, Boxall et al. 2003, Johnson et al. 2006, Matthiessen et al. 2006). Other, less common routes of entry for pharmaceutical compounds into the environment include landfill leachates, fisheries, and drug manufacturers (Holm et al. 1995, Heberer 2002, Kolodziej et al. 2004).

1.3.2 Environmental Fate

Information about the fate and behavior of pharmaceuticals and their metabolites in the environment is limited (Kolpin et al. 2002). Due to the low volatility of pharmaceuticals, they are assumed to distribute in the environment mainly through aqueous transport and food chain dispersal. During the wastewater removal process, adsorption to suspended solids and biodegradation are important processes (Kummerer 2004). Acidic pharmaceuticals, such as ibuprofen, naproxen, diclofenac, and indomethacin, are characterized by having pKₐ values ranging from 4.9 to 4.1. These compounds are predicted to occur mainly in the dissolved phase in wastewater. Therefore, adsorption of acidic pharmaceuticals to sludge is not likely to be an important route of elimination from wastewater and surface water (Fent et al. 2006). Basic pharmaceuticals and hydrophobic compounds, however, can adsorb to sludge significantly (Golet et al. 2002, Ternes et al. 2002, Fent et al. 2006). Biodegradation is assumed to be the most important elimination process for pharmaceuticals occurring mainly in the dissolved phase (Fent et al. 2006). Elimination rates based on measurements of influent and effluent concentrations during the WWTP process can vary greatly. The average elimination rates for
carbamazepine vary from only 7 to 8% (Ternes 1998, Carballa et al. 2004), while the elimination rates for acetylsalicylic acid, propranolol, and salcyclic acid are up to 81%, 96%, and 99% (Ternes 1998, Ternes et al. 1999, Heberer 2002) respectively.

1.3.3 Environmental Regulations

Although scientists have been aware of the presence of pharmaceutical compounds in wastewater effluent since the mid-1970s, only in the past two decades have regulatory agencies issued detailed guidelines on how drugs should be assessed for possible unwanted effects in the environment. In 1995, under European Union (EU) Directive 92/18 and the corresponding “Note for Guidance” (EMEA 1998), the EU established the first requirement for ecotoxicity testing of human and veterinary pharmaceuticals as a criterion for registration. The European Commission released a draft policy (Directive 2001/83/EC) indicating that an authorization of a product designed for human medicinal use must be accompanied by an environmental risk assessment. In 1998, the U.S. Food and Drug administration set fourth guidelines requiring applicants in the U.S. to produce an environmental risk assessment report if the expected discharge concentration of the active ingredient of the pharmaceutical in the aquatic environment is \( \geq 1 \, \mu g/L \) (Fent et al. 2006). The European Medicines Agency (EMEA) developed guidelines in 2006 with the goal of estimating potential environmental risks of human pharmaceuticals using a tiered methodology (Christen et al. 2010). While the last decade has witnessed increased public awareness and regulatory scrutiny of pharmaceuticals in the environment, little is known about the Ecotoxicological effects of these chemicals on aquatic organisms (Länge and Dietrich 2002, Fent et al. 2006).
1.3.4 Ecotoxicity

There is concern over the human health and ecological consequences of pharmaceutical compounds in surface waters because, unlike many traditional priority contaminants, these chemicals are designed to be biologically active in target organisms at relatively low concentrations (Rodriguez-Mozaz and Weinberg 2010). Many mechanisms/modes of action (MOA) by which pharmaceuticals act on a target species are also present in several non-target aquatic species (Länge and Dietrich 2002, Huggett et al. 2004, Gunnarsson et al. 2008). Data from previous research suggest that enzyme/receptor systems by which pharmaceuticals elicit therapeutic effects in humans are also present in teleost systems (Huggett et al. 2003). β-blockers, for example, are compounds used in therapies for the treatment of angina, glaucoma, heart failure, high blood pressure and other related conditions (Toda 2003). These pharmaceuticals elicit beneficial effects, such as a decrease in heart rate, by acting as antagonists of the β-adrenergic receptors found in cardiac and smooth muscle tissues (Rxlist 2013). In teleost systems, β-adrenergic receptors have been identified in tissues including the heart (Keen et al. 1993, Gamperl et al. 1994), branchial vascular tissue (Payan and Girard 1977), aorta (Klaverkamp and Dyer 1974), gill arches (Haywood et al. 1977), gill filaments (Burleson and Milsom 1990), liver (Reid et al. 1992), lymphoid organs (Nickerson et al. 2003), red and white muscle (Lortie and Moon 2003), and brain (Zikopoulos and Dermon 2005). When fish are exposed to β-blockers, it is the physiological processes regulated by the β-adrenergic receptors that are most likely to be affected (Owen et al. 2007). Consequently, aqueous exposure to β-blockers can result in decreased heart rate (Fraysse et al. 2006), reduced growth (Huggett et al. 2002), and reduced fecundity (Huggett et al. 2002) in teleosts. Fish are not the only aquatic
organisms at risk of exposure to pharmaceuticals in surface waters. Several pharmaceuticals can impair reproduction in freshwater crustaceans (Ferrari et al. 2003, Henry et al. 2004, Flaherty and Dodson 2005, Dzialowski et al. 2006), inhibit growth in aquatic plants and algae (Lützhøft et al. 1999, Brain et al. 2004), and alter development in amphibians (Foster et al. 2010).

Pharmaceutical compounds and their metabolites are continually discharged into surface waters through WWTP effluent. These compounds can act as truly persistent contaminants as their rate of replacement compensates for their degradation rate. Thus, aquatic organisms face potential lifetime exposure to pharmaceuticals contained in wastewater discharge (Daughton and Ternes 1999, Brooks et al. 2009). Taking into consideration that many drugs are designed to affect specific pathways in target organisms at low doses, that mechanistic pathways can be highly conserved across phyla, and that many aquatic organisms face chronic exposure to wastewater effluent, acute toxicity test data is likely inadequate for predicting ecological risks (Huggett et al. 2004, Ankley et al. 2007). Chronic toxicity data for pharmaceuticals on aquatic species are lacking, especially with respect to potential disturbances in endocrine function (Fent et al. 2006, Ankley et al. 2007, Sanderson and Thomsen 2009, Christen et al. 2010). Having chronic toxicity data for potential endocrine disrupting compounds (EDCs) is of particular importance because EDCs can elicit adverse effects over a prolonged period of time at very low concentrations (Brian et al. 2005, Durhan et al. 2006, Ankley et al. 2007). Additionally, multiple generation exposure of aquatic organisms to some EDCs may cause developmental and reproductive changes that have a much larger impact on a species than was indicated from shorter term exposures (Cripe et al. 2010).
1.4 Synthetic Steroid Hormones: Potent Endocrine Disrupting Compounds

Synthetic steroid hormones, commonly used in oral contraceptives, hormone replacement therapy, and livestock production, are present in the aquatic environment and can act as potent EDCs when exposed to aquatic organisms (Durhan et al. 2006, Vajda et al. 2008, Viglino et al. 2008, Cripe et al. 2010). Surface waters receive synthetic hormones from a number of sources, including discharge from WWTPs, sewage runoff from livestock farms, and excrement from fish hatcheries (Ternes et al. 1999, Kolodziejski et al. 2004, Chang et al. 2008, Chang et al. 2011). Several steroid hormones have been shown to reduce fecundity or cause intersex in fish at concentrations (1 – 50 ng/L) relative to those detected in WWTP effluent, runoff from beef cattle feeding operations, and even streams (Ankley et al. 2003, Kolodziej et al. 2003, Durhan et al. 2006, Jobling et al. 2006, Fernandez et al. 2007, Santos et al. 2007, Zeilinger et al. 2009). 17-α-ethinylestradiol (EE2), a synthetic estrogen, has been detected in WWTP effluent at concentrations up to 7 ng/L (Desbrow et al. 1998) and has induced vitellogenin, an egg yolk precursor protein, in male rainbow trout at a 100 pg/L exposure level (Purdom et al. 1994). Metabolites of the synthetic androgen trenbolone acetate have been detected in beef feeding lot drainage discharge at concentrations up to 120 ng/L (Durhan et al. 2006). One of the metabolic products of this synthetic hormone caused masculinization and decreased egg production in female fish exposed to concentrations of the compound ≥50 ng/L (Ankley et al. 2003). The presence of synthetic steroid hormones in wastewaters of some streams and rivers is associated with sexual abnormalities in fish (Allen et al. 1999, Kirby et al. 2004). (Jobling et al. 2002) report that all male fish sampled from waterways receiving wastewater effluent in the U.K. contained both male and female reproductive tissues. EE2 and other estrogenic chemicals
are linked to the feminization of fish in waste water effluent dominated waters (Sumpter and Johnson 2008).

1.5 Synthetic Progestins

Whereas much attention of eco-toxicological studies focused on synthetic steroid hormones has been directed towards estrogens, the efficacy of combination estrogen/progestin oral contraceptives in humans is apparently derived from progestins (Benagiano et al. 2004, Erkkola and Landgren 2005). Additionally, synthetic progestins are likely to exist in the environment at higher concentrations than estrogens because birth control medications usually contain 3 to 100 times more progestin than estrogen (Zeilinger et al. 2009).

Synthetic progestins closely mimic progesterone, a steroid hormone that plays a critical role in establishing and maintaining pregnancy in humans and other species (Spencer and Bazer 2002). Progesterone is secreted by the corpus luteum within the ovary and is involved in preparation of the endometrium for pregnancy, the regulation of specific uterine functions during the menstrual cycle, and implantation (Spencer et al. 2004). In addition to functions it carries out during pregnancy, progesterone plays important roles in oocyte meiotic maturation, postnatal development plasticity of the mammary gland, and sperm motility (Conneely et al. 2002, Thomas et al. 2009). Two nuclear progesterone receptors (nPRA and nPRB) and three membrane progesterone receptor subtypes (mPRα, mPRβ, mPRγ) mediate the physiological effects associated with progesterone (Kastner et al. 1990, Zhu et al. 2003). Recent research indicates that nuclear progesterone receptor alpha (nPRA) is required to elicit progesterone dependent responses necessary for ovarian and uterine functions leading to female fertility,
while nuclear progesterone receptor beta (nPRB) is required to elicit responses associated with mammary gland development (Conneely et al. 2003). These receptors act through genomic mechanisms, resulting in alteration in gene transcription, as opposed to membrane progesterone receptors (mPRs), which are located on the surface of cells and induce effects via nongenomic mechanisms that occur rapidly (Revelli et al. 1998, Watson and Gametchu 1999, Falkenstein et al. 2000). In the case of fish and amphibians, mPRs induce oocyte maturation over a prolonged (6-18h) period of time (Thomas 2008). The mPRα has been associated with progesterone regulation of sperm motility (Uhler et al. 1992, Luconi et al. 2004) and uterine function (Thomas 2008) in humans. Although little information is available on binding and signaling characteristics of β and γ subtypes, data suggest that these receptors are involved in gamete transport in mammalian fallopian tubes (Nutu et al. 2009).

1.5.1 Therapeutic Mechanism of Action

Synthetic progestins prevent pregnancy through several different mechanisms within various target tissues (Flores-Herrera et al. 2008). One such mechanism occurs through the inhibition of FSH and LH surges that stimulate ovulation. Estradiol provides positive feedback to the hypothalamic distribution of gonadotropin releasing hormone (GnRH) responsible for stimulating follicle-stimulating hormone (FSH) and luteinizing hormone (LH) production in the pituitary gland, which in turn signals egg maturation and release (Richter et al. 2002). Synthetic progestins disrupt the positive feedback of estradiol to the hypothalamus, thus preventing the release of a mature egg (Letterie 1998). Another way by which synthetic progestins are able to prevent pregnancy is through several changes in the cervical mucus. These changes include
reducing the amount of mucus produced mid-cycle, increasing mucus thickness and cell content, and altering the molecular structure of the mucus. Each change to the cervical mucus acts to prevent sperm penetration, and reduce sperm motility in the rare case that sperm infiltrate the cervical canal (McCann and Potter 1994). Synthetic progestins also render the uterine unsuitable for implantation by suppressing the development and activity of the endometrium (McCann and Potter 1994, Erkkola and Landgren 2005). Finally, synthetic progestins may reduce the number of cilia, and the frequency and intensity of cilia action on the tubal epithelium, thereby inhibiting the transport of the fertilized egg from the oviduct to the uterus (McCann and Potter 1994, Flores-Herrera et al. 2008).

1.5.2 Role of Progesterone in Fish

In female fish, progestins play a critical role in oogenesis (Miura et al. 2007), regulation of oocyte maturation (Nagahama and Yamashita 2008), and, in some species, ovulation (Pinter and Thomas 1999). Progestins are associated with sperm motility (Tubbs and Thomas 2008) and initiation of spermiation (Ueda et al. 1985) in males. Gonadotropin (GTH) initiates the meiotic maturation of fish oocytes by acting on ovarian follicular cells. This action causes follicular cells to produce maturation-inducing hormone (MIH), a substance that interacts directly with the oocyte to trigger oocyte maturation (Nagahama 1997). Two MIH hormones, both progestins, have been identified. These hormones are 17,20β-dihydroxypregn-4-en-3-one (17,20β-P) (Nagahama 1997, Berg et al. 2005) and 17,20β,21-trihydroxypregn-4-en-3-one (17,20β,21-P) (Thomas and Das 1997). Although nuclear and membrane-bound progestin receptors mediate the actions of MIH in fish, research data suggest that teleost reproductive
functions such as oocyte final maturation and sperm motility are associated with mPRα
using zebrafish oocytes by Thomas et al. (2004) suggest that the mPRβ subtype might be
involved in the control of oocyte maturation in zebrafish.

1.5.3 Toxicity of Synthetic Progestins in Fish

Many pharmaceuticals, including those that contain progestins, have mechanisms of
action that are conserved between mammalian and teleost systems (Huggett et al. 2004).
Because synthetic progestins have the ability to mimic natural progestins in fish, and thus
disrupt reproductive and developmental processes, these compounds pose a risk to fish
communities. Previous research indicates that synthetic progestins are capable of inhibiting
reproduction in fathead minnows at concentrations as little as 0.8 ng/L (Zeilinger et al. 2009)
and completely halting reproduction at concentrations ranging from 85 – 100 ng/L (Paulos et al.
2010, Runnalls et al. 2013). A commonly used synthetic progestin, levonorgestrel, induced
androgenic effects in the three-spined stickleback at concentrations ≥40 ng/L (Svensson et al.
2013).

1.5.4 Medroxyprogesterone Acetate

The synthetic progestin, medroxyprogesterone acetate (MPA), is widely used as an
injectable contraceptive and as a therapy for breast cancer and hormone replacement. Over 50
million women worldwide use the form of MPA administered as an intramuscular injection,
depot MPA (DMPA) (Hapgood 2013). MPA has been detected in wastewater effluent at
concentrations up to 18 ng/L (Chang et al. 2009) and in surface waters up to 1 ng/L (Kolodziej et al. 2004). In mammals, MPA has been shown to interact with receptors for progesterone (Winneker et al. 2003), androgen (Hackenberg et al. 1993, Bentel et al. 1999), and estrogen (Di Carlo et al. 1983). Research by Peterson et al. (University of North Texas Aquatic Toxicology Laboratory, University of North Texas, TX, USA, unpublished data) indicates that MPA can inhibit fathead minnow larvae growth at concentrations > 500 µg/L over a 7-day exposure period. Like many other steroid hormones, MPA is relatively hydrophobic (log Kow = 4.09) giving it the ability to partition into the lipid portion of organisms and bioaccumulate (Lindenmaier et al., 2005).

1.6 Importance of BCF Risk Assessments

Bioaccumulation is a term commonly used to describe the process by which a chemical achieves a higher concentration in an organism than in the medium used for respiration (e.g., water for a fish or air for a mammal), the diet, or both (Gobas et al. 2009). The tissue-residue approach for toxicity assessment (TRA) evaluates toxicity based on chemical concentrations in tissue rather than those in the exposure medium (e.g., air, water). Under this concept, tissue burdens provide an exposure metric that is more strongly correlated with effects than other metrics, such as water concentrations (McCarty and Mackay 1993, Meador et al. 2008). Taking this concept into consideration, xenobiotics that bioaccumulate may trigger certain toxicological responses, such as reduced fecundity, as a result of increased tissue burden over an extended period of time. Therefore, bioaccumulation is an important factor in the evaluation of risks that compounds might pose to the environment (Arnot and Gobas 2006,
Bioconcentration factor (BCF) is a quantity used to express the degree to which a compound accumulates in an aquatic organism based on laboratory controlled aqueous exposure to that chemical (Gobas et al. 2009). BCF assessments are useful in understanding the chronic risks posed by contaminants to aquatic organisms in several ways. These assessments allow for a more clear estimation of risk based on exposure (Daughton and Brooks 2011). Tissue specific BCF data provide insight into the toxicokinetics of compounds in fish, furthering understanding of how these compounds target certain organs (Länge and Dietrich 2002, Brooks et al. 2009). In the case of pharmaceuticals, plasma concentrations are particularly important because they are used as the standard by which internal exposure is measured in human and veterinary target organism assessments (Huggett et al. 2003, Owen et al. 2007, Brooks et al. 2009). BCF data on MPA in fish will be useful because other factors besides hydrophobicity and polarity might influence the rate of uptake of pharmaceuticals into fish blood or tissue (Schultz et al. 2010a, Daughton and Brooks 2011). The uptake of sex steroid hormones by fish is mediated via binding to sex-steroid binding globins (SSBG) in the gills (Scott et al. 2005, Miguel-Queralt and Hammond 2008). Fick et al. (2010) found that the synthetic progestin levonorgestrel had a measured fish plasma BCF of 12000, a number that exceeds the predicted value by more than 200-fold. SSBG might have been responsible for the much greater than expected BCF.

1.7 Hypotheses

This study was designed to test the following working hypotheses:
H₀₁: After 7d of exposure to MPA at a concentration of 100 µg/L followed by 7d of depuration, MPA will be detected in carp tissue.

Because MPA mimics progesterone, a natural hormone that is rapidly metabolized in vertebrates, this compound will not accumulate enough to be detected in the tissue of carp during the depuration phase of the experiment.

H₀₂: Tissue concentrations of MPA will NOT be ordered as: brain>liver>plasma>muscle.

In mammals, progesterone receptors are widely distributed in the brain (Graham and Clarke 1997), and therefore brain tissue might be a target for compounds, such as MPA, that interact with these receptors. MPA will probably concentrate the least in muscle tissue because muscle is not as lipid rich as liver and brain tissue. Plasma will likely concentrate MPA more than the muscle because plasma is the primary route by which pharmaceuticals are distributed within the body (Owen et al. 2007, Brooks et al. 2009, Daughton and Brooks 2011).
CHAPTER 2

METHOD DEVELOPMENT

The seven sections of this chapter cover all of the experimental procedures and materials used to conduct the 14-day BCF study. Section 1 includes the different chemicals and reagents used. The details on fish exposures, experimental design, and sample collection are covered in sections 2-4. As this is the first study to determine accumulation of MPA in fish tissue, section 5 will cover the processes by which the methods for tissue extraction and analysis were developed. Section 6 covers quality control procedures, and the last section explains BCF calculations.

2.1 Chemicals and Reagents

The test chemical, Medroxyprogesterone acetate (MPA, 17α-Acetoxy-6α-methylprogesterone, CAS#71-58-9), was purchased from Sigma-Aldrich (St. Louis, MO). Medroxyprogesterone-d3 (MP-d3, CAS#162462-69-3), also acquired from Sigma-Aldrich, was used as an internal standard.

HPLC grade dichloromethane (DCM), methanol (MeOH), dimethyl formamide (DMF), Hexane (HEX), Acetonitrile (ACN) and acetone were acquired from Fisher Scientific (Houston, TX). Milli-Q water was obtained from the Milli-Q Water System (Millipore, Billerica, MA) within the laboratory.
2.2 Fish Exposures

2.2.1 Animals and housing

Juvenile common carp (Cyprinus carpio) were cultured at the University of North Texas aquatic toxicology facility. The average (mean ± SD) fish weight was 2.6 ± 1 g, and total fish loading expressed as grams of fish tissue per liter of water in exposure and control tanks was less than 2 g/L. The carp were maintained in a 16:8-h light/dark cycle and fed flake food once a day. Food and waste residues were siphoned from exposure chambers approximately two to three hours after feeding.

2.2.2 Exposure System

Fish exposures were accomplished using a continuous flow-through system that incorporated two 20 L exposure chambers (Fig 1). Tap water (City of Denton, TX) was initially filtered through activated charcoal units and subsequently stored in a 500 gallon plastic storage silo. The storage silo was re-filled with dechlorinated tap water every 3-4 days. A peristaltic pump (Masterflex L/S, Cole Parmer, Veron Hills, IL) delivered dechlorinated water at a flow rate of 120 ml/min from the storage silo into mixing chambers, where stock solution was introduced at 5 µl a minute from 30 ml polycarbonate syringes (Becton Dickinson, Franklin Lakes, NJ) using a syringe pump (KDS 220 Multi-Syringe Infusion Pump, KD Scientific, Holliston, MA). Stock solutions of MPA were prepared at a concentration of 2.4 g/L, resulting in a 24,000X dilution factor when the solution was introduced into mixing chambers. High concentration stock solution was prepared in DMF and stored in a 250 ml glass volumetric flask (Corning, Tewksbury, MA) at 4°C. This stock solution was used for the entirety of the study. The target
concentration of MPA in water was delivered from the mixing chambers to the exposure tanks using a peristaltic pump (Masterflex L/S, Cole Parmer, Veron Hills, IL). Complete tank turn over with de-chlorinated tap water occurred approximately 9 times per 24 hours.

![Fig. 1. Continuous fish flow-through exposure system used to conduct BCF test.](image)

2.3 Experimental Design

Carp (n=28) were randomly distributed between two tanks and exposed to 100 µg/l MPA (in dimethyl formamide, DMF<0.003%) for 7 days followed by a depuration phase where the fish were held in clean water for an additional 7 days. To account for any possible effects of
the carrier solvent (DMF), the experiment included a solvent control (n=14) that introduced the fish to DMF by the same exposure method as that of the MPA exposed carp.

2.4 Sample Collection

On days 1, 3, 7, and 14, three fish from each tank were sampled for plasma and tissues (muscle, liver, and brain). Fish were anesthetized with tricaine methanesulfonate (MS-222) prior to removal of any blood or tissue samples. To insure there was enough plasma to analyze, blood samples were combined from the three fish sampled from each tank on each sampling day. Blood was taken from the caudal vein using a heparinized capillary tube (Fisher Scientific) and subsequently placed in a microfuge tube with heparin. Following centrifugation at 2500 g, plasma was taken from the sample and deposited in another heparinized microfuge (Fisher Scientific) tube. Plasma and tissues were stored at -80°C for further processing. To determine the realized exposure concentrations, 5 water samples were collected from each of the 20L test tanks on days 1, 3, and 7 of the 7 day uptake phase of the experiment. On day three of the experiment, the syringe pump stopped infusing stock solution into the mixing chambers due to an error in setting the syringe volume on the pump computer system. This error was fixed approximately 8-10 hours after the problem occurred. The stop in stock solution infusion likely occurred before water samples were collected on day three. For this reason, only samples from days 1 and 7 of the uptake phase were used to calculate exposure concentrations.
2.5 Analytical Methodology

2.5.1 Sample Preparation

2.5.1.1 Water

No extraction step was required for the collected water samples. These samples were only subject to the addition of internal standard and filtration. To remove particulates that could damage the analytical equipment, water samples were filtered into auto sampler vials through 0.45 µm polytetrafluoroethylene (PTFE) syringe filters (Millipore, Billerica, MA) prior to analysis by LC/MSD.

2.5.1.2 Extraction of MPA from Tissues

There was no established method prior to the onset of this study to extract MPA from fish tissues. Several different methods were tested during the course of this research. The first attempt to extract MPA from spiked tissues followed a method that has been used to extract pharmaceuticals, including synthetic progestins, from fish tissues in other studies (Garcia et al. 2012, Nallani et al. 2012). This extraction method (henceforth referred to as the “initial extraction method”), and the modifications made to this method for successful MPA extraction are described below.

Tissues were removed from storage and allowed to thaw. Once thawed, each tissue was weighed in 5 ml polypropylene vials (VWR International, Sugar Land, TX). Fish tissue weights depended on tissue type. Approximately 6-10 mg of muscle, 3-9 mg of brain, and 1-3 mg of liver were taken for extraction. In the 5 mL vial containing the tissue analyzed, 4 mL of the extraction solvent (1+1 (v/v) hexane (HEX): ethyl acetate (EA)) was added followed by the
addition of internal standard. This mixture was homogenized for 3 minutes with a Mini-
Beadbeater™ (Biospec Products, Bartlesville, OK) using 1 mm glass beads (Biospec Products,
Bartlesville, OK). Following homogenization, the resulting homogenate was transferred to a 15
mL glass conical test tube (Kimble Chase, Vineland, NJ) along with 1 ml of Milli-Q water. The
test tubes were mixed using a vortex unit (Fisher Scientific) for approximately 1 minute and
then centrifuged at 2000 rpm for 20 minutes. After centrifugation, the solvent layer was
removed and placed in a 15 mL scintillation vial (Fisher Scientific) and evaporated under
nitrogen to dryness. The contents of the scintillation vials were washed with 1 ml of extraction
solvent and transferred to a pre-weighed 2 mL amber glass auto sampler vial. After
evaporation under nitrogen, the amber vial was weighed again to determine the solvent-
extractable residue weight. The contents of the vial were resolubalized in MeOH and 0.1%
formic acid. Tissue samples were filtered into auto sampler vials through 0.45 µm
polytetrafluoroethylene (PTFE) syringe filters (Millipore) prior to analysis by LC/MSD.

The initial extraction method described above yielded poor extraction recoveries when
used to extract MPA from carp tissues. Extraction recoveries from spiked muscle tissues ranged
from 30-60 percent. Based on these recoveries, it was assumed that the polarity of the
extraction solvent did not properly match that of the test chemical or the internal standard. To
find a solvent that could be used to effectively extract MPA from tissues, spike recoveries were
compared among four extraction solvents that differed by polarity. The solvents compared are
ranked from most polar to least polar in the following order: 2:1 Acetonitrile (ACN):EA > EA >
2:1 HEX:EA > 3:1 HEX:EA. These solvents were chosen because they represented a range of
polarities that extended in both increasing and decreasing polarity directions in relation to the
extraction solvent used initially (1:1 HEX:EA). For each solvent, MPA extractions were performed on three carp muscle samples following the steps of the initial extraction method. Extractions from spiked tissues using EA yielded the highest and most consistent extraction efficiencies. Using this solvent, the extraction efficiency expressed as percent recovery (mean ± SD) of MPA was 68 ± 2.5 percent. This recovery was too low, as the desired extraction efficiency for this study was between 80 and 120 percent. Data gathered from comparing various solvents (2:1 ACN:EA, EA, 2:1 HEX:EA, and 3:1 HEX:EA) and from Rambaud et al. (2005), a study that successfully extracted MPA from bovine hair, indicated that DCM would be effective at extracting MPA from fish tissue. This solvent produced acceptable extraction efficiencies with MPA spiked tissues (muscle, brain, liver, and plasma). The extraction efficiency, expressed as percent recovery (mean ± SD) of MPA from muscle (n=4), brain (n=4), liver (n=4) and plasma (n=4) was 83.3 ± 5.1 percent, 110.1 ± 11.3 percent, 113.3 ± 11.2 percent, and 118.9 ± 8.4 percent, respectively. Because these recoveries were acceptable, DCM was chosen as the extraction solvent.

Due to the volatile nature of DCM, changes were made to the initial extraction method. Because DCM would warp 5 ml polypropylene vials using a Mini-Beadbeater™, tissue homogenization was performed in a 15 mL scintillation vial using a handheld, electric Fisher Tissuemiser (Fisher Scientific, Pittsburgh PA). To avoid cross contamination, the Fisher Tissuemiser shaft tip was rinsed in acetone after homogenization of each tissue.

2.5.1.3 Extraction of MPA from Plasma

8-10 µL of thawed plasma was placed in a glass conical test tube with 5 mL of extraction solvent (dichloromethane [DCM]). Contents of the test tube were spiked with internal standard
followed by addition of 1 mL of Milli-Q water. Each test tube was vortexed for 10-20 seconds and centrifuged for 20 minutes at 2000 rpm. After centrifugation, the solvent layer was transferred to a glass scintillation vial. Five ml of extraction solvent was added to the test tube once more, and the test tube was vortex and centrifuged a second time. The solvent layer was removed from the test tube and added to the scintillation vial that contained the previous addition of solvent. The contents of the scintillation vial were evaporated under nitrogen. Once the scintillation vial was completely dry, it was rinsed with 1 mL of extraction solvent. The extraction solvent was transferred to a 2 mL amber glass auto sampler vial and nitrogen-evaporated. Contents of the vial were reconstituted in methanol and 0.1% formic acid. Plasma samples were filtered into auto sampler vials through 0.45 µm polytetrafluoroethylene (PTFE) syringe filters (Millipore) prior to analysis by LC/MSD.

2.5.2 LC/MSD Analysis

Samples were quantified using an Agilent 1100 LC coupled to an Agilent SL ion trap mass spectrometer. The target analyte was separated using a Nestek Ultra II C18 column (150 x 2.1 mm, 5 µm particle size). The HPLC was maintained at a flow rate of 0.2 ml/min, and the injection volume was 8 µl. Water (A) and MeOH (B), both containing 0.1% formic acid, were used as mobile phases. Gradient conditions of the column were initiated with 50% A, followed by a linear increase to 90% B in 9 minutes. After it reached 90% B, the mobile phase was held at this ratio for 5 minutes. During the final 0.1 minutes of the run, gradient conditions were decreased to 50% B. Spectrometry was performed in electrospray positive ionization mode with nebulizer pressure, dry gas flow rate, dry gas temperature, and capillary voltage conditions
set to 30 psi, 8 L/min, 350 °C, and 3.5 kV, respectively. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode to monitor the following transitions: MPA (387>327) and MP-d3 (348>126). MPA quantification was achieved using an eight point calibration curve (500 ppb to 4ppb).

During sample analysis, the internal standard would occasionally have poor responses that deviated much lower (approximately 60-80%) than usual. These unusually low responses were presumably due to variable levels of ion suppression and would result in elevated estimates of MPA concentration. To compensate for this problem, three separate injections were made for each sample. If any one of these three replicate injections had an internal standard response that was lower than usual, the sample was re-run with two additional replicate injections taken from the sample. All of the replicate injections from that sample over both runs were then compared to determine if the low response was due variable ion suppression or if the low response truly represented an unusually low internal standard concentration in the sample.

2.6 Quality Control

An eight point calibration curve (4-500 µg/L) with linear curve fit between response ratio and the concentration was developed for the analyte (medroxyprogesterone acetate). Only curves with a coefficient of determination ($r^2 \geq 0.99$) were used for quantification.

As a quality control procedure, control (unexposed) tissues and spiked matrices were included in sample batches. Matrix spikes were prepared by spiking each of the matrices (water, muscle, brain, liver, and plasma) with a known concentration (250 ng/mL) of MPA.
followed by preparation and quantification of the compound using the methods previously described.

2.7 BCF Estimation

Tissue-specific BCFs were be determined using two separate approaches. First, the toxicokinetic (BCF<sub>k</sub>) method will calculate the ratio between uptake (k<sub>1</sub>) and depuration (k<sub>2</sub>) rate constants. Based on Newman (1995), the uptake and depuration rate constants were determined by a sequential method that combined linear and nonlinear regression models using the following equation:

\[
C_f = C_w \cdot \left(\frac{k_1}{k_2}\right) \cdot (1 - e^{k_2 \cdot t})
\]

Where (C<sub>f</sub>) is chemical concentration in the fish, (C<sub>w</sub>) is the chemical concentrations in the water and (t) is time. A simple linear curve fit model (lnC<sub>f</sub> = a(t) + b) is used to calculate k<sub>2</sub>. The second approach (proportional BCF (BCF<sub>p</sub>)) determines BCF through calculating the ratio of the chemical concentration in an organism at steady-state equilibrium to the chemical concentration in ambient water. Tissue lipid weights were used to express lipid normalized BCF<sub>p</sub>s.
CHAPTER 3

ACCUMULATION OF MEDROXYPROGESTERONE ACETATE IN FISH

3.1 Abstract

The steroid hormone medroxyprogesterone acetate (MPA), commonly used in oral and injectable contraceptives, has been detected in surface and wastewaters near urban and agricultural areas in several rivers of the world. Although there are no data on the reproductive effects of this compound in fish, other synthetic hormones have been shown to inhibit fish reproduction at concentrations close to or below those that have been detected in the aquatic environment. The objectives of this study are to examine the accumulative potential and tissue distribution of MPA in fish. A freshwater species, the common carp (Cyprinus carpio), was exposed to 100 μg/L of MPA for a 7-day period followed by a depuration phase in which fish were maintained in dechlorinated tap water for an additional 7 days. Tissues (muscle, brain, plasma, and liver) were sampled during the uptake (days 1, 3, and 7) and depuration (day 14) phases of the experiment. MPA concentration in each tissue was determined by liquid chromatography/mass spectrometry (LC/MS). Tissue-specific bioconcentration factors (BCF) ranged from 4.3 to 37.8 and uptake was greatest in the liver > brain > plasma and lowest in the muscle. From a regulatory standpoint, MPA shows little tendency to bioaccumulate in fish.

3.2 Introduction

Synthetic steroid hormones, commonly used in oral contraceptives, hormone replacement therapy, and livestock production, are present in the aquatic environment and can act as potent endocrine disruptors when exposed to aquatic organisms (Durhan et al. 2006,
Peterson et al. 2008, Vajda et al. 2008, Viglino et al. 2008, Cripe et al. 2010). Surface waters receive synthetic hormones from a number of sources, including discharge from waste water treatment plants (WWTPs), sewage runoff from livestock farms, and excrement from fish hatcheries (Ternes et al. 1999, Kolodziej et al. 2004, Chang et al. 2008). Several steroid hormones have been shown to reduce fecundity or cause intersex in fish at concentrations (1-50 ng/L) relative to those detected in WWTP effluent, runoff from beef cattle feeding operations, and even streams (Ankley et al. 2003, Kolodziej et al. 2003, Durhan et al. 2006, Jobling et al. 2006, Fernandez et al. 2007, Santos et al. 2007, Zeilinger et al. 2009). 17-α-ethinylestradiol (EE2), a synthetic estrogen, has been detected in WWTP effluent at concentrations up to 7 ng/L (Desbrow et al. 1998) and has induced vitellogenin, an egg yolk precursor protein, in male rainbow trout at a 100 pg/L exposure level (Purdom et al. 1994). The presence of synthetic steroid hormones in wastewaters of some streams and rivers is associated with sexual abnormalities in fish (Allen et al. 1999, Kirby et al. 2004). Jobling et al. (2002), report that all male fish sampled from waterways receiving wastewater effluent in the U.K. contained both male and female reproductive tissues. EE2 and other estrogenic chemicals are linked to the feminization of fish in waste water effluent dominated waters (Sumpter and Johnson 2008).

Whereas much attention of eco-toxicological studies focused on synthetic steroid hormones has been directed towards estrogens, the efficacy of combination estrogen/progestin oral contraceptives in humans is apparently derived from progestins (Erkkola and Landgren 2005). Additionally, synthetic progestins are likely to exist in the
environment at higher concentrations than estrogens because birth control medications usually contain 3 to 100 times more progestin than estrogen (Zeilinger et al. 2009).

In mammals, synthetic progestins prevent pregnancy through several different mechanisms within various target tissues (Flores-Herrera et al. 2008). These mechanisms include the prevention of follicle stimulating hormone (FSH) and luteinizing hormone (LH) surges that stimulate ovulation (Letterie 1998, Richter et al. 2002), the alteration of cervical mucus cell content and molecular structure (McCann and Potter 1994), and the reduction in the number of cilia, and the frequency and intensity of cilia action on the tubal epithelium, thereby inhibiting the transport of the fertilized egg from the oviduct to the uterus (McCann and Potter 1994, Flores-Herrera et al. 2008). In female fish, natural progestins play a critical role in oogenesis (Miura et al. 2007), regulation of oocyte maturation (Nagahama and Yamashita 2008), and, in some species, ovulation (Pinter and Thomas 1999). Progestins are associated with sperm motility (Tubbs and Thomas 2008) and initiation of spermiation (Ueda et al. 1985) in males. Because synthetic progestins have the ability to mimic natural progestins in fish, and thus disrupt reproductive and developmental processes, these compounds pose a risk to fish communities. Previous research indicates that synthetic progestins are capable of inhibiting reproduction in fathead minnows at concentrations as little as 0.8 ng/L (Zeilinger et al. 2009) and completely halting reproduction at concentrations ranging from 85 ng/L – 100 ng/L (Paulos et al. 2010, Runnalls et al. 2013).

The synthetic progestin, medroxyprogesterone acetate (MPA), is widely used as an injectable and oral contraceptive and as a therapy for breast cancer and hormone replacement. MPA has been detected in wastewater effluent at concentrations up to 18 ng/L (Chang et al.
2009) and in surface water up to 1 ng/L (Kolodziej et al. 2004). In mammals, MPA has been shown to interact with receptors for progesterone (Winneker et al. 2003), androgen (Hackenberg et al. 1993, Bentel et al. 1999), and estrogen (Di Carlo et al. 1983). Like many other steroid hormones, MPA is relatively hydrophobic (Table 1) giving it the ability to partition into the lipid portion of organisms and bioaccumulate (Lindenmaier et al. 2005). Xenobiotics that bioaccumulate may trigger certain toxicological responses, such as reduced fecundity, as a result of increased tissue burden over an extended period of time (Nallani et al. 2012). From a regulatory perspective, bioaccumulative potential is an important aspect in determining the risk a compound poses to aquatic organisms. For these reasons, the objectives of the current study are to determine the tissue specific and plasma bio concentration factor (BCF) of MPA in fish.

3.3 Materials and Methods

3.3.1 Chemicals and Reagents

The test chemical, Medroxyprogesterone acetate (MPA, 17α-Acetoxy-6α-methylprogesterone, CAS#71-58-9), was purchased from Sigma-Aldrich (St. Louis, MO). Medroxyprogesterone-d3 (MP-d3, CAS#162462-69-3), also acquired from Sigma-Aldrich, was used as an internal standard. HPLC grade methanol, dichloromethane, and dimethylformamide were obtained from Fisher Scientific (Houston, TX). Milli-Q water was obtained from the Milli-Q Water System (Millipore, Billerica, MA) within the laboratory.

3.3.2 Fish Exposure and Study Design
Juvenile common carp (*Cyprinus carpio*) were cultured at the University of North Texas aquatic toxicology facility. The carp were maintained in a 16:8-h light/dark cycle and fed flake food once a day. Fish exposures were accomplished using a continuous flow-through system that incorporated two 20 L tanks. Each tank received the test chemical from a mixing chamber that was dosed with MPA by direct infusion from a syringe pump, and both tanks drained via overflow into a common drain pan. Complete tank turn over with de-chlorinated tap water occurred approximately 9 times per 24 hours. Carp (n=28) were randomly distributed between the two tanks and exposed to 100 µg/l MPA (in dimethyl formamide, DMF<0.003%) for 7 days followed by a depuration phase where the fish were held in clean water for an additional 7 days. To account for any possible effects of the carrier solvent (DMF), the experiment included a solvent control (n=14) that introduced the fish to DMF by the same exposure method as that of the MPA exposed carp.

3.3.3 Tissue and Water Sample Collection

On days 1, 3, 7, and 14, three fish from each tank were sampled for plasma and tissues (muscle, liver, and brain). Fish were anesthetized with tricaine methanesulfonate (MS-222) prior to removal of any blood or tissue samples. To insure there was enough plasma to analyze, blood samples were combined from the three fish sampled from each tank on each sampling day. Blood was taken from the caudal vein using a heparinized capillary tube and subsequently placed in a microfuge tube with heparin. Following centrifugation at 2500 g, plasma was taken from the sample and deposited in another heparinized microfuge tube. Plasma and tissues were stored at -80°C for further processing. To determine the realized exposure
concentrations, 5 water samples were collected from each of the 20L exposure tanks on days 1 and 7 of the 7-day uptake phase.

3.3.4 Preparation of Tissue and Water Samples

Tissues were removed from storage and allowed to thaw. Once thawed, they were blotted dry and approximately 6-10 mg of muscle, 3-9 mg of brain, and 1-3 mg of liver were taken for extraction. In a 15 mL scintillation vial containing the tissue analyzed, the extraction solvent (dichloromethane [DCM]) was added along with the internal standard. This mixture was homogenized with a Tissuemiser for approximately 1 minute. Following homogenization, the resulting homogenate was transferred to a glass conical test tube along with 1 ml of Milli-Q water. Test tubes were vortexed for approximately 1 minute and then centrifuged at 2000 rpm for 20 minutes. After centrifugation, the solvent layer was removed and placed in a scintillation vial and evaporated under nitrogen to dryness. Contents of the scintillation vials were washed with 1 ml of extraction solvent and transferred to a pre-weighed 2 mL amber glass auto sampler vial. After dry, the amber vial was weighed again to determine the lipid weight. Contents of the vial were resolubalized in MeOH and 0.1% formic acid.

8-10 µL of thawed plasma was placed in a glass conical test tube with 5 mL of extraction solvent. Contents of the test tube were spiked with internal standard followed by addition of 1 mL of Milli-Q water. Each test tube was vortexed for 10 – 20 seconds and centrifuged for 20 minutes at 2000 rpm. After centrifugation, the solvent layer was transferred to a glass scintillation vial. 5 ml of extraction solvent was added to the test tube once more, and the test tube was vortex and centrifuged a second time. The solvent layer was removed from the test
tube and added to the scintillation vial that contained the previous addition of solvent. The contents of the scintillation vial were dried under nitrogen. Once the scintillation vial was completely dry, it was rinsed with 1 mL of extraction solvent. The extraction solvent was transferred to a 2 mL amber glass auto sampler vial and nitrogen-evaporated. Contents of the vial were reconstituted in methanol and 0.1% formic acid.

No extraction step was required for collected water samples. These samples were only subject to addition of internal standard and filtration. As a clean-up step, tissue, plasma, and water samples were filtered into auto sampler vials through 0.45 µm polytetrafluoroethylene (PTFE) filters prior to analysis by LC/MSD.

3.3.5 LC/MSD Analysis

Samples were quantified using an Agilent 1100 LC coupled to an Agilent SL ion trap mass spectrometer. The target analyte was separated using a Nestek Ultra II C18 column (150 x 2.1 mm, 5 µm particle size). The HPLC was maintained at a flow rate of 0.2 ml/min, and the injection volume was 8 µl. Water (A) and methanol (B), containing 0.1% formic acid, were used as mobile phases. Gradient conditions of the column were initiated with 50% A, followed by a linear increase to 90% B in 9 minutes. After it reached 90% B, the mobile phase was held at this ratio for 5 minutes. During the final 0.1 minutes of the run, gradient conditions were decreased to 50% B. Spectrometry was performed in electrospray positive ionization mode with nebulizer pressure, dry gas flow rate, dry gas temperature, and capillary voltage conditions set to 30 psi, 8 L/min, 350 °C, and 3.5 kV, respectively. The mass spectrometer was set on multiple reaction
monitoring mode (MRM) for the following ions: MPA (387>327) and MP-d3 (348>126). MPA quantification was achieved using an eight point calibration curve (500 ppb to 4 ppb).

3.3.6 BCF Estimation

BCFs were determined using two separate approaches: the kinetic BCF (BCF\textsubscript{k}) method and proportional BCF (BCF\textsubscript{p}) method. Following procedures previously described by Newman (1995), BCF\textsubscript{k}'s were calculated as the ratio between uptake (k\textsubscript{1}) and depuration (k\textsubscript{2}) rate constants. These rate constants were determined by a sequential approach that combined linear and nonlinear regression models. BCF\textsubscript{p}'s were calculated as the ratio of the chemical concentration in each fish tissue at steady-state equilibrium to the chemical concentration in the water.

3.4 Results

3.4.1 Water Quality and MPA Concentrations

Water quality parameters (mean ± SD) were measured in each exposure and control tank during each sampling day of the 14-day study. Temperature, dissolved oxygen, pH, and conductivity in exposed and control tanks were 22.0 ± 0.2°C, 7.4 ± 0.3 mg/L, 7.8 ± 0.2, and 325.6 ± 6.6 μS, respectively. The average measured MPA concentration (mean + SD) in the exposure tanks during the uptake phase of the experiment was 118 ± 17.3 μg/L (n=20) (Figure 2). This value is approximately 118% of the nominal exposure concentration (100 μg/L). MPA was not detected (<4 μg/L) in any of the solvent control water samples (n=10).
3.4.2 MPA Concentrations in Fish Tissues and BCFs

Concentrations (ng/g wet weight) of MPA in carp muscle, brain, liver, and plasma (ng/ml) over the 14-day experiment are summarized in Figure 3. With MPA concentrations ranging from 955.6 to 6515.6 ng/g, liver had the greatest uptake, followed by brain (470.5 – 2160.8 ng/g), then plasma (160.0 – 1549.3 ng/ml), and lastly muscle (150.5 – 780.2 ng/g). Brain displayed the greatest accumulation on day three of the exposure period, unlike the other tissues, which had greatest MPA concentrations on day 7. Plasma showed the most dramatic increase in MPA concentrations, with a seven fold increase in accumulation from the start of the experiment to day-3. There were no detectable levels (<89 ng/g for muscle, <114 ng/g for brain, <114 ng/g for liver, and <160 ng/ml for plasma) of MPA in tissues of fish from solvent control or depurated fish.

The wet weight kinetic BCFs for MPA in carp tissues ranged from 10.9 to 37.8 (Table 2), a range that is similar to that of the 7-day proportional BCFs (4.3-32.0). Lipid normalized proportional BCFs were approximately one order of magnitude higher than the wet weight values. Expressing BCFs based on lipid weights are useful for comparing accumulation data derived from different species (Meador et al. 2008).

3.4.3 Spike Recoveries

To assess the extraction and analytical method efficiency, each of the matrices (water, muscle, brain, liver, and plasma) was spiked with a known concentration (250 µg/L) of MPA. Tissue spikes were subject to the extraction and quantification methods previously described. Because spiked water samples did not require an extraction step, these samples were only
passed through syringe filters before analysis. Four replicate spikes were analyzed for each matrix. Method efficiency, expressed as percent recovery (mean ± SD) of MPA from water, muscle, brain, liver and plasma are 80.3 ± 8.7 percent, 83.3 ± 5.1 percent, 110.1 ± 11.3 percent, 113.3 ± 11.2 percent, and 118.9 ± 8.4 percent, respectively.

3.5 Discussion

Although synthetic progestins have been frequently detected in surface and wastewaters, data on the toxicity and accumulation of these compounds is insufficient to estimate the risk that they pose to aquatic ecosystems (OECD 1996). So far, the only toxicity test performed on an aquatic species using MPA was by Petersen et al. (University of North Texas Aquatic Toxicology Laboratory, University of North Texas, TX, USA, unpublished data). Their study indicated that fathead minnow larval growth was inhibited after aqueous exposure to 500 µg MPA/L. This exposure concentration is much higher than what has been detected in the environment, however, aquatic toxicity data from studies on other synthetic progestins indicate that reproductive endpoints are likely to be sensitive to MPA concentrations close to or below those that have been detected in wastewaters.

3.5.1 MPA Tissue-Specific Accumulation and BCFs

This study is the first to determine the bioaccumulative potential of MPA in fish. Laboratory derived wet weight tissue-specific BCFs ranged from 4.3 to 37.8, indicating that MPA has little ability to bioaccumulate in common carp. So far LNG and NET are the only other synthetic progestins for which fish tissue accumulation data are available. Nallani et al. (2012)
reported tissue specific BCFs for NET ranging from 2.5 to 40. These values are remarkably close to the BCFs for MPA. LNG, however, has a field derived fish plasma BCF that is over 1700-fold higher than that of MPA (Fick et al. 2010). The estimated BCF of LNG is 46, but Fick et al. (2010) reported a plasma BCF of 12000 in fish exposed to sewage effluents. The authors of the study stated that higher than expected uptake might have been mediated through sex-steroid binding globulins (SSBG) found in the gills. Due to the low BCF values we reported, SSBG does not appear to be involved in sequestering MPA within carp. This observation correlates well with mammalian data as MPA does not bind with SSBG in humans (Rxlist 2013).

From a regulatory standpoint, MPA is well below the lower BCF threshold (1000) that the EPA uses to characterize a chemical as a priority contaminant and one that poses a risk to humans and ecosystems. However, a BCF is only one of the many factors taken into consideration when determining a contaminant’s environmental threat based on its persistence in the environment (P), accumulation in biological organisms (bioaccumulation (B)) and Toxicity (T). Chemicals that display reproductive toxicity towards aquatic organisms and have high predicted environmental concentrations may meet “PBT” criteria that warrant an environmental risk assessment (USEPA 1999). Though no data are available on the reproductive effects of MPA in fish, other progestins, including natural progesterone, are capable of hindering or completely halting reproduction in fish at concentrations close to or below those that have been detected in the environment (Zeilinger et al. 2009, Paulos et al. 2010).

MPA concentrations were greatest in the liver > brain > plasma and lowest in the muscle. A similar tissue distribution pattern was obtained for NET in channel catfish (*Ictalurus*...
punctatus) and fathead minnow. NET concentrated from greatest to least in the various tissues of these species as follows: kidney > liver > plasma > gill > brain > muscle (Nallani et al. 2012). Greater uptake likely occurred in the liver because MPA is fairly lipophilic (log Kow = 4.09) such that it can passively diffuse through lipid membranes, and thus displays more affinity towards fatty tissues. Furthermore, mammalian pharmacology data indicates that MPA is extensively metabolized in the liver. The BCF difference between the tissue with the highest (liver) and that with the lowest (muscle) MPA concentration was less than 28. This difference is marginal considering that tissue uptake differences for other compounds in fish often vary by two or more orders of magnitude. Like MPA, NET displayed relatively low uptake variation among different tissues (Nallani et al. 2012).

3.5.2 Tissue Partition Coefficients

Partition coefficients, calculated from the ratio of a chemical’s concentration in plasma to that in tissue or water, are useful in the risk assessment of aquatic contaminants in several ways. One important use of partition coefficients is to establish tissue burdens across several different tissues based on the concentration detected in a single tissue. For example, if plasma concentrations of a compound are known, concentrations in the liver, brain, and muscle can be determined through partition coefficients. These ratios are also helpful in field studies as plasma:water coefficients can be used to predict plasma levels of a compound in fish based on concentrations that have been detected in water. Lastly, partition coefficients can be utilized to predict the biological effects of an aquatic contaminant on fish based on mammalian data. The fish plasma model developed by Huggett et al. (2004), for instance, calculates a
pharmaceutical’s fish steady state plasma concentration (FssPC) based on the drug’s predicted/measured environmental concentration and partitioning between blood and water. The FssPC is used in conjunction with human plasma data to produce an effect ratio that indicates potential for pharmacological response in fish. BCF data from the 7-day exposure period were used to calculate partition coefficients for MPA in common carp tissue and plasma. These coefficients (blood:water, blood:muscle, blood:brain, and blood:liver) are presented in Table 3. The data indicates fish plasma MPA concentrations will be slightly higher than muscle concentrations, similar to brain concentrations, and lower than liver concentrations.

3.5.3 Conclusions

MPA shows little potential to accumulate in fish tissue. Because this compound has been detected in the aquatic environment and is likely to interact with fish progesterone receptors, further research is needed on the reproductive effects of MPA in fish. The results from this study will provide part of the framework needed to predict the human health and ecological risks posed by MPA.

3.6 Chapter References


Peterson, L. H., C. F. Gomez, and D. B. Huggett. 2008. EFFECTS OF MEDROXYPROGESTERONE AND PROGESTERONE ON LARVAL GROWTH AND SURVIVAL IN FATHEAD MINNOWS (Pimephales promelas)


Table 1

Physical and Chemical properties of Medroxyprogesterone acetate (retrieved from EPISUITE [USEPA, 2012])

<table>
<thead>
<tr>
<th>Property</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAS no.</td>
<td>71-58-9</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>386.52</td>
</tr>
<tr>
<td>Partition coefficient (log $K_{ow}$)</td>
<td>4.09</td>
</tr>
<tr>
<td>Log $D_{ow}$ (log P @ pH 7.4) $^a$</td>
<td>4.17</td>
</tr>
<tr>
<td>Log $D_{ow}$ (log P @ pH 5.5) $^a$</td>
<td>4.17</td>
</tr>
<tr>
<td>Water solubility @ 25°C (mg/L)</td>
<td>1.20</td>
</tr>
<tr>
<td>Vapor pressure @ 25°C (mm HG)</td>
<td>5.61E-007</td>
</tr>
<tr>
<td>Henry’s law constant @ 25°C (atm-m³/mol)</td>
<td>1.45E-009</td>
</tr>
<tr>
<td>Estimated Half Lives (hr):</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>1.44e+003</td>
</tr>
<tr>
<td>Soil</td>
<td>2.88e+003</td>
</tr>
<tr>
<td>Sediment</td>
<td>1.3e+004</td>
</tr>
<tr>
<td>Environmental Persistence $^b$ (hr)</td>
<td>2.4e+003</td>
</tr>
<tr>
<td>Structure</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Retrieved from ACD/PhysChem Suite (ACD/Labs 2012)

$^b$ Using emission rates of 1000 kg/hr
Fig. 2. Medroxyprogesterone acetate (MPA) concentration (median, 75th percentile, 25th percentile, high value, low value, n=20) in water during 7-day uptake phase.
Fig. 3. Medroxyprogesterone acetate (MPA) concentration (mean ± SEM, n=5-6) in muscle, brain, liver, and plasma of common carp exposed to 118 µg MPA/L

Table 2

Kinetic and proportional BCFs for muscle, brain, liver, and plasma tissues of common carp exposed to 118 µg medroxyprogesterone acetate/L

<table>
<thead>
<tr>
<th>Tissue</th>
<th>BCFk</th>
<th>7 dBCF</th>
<th>Wet wt. basis</th>
<th>Lipid Normalized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle</td>
<td>10.9</td>
<td>4.3</td>
<td>90.1</td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>13.0</td>
<td>7.0</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>14.4</td>
<td>10.7</td>
<td>99.0</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>37.8</td>
<td>32.0</td>
<td>269.1</td>
<td></td>
</tr>
</tbody>
</table>
Table 3

Partition coefficient for medroxyprogesterone acetate in different compartments based on tissue bioconcentration in common carp

<table>
<thead>
<tr>
<th>Tissue Compartments</th>
<th>Partition coefficient a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood:water</td>
<td>7.0</td>
</tr>
<tr>
<td>Blood:muscle</td>
<td>1.6</td>
</tr>
<tr>
<td>Blood:brain</td>
<td>0.6</td>
</tr>
<tr>
<td>Blood:liver</td>
<td>0.2</td>
</tr>
</tbody>
</table>

a Calculated from the ratio of concentration of MPA in plasma to that in water or tissue during uptake phase.
CHAPTER 4
CONCLUSIONS AND FUTURE AREAS OF RESEARCH

The overall aim of this thesis research is to determine the laboratory derived BCF of MPA in tissues (muscle, brain, liver, and plasma) of common carp. Based on the results from the BCF experiment, MPA displays potential to accumulate in fish tissue, but this accumulation potential is considered low from a regulatory standpoint.

Lipid normalized BCFs for MPA are about an order of magnitude higher than wet weight BCFs and uptake was highest in liver tissues, indicating that progestin shows preference for lipid-rich fractions. These distribution patterns correlate with the lipophilic nature of MPA as it has a log $K_{ow}$ of 4.09.

Compared to Norethindrone (NET), the only other synthetic progestin for which there are fish BCF data available, MPA shows similar tissue accumulation, yet the partition coefficient for MPA (4.09) is considerably higher than that of NET (2.97) (Nallani et al. 2012). One possible explanation for this phenomenon is that fish more quickly metabolize MPA than NET. In mammals, MPA is rapidly metabolized (Rxlist 2013). Furthermore, SSBG in the gills of zebrafish have been shown to actively sequester NET (Miguel-Queralt and Hammond 2008). In mammals, MPA does not interact with SSBG (Rxlist 2013). Therefore, SSBG in fish might explain the fact that MPA and NET have close to equal fish BCF values despite MPA’s higher log $k_{ow}$.

Whereas MPA displays low bioaccumulation in fish tissues, the presence of this compound in surface waters still poses potential risks to fish communities. Synthetic progestins likely elicit reproductive effects in fish through interaction with steroid hormone receptors. In humans, MPA is capable of binding to the human uterine progesterone receptor with nearly
three times the affinity of natural progesterone and over twice the affinity of NET (Winneker et al. 2003), a synthetic progestin that can impair egg production in fathead minnow at concentrations as low as 1.2 ng/L (Paulos et al. 2010). Like several other synthetic progestins, MPA has multiple hormonal properties. In addition to binding to the progesterone receptor, MPA has also been shown to bind to the androgen receptor in humans (Winneker et al. 2003) and reduce binding affinity of estrogen for the estrogen receptor in rats (Di Carlo et al. 1983). Other synthetic progestins have been reported to decrease plasma 17β-estradiol levels and cause the appearance of male secondary morphological characteristics in fathead minnows at exposure concentrations ≤ 30 ng/L (Zeilinger et al. 2009, Paulos et al. 2010).

Bioaccumulation is only one piece of the puzzle in PBT assessments used by regulatory agencies to determine the environmental risk posed by an aquatic contaminant. MPA is a potent steroid hormone, yet nothing is known about the reproductive effects of MPA on aquatic organisms. Reproductive effects are of particular concern with regards to fish because the development and physiology of mammals and aquatic vertebrates is similar. Consequently, the target molecules of pharmaceutical compounds are likely to be comparable between fish and mammals (Gunnarsson et al. 2008).

Future research should be aimed at generating data on the chronic reproductive effects of MPA on fish with a particular focus on how the compound affects fecundity, plasma steroid hormone levels, and steroid receptor expression. Furthermore, data are needed on concentrations of MPA in fish tissues sampled from areas likely receiving pharmaceutical compounds, such as surface waters near WWTP discharges. These data would aid in further
prioritizing risk based on exposure. Field plasma samples would be useful in determining risks posed by MPA based on mammalian pharmacology data (Huggett et al. 2004).

In conclusion, the presence of synthetic progestins in surface waters is potentially harmful to aquatic communities. More data are needed on the eco-toxicological effects of MPA in order to assess risks that this compound poses to the environment. This research should aid future efforts in characterizing and prioritizing threats posed by synthetic progestins to aquatic ecosystems.
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