DETERMINATION OF BIOCONCENTRATION POTENTIAL OF SELECTED

PHARMACEUTICALS IN FATHEAD MINNOW, *Pimephales promelas*,

AND CHANNEL CATFISH, *Ictalurus punctatus*

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The primary objective of this work was to determine the tissue-specific bioconcentration factors (BCFs) of the selected pharmaceuticals: norethindrone (NET), ibuprofen (IBU), verapamil (VER), clozapine (CLZ) and fenofibrate (FFB) in two freshwater fishes: fathead minnow and channel catfish. BCF tests on fathead followed the standard OECD 42-day test while a 14-day abridged test design was used in catfish exposures. Additional objectives included a) comparing the measured BCFs to the US EPA's BCFWIN model predicted values, b) comparing the BCF results from the standard and reduced tests, and c) prediction of chronic risk of the pharmaceuticals in fish using the human therapeutic plasma concentrations.

The results indicated that all the pharmaceuticals, except IBU, have the potential for accumulation in fish. Estimated BCFs for NET, VER and FFB may not be significant in view of the current regulatory trigger level (BCF $\geq$ 2000); however, CLZ's BCF in the liver had approached the criterion level. Significant differences were noticed in the tissue-specific uptake levels of the pharmaceuticals with the following general trend: (liver/kidney) > (gill/brain) > (heart/muscle) > plasma. IBU uptake was highest in the plasma.

When compared to the measured BCFs, predicted values for NET, IBU, VER and FFB were slightly overestimated but did not differ largely. However, the measured BCF of CLZ in the liver was approximately two-orders of magnitude higher than the predicted level. The tissue-BCFs for the two species were not widely different indicating the potential usefulness of the reduced BCF test. Comparison of fish and human plasma levels indicated that NET, CLZ and VER have the potential to cause chronic effects in fish.

ACKNOWLEDGMENTS

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5-HT	5-hydroxytryptamine
ACE	Angiotensin converting enzyme
ACN	Acetonitrile
ACUC	Animal Care and Use Committee
ADME	Absorption distribution metabolism
AF	Assessment factor
ALDH2	Aldehyde dehydrogenase 2
API	Active pharmaceutical ingredient
API-ESI	Atmospheric pressure ionization-electrospray interface
BAF	Bioaccumulation factor
BCF	Bioconcentration factor
BEI	Bioequivalence index
BF3/MeOH	Borontrifluoride/methanol
BSAF	Bio-sediment accumulation factor
BSI	Brain somatic index
CAFO	Confined animal feeding operation
CDER	Center for Drug Evaluation and Research
CID	Collision induced dissociation
CONCAL	Continuing calibration standard
COX	Cyclooxygenase
CSI	Cardio somatic index
CYP450	Cytochrome P450
DCM	Dichloromethane
DDD	Daily defined dose

DMF	N, N-dimethylformamide
DOM	Dissolved organic matter
DSL	Domestic substance list
EC	Environment Canada
ECOSAR	Ecological structure activity relationship
EDC	Endocrine disruption compound
EE2	17 alpha-ethinyl estradiol
EIC	Expected introduction concentration
ELS	Early life stage
EMEA	European Medicines Agency
EPI	Estimation program interface
ER	Effect ratio
ERA	Environmental risk assessment
FONSI	Finding of no significance impact
FPC	Fish plasma concentration
FPM	Fish plasma model
FWMF	Food web magnification factor
GC/MS	Gas chromatography/mass spectrometry
GnRH	Gonadotropin releasing hormone
GREAT-ER	Geo-reference regional exposure assessment tool for European rivers
HI(Q)	Hazard index (quotient)
HIS	Hepatic somatic index
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
HPLC	High performance liquid chromatography
HTPC	Human therapeutic plasma concentration

IDMS	Isotopic dilution mass spectrometry
ISTD	Internal standard
LC/MS/MS	Liquid chromatography tandem mass spectrometry
LC-50	Lethal concentration 50
LTQ-MS	Linear quadruple-mass spectrometry
MDL	Method detection limit
MDR	Multi drug resistance
MEC	Measured environmental concentration
MEEC	Maximum expected environmental concentration
MOA	Mode of action
MRM	Multiple reaction monitoring
MS-222	Tricaine methanesulfonate
MSD	Mass selective detector
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
MXR	Multi xenobiotic resistance
NCCOS	National Centers for Coastal Ocean Science
NOEC	No observed effect concentration
NOM	Natural organic matter
NSAID	Non-steroidal anti-inflammatory drug
NSI	Nephritic somatic index
OECD	Organization for Economic Cooperation and Development
OTC	Over the counter
PBPK	Physiologically based pharmacokinetic model
PBT	Persistent bioaccumulative toxic
PCB	Polychlorinated biphenyl

PCP	Personal care product
PEC	Predicted environmental concentration
PER	Plasma effect ratio
PFTBA	Perfluorotributylamine
PhAC	Pharmaceutically active compounds
PhATE	Pharmaceutical assessment and transport evaluation
PIER	Pharmaceuticals in the environment
PNEC	Predicted no effect concentration
POP	Persistent organic pollutant
PPAR	Peroxisome proliferator activated receptor
ppb	Parts per billion
PTFE	Polytetrafluoroethylene
PVDF	Polydivinylidene fluoride
QSAR	Quantitative structure activity relationship
REACH	Registration Evaluation and Authorization of Chemicals
RQ	Risk quotient
RT	Retention time
SIM	Selected ion monitoring
SPE	Solid phase extraction
SPMD	Semi permeable membrane device
SPME	Solid phase micro extraction
SRC	Syracuse Research Corporation
SSRI	Selective serotonin reuptake inhibitor
STP	Sewage treatment plant
TGD	Technical Guidance Document

TIC   Total ion current

TMF   Trophic magnification factor

UNEP   United Nations Environmental Program

WWTP   Wastewater treatment plant

CHAPTER 1

INTRODUCTION

Pharmaceuticals are bioactive compounds designed to target specific receptors and elicit certain biological effects in mammals. In addition to the main intended therapeutic effect, drugs often incur several secondary pharmacodynamic activities. The biological targets are phylogenetically conserved across species and unintended exposure to the medicinal products could trigger pharmacological interactions (similar those in mammals) in the non-target organisms (Länge and Dietrich, 2002). The implications of such interactions in the aquatic and terrestrial species have been recognized (Jobling et al., 1998; Oaks et al., 2004). Nevertheless, increased efforts are warranted to thoroughly understand the extent and magnitude of this "emerging" ecological issue. The global consumption of pharmaceuticals has risen substantially over the last decade and is not expected to lessen in the future due to the recent advances in biotechnology that may lead to "individualized" therapeutic options (Williams, 2005).

After consumption, pharmaceuticals and/or their metabolic products are released to the environment, primarily through effluents from sewage treatment plants (STPs). Because STPs are not designed to remove pharmaceuticals (organic molecules with several polar functional groups), trace levels of residual pharmaceuticals have been detected in different environmental compartments (Richardson and Bowron, 1985; Buser et al., 1998a; Ternes, 1998; Stumpf et al., 1999; Ayscough, 2000; Heberer, 2002; Kolpin et al., 2002). The environmental entry of these micropollutants can be direct, if the discharge is unregulated (e.g. intensive animal feeding operations and aquaculture facilities) (Boxall et al., 2004). Unlike the agricultural chemicals that are used seasonally, the environmental release of pharmacologically active compounds (PhACs) is continuous, resulting in their (pseudo-) persistence nature, especially in the aquatic

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environment. Such low-level, continuous and long-term (chronic) aquatic exposures to these "emerging contaminants" is of great concern due to the potential ecological impacts as well as possible human health effects.

Environmental regulation of pharmaceuticals is not currently in practice as the protection of the public health receives higher priority. However, all new drugs need environmental risk assessment (ERA) prior to their approval. It is suggested that such ERAs should also be conducted on the "existing" pharmaceuticals due to their frequent detection in the environment (Adler et al., 2008). With over 3000 existing pharmaceutical products and an equal number of drugs under development, prioritization of pharmaceuticals for risk analysis becomes crucial. As it is not practical to test all the compounds on every single species, ERAs often rely on the extrapolation of results from short-term acute toxicity tests on few surrogate species (algae, daphnia and fish) or on the models based on the physicochemical characteristics of compounds. Although such strategies may be useful in predicting the environmental risk of priority pollutants, their utility is limited in terms of risk predictions for pharmaceuticals. Some authors suggested the mode of action (MOA) or mechanism based approach as the most desirable strategy for understanding the potential risk to non-target species (Seiler, 2002). However, this requires in-depth knowledge of comparative physiology and biochemistry, to evaluate the potential differences in the pharmacological interactions and hence varied effects on ecologically relevant species.

Two potentially useful strategies for predicting chronic risk for aquatic organisms include a) utilizing the widely available mammalian pharmacological data, and b) using measured/predicted bioconcentration potential as a tool for priority setting. Recent studies have indicated a high degree of homology between human and aquatic vertebrate biological target

systems and hence fish and amphibians could be sensitive to the unintended exposure to pharmaceutical products (Huggett et al., 2003; Gunnarsson et al., 2008; Christen et al., 2010). Investigation of the utility of the above suggested approaches in prioritizing pharmaceuticals that may pose potential risks to fish forms the basis for this dissertation.

As part of the drug safety studies, extensive information is generated in terms of pharmacological and toxicological data. One such important parameter is the human therapeutic plasma concentration, often related to the dose and the resultant pharmacological activity. If plasma concentration of a drug in fish reaches that of mammals, then an equivalent pharmacological activity may be expected in the two species. Utilizing this principle, a fish plasma model (FPM) model has been developed that compares the human therapeutic plasma levels to the fish plasma concentrations, yielding an effect ratio (ER) (Huggett et al., 2003). FPM incorporates a log P based model (Fitzsimmons et al., 2001) to predict the fish plasma concentration using the measured or predicted exposure concentrations. Improvements in such prediction may be obtained with using log Dow (log P @ pH 7) values. In the FPM model, ER<1 for a particular drug indicates possible chronic risk to fish and hence needs to be prioritized for ERA. The model was initially tested and validated on 28 pharmaceutical compounds (Huggett et al., 2004). Assuming similar pharmacodynamic effects in mammals and fish (i.e. equal plasma concentrations), a recent study used the FPM to determine critical environmental concentrations of 500 pharmaceuticals (Fick et al., 2010b). Although extensive validation of this model is necessary, it has the potential for its use in prioritization schemes.

Bioconcentration has been an important regulatory end point in ERAs for priority pollutants (Arnot and Gobas, 2006; Nordberg and Rudén, 2007). Measured or predicted potential for bioconcentration is also used in tiered ERA frameworks for pharmaceuticals (Bound and

Voulvoulis, 2004). In fact, a compound with bioaccumulative potential is automatically chosen

for chronic risk studies, bypassing the initial stages of testing/screening. In spite of their valuable

use in priority setting, the available data on experimentally determined bioconcentration

measures (expressed in terms of bioconcentration factor, BCF) are limited. This is due to the

cost, time and animal use constraints associated with the standard BCF test guidelines (OECD

305) that requires large number of animals with long-duration of exposures (Weisbrod et al.,

2007). The potential use of a reduced BCF test design with fewer animals and shorter duration

has been suggested (Springer et al., 2008). However, it is not known whether such reduced

testing approaches may be used to estimating BCFs for pharmaceuticals. It is, therefore,

necessary to determine whether the measured pharmaceutical BCFs using reduced test designs

will be similar to those obtained with the standard OECD test.

In the absence of measured data, BCFs are predicted using compound-specific

quantitative structure activity relationship (QSAR) models (e.g. BCFWIN). The predicted BCFs

for traditional environmental toxicants are often in good agreement with the measured values.

However, pharmaceuticals are chemically distinct from the conventional pollutants in that they

have polar functional groups and often are subjected to extensive metabolism. Although these

characteristics are accounted for using appropriate correction factors, it is not known whether

such models can be used to predict BCFs for pharmaceuticals.

1.1   Aims and Objectives

The overall aim of this work was to examine the usefulness of the existing models to

predict bioconcentration potential of pharmaceuticals in fish. To achieve this primary objective,

BCFs for five pharmaceuticals: norethindrone, ibuprofen, verapamil, clozapine and fenofibrate

were experimentally determined in two fresh water species fathead minnow (*Pimephales promelas*) and channel catfish (*Ictalurus punctatus*). BCF tests were conducted using two testing approaches, one following the standard OECD test guidelines (fathead tests) and other with a reduced test design (catfish tests), to achieve the following objectives.

Objective 1: To compare the measured and predicted tissue BCFs of pharmaceuticals

> Hypothesis: BCFs of pharmaceuticals can be predicted using the US EPA's BCFWIN model

Objective 2: To compare the measured and predicted fish plasma BCFs

> Hypothesis: Fish plasma BCFs can be predicted using the log K_{ow} based model

Objective 3: To compare the measured BCFs from the standard long-term and the reduced test design

> Hypothesis: The BCF test results from the long-term and reduced test design will be similar

Objective 4: To predict the potential chronic risk of the select pharmaceuticals in fish using the mammalian pharmacological data

> Hypothesis: Fish plasma model derived ERs can be used to predict the pharmaceuticals' chronic risk to fish

1.2   Dissertation Structure

The dissertation consists of seven chapters. The first chapter provides the general background information, aims and objectives of the research. Extensive review of the available literature was conducted on the emergence of pharmaceuticals as environmental contaminants (Chapter 2) with detailed information on the usage, sources, occurrence, fate, transport, ecotoxicity and the current regulatory aspects. A comprehensive review on bioconcentration is outlined in Chapter 3, covering the basic terminology, regulatory importance, prediction of bioconcentration using models and the prior research on bioconcentration of pharmaceuticals in the aquatic organisms.

Chapter 4 covers the materials and method section providing the details on the pharmaceuticals and the fish species chosen for this work. BCF testing methods on fathead minnow and channel catfish using a continuous flow-through exposure system are included. This chapter also consists of information on the analytical methods including extraction, cleanup and instrumental analysis. Finally, details on the data collection and statistical analyses are provided. Chapters 5 through 8 cover the bioconcentration studies conducted on the pharmaceuticals considered in this project. These chapters were formatted to represent a typical manuscript style that consists of introduction, experimental details, results, discussion, summary/conclusions and references.

Chapter 9 consolidates the results from all the BCF experiments. This chapter discusses the objectives and hypotheses of this dissertation and is divided into three primary sections. In the first section, experimentally determined tissue and plasma BCFs are compared to the predicted levels (Objectives 1 & 2). The second section details the differences in the long-term and reduced BCF test results (Objective 3). The potential chronic risks to fish as predicted using the fish plasma model are presented in the third section (Objective 4). This section also covers the elimination half-lives of the pharmaceuticals in the fish.

Finally, the overall conclusions from this research and future research directions are provided in the Chapter 10.

CHAPTER 2

PHARMACEUTICALS: EMERGING ENVIRONMENTAL CONTAMINANTS

This chapter provides a background on the emergence of pharmaceuticals as a new class

of environmental contaminants. The first section provides detailed information on the usage,

sources and environmental occurrence of active pharmaceutical ingredients (APIs). The second

section details the fate and transport of pharmaceutical products in the environment. The third

section presents the significance of the environmental presence of pharmaceuticals and their

potential ecotoxicological impacts. The final section covers the current regulatory guidelines for

the environmental risk assessment (ERA) of pharmaceutical products.

2.1   Usage, Sources and Occurrence

2.1.1   Usage of Pharmaceuticals

A pharmaceutical (also frequently referred to as a drug or a medicinal product) can

broadly be defined as a compound of known chemical structure, which when administered into a

host organism, produces biological effect(s). The US Food and Drug Administration (US FDA)

classifies a substance as a "drug" if it is intended to diagnose, treat, cure, mitigate or prevent

disease(s). Pharmaceuticals comprise a diverse group of human and veterinary drugs as well as

bioactive food supplements. They have been in use for several decades now and have

undoubtedly improved the quality of life with dramatic increases in the life expectancy of

people. However, the longevity in human lives is also aided by improved nutritional and sanitary

conditions. Establishment of healthier societies in the developed nations has sparked the health

care spending globally, with a parallel increase in scientific advances in the medical field. There

are about 4000 different therapeutic substances in use in the European Union, EU (Mompelat et

al., 2009) and within the United Kingdom (UK) itself, there are approximately 3000 pharmaceutical products available for human use (Fent et al., 2006; Madden et al., 2009). In addition to the pharmaceuticals that are already in the market, there are about 4500 potential new drugs in the "pipeline" or under the process of development (Khetan and Collins, 2007).

Substantial amounts of pharmaceuticals are consumed worldwide and their usage in several hundreds to thousands of tons per year parallels that of the agrochemicals (Daughton and Ternes, 1999). For example, in Denmark, about 200 tons of antibiotics are administered annually for human and veterinary therapeutic uses, and an estimated 185 tons of insecticides are sprayed over the same period (Halling-Sørensen et al., 1998). To provide another perspective on the extensive usage of pharmaceuticals, approximately 23,000 and 13,200 tons of antibiotics per year are produced in the United States (US) and the EU respectively, and heavily used in animal husbandry as well as in treating certain human diseases (Sanderson et al., 2004). Also, several pharmaceuticals of various therapeutic classes such as hormones (contraceptives, osteoporosis and thyroid treatments), neuroactive compounds, analgesics, anti-inflammatory compounds, cholesterol reducing agents, cardiovascular drugs, diuretics, gastrointestinal (GI) drugs, anticancer therapeutics, antifungal and antimalarial agents, muscle relaxants, cough suppressors and bronchi dilators, antidiabetic compounds, bone modulators and nutritional supplements are used in large quantities (10,000-70,000 kg yr^{-1}), with some classes often exceeding hundreds of tons of annual usage (Jones et al., 2002; Sanderson et al., 2004; Fent et al., 2006; Khetan and Collins, 2007).

The consumption of pharmaceuticals has been increasing consistently over several decades and is expected to rise further in the future. In the US, for example, the annual pharmaceutical sales has increased by 62% in 5 years (from 2000 to 2004) with a value of

around $250 billion and is expected to reach $330 billion by 2010 (Khetan and Collins, 2007). The expanding global human population, higher usage of drugs by older people, scientific advances in biotechnology leading to individualization of drug therapy, expiration of patents resulting in higher per capita consumption of drugs are some of the factors predicted to result in higher usage of therapeutic drugs (Williams, 2005; Khetan and Collins, 2007). According to a 2007 report, people aged 65 or older purchased prescriptions worth of $ 82 billion and 65% of this dollar amount was spent on top five prescription categories viz., antidiabetic and antihyperlipidimic agents ($19 billion), cardiovascular drugs ($18 billion), neuroactive compounds ($8 billion), gastrointestinal medications ($7 billion) and hormonally active substances ($5 billion) (http://www.medscape.com/viewarticle/718339). The increase in the usage of pharmaceuticals is also often related to the socio-economic status as well as different marketing strategies by the industry in highlighting the use of the "life-style" drugs used to treat the rare disorders related to personality, anxiety, irritability syndrome, impotency, eating, childhood related, cognitive etc., that were not common, for example, a decade ago (Triggle, 2010).

2.1.2   Sources

The principal source by which APIs or their metabolites enter into the environment is through the usage and excretion of human pharmaceuticals by patients. Pharmaceuticals disposed via domestic wastewater are treated at the local waste water treatment plants (WWTPs; also called sewage treatment plants, STPs), and the effluents from the plants are subsequently released into the aquatic and/or terrestrial environments. Thus, STPs become the single most important point sources of pollution for these contaminants of environmental concern. This is

because, STPs have historically been used to improving dissolved oxygen (DO) levels in water bodies, reduce nutrient loads and to remove some biodegradable organic compounds. Therefore, by design, they are not equipped to remove pharmaceutical products.

The consumption and release of veterinary drugs (particularly, antibiotics and steroid hormones) from confined animal feeding operations (CFAOs) and other intensive large-scale animal farming practices, is also considered a major source of pharmaceuticals in the environment (Boxall et al., 2004). Pharmaceutical products that are excreted after veterinary uses often do not get treated in STPs and hence their environmental entry is rather direct, for example via land applications. Another predominant point source of pollution comes from the aquaculture facilities. In this case, pharmaceuticals and/or their byproducts are discharged directly into the surface waters, again without prior treatments at STPs (Lalumera et al., 2004).

The other less significant sources of pharmaceuticals in the environment include discharges from the manufacturing and heath care facilities although such release are often tightly regulated (Williams, 2005; Kümmerer, 2009). However, due to less stringent implementation of environmental regulations in the developing nations, significant amounts of pharmaceuticals of various therapeutic classes are being detected in the effluents from the manufacturing facilities (Larsson et al., 2007). Another common but unregulated entry of pharmaceuticals occurs through the household disposal of outdated and unused medicines via wastewater (toilet flush) or domestic trash (Bound et al., 2006). Pharmaceuticals disposed through domestic trash often end up in landfill sites. Landfill leachates constitute another potential source of APIs and their products in the environment (Stumpf et al., 1999). There are other rare instances like transportation accidents and unintended release of pharmaceutical products from improper storage practices. The other occasional but very significant source of

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drug entry into the surface waters is through the release of untreated sewage from STPs directly into the surface waters. This is particularly true with the treatment facilities that are not equipped to deal with large amounts storm water, resulting in the release of millions of gallons of untreated effluent into water bodies.

2.1.3   Occurrence

The occurrence of pharmaceuticals in different environmental compartments is perhaps the most studied aspect in terms of the ongoing efforts to understand the extent, magnitude and effects of these emerging contaminants. Clofibric acid, a major metabolite of the fibrate class of drugs, was the first compound to be detected in the treated wastewater (Keith et al., 1981). This was followed by a number of reports on the detection of other pharmaceutical products in the environment (Keith et al., 1981; Rogers et al., 1986; Fotsis and Adlercreutz, 1987; Aherne and Briggs, 1989). However, starting 1990s, the scientific reports on the identification of APIs in different environmental compartments have consistently been increasing (Williams, 2005). In fact, the increase in the number of published articles has been exponential. For example, in a recent review article on the environmental occurrence of pharmaceutical products, the authors indicated that more than half of the studies cited in the article were published between 2007 and 2009 (Mompelat et al., 2009). Although majority of the reports focused on the environmental presence of pharmaceuticals in the US and the EU, pharmaceutically active compounds (PhACs) have also been detected in the aquatic environments in Australia, Brazil, Canada, China, Japan and South Korea (Heberer, 2002).

Numerous systematic studies/reviews on this subject are now available in the peer-reviewed literature. It is beyond the scope of this dissertation to discuss the global occurrence

data from several hundreds of published papers. However, such efforts to develop publicly accessible databases on the environmental occurrence and/or ecotoxicity of pharmaceuticals are currently in progress. For example, National Centers for Coastal Ocean Science (NCCOS)'s Center for Coastal Environmental Health and Biomedical Research has developed a project on pharmaceuticals in the environment (PIER), a database on the environmental occurrence and toxicity drugs (Cooper et al., 2008). A search can be conducted in this database by chemical abstract service registry (CAS) number, pharmaceutical type, contaminant name or molecular formula. A few non-web based databases or small compilations on the environmental occurrence of pharmaceuticals are also available. Ayscough (2000) previously compiled data on the compartment-specific concentrations of various pharmaceuticals. Similar efforts on data accumulation were previously reported by Williams (2005) and more recently by Richman and Castensson (2008). Williams (2005) developed a relational database consisting of the reports on the environmental occurrence of pharmaceuticals from 700 citations published from 1975 through 2004. The author reported a spike in the number studies conducted after 2001, with an average of 20 per year as opposed to one per year from 1975 through 1995. In another report, a Medline search for the environmental occurrence of pharmaceuticals in different environmental compartments was conducted and the data was reported for 61 different drugs (Richman and Castensson, 2008). The authors in this report pointed out that the proportion of pharmaceuticals detected in ground and drinking waters was small (≤15%). Similar trend was also reported by Williams (2005).

It is evident from these assemblages of environmental occurrence data that tremendous amounts of effort have been devoted to detecting APIs in rivers, streams, estuaries and lakes compared to reports pertaining to terrestrial environmental concentrations. It is not surprising to

witness such a trend in the literature, as the analysis of pharmaceuticals in water samples is less likely to pose analytical challenges when compared to conducting sludge, soil or biota analyses. However, the importance of understanding the non-aqueous partitioning of pharmaceuticals in predicting the environmental fate and distribution is widely being recognized (Jones-Lepp and Stevens, 2007).

In general, pharmaceuticals of various therapeutic classes in WWTP effluents and surface waters are present in the ng l^{-1} to µg l^{-1} range. Due to the presence at such trace levels, pharmaceuticals (together with personal care products, PCPs) are commonly referred to as micro-pollutants. The environmental occurrence data on the APIs in the influent and effluent waters, sewage sludge, landfill leachates, rivers, streams, sea water, soils, agricultural crops, sediment, biota, groundwater and drinking water are available in several reviews/research articles (Halling-Sørensen et al., 1998; Ternes, 1998; Daughton and Ternes, 1999; Heberer, 2002; Kolpin et al., 2002; Calamari et al., 2003; Hilton and Thomas, 2003; Ternes et al., 2004b; Williams, 2005; Fent et al., 2006; Jjemba, 2006; Khetan and Collins, 2007; Focazio et al., 2008; Kinney et al., 2008; Kümmerer, 2009). There are approximately 160 medicinal compounds and about 30 by products present in different environmental compartments (Kümmerer, 2009; Mompelat et al., 2009). The widespread detection of trace amounts of pharmaceutical products in the environment is achieved by improved analytical techniques capable of measuring compounds at low parts per trillion (ppt) levels. However, the number of pharmaceuticals detected in the environment is much smaller ($< 5\%$) than the total number of drugs approved for medicinal use. The occurrence of the reminder of the pharmaceutical products has not been reported, not because of their absence in the environment, but rather the absence of such studies. This is, in general, due to the non-availability of sensitive analytical techniques and/ or increased attention

on certain therapeutic classes. The focused surveys and monitoring programs on the environmental presence of pharmaceuticals (e.g. Kolpin et al., 2002) are driven by several factors such as the available data on the usage (consumption in tons per year), environmental persistence, toxicity and the region-specific regulatory practices. Such prioritization could be helpful in monitoring ecologically relevant pharmaceuticals (Batt et al., 2008) such as analgesics and anti-inflammatory drugs, antineoplastic, antitumor and immunosuppressant agents, neuroactive drugs and other general stimulants, lipid lowering compounds, antimicrobial compounds, steroidal hormones, antihypertensive and cardiovascular medicines, antacids, X-ray contrast media, antidiabetic drugs, bronchodilators, anticoagulants and anesthetics.

Recently, comprehensive reviews on the environmental occurrence and/ or toxicity of pharmaceuticals have been presented (Mompelat et al., 2009; Santos et al., 2010). Overall, these studies found that non-steroidal anti-inflammatory drugs (NSAIDs), antibiotics, lipid regulators, β-blockers, antiepileptic drugs and steroid hormones constitute the major therapeutic classes of drugs frequently reported as being detected in the environment.

2.2   Fate and Transport

Environmental fate and transport of a chemical are predominantly dictated by its physical and chemical properties, which are in turn governed by the structure of the molecule. The most important characteristics that help in predicting the environmental behavior of compounds include molecular weight, water solubility, acid dissociation constant (pK_a), volatility (Henry's law constant), and the distribution coefficients (octanol-water partitioning coefficient, K_{ow}; soil-water partitioning coefficient, K_d or K_d normalized to total soil organic carbon content, K_{oc}). For priority pollutants like organochlorine pesticides and other industrial chemicals, hydrophobicity

is often used to predict the environmental fate and distribution. However, pharmaceuticals are structurally different and often carry multiple polar functional groups with higher solubility. The structural characteristics of pharmaceuticals are also manipulated in such a way that the compound or its active metabolite reaches the intended biological target for triggering a specific mode of action (MOA). The molecular functionalities (acidic, basic or neutral) of pharmaceuticals result in their existence as cationic, anionic or sometimes zwitterionic entities in the environment (Kümmerer, 2009). Due to these properties, prediction of the environmental fate and transport of pharmaceuticals using the hydrophobicity derived traditional modeling approaches, is not feasible.

After consumption, pharmaceuticals are excreted through renal and biliary routes into the domestic wastewater. As detailed in the "sources" section of this chapter, this pathway constitutes the major environmental entry route for pharmaceuticals or their active products (Jjemba, 2006). Environmental fate of a chemical is the study of the processes that lead to the formation of different transformation products of the parent molecule. These transformations can be triggered by chemical, biological or photo-induced reactions. Structural alterations in the compound are produced by biotic, abiotic or a combination of the two processes. Therefore, in most cases, estimation of the environmental fate of a chemical is a measure of the extent to which the original compound is depleted, as it gets distributed in the environment.

2.2.1   Biotic Transformations

Biological transformation of pharmaceuticals occurs in two different ways, a) within the animal body through the action of enzymes (for e.g., cytochromes) and/or by the microorganisms present in the digestive tract, and b) transformations aided by bacteria in the environment. While

the former is commonly referred to as metabolism, biotransformation in the environment is termed biodegradation. Metabolism of drugs is aided by phase I and phase II reactions. Phase I reactions (hydrolysis, oxidation, hydroxylation and reduction) render polar functional groups to the parent compound and generate more reactive product(s). Phase I products are generally less toxic than the parent drug, but there are instances where the metabolic product can be more toxic (e.g. N-hydroxylation of acetaminophen). Hydrophilicity of phase I products is further enhanced by phase II conjugation reactions with the help of glucuronic acid, sulfate, acetyl, glutathione and amino acids. The highly polar conjugated products are then excreted through urine and feces. Therefore, the extent to which a parent compound and/or its metabolite(s) are excreted depends on the compound-specific metabolism. High proportions (>70%) of administered drugs are sometimes excreted unchanged (Ternes, 1998; Hirsch et al., 1999). The excreted phase II conjugated products are sometimes converted back to the parent compound with aid of various treatment processes that occur in STPs (Halling-Sørensen et al., 1998; Daughton and Ternes, 1999; Fent et al., 2006).

Biodegradation or more correctly, microbial degradation (*ex vivo*) constitutes an important attenuation process for pharmaceutical products. This degradation is often part of the secondary treatment in the STP processes and involves the use of aerobic activated sludge treatments and/or anaerobic sewage sludge digestion. It is often desirable that biodegradation results in the complete mineralization. However, in addition to the differences in the chemical characteristics of pharmaceutical compounds, the extent of biodegradation (low, moderate or high) and hence the removal efficiency, depends on a variety factors including sludge age, hydraulic retention times, season, differences in STP construction and treatment technologies, amount of biomass, temperature, pH and oxygen saturation levels (Ternes, 1998; Ternes et al.,

1999; Carballa et al., 2005; Clara et al., 2005; Kummerer et al., 2005; Williams, 2005; Fent et al., 2006). The elimination rates of pharmaceutical products in STPs varies widely (0-99%). For example, very high removal efficiencies (ca. 95%) have been reported for NSAIDs (Thomas and Foster, 2004) while an antiepileptic drug, carbamazepine is poorly (<10%) eliminated (Metcalfe et al., 2003; Clara et al., 2004).

2.2.2   Abiotic Transformations

Pharmaceuticals or their active products are subjected to two major kinds of abiotic mechanisms viz., adsorption and photodegradation. Adsorption or simply sorption to suspended solids, particulates, sediments, colloids and/or natural organic matter (NOM) is a major elimination pathway of pharmaceutical products within the STPs as well as in surface waters. As discussed previously, sorption properties of legacy contaminants (like organochlorine compounds) can be predicted by their K_{ow} values (Rogers, 1996). However, pharmaceuticals are usually charged and hence the sorption characteristics are controlled by non-hydrophobic mechanisms like electrostatic interactions. Therefore, K_d could be a better predictor of sorption capacity (Ternes et al., 2004a). Antibiotics (e.g. tetracyclines) and steroid hormones (e.g. ethinyl estradiol, EE2) are the two best known classes of compounds that are known to sorb to suspended particles, sewage sludge or sediment (Ternes et al., 1999; Boxall et al., 2002). Pharmaceuticals with high sorption capacity contribute to reduced contaminant load in the aquatic environments. However, sewage sludge is often applied on land, creating a potential entry of the sorbed products into the terrestrial environment.

Photodegradation is another major transformation process for pharmaceuticals in the environment (again, both in STPs and natural waters). Photolysis of compounds can occur either

directly or indirectly. In the direct photolysis, a compound absorbs an appropriate radiation (depending on the absorption spectrum of the compound) directly from the sunlight. Indirect photolysis occurs when the dissolved photosensitizers (nitrates, humic acids etc.) generate free radicals which in turn trigger the photo-induced reactions. In addition to the individual absorption profiles of compounds, photodegradation depends on various factors like intensity of radiation (and hence season and latitude), dissolved organic matter (DOM), nutrient loads and water depth. There are several classes of pharmaceutical compounds that yield photo-induced transformation products (Petrovic and Barceló, 2007). Some examples include diclofenac (Buser et al., 1998b), and other NSAIDs (Packer et al., 2003), triclosan (Lindström et al., 2002; Singer et al., 2002), paroxetine (Cunningham et al., 2004) and ranitidine (Latch et al., 2003).

The transport of pharmaceuticals is studied in terms of their partitioning into different environmental compartments. As most pharmaceuticals are relatively non-volatile, their disposition in the environment is believed to occur primarily via aqueous transport. The partitioning into aqueous or solid phase depends on the compound-specific distribution coefficients. Compounds that tend to stay in the dissolved phase are transported into surface waters through the effluent discharge from STPs, while those with high sorption capabilities end up in the terrestrial environments, particularly if the treated sludge is used for land applications. Pharmaceutical products residing in soil environments may reach the groundwater upon leaching (Heberer, 2002) or enter surface waters again after run-off events. A final less studied aspect of transport of pharmaceuticals within the environment is via the food chain transfer. This is usually studied in terms of the bioaccumulation potential in the organisms. Very few studies have previously been conducted on estimating the bioaccumulation potential of pharmaceuticals in the

aquatic and terrestrial species (Oaks et al., 2004; Schwaiger et al., 2004; Mimeault et al., 2005; Brown et al., 2007; Giltrow et al., 2009; Ramirez et al., 2009; Fick et al.).

2.3   Environmental Significance and Ecotoxicity

2.3.1   Significance

The presence of human and veterinary pharmaceuticals in the aquatic and terrestrial environments has raised increased concerns over the potential ecotoxicological effects. These concerns are primarily triggered by a few important discoveries on the effects of pharmaceuticals on the non-target organisms as detailed in the following paragraphs.

More than 97% decline in the population of three species of oriental vultures in Pakistan and India resulted after they fed on the carcasses of domestic animals treated with diclofenac (Oaks et al., 2004). The species is now critically endangered and efforts are being made to avoid the risk of extinction. Diclofenac, an NSAID, is both a human and a veterinary drug used to alleviate pain, inflammation and fever. However, the oriental vultures are extremely sensitive to diclofenac and mass mortality occurred from renal failure due to the acute poisoning. The unexpected decline in the vulture population has demonstrated our current lack of understanding of the potential catastrophic effects of pharmaceutical products on wildlife.

There has been an increasing evidence of endocrine disruption in fish exposed to natural and synthetic estrogens. Several field and laboratory studies have reported inter-sex condition in fish exposed to estrogen compounds (Jobling et al., 1998; Jobling et al., 2006; Kidd et al., 2007). Some pharmaceuticals, especially oral contraceptive medicines are potent endocrine disrupting compounds (EDCs). The potential for such effects from the drugs of other classes are largely

unknown except for few compounds such as fluoxetine (Mennigen et al., 2008) and triclosan (Veldhoen et al., 2006).

Antibiotics are heavily used as human and veterinary drugs. This wide-spread usage of broad-spectrum antibiotics may result in the development of multi-drug resistant (MDR) bacteria, heightening the concerns over the ineffective treatment options and the subsequent issues related to the public health (Schweizer, 2001; Teuber, 2001; Sengeløv et al., 2003).

The other potential ecological consequences resulting from the intensive use and disposal of pharmaceuticals are outlined here. Antineoplastic drugs constitute an important treatment option for various types of cancers. They act as alkylation agents and have the potential to cause genotoxic effects in the non-target species. Some pharmaceuticals (e.g. verapamil) have the ability to inhibit the multi xenobiotic resistance (MXR), a common protective mechanism that expels toxic compounds from the body (Daughton and Ternes, 1999). Impairment of this phylogenetically conserved mechanism, especially in the organisms that are at the bottom of the food chain (e.g. filter feeders), could jeopardize the structure of the aquatic community. And finally, it is currently debated whether environmental exposure to pharmaceuticals (through food chain transfer or drinking water) is a threat to human health. Pharmaceuticals have been detected in the drinking water (Mompelat et al., 2009), but the potential health concerns resulting from such exposure are considered negligible (Schwab et al., 2005; Johnson et al., 2008).

The above mentioned well understood health hazards on the non-target species resulting after human and veterinary pharmaceutical usage, have spurred interest among research scientists and environmental regulators to understand the possible ecotoxicological implications resulting from several other classes of pharmaceuticals and their byproducts.

2.3.2 Ecotoxicity

Assessment of ecotoxicity of pharmaceuticals is highly desirable due to the following general facts about this special class of environmental contaminants.

Human and veterinary pharmaceuticals are bioactive compounds that are designed to target specific receptors or enzymes and trigger certain biological effects. Such receptor/enzyme systems are phylogenetically conserved across species (Gunnarsson et al., 2008), suggesting the possible similar pharmacological interactions in the non-target organisms. Also, from the mammalian drug safety studies, it is known that pharmaceutical usage often results in several side-effects, in addition to the main desired effect. These side-effects in the mammalian systems could turn into major adverse effects in the unintentionally exposed aquatic and terrestrial organisms.

Most drugs are developed to resist changes until they reach the intended target(s) in the body. This is often achieved by the introduction of certain functional groups in the molecule that help slow the metabolic activity and enhance the potency of the drug. Such a pharmacologically intended drug design, unfortunately, becomes a threat to the environment due to the enhanced persistence (e.g. synthetic estrogens).

Unlike the agrochemicals that are used seasonally, pharmaceuticals are disposed continuously into the environment. This long-term (often the entire-life cycles), uninterrupted chronic aquatic exposure is of great concern because the subtle effects resulting from such exposures are often not detected until combined with ecologically unfavorable circumstances which may occur only transiently (Daughton and Ternes, 1999). Knowledge of such potential ecological impacts is lacking and a thorough understanding through concerted research efforts is warranted to improve our ability to predict the subtle combined effects of multiple stressors.

It is now quite evident that pharmaceuticals have a profound potential to disrupt or impair health status of the aquatic and terrestrial life forms. As described previously in the vulture decline example, previously unknown adverse effects of medicinal drugs can create imbalances in the wild animal populations. Population declines or extinction (in worst cases) of a particular species in a community ultimately causes irreversible damage to the ecosystem.

To prevent such unfavorable events, it is desirable to have reliable ecotoxicity data on various pharmaceutical products in use. However, it is not practical to generate such data on every single species present in the environment. Hence, ecotoxicological tests are frequently performed on few surrogate species (algae, daphnia and fish) that are believed to represent the diversity of the wild species in the natural environment. However, such standard ecotoxicology test methods (e.g., Organization for Economic Cooperation and Development, OECD or US Environmental Protection Agency, US EPA) are often short-term (24 – 96 hr.) acute toxicity studies with survival as the major endpoint. Except in situations where large amounts of untreated waste are discharged accidentally (which is a rare event), the results from the laboratory generated short term exposure studies may not be useful for extrapolation to understand/predict the implications resulting from the real exposures in the wild life. This is because the ecologically relevant exposure levels of pharmaceuticals are usually several orders of magnitude lower than the concentrations at which the acute effects are seen in the laboratory tests. Also, the non-target species, especially aquatic organisms, are exposed to pharmaceutical residues throughout their life and hence the test durations utilized in standard toxicity methods do not correspond to the chronic exposures in the field.

Prolonged and low exposure levels (chronic exposure) of pharmaceuticals often result in subtle effects in the organisms, such as growth, reproduction or behavior. However, except for a

few drug classes (e.g., estrogens and antimicrobials), data on such sub-lethal end points are generally absent. The primary impediments in obtaining the more useful chronic toxicity data are, the requirements of large number of test species, long exposure durations (sometimes multi-generations) and documentation of a suite of relevant physiological end points. Studies of this kind require large investments in terms of cost, time and animal resources. In spite of these difficulties, there is growing interest in understanding the subtle, long-term effects of pharmaceuticals on non-target organisms, preferably through direct chronic tests or by predictions using thoroughly validated models.

Although acute toxicity test results do not sufficiently address the current ecological concerns, they are frequently utilized in the risk estimations after applying certain safety factors. Incorporation of large safety margins (in thousands) is essential to compensate for the uncertainties existing in the extrapolation of acute toxicity results generated on few surrogate species (Khetan and Collins, 2007). A large proportion of ecotoxicological literature on the effects of pharmaceuticals is limited to acute toxicity data, with relatively few investigations focused on chronic studies. The remainder of this section will focus on providing our current understanding the acute and chronic ecotoxicological effects of pharmaceuticals.

2.3.2.1 Acute Effects

Acute toxic effects of pharmaceutical compounds of various therapeutic classes on different taxa have previously been reviewed (Halling-Sørensen et al., 1998; Webb, 2004; Crane et al., 2006; Fent et al., 2006; Jjemba, 2006; Khetan and Collins, 2007; Li and Randak, 2009; Madden et al., 2009; Sanderson and Thomsen, 2009; Santos et al., 2010). Although it is not the intent of this dissertation to provide all the currently available measured or predicted acute

toxicity levels of approximately 364 different pharmaceutical products (Madden et al., 2009), the toxicity of pharmaceuticals of major the therapeutic classes that are considered of ecological concern will be addressed in this section.

Halling-Sørensen et al., (1998) compiled the acute toxic effects of 12 human medicines and 27 veterinary drugs on micro-organisms, phytoplankton, crustaceans, fish and plants. For the human drugs, toxicity data for four steroid hormones, 3 antibiotics and one drug each from analgesic, beta-blocker, neuro-active, psychomotor and lipid lowering agents were provided. In this list, except for one compound (a neuroactive agent), all the veterinary medications are antibiotics. Antibiotics are the most used drug classes in animal operations especially in intensive farming. In the human drugs category, steroid hormones were reported to be toxic even at low parts per billion (ppb) levels while the toxicity of all other drug classes varied between 1 and 300 mg l^{-1}. The only exception to this was the reported reproductive sensitivity (NOEC=10 µg l^{-1}) of *Daphnia magna* to clofibrate . In the antibiotics listed, bromocyclen was reported to have reproductive effects in crustaceans when exposed to concentration above 100 µg l^{-1}. Antiparasitic agent ivermectin and its residues were shown to be extremely toxic to crustaceans (NOECs between 0.01-9 µg l^{-1}) while streptomycin was reported to inhibit the growth of blue-green algae at 9 µg l^{-1}.

The acute toxicity results of more than 100 pharmaceuticals are available in another comprehensive review by Webb (2004). Antimicrobials dominated the list of the pharmaceutical compounds compiled with their toxicity information. Antidiabetic, neuroactive, analgesic and antipyretic, steroid hormones, antihypertensive, anti-ulcerative and cardiovascular agents constituted other prominent drug classes. The acute toxicity data was reported on different species and the general order of species sensitivity was: algae > daphnia > fish. While more than

80% of the compounds had low acute toxicity levels (1-1000 mg l^{-1}), certain drugs in antibiotic, anti-depressive, antipsychotic, cytostatic and bone modulator categories had higher toxicity.

In another review by Fent et al., (2006), the most acutely toxic drugs representing each of the major therapeutic classes were identified along with the indication of the most sensitive species (in some instances). For example, diclofenac was identified as the most toxic (<100 mg l^{-1}) drug among the NSAIDs and algae was reported to be the most sensitive species. Propranolol topped the toxicity list among the β-blockers and was shown to be toxic (<1 mg l^{-1}) to both algae and daphnia. Clofibrate and gemfibrozil represented the most studied compounds in antilipidemic drugs class. In the neuroactive drug category, fluoxetine (Prozac) was reported to be most toxic (< 0.05 mg l^{-1}) to algal species and diazepam (EC50 of 10 µg l^{-1} in *Hydra vulgaris*) and carbamazepine (LC50 of 45 µg l^{-1} in fish) were other two reported toxic compounds in this class. Overall, the authors noted wide ranges of differences in toxicity results within the same species, sometimes by two to three orders of magnitude. Such wide differences in the reported data were attributed to differences in exposure concentrations, sensitivities of species, degree of conformity with the standard protocols and laboratory performance. From this data, it is concluded that the occurrence of acute toxicity in aquatic species is highly unlikely due to the broad differences (at least 1000 times) between acute levels and measured environmental concentrations.

Sanderson & Thomsen (2009) collected acute toxicity data on 275 pharmaceutical compounds from the available ecotoxicological data in the literature. They reported that about 64% of the pharmaceuticals in this list had acute toxicity levels between 10 and 100 mg l^{-1} while less than 4% of the drugs showed acute effects below 1 mg l^{-1}. These findings suggest that acute toxicity effects of most pharmaceuticals occur through non-specific narcotic MOA. The authors

also indicated that in comparison with fish, algae and invertebrates appeared more sensitive towards pharmaceuticals. In a recent review, Santos et al., (2010) provided a comprehensive review of ecotoxicological data on the pharmaceuticals belonging to the major therapeutic classes such as NSAIDs, steroid hormones, antibiotics, hypolipidimic, neuroactive drugs, β-blockers, antidepressants and cytostatic agents. In this review, the authors noted that examining non-traditional measurements like morphology, population growth inhibition and growth rate result are more sensitive endpoints compared to the standard end points such as lethality. Again, like previous reviews, each therapeutic class was dominated by 4 or 5 of the most studied compounds indicating that the available ecotoxicological data on the currently used pharmaceutical compounds is limited.

2.3.2.2 Chronic Effects

The efforts to document more relevant and useful chronic toxic effects of pharmaceuticals on aquatic organisms are limited. Studies reporting chronic toxicity levels have recently been conducted (Crane et al., 2006; Fent et al., 2006; Sanderson and Thomsen, 2009; Santos et al., 2010). However, as expected, the available data on the acute toxicity of the pharmaceuticals far exceeds the existing chronic data. For example, Sanderson and Thomsen (2009) indicated that acute toxicity data generated following the OECD testing guidelines are available for 275 compounds, while screening for chronic effects had been done for less than two dozen pharmaceuticals.

The interest in unraveling the long-term effects of pharmaceuticals on the non-target organisms has primarily been triggered from the well-understood effects of steroid hormones, synthetic estrogens in particular. EE2 has been proved to be extremely toxic in terms of its

reproductive effects on fish even at concentrations as low as 1 ng l^{-1} (Länge et al., 2001).

Although the value of understanding the target-specific toxicological effects has been

recognized, the current ecotoxicological testing methods are not designed for such MOA based

testing. The other prominent drug class with proven chronic effects is antibiotics, the major

concern being the development of resistance. In general, antibiotics are very toxic to

microorganisms and algae (Jones et al., 2002). Erythromycin, sulfamethaxazole, amoxicillin,

ampicillin, oxytetracycline are the most studied compounds in this category and have chronic

toxicity levels in the order of µg l^{-1} (Santos et al., 2010).

There are also reports of chronic effects on non-target species from other therapeutic

drug classes such as NSAIDs, β-blockers, blood-lipid regulators, antidepressants, antiepileptics

and antineoplastics. Diclofenac, ibuprofen and naproxen are the most studied NSAIDs in terms

of their chronic effects on the aquatic organisms. Diclofenac resulted in the histopathological and

cytotoxic alterations in rainbow trout at exposure concentrations of 1-5 µg l^{-1} (Schwaiger et al.,

2004). In the cardiovascular drug category, propranolol exposure for 4 weeks at 0.5 µg l^{-1} caused

reproductive effects (reduced egg production) in fish (Huggett et al., 2002). Clofibric acid and

gemfibrozil are the two main blood-lipid regulating agents that have the potential to cause

chronic effects at sub ppb levels and gemfibrozil has bioaccumulative potential in fish (Ferrari et

al., 2003; Mimeault et al., 2005). Among various neuroactive drugs, chronic effects of

fluoxetine, sertraline, paroxetine, citalopram, fluvoxamine and carbamazepine have been

reported. Carbamazepine was found to be toxic to invertebrates even at low µg l^{-1} exposures

(Ferrari et al., 2003) while the selective serotonin reuptake inhibitors (SSRIs) were shown to

have reproductive toxic effects (ranging from 0.032-56 µg l^{-1} l) in invertebrates (Fong, 1998;

Brooks et al., 2003). Tamoxifen, a cytostatic drug has been shown to inhibit the growth in

crustaceans at sub ppb exposure levels. The current literature suggests that there is very little need for concern for the toxicological issues related to X-ray contrast media. Santos et al., (2010) recently provided a comprehensive review on the chronic toxicity effects of the above mentioned drug classes on microorganisms, phytoplankton, zooplankton, plants and fish.

Due to the growing concerns over the potential chronic toxic effects of pharmaceuticals, especially on the aquatic species, there has been steady increase in the number of long-term exposure studies to measure the subtle end points. However, recent estimates indicate that the ecotoxicological data is available for less than 1% of the currently available human and veterinary pharmaceuticals (Sanderson et al., 2004). Also, the significance of compilation of publicly available ecotoxicological effects of pharmaceuticals has recently gained prominence (Molander et al., 2009). This indicates the need to further enhance our efforts to better understand less familiar toxicological effects (e.g. endocrine disruption, inhibition of lipid biosynthesis) on the non-target organisms.

Finally, it should be noted that pharmaceuticals have been in use for several decades now and have always been present in the environment. So, the presence of these compounds in the environment is not new, although the improvement in analytical technologies has expanded the detection capabilities enabling identification of several classes of human and veterinary drugs down to low ppt levels. The real new or "emerging" issues are related to the potential implications resulting from the presence of pharmaceuticals in the environment. The unfolding of these previously unknown ecotoxicological impacts is what qualifies pharmaceuticals as "emerging contaminants."

2.4   Environmental Risk Assessment (ERA)

The consequences of the presence of pharmaceutical products in the environment need a careful evaluation in view of the growing concerns over their potential implications. Consequently, regulatory agencies (FDA and European Medicines Agency, EMEA) have implemented the guidelines for conducting ERA of pharmaceutical substances. In the present section, the current regulatory strategies and/or practices to evaluate the ERA of pharmaceuticals are addressed. This section is also intended to provide an overview of the major differences in the international ERA procedures.

The framework for ERA of pharmaceuticals is based on the same principles applied in the traditional chemical risk assessments that involve assessments of exposure, effects and risk characterization. Analogous to the hazard index (HI) in the traditional risk assessment methods, a risk quotient (RQ) is calculated by the ratio between a predicted or measured environmental concentration (PEC or MEC) and a predicted no effect concentration (PNEC). While PEC or MEC provide a measure of exposure, derivation of PNEC covers the effects assessment. In the absence of MECs (which is always true, as it is impractical to measure the environmental concentrations of every single medicinal product), the exposure concentrations are predicted using a variety of factors including the data on sales, usage, expected route of entry into the environment, amount of waste generated per inhabitant etc. (Carlsson et al., 2006). In addition to these, PECs are also adjusted for compound-specific characteristics such as physicochemical properties, fate and transport in the environment, all determined using the standard OECD methods (Dietrich et al., 2006) . PNECs are computed from the measured no observed effect concentrations (NOECs) for the most sensitive species after applying relevant safety margins (termed assessment factors, AFs). Finally, RQ is obtained from the ratio of P(M)EC to PNEC.

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Large AFs are frequently applied to account for the uncertainties in computing RQs, which ultimately, are used in the decision making process i.e. to continue or stop further assessments and/or devising a risk management strategy to mitigate the perceived risks to aquatic and terrestrial species. The risk management strategies (e.g. upgrading STP processes) are often costly and hence proper care should be exercised in determining RQs.

ERA of pharmaceuticals requires extensive information on the measured or predicted environmental behavior of compounds and a suite of standard ecotoxicity tests on species (often algae, daphnia and fish) representing at least three trophic levels. The ERA requirements for predicting risk posed to the terrestrial species are similar with a few major differences. For example, as per the EU guidelines, a PEC of 100 μg kg^{-1} of the medicine in the soil triggers a complete ERA of the compound. The species on which the toxicity tests are conducted to measure NOECs include earthworms, microbes and plants, again the species representing three trophic positions. In summary, due to the enormous value in determining the possible risk to aquatic or terrestrial species from the unintended exposure to pharmaceutical products, it is imperative to develop accurate models that are useful in predicting the fate, transport and ecotoxicity of pharmaceuticals. The remainder of this section will focus on the current regulatory practices in the ERA of pharmaceuticals.

2.4.1 EMEA Approach

In the EU, human and veterinary drugs that require product authorization must submit an ERA and the process is currently regulated by Directives 2004/27/EC and 2004/28/EC respectively. Guidelines also currently exist to assess the impacts of drugs derived from genetically modified organisms (Kampa et al., 2010). These guidelines came into effect after

2006 and require a thorough ERA submission on all new drugs intended for marketing in the EU. Therefore, pharmaceutical products that were authorized prior to 2006 are excluded from such rigorous ecotoxicological tests. It has been reported that the drugs that escaped ecotoxicological requirements (i.e. those marketed before 2006) are the ones that are frequently detected in the environment, emphasizing the need to assess the risks of both new and "existing products" (Adler et al., 2008).

The ERA follows a tiered approach consisting of two phases, I and II, with phase II further subdivided into tiers A & B (EMEA, 2006). In the initial exposure assessment (Phase I), PEC in the aquatic environment or more correctly, in surface water (PEC_{sw}) is estimated using the equation:

$$PEC_{sw} = (DOSE_{ai} * F_{pen}) \div (WASTE_{inhab} * DILUTION * 100)$$

Estimation of conservative PEC for surface water. $DOSE_{ai}$: maximum daily dose of active ingredient per inhabitant ($mg.hab^{-1}.day^{-1}$; F_{pen}: percentage of market penetration (default value=1%); $WASTE_{inhab}$: amount of wastewater per inhabitant per day (default value=200 l); DILUTION: dilution factor from STP to surface water (default value =10).

The resultant PEC is considered "conservative" because of the assumption of the worst-case scenarios such as no metabolism, no degradation in STPs and maximum daily dose and market penetration. If the $PEC_{sw} < 0.01$ µg l^{-1} and there are no additional environmental concerns (such as bioaccumulation, endocrine disruption or genotoxicity) the substance is considered safe and no further testing is necessary. On the contrary, if $PEC_{sw} \geq 0.01$ µg l^{-1}, the compound is tested in phase II tier A assessment for environmental fate and effects. At this stage, a modified or refined PEC_{sw} is computed for the compound with no assumptions. Therefore, a refined PEC is obtained from the measured or modeled environmental fate and transport data (including human metabolism), as represented in the following equation:

$$PEC_{sw} = (DOSE_{ai} * F_{excreta} * F_{stp} * F_{pen}) \div (WASTE_{inhab} * FACTOR * DILUTION * 100)$$

Estimation of refined PEC for surface water. $F_{excreta}$: fraction of active substance excreted; F_{stp}: fraction of emission directed to surface water; FACTOR: factor accounting for sorption; F_{pen} is modified as F_{pen} = consumption $*100 \div$ DDD$*$ hab$*365$ where consumption: consumption of active substance (mg yr^{-1}); DDD: daily defined dose i.e. average daily dose per person; inhab: no. of inhabitants.

As detailed in the introductory paragraph in this section, a PNEC (surface water) is then computed using the standard OECD acute ecotoxicity tests (Dietrich et al., 2006) on the three aquatic species. NOEC for the most sensitive species is considered and appropriate AFs (10-1000) are applied to account for the uncertainties such as inter and intra species differences in sensitivity, extrapolation of acute toxicity results or extrapolation of data from laboratory to the field conditions. In this tier, a PNEC for microorganisms is also determined. This is important because pharmaceuticals that are toxic to bacterial population in STPs could influence the overall elimination process and hence the resultant assessment. Risk quotients are computed for both microorganisms (RQ_{micro}) and aquatic species (RQ_{aqu}). If neither of the RQs is above 1 and log K_{ow} of the compound is below 3, it is assumed that the substance is unlikely to cause any adverse effects. However, if either of the RQs is greater than 1, then extended tier B assessments are required. In tier B of phase II, compartment-specific tests and assessments such as bioaccumulation, terrestrial fate and sediment effects are performed.

Unlike the human medicinal products, guidelines for the risk analysis of veterinary drugs have been in place since 1990s. In the phase I stage, PECs are calculated for both aquatic and terrestrial environments depending on the intended use of the drug (aquaculture or farm animals). If the predicted or measured levels are more than 1 µg l^{-1} (aquatic) or 100 µg kg^{-1} (terrestrial), then phase II assessments are deemed necessary. Phase II assessments for veterinary medicines are similar to those applied for the human drug assessments in surface waters. It is important to

point out here that the market authorization principles for veterinary and human drugs differ by the risk-benefit analysis. Unlike veterinary medicines, health benefits of human medicinal drugs invalidate their exclusion (based on the ecotoxicological assessments) from the market.

2.4.2 US EPA/FDA Approach

ERA requirements for medical products have been in existence in the US since 1970s. All new drug applications submitted to FDA must include a dossier on environmental risk (FDA, 1998). In the FDA approach, the compound of concern is first inspected for all the possible depletion mechanisms (see the section on "fate") in the environment. If the extent of depletion meets the threshold level, then no further assessments are necessary, except the microbial inhibition test. This provision is helpful in avoiding the complex ERA process, if the environmental depletion of a chemical is rapid. For the compounds that do not deplete appreciably, an expected introductory concentration, EIC is computed using the equation below. The EIC is similar to the PEC calculated in the EU approach.

$$EIC = (A * D) \div (B * C)$$

Expected Introductory Concentration (FDA, 1998). A: amount of API produced for direct use (kg yr^{-1}); B: effluent quantity entering publicly owned STPs (l day^{-1}); C: 365 (days in yr.); D: conversion factor (10^6 µg kg^{-1}).

If the EIC of the compound is lower than 1 µg l^{-1} in the WWTP effluent or below 0.1 µg l^{-1} in surface water, the microbial test results are negative (i.e. biodegradation efficiency is not adversely affected), and log K_{ow} of the compound less than 3.5, the chemical is considered to pose no environmental risk. Such result for a chemical in the initial screening is given "finding of no significant impact" (FONSI) status and is excluded from further testing. On the other hand,

if the EICs are higher than the threshold levels, the compound is required to proceed for tiered risk assessment. After this initial screening, the ERA of pharmaceuticals is conducted in 3 tiers. The first two tiers are acute toxicity tests while last tier requires chronic toxicity testing.

In tier 1, an acute toxicity test is performed on a single species to determine LC50 or EC50. If the ratio between the median lethal toxicity and the maximum expected environmental concentration (MEEC) is greater than or equal to 1000, and no sub-lethal effects are noticed, then further assessments are not required. If not, the compound proceeds to tier 2, where acute toxicity tests are conducted on three species (similar to the EU approach in phase II) to determine the toxicity level for the most sensitive species. If the ratio of the toxicity level to the MEEC is greater than or equal to 100, and no sub-lethal effects are observed, again further assessments are ended. On the other hand, observation of sub-lethal effects in tier 1, 2 or the high hydrophobicity (log $K_{ow} > 3.5$) qualifies the compound for chronic toxicity assessments in tier 3. If the ratio between the toxicity level and the MEEC is greater than equal to 10 with no other effects observed, the assessment can be concluded. If not, Center for Drug Evaluation and Research (CDER) should be consulted for further action.

2.4.3   Environment Canada (EC) Approach

Unlike the regulations in the US and the EU, EC does not have separate risk assessments for pharmaceutical products. Any substance that is not listed in the domestic substance list (DSL) must go through ERA. However, in place of PECs, the quantity of substances imported or manufactured in a year is computed and if such quantities exceed the threshold levels, the specific compounds go through a complex ERA process consisting of several notification and schedule procedures. If the production/import of a chemical is greater than 10,000 kg yr^{-1} or

greater than 50,000 kg accumulation, then it is required to conduct acute toxicity tests on the compound (Dietrich et al., 2006). Conformity with stipulated regulations enables the chemical to be added to the DSL.

2.4.4   Concerns over the Current ERA Approaches

Two important concerns have been expressed regarding the present ERA frameworks. First, the current guidelines are designed to assess risk for single compounds only. Our current knowledge on the toxicity of mixtures of compounds (which is quite relevant, especially for pharmaceuticals) is limited. Few studies have highlighted the additive toxicity effects resulting from the compounds of similar therapeutic class (Silva et al., 2002; Cleuvers, 2004; Pomati et al., 2008). The other important aspect is the increased focus on understanding the risks of parent compounds with little attention on the major degradation products. The transformation products may sometimes be equally or more toxic than the parent compound (Boxall et al., 2004). Therefore, future risk assessment strategies should be directed to address these important issues.

CHAPTER 3

BIOCONCENTRATION

The first section of this chapter provides definition of basic terms and discusses the

significance of bioconcentration measure as an important end point in ERAs. The second section

covers the modeling approaches to estimate bioconcentration factors (BCFs). Third section of the

chapter provides different strategies on prioritizing pharmaceuticals for tiered ERAs. This

section also discusses the usefulness of the existing mammalian pharmacological and

toxicological information to predict chronic risk with a detailed discussion on the fish plasma

model (FPM). The final section presents the previously reported laboratory and field exposed

bioconcentration studies and discusses the importance of experimentally determined BCF

measurements.

3.1   Terminology and Regulatory Importance

3.1.1   Bioconcentration

        Bioconcentration is defined as a process by which a chemical is retained in an aquatic

organism following its absorption by respiratory and dermal routes from the surrounding water.

Therefore, "non-dietary" bioaccumulation (defined below) of water-borne chemicals in aquatic

species is referred to as bioconcentration. The potential for a chemical to bioconcentrate is often

expressed in terms of its bioconcentration factor (BCF) and is measured under controlled

laboratory conditions. BCF is calculated either as the ratio of chemical concentration in the

organism (C_b) to that in water (C_w) at steady state or using the ratio of the rate of chemical

uptake (k_1) and rate of elimination (k_2). Chemical concentration in the organism is expressed as

wet weight or lipid weight basis and when the former is used BCF has $l.kg^{-1}$ units. However, for better comparison across different species it is often useful to estimate lipid-normalized BCFs.

$$BCF_{steadystate} \ = \ C_b \div C_w \ \text{ and } \ BCF_{kinetic} = k_1 \div k_2$$

3.1.2   Bioaccumulation

Bioaccumulation is a process that leads to higher concentration of a chemical in organisms than in the surrounding environmental media. It is typically a field measured parameter and hence all possible sources and routes of exposures (respiratory, dermal, and dietary) are considered. Bioaccumulation results when the rate of intake exceeds the organism's ability to eliminate. The extent to which a chemical can bioaccumulate in an organism is expressed in terms of its bioaccumulation factor, BAF (the ratio of chemical concentration in an organism to that in water) or biota-sediment accumulation factor, BSAF (the ratio of concentration of a chemical in an organism to that in sediment). In both estimates, it is a common practice to normalize the organism's chemical concentration on lipid basis. Also, chemical concentration in the sediment is expressed on organic carbon basis. These normalization principles are helpful to extrapolate the accumulation patterns across species.

3.1.3   Biomagnification

US EPA defines biomagnification as the "result of the process of bioaccumulation and biotransfer by which tissue concentrations of chemicals in organisms at one trophic level exceed tissue concentrations in organisms at the next lower trophic level in a food chain." This is frequently measured under field conditions and is expressed in terms of biomagnification factor (BMF). BMF is a simple ratio between the concentration of a chemical in an organism (C_b) to

that in its diet (C_d). The other less commonly used field based estimates are food web

magnification factor (FWMF) and trophic magnification factor (TMF). These indices are used to

examine a chemical's potential to biomagnify in a food web (Arnot and Gobas, 2006).

3.1.4   Regulatory Importance of BCF/BAF Assessments

Ecological and human health hazards resulting from the use and subsequent release of

man-made chemicals into the environment have been known at least since 1960s. Currently,

there are about 100,000 existing chemicals and around 2000 new chemicals are added each year

(Arnot and Gobas, 2006; Nordberg and Rudén, 2007). Among these, the compounds with

persistent (P), bioaccumulative (B) and toxic (T) characteristics are of major concern. Regulatory

efforts such as implementation of Registration, Authorization and Evaluation of Chemicals,

REACH, are in progress to identify, ban or restrict the use of these PBT substances (Weisbrod et

al., 2007). Similar assessments for PBT characteristics of approximately 23,000 chemicals

included in the DSL are being conducted in Canada.

Evaluation of bioaccumulation potential of a chemical is important due to several

reasons. The potential for biological accumulation, serves as a valuable tool to predict the

occurrence of possible adverse effects on the wild life (e.g. accumulation of DDE in fatty tissues

of brown pelican resulting in eggshell thinning). BAFs are also frequently used to monitor

environmental contamination. And finally, BAF/BCF assessments are increasingly used in

establishing regulatory criteria (Mackay and Fraser, 2000; Weisbrod et al., 2009b). Although

region-specific regulatory guidelines based on bioaccumulation existed before, more emphasis

has been given to conducting such assessments since the 2001 United Nations (UN) Stockholm

Convention on the persistent organic pollutants (POPs), called POPs protocol. The summary of

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the bioaccumulation screening criteria used by various regulatory agencies around the world is

provided in Table 1. If a compound of interest exceeds the threshold level, it is subjected to a

comprehensive evaluation including environmental risk and management.

Table 1

Bioaccumulation assessment endpoints and criteria applied by regulatory agencies (adapted and
modified from Arnot & Gobas, 2006).

Regulatory Agency/program	Category	Bioaccumulation endpoint	Criteria
US EPA	bioaccumulative	BCF	1000-5000
US EPA	very bioaccumulative	BCF	$\geq$5000
REACH	bioaccumulative	BCF	$\geq$2000
REACH	very bioaccumulative	BCF	$\geq$5000
EC	--	BCF	$\geq$5000
EC	--	BAF	$\geq$5000
EC	--	Kow	$\geq$100,000
UNEP	--	Kow	$\geq$100,000
UNEP	--	BCF	$\geq$5000

US EPA: United States Environmental Protection Agency; REACH: Registration, Evaluation and Authorization of
Chemicals; EC: Environment Canada; UNEP: United Nations Environment Program.

The significance of including bioaccumulation/bioconcentration assessments in the ERAs

of pharmaceuticals has been recognized. For example, in most of the ERA frameworks, all

medicinal products are initially screened for their hydrophobicity and those with log K_{ow}s greater

than 3 (EMEA guideline) or 3.5 (FDA guideline) are automatically qualified for chronic toxicity

(usually, the last phase) testing. Some studies have suggested the use of environmental hazard

assessments for pharmaceuticals (Carlsson et al., 2006). For example, in a risk assessment

conducted on selected pharmaceuticals in Sweden, two criteria were used to classify the

compounds, environmental risk and environmental hazard. While environmental risk was the

same as the other ERA assessments for pharmaceuticals, hazard assessment required the

determination of PBT profiles (Carlsson et al., 2006). It is also known that aquatic organisms are

continuously exposed to low levels of pharmaceuticals, and some of them have potential to

bioaccumulate (Paterson and Metcalfe, 2008). Such accumulation could result in the buildup of concentrations to dangerous levels, ultimately resulting in profound toxic effects. This assumption (which could in fact be true for certain compounds) is the driving force to gain increased knowledge on bioaccumulative potential of pharmaceuticals.

In a recent study, bioaccumulation potential of pharmaceutical products was used as one of the criteria to prioritize compounds for further risk assessments (Besse and Garric, 2008). However, the authors used log K_{ow} values of the pharmaceuticals to predict the potential for bioaccumulation despite the fact that such strategy may not be applicable to the charged pharmaceutical products (Fent et al., 2006). But, the current guidelines for the screening of pharmaceutical products for bioaccumulation use K_{ow} with a BCF trigger level of 2000 (EU technical guidance document, TGD). Although measured BCFs are the most desirable in terms of accurate prediction of potential risk, dependence on log K_{ow} for such tasks is inevitable in view of the number of compounds (ca. 3000) that require such assessments as well as the desire to reduce animal use in testing. Therefore, it is important to develop new or refine the existing BCF models using measured BCF data obtained for compounds that are representative. The next section focuses on currently available BCF models.

3.2   BCF Models

The potential for bioaccumulation of a compound is commonly assessed using the OECD Technical Guidance (TG) 305 test. This test requires a large number of animals (fish) and long duration exposure regimes. Conducting this test on all the chemicals including pharmaceuticals is not feasible. To provide an example, 1240 chemical substances in the Canadian DSL were identified as candidates with potential bioaccumulating capability. If each chemical is subjected

to the standard OECD BCF testing, it was estimated that such an effort would require 82-work years (Weisbrod et al., 2007). However, the current regulations (e.g. REACH) require such assessments and hence it is imperative to rely on models or rapid *in vitro* testing approaches to predict BCFs.

Most of the currently available BCF models are based on hydrophobicity (K_{ow} or P) of compounds. These models often assume a linear relationship between the logarithmic transformations of BCF (log BCF) and partition coefficient (log P). Such a relationship was found to exist for a number of compounds (Veith and Kosian, 1983). Models based on the hydrophobicity as the sole predictor may be applicable to compounds which are non-ionic, low molecular weight compound and have little or no degradation (Weisbrod et al., 2007).

BCF models have been improved by including several correction or description factors using quantitative structural activity relationship, QSAR approaches (Meylan et al., 1999; Lu et al., 2000; Dearden and Shinnawei, 2004). Such improvements in the models enhanced the predictability of BCFs. Although there are several QSAR based models reported in the literature, further discussion (brief) will be restricted to one prominent model, BCFWIN developed by the USEPA and Syracuse Research Corporation (SRC). BCFWIN is part of the Estimation Programs Interface (EPI) Suite, a publicly available program integrated into the PBT profiler. The program is very extensive with around 700 chemicals including some ionic compounds (Meylan et al., 1999). The database has been based on the previous studies the quality of which was not evaluated, although certain criteria were incorporated to minimize the uncertainty. In spite of this drawback, a good correlation (r^2=0.73) was observed between the measured and estimated BCFs (Weisbrod et al., 2007)

Pharmaceuticals, contrary to non-ionic hydrophobic contaminants, tend to be larger

molecules with multiple functional groups and often exist in their ionized state at environmentally relevant pH levels. It is, therefore, necessary to consider the pH dependent distribution coefficient, log D_{ow} (log K_{ow} at pH 7) for bioconcentration assessments of pharmaceutical products. However, several of the current ecotoxicity models may not use log D_{ow} leading to possible over-prediction of bioconcentration potential. Hence proper care should be exercised in accounting for the ionization of pharmaceutical products, especially when the data is used in risk assessment models.

In addition to the partitioning coefficient (log P or D_{ow}), the extent of bioconcentration of chemicals in the organisms (especially in fish), is dependent on differences in absorption, distribution, metabolism and elimination (ADME) processes, steric hindrance (across gills), biological availability, inter and intra species differences and environmental factors (Barron, 1990). Hence, an alternative approach that accounts for all these factors is the physiologically based pharmacokinetic model (PBPK). PBPK model is often used in the pharmaceutical research and development (R&D) and in human health risk assessments. This model requires prior knowledge of the anatomy, physiology and biochemistry of the species concerned. It consists of different compartments of organs or group of organs mediated by blood flow. In order to conduct such predictions, differential equations are written that represent parameters such as rate of perfusion of tissue, ventilation rate or stroke volume. Hence the ADME profile of a particular chemical in the exposed animal can accurately be modeled by determining the fate of the chemical as it distributes among different organs. This is possible because the concentration of a chemical at a target tissue depends on the partitioning between blood and tissue, extent of blood flow to the tissue, metabolism in the tissue and blood flow out of the tissue. Because of these considerations, PBPK model can successfully be applied between species, body sizes and

exposure conditions (Barron, 1990; Kedderis and Lipscomb, 2001). However, requirement of many parameters and huge amounts of data (which in turn require many experiments and/or complex computational approaches) are the primary reasons for its decreased potential for use in ecological risk assessments.

In summary, assessment of bioconcentration has been considered an important endpoint in risk assessment of pharmaceuticals and there is a growing need to generate reliable and useful bioconcentration data. Such an exercise requires the use of validated models. However, the existing models developed to address the risk assessments of hydrophobic compounds, may not be applied directly to pharmaceuticals. Suitable corrections should be made in the models to account for the ionic character of pharmaceuticals and hence better risk predictions. Finally, the potential for using PBPK model in ERAs should be explored.

3.3   Prioritization of Pharmaceuticals

Implementation of the guidelines for conducting ERA of pharmaceuticals has prompted research community and regulatory agencies to devise efficient prioritization strategies to identify the most important pharmaceutical products that are likely to be present in the environment and that may cause significant adverse effects in the non-target species. Pharmaceutical ERA frameworks often have an initial screening step where an RQ (PEC/PNEC) is computed. RQ is an important factor in deciding whether a particular chemical needs to go through further risk assessments. Therefore, any factor that influences PEC or PNEC is considered in prioritization schemes.

The prioritization strategies often depend on factors such as the annual pharmaceutical sales (Stuer-Lauridsen et al., 2000; Jones et al., 2002; Grung et al., 2008), therapeutic dose ,

consumption amounts (Carlsson et al., 2006), demographics, extent of metabolism (Huschek et al., 2004; Zuccato et al., 2005), environmental fate (Boxall et al., 2004) or measured or modeled occurrence data (Anderson et al., 2004). The environmental concentrations of pharmaceuticals may be better predicted using watershed based approaches that account for the diversity in the environment. Two prominent predictive models in this category are the pharmaceutical assessment and transport evaluation (PhATE) and geo-referenced regional exposure assessment tool for European rivers (GREAT-ER) (Feijtel et al., 1997; Anderson et al., 2004). On the other hand, prioritization of pharmaceuticals on the effect basis relies on the available information on ecotoxicity (measured or predicted using PBT profiling or ecological structure activity relationship, ECOSAR) (Sanderson et al., 2004; Carlsson et al., 2006) pharmacological data (Huggett et al., 2003; Kostich and Lazorchak, 2008) or bioconcentration (Nordberg and Rudén, 2007; Besse and Garric, 2008) . Extensive reviews are available on various prioritization strategies required for the selection pharmaceuticals for risk assessments (Besse and Garric, 2008; Cooper et al., 2008). Utilizing the widely available pharmacological and toxicological data on pharmaceuticals for prioritization has been gaining prominence (Länge and Dietrich, 2002) and is further discussed here.

3.3.1   Prioritization using Mammalian Data

As previously noted, large numbers of pharmaceuticals are already in the market and the usage is projected to increase in the future. The current ERA approaches predict the pharmaceutical NOEC levels after applying certain safety margins on acute toxicity results. However, such an approach is questioned because drugs are bioactive compounds and their actual chronic toxicity levels differ widely from the predicted levels (Dietrich et al., 2006).

However, efforts to test each drug for chronic toxicity on aquatic species are counterbalanced by cost, time and animal use constraints. An alternative approach is therefore required for prioritization. One such approach is to utilize readily available mammalian pharmacokinetic and pharmacodynamic data as a useful tool in prioritizing pharmaceuticals (Länge and Dietrich, 2002; Seiler, 2002).

Länge and Dietrich (2002) provided a step-by-step approach for using mammalian data in prioritization schemes. Briefly, the proposed concept requires a case-by-case testing strategy to screen for the presence of similar targets (enzymes/receptors) in the non-target species, identification of drug targets (e.g. organs) that are most susceptible to adverse (toxic) effects, use of differences between acute and chronic mammalian toxicity levels as an indicator of possible chronic effects in the non-target organisms and finally, comparison of effective mammalian plasma concentrations with the environmental exposure levels. Using similar concepts, a fish plasma model (FPM) has been developed as a tool to predict the chronic toxicity of pharmaceuticals in fish (Huggett et al., 2003) and is discussed in detail here.

3.3.2 Fish Plasma Model (FPM)

USFDA requires extensive drug safety studies on pharmaceutical products as part of the approval process. Extensive information is obtained on the physiochemical characteristics of the compound followed by several studies to determine the pharmaco (toxico) kinetic and pharmaco (toxico) dynamic profiles. These studies enable accurate determination of ADME profiles of the compound as well as establishment of reliable analytical methods, especially for plasma. FPM is intended to use this wealth of information on pharmaceuticals and predict if similar responses result in aquatic organisms, particularly in fish. This is particularly useful in view of the studies

supporting the presence of functionally conserved drug targets in various aquatic species, most

importantly in vertebrates as shown in Table 2 (Huggett et al., 2003). Gunnarsson et al., (2008)

recently reported functionally identical drug targets in fish (86%), invertebrates (61%) and algae

(35%) as compared to mammals. These studies indicate that fish are more susceptible to

pharmacological interactions and hence are required to be included in ERAs.

Table 2

Receptor/enzyme identity (%) in fish compared with those present in mammalian species for
various therapeutic classes (Huggett et al., 2003; Gunnarsson et al., 2008).

Pharmaceutical class	Target organ/system	Target receptor/enzyme	Identity (%)
SSRI	CNS	5-HT (serotonin)	72
α, β adrenergic receptor	Heart, lung, smooth	β- receptor	63
agonists/antagonists	muscle	α- receptor	61
Statins	Liver	HMG-CoA reductase	77
Calcium channel blockers	Heart & smooth muscle	Calcium channels	98
ACE inhibitors	Kidney	ACE	47
Cholinergic antagonists	Heart, lung, bladder	Acetylcholine receptor	74
NSAIDs	Multiple systems	COX enzymes	67
PPARs agonists	Liver	PPARs	47
Androgens	Testis and other systems	Androgen receptor	45
Estrogens	Testis and other systems	Estrogen receptor	47
Aromatase inhibitors	Testis	CYP450	50
Anticonvulsants	CNS	Sodium channel	62
Anti-addictives	--	ALDH2	69
Immunosuppressants	--	inosine monophosphate dehydrogenase	90

SSRI: selective serotonin reuptake inhibitor; CNS: central nervous system; 5-HT: 5-hydroxytyptamine; HMG-CoA: 3-hydroxy-3-methyl-glutaryl coenzyme A; ACE: angiotensin-converting enzyme; NSAID: non-steroidal anti-inflammatory drug; COX: cyclooxygenase; PPAR: peroxisome proliferator-activated receptor; CYP450: cytochrome P450 enzyme system; ALDH2: aldehyde dehydrogenase 2 family

The most important assumption in FPM is that the receptor/enzyme systems are

evolutionarily conserved, which allows the biochemistry to "read-across" the taxonomic lines,

and species sharing similar drug targets respond similarly. However, Huggett et al., (2003)

suggested that inclusion of certain correction factors could address the species-specific changes

in receptor responses. A brief description of the model, applicability and refinements are

discussed next.

The mammalian therapeutic effect level of a pharmaceutical is related to the dose and circulating plasma concentrations. If the aquatic exposure levels and plasma concentrations of the particular pharmaceutical are known, the likelihood of occurrence of similar pharmacological interactions in the non-target organisms could be predicted. This essentially is the crux of the FPM. Such information on whether there is potential for pharmacological interactions in the non-target aquatic species (mainly, fish) is useful in prioritizing pharmaceuticals for ERAs. In this theoretical model, established values of human therapeutic plasma concentration (H_TPC) are compared to the predicted steady-state plasma concentrations in fish ($F_{SS}PC$) as detailed in the following equations:

$$\text{plasma exposure ratio, PER} = H_TPC / F_{SS}PC$$

$$F_{SS}PC = EC * P_{b:w}$$

EC is the measured/predicted environmental concentration and $P_{b:w}$ is the measured plasma bioconcentration or predicted value using the following equation:

$$\log P_{b:w} = 0.73 \text{ x } \log K_{ow} - 0.88 \text{ (Fitzsimmons et al., 2001)}$$

PER thus provides an estimate of likelihood of aquatic concentrations achieving a level that could potentially trigger pharmacological interactions. It should be noted, however, that this model cannot be used to predict whether such pharmacological interactions result in adverse effects or not.

As indicated earlier, in order to account for the differences in the receptor responsiveness in mammalian and aquatic vertebrate systems, a correction in terms of functional equivalency ratio (FER) should be incorporated in the model. However, such determination in differences in the response of biological targets requires extensive understanding of the comparative (humans

vs. fish) physiology and pharmacology. In the absence of such information, FER of 1 is assumed. And finally, bioequivalence index (BEI) is computed from the ratio of PER and FER.

Huggett et al., (2003) applied this model (assuming FER of 1) for 28 pharmaceuticals that are frequently detected in the environment and reported an estimated PER (also called effect ratio, ER) of greater than 1000 for most of the compounds. Some compounds (e.g. EE2) had ER less than 1, indicating that this compound should be prioritized for chronic toxicity testing. Therefore, lower the ER, the greater the potential for a pharmacological response in fish. In a recent study, critical environmental concentrations of about 500 pharmaceuticals were predicted using this model (Fick et al., 2010b). In estimating these critical concentrations, the authors assumed a similar magnitude of pharmacological responses in humans and fish when experiencing similar plasma concentrations (i.e. $H_TPC = F_{SS}PC$).

The main concerns with the extrapolation of mammalian data to aquatic vertebrates are: a) the concentration of a drug that would elicit a pharmacodynamic response in mammals, in general, is several magnitudes greater than the environmentally relevant levels, b) differences in expression and specificity of biological target molecules (enzymes/ receptors), c) differences in pharmacodynamic activity in terms of pharmaceutical's major (intended) and secondary (side) effects. In other words, drugs that provide desirable human therapeutic effects could cause similar effects at higher magnitude or the side effects (considered unimportant) in humans, could turn into major effects for aquatic species. All these considerations would suggest the requirement of a "mechanism-based" or MOA based approach to the experimental determination of potential ecological risks of pharmaceuticals (Seiler, 2002). Again, approaches like this are often resource demanding.

3.4   Bioconcentration of Pharmaceuticals

The extent to which a chemical bioconcentrates in an animal is an important indicator of possible adverse effects on the organism due to long-term exposure. This is the reason why regulatory agencies (e.g. REACH) require the data on BCFs of chemicals for prioritization/ screening. The ERAs of pharmaceuticals also consider BCF as important regulatory end point. However, the current efforts for estimating bioaccumulative potential of pharmaceuticals are far less than those focused towards assessing priority pollutants. For example, a simple PubMed search on bioaccumulation of polychlorinated biphenyls (PCBs) provided 402 articles, while a similar search (at the same time) on pharmaceuticals has yielded only 13 studies (ca. 3 % of the total reports on PCBs). Next, the available field and laboratory studies on bioconcentration of pharmaceuticals in aquatic species (prominently in fish) are discussed here.

Schwaiger et al., (2004) determined BCF levels for diclofenac, an NSAID, in different tissues (liver, kidney, gill and muscle) of rainbow trout (Onorhynchus mykiss) exposed to concentrations ranging from 1-500 µg l^{-1} for 28 days. They observed wide variability in BCF levels among the tissues with the highest accumulation in the liver. In another study, gold fish (*Carassius auratus*) exposed to gemfibrozil, a lipid regulator, for 14 days at environmentally relevant concentrations (1.5µg l^{-1}) resulted in plasma BCF of 113 (Mimeault et al., 2005). A recent study (Mehinto et al., 2010) reported diclofenac BCFs between 509 and 657 in the bile of rainbow trout exposed to environmentally relevant levels (0.5-25 µg l^{-1}). This study has also reported reduction in the expression of COX enzymes, inhibition of CYP1A1 in kidney and tubular necrosis even at 1 µg l^{-1} exposure level. Paterson et al., (2008) exposed Japanese medaka (*Oryzias latipes*) to the anti-depressant fluoxetine at 0.64 µg l^{-1} for seven days and reported a steady-state and kinetic BCF values of 74 and 80 respectively. The authors suggested the need

for generation of tissue-specific bioconcentration of pharmaceuticals for better prediction of toxic effects. In a recent study, Owen et al., (2009) reported a plasma BCF value of 0.59 for rainbow trout exposed to propranolol, a cardiovascular drug. A few studies have determined bioaccumulation potential of steroid hormones in the aquatic species of different trophic levels, algae (Lai et al., 2002), daphnia (Gomes et al., 2004) and fish (Länge et al., 2001). These studies reported BCFs (660-27,228) for algae, invertebrate and fish.

Some studies measured pharmaceutical levels in plasma and tissues of fish exposed to effluent waters from WWTPs (Brown et al., 2007; Fick et al., 2010a) or from fish residing in effluent dominated rivers (Brooks et al., 2005; Ramirez et al., 2009; Schultz et al.). A few studies have reported the use of solid phase micro extraction (SPME) and semi-permeable membrane devices (SPMDs) as surrogate for predicting bioaccumulation of pharmaceuticals in fish tissues (Barber et al., 2006; Zhang et al., 2010). The studies focusing on the bioaccumulation of pharmaceuticals in the terrestrial environment are limited (Barber et al., 2006; Wu et al., 2010).

In summary, few studies have previously reported bioconcentration potential for pharmaceuticals and systematic BCF studies using the standard test guidelines (OECD 305 test) are absent. Most studies measured plasma BCFs and very few studies have focused on tissue-specific or whole fish measurements. In the absence of estimated BCF levels for pharmaceuticals, it may be difficult to test the usefulness existing BCF models (e.g. BCFWIN). Therefore, an attempt has been made in this dissertation work, to generate experimentally determined BCF levels for five compounds and compare the results with the predicted levels.

CHAPTER 4

MATERIALS AND METHODS

This chapter covers the experimental section and is subdivided into 9 sections. The first section provides the basis for selecting the pharmaceuticals and the fish species used in this work. Different chemicals and reagents used are listed in section 2. The experimental details on fish exposures and data collection are covered in the third, fourth and fifth sections. The sixth section provides comprehensive details on the analytical methods used. BCF estimations, quality control and data analysis are covered in the subsequent sections.

4.1   Selection of Pharmaceuticals and Test Species

4.1.1   Pharmaceuticals

Five pharmaceutical compounds (ibuprofen, verapamil, norethindrone, clozapine and fenofibrate) were identified based on their log D_{ow} (log P at pH 7) values, ionic moiety (acid, base or neutral), therapeutic use, homology of drug targets between mammals and fish, and environmental concentrations (Table 3). The list consisted of one neutral compound (norethindrone) and two compounds each represented acidic (ibuprofen & fenofibrate) and basic groups (verapamil and clozapine). The partition coefficients, log D_{ow}s covered a wide range from 1 to 5. The pharmaceuticals selected represented over the counter (OTC) (ibuprofen and norethindrone) as well as the prescription drugs. Most importantly, homology between human and aquatic vertebrate biological targets was considered for the selection. And finally, the reported measured surface water concentrations (maximum, if reported) of the pharmaceuticals were taken into consideration. The selection of the compounds was influenced equally by all the factors indicated here.

Table 3

Description of pharmaceuticals selected for the study.

Drug	Class	log Dow	Therapeutic use	Target system	Receptor/enzyme (Identity in fish, %) #	MEC ng/L
Ibuprofen	Acid	1.06a	Anti-inflammatory	Various	COX (67)	5-3400
Verapamil	Base	2.41b	Antihypertensive	Heart & smooth muscle	Calcium channel (98)	14-190
Norethindrone	Neutral	3.15b	Contraceptive	Reproductive	Progestin receptor *	10-872
Clozapine	Base	3.23c	Antipsychotic	CNS	Dopamine receptor*	< 2
Fenofibrate	Acid	5.10d	Hypolipidemic	Liver	PPAR (47)	30-160

log D_{ow}: log P at pH 7; [a] (Avdeef et al., 1998), [b] (www.cerep.com), [c] (Sköld et al., 2006), [d] (Yamashita et al., 2009) ; [#] adapted from Huggett et al., 2003; MEC: measured environmental concentration; CNS: central nervous system; COX; cyclooxygenase; * present in fish but identity not reported; PPAR: peroxisome prolifator-activated receptor; MECs (range) were obtained from Kolpin et al., 2002; Batt et al., 2008; Boxall et al., 2004; Woldegiorgis et al., 2007; Andreozzi et al., 2003.

4.1.2   Selection of Test Species

Two fresh water fish species, fathead minnow (*Pimephales promelas*) and channel catfish (*Ictalurus punctatus*) were chosen for conducting bioconcentration studies. The fathead minnow has been a widely used small fish model for regulatory ecotoxicological studies (Ankley and Villeneuve, 2006) and represents the ecologically important Cyprinid family which inhabits both lotic and lentic environments. The fish can feed on a wide range of diet and they tolerate a wide range of water quality characteristics. With relatively rapid life cycles, fathead minnow are easy to handle and are increasingly becoming the aquatic vertebrate model for ecotoxicity tests to assess the potential risks of chemicals on the environment.

Channel catfish are readily available from local fish farming facilities due to their wide spread distribution and culturing across the North America. Catfish are also omnivores that can live both in clear and turbid waters. In addition to their utility as a sentinel organism to monitor water quality, the fish species are also frequently used in bioconcentration testing (Weisbrod et

al., 2009a). Most importantly, the size of the fish facilitated sampling large blood volumes for plasma BCF work.

4.2   Chemicals and Reagents

Norethindrone (NET, (17α)-17-hydroxy-19-norpreg-4-en-20-yn-3-one, CAS#68-22-4), norethindrone-d6 (NET-d6, 4-estren-17α-ethynyl-17β-ol-3-one-d6), ibuprofen (IBU, 2-(4-isobutylphenyl)-propionic acid, CAS # 15687-27-1), ibuprofen-methyl-d3(IBU-d3, 2-(4-isobutylphenyl)-propionic acid-d3, CAS#121662-14-4), verapamil hydrochloride (VER, α-[3-[[2-(3,4-dimethoxyphenyl)ethyl]methylamino]propyl]-3,4-dimethoxy-α-(1-methylethyl)-benzeneacetonitrile hydrochloride, CAS # 23313-68-0), verapamil-d6-hydrochloride (VER-d6, α-[3-[[2-(3,4-dimethoxyphenyl)ethyl] methylamino]propyl]-3,4-dimethoxy-α-(1-methylethyl)-benzeneacetonitrile-d6 hydrochloride), clozapine (CLZ, 8-chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo[b,e][1,4]diazepine, CAS#5786-21-0), clozapine-d8 (CLZ-d8, 8-chloro-11-(4-methyl-1-piperazinyl-d8)-5H-dibenzo[b,e][1,4]diazepine), fenofibrate (FFB, 2-[4-(4-chlorobenzoyl)phenoxy]-2-methyl-propanoic acid 1-methylethyl ester, CAS#49562-28-9) and fenofibrate-d6 (FFB-d6, 2-[4-(4-chlorobenzoyl)phenoxy]-2-methyl-propanoic acid-d6 1-methylethyl ester) were all purchased from Toronto Research Chemicals (TRC; North York, ON, Canada).

HPLC grade solvents acetonitrile (ACN), methanol (MeOH), hexane (HEX), ethyl acetate (EA), dichloromethane (DCM), dimethyl formamide (DMF) and acetone were obtained from Fisher Scientific (Houston, TX). N-methyl-N-(trimethyhlsilyl) trilfluoroacetamide (MSTFA, CAS# 24589-74-4), 14% boron trifluoride in methanol (BF3/MeOH, CAS# 373-57-9) were procured from Thermo Scientific (Rockford, IL). Formic acid (CAS# 64-18-6), ammonium

acetate (CAS# 631-61-8), tricaine mesylate (MS-222, ethyl 3-aminobenzoate methanesulfonic acid, CAS# 886-86-2), potassium chloride (CAS# 7447-40-7) were purchased from Sigma-Aldrich (St. Louis, MO). Milli-Q water (18.3 M Ω. cm) was obtained in the lab from Milli-Q water system (Millipore, Billerica, MA).

4.3   Fish Exposures

4.3.1   Animals and Housing

Adult fathead minnow of either sex (ca. 3 g) were obtained from the University of North Texas (UNT) aquatic toxicology facility. Juvenile channel catfish of either sex (ca. 50 g) were procured from a commercial supplier (Pond King Inc., Gainesville, TX). All experimental fish were allowed to acclimatize for at least a week to the standard laboratory conditions of $25 \pm 2°C$ and 16:8 hour light: dark cycle. Fathead minnow were fed brine shrimp flakes (Ocean Start International, OSI, Snowville, UT) twice daily. Channel catfish were fed pellets of AquaMax grower diet (PMI International LLC, Brentwood, MO) to satiation once daily. The food residues in the tanks were siphoned off approximately two hours after feeding.

4.3.2   Flow-Through Exposure System

In the aquatic toxicology laboratory, tap water (City of Denton, TX) was initially passed through activated charcoal units and then fed to dechlorination tanks. A continuous flow-through system (Fig 1) was used for all the exposure experiments. A peristaltic pump (Masterflex® L/S®, Cole-Parmer®, Veron Hills, IL) was used to deliver dechlorinated tap water (ca. 150 ml min^{-1}) into mixing chambers where stock solutions of the analytes or dimethyl formamide (DMF, solvent control) in 30 ml polycarbonate syringes (Becton Dickinson, Franklin

Lakes, NJ) were infused at 5 µl min^{-1} using a syringe pump (PHD 2000, Warner Instruments, Hamden, CT). Vinyl tubing (Watts, Andover, MA) was used in all the exposure experiments. The stock solutions of the analytes in syringes were prepared in accordance with the dilution (30,000X) in mixing boxes. High concentration (10 mg ml^{-1}) stock solutions were prepared either in DMF or Milli-Q water and stored in 500 ml amber bottles (Fisher Scientific, Houston, TX) at 4°C. The same stock solutions were used for the entire exposure duration of the experiments. From mixing boxes, the targeted concentration of a chemical was gravity fed into exposure tanks. The exposure chambers were either 20 (fathead tests) or 60 liter (catfish tests) tanks with approximately 10 and 4 volume replacements per day, respectively. All fish tanks were aerated during the course of the experiments.
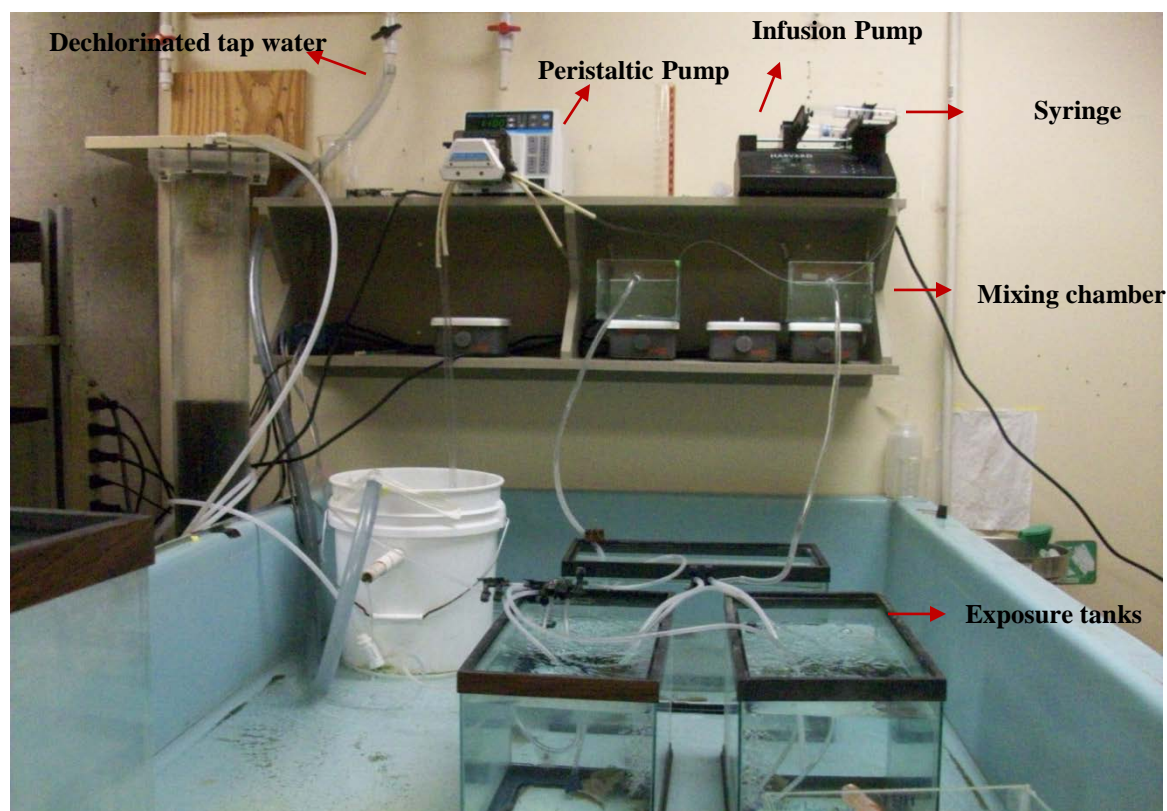
Fig. 1. A continuous fish flow-through exposure system used for conducting BCF tests.

4.4　　BCF Experiments

For each of the five test compounds, two separate BCF experiments were performed.
BCF tests on Fatheads followed the standard OECD 305 BCF testing guidelines (OECD, 1996)
with some modifications while those conducted on catfish involved a reduced sampling design
(Springer et al. 2008). In both tests, fish were exposed to a single test concentration based on its
7-day no observed effect concentration (NOEC) obtained from the early life stage (ELS) tests
conducted on fathead larvae (EPA test method 1000.1) or on the standard 96 hr LC-50
concentration. These ELS studies on the selected pharmaceuticals were a component of the BCF
project (Pfizer Global Research & Development) but were not part of this dissertation. The ELS
research work was conducted by other researchers in our laboratory, and the NOEC values were
adopted from those exposure studies. As a general approach, ten percent of the test chemical's
NOEC (survival) level was chosen for fish exposure experiments. In addition to NOEC level for
each test chemical, the analytical requirements (detection limits) also dictated the final selection
of exposure concentration. The exposure levels of pharmaceuticals are summarized in Table 4.

Table 4

Pharmaceuticals' exposure concentrations (μg l^{-1}) used in the BCF tests.

Pharmaceutical	Exposure concentration (μg l^{-1})
Ibuprofen (IBU)	250
Verapamil (VER)	500
Norethindrone (NET)*	100 or 50
Clozapine (CLZ)	50
Fenofibrate (FFB)	25

* catfish were exposed to 100 μg l^{-1} and Fathead minnow were exposed to 50 μg l^{-1}

Except for norethindrone, the exposure levels (μg l^{-1}) of all the pharmaceutical were similar in
both fathead and catfish tests. A solvent control (DMF, 0.003% in exposure tanks) was also
included in all the exposure experiments to account for any possible side effects. Hereafter,

solvent control will be referred to as "control." All flow-through experiments started at least two days before the fish addition in order to allow the exposure tanks to equilibrate with the target test concentration.

4.4.1 Fathead Tests

After a week's acclimation, fish were randomly distributed into control (n=10) and exposure (n=40-50) groups. The 42-day BCF tests included 28 days of exposure followed by depuration in clean tanks for 14 days. Fish were sampled (n=4-6) at five time points during exposure (days 1, 3, 7, 14 and 28) and twice during depuration (days 35 and 42).

4.4.2 Catfish Tests

BCF tests conducted on the catfish were of short duration with reduced sampling design. As explained previously, these tests were intended two serve two purposes, a) to provide large volumes of blood enabling the quantification of pharmaceuticals in the fish plasma and b) to compare the BCF test results to those obtained from the standard OECD tests performed with fathead minnow. Additionally, these tests were helpful in determining the inter-species differences in the bioconcentration potential of the test compounds. The reduced BCF test design consisted of fish (n=30) exposure to the test chemical for 7 days followed by 7day-depuration phase. During the 14-day test duration, fish (n=3-5) were sampled after days 1, 3, 7 (exposure) and 10 and 14 (depuration). A control group (n=6) was also included in all the experiments.

4.5   Data Collection

In all the experiments, fish were monitored on daily basis for signs of stress, if any, throughout the test duration. A fish mortality record was maintained for each test and mortalities greater than 10% in the control group required reinitiation of BCF tests. Dissolved oxygen (DO), temperature and pH levels in the exposure tanks were monitored at least twice a week.

4.5.1   Measured Test Concentrations

In each experiment, water samples from fish tanks were sampled at least four times during the exposure phase to measure the chemical concentration. Time-weighted average measured test concentrations were used to calculate BCFs.

4.5.2   Body Weight and Length

At the end of each sampling day, individual weights and lengths were recorded immediately after anesthetizing fish in a solution of MS-222 (100 mg/l). Data on fish weight (g) and length (cm) were used to calculate condition factor (K), which is indicative of overall well-being of fish and fish with K value ≥1.0 were considered healthy and stress-free from chemical exposure (Jones et al., 1999). Condition factor was calculated using the following formula:

$$K = [(\text{weight, g}) \div (\text{length, cm})^3] \times 100$$

4.5.3   Plasma and Tissue Sampling

Blood was collected from the caudal vein/arterial complex (catfish only) using a heparinized syringe and transferred into plasma separation tubes containing lithium heparin (Becton Dickinson, Franklin Lakes, NJ). Plasma was separated by centrifugation at 12,000 x g

for 5 min in a micro centrifuge system (Eppendorf 5418, Hamburg, Germany) and frozen for further analysis. Muscle and liver tissues of fish were collected in all the BCF tests. Based on the known human pharmacological effect of each test chemical, additional tissues (gill, kidney, heart or brain) were also collected. All fish handling methods followed the Institute's Animal Care and Use Committee (ACUC) guidelines. Individual tissues were weighed and stored at -20°C for chemical analysis. Liver, kidney, heart and brain tissue weights were used to calculate hepatic somatic index (HSI), nephritic somatic index (NSI), cardio somatic index (CSI) and brain somatic index (BSI). These indices were used to get additional information on the physiological status of the exposed fish. All the indices were calculated using the following formula:

$$\text{Tissue index} = [\text{tissue weight (g)} \div \text{body weight (g)}] \times 100$$

4.6   Analytical Methodology

4.6.1   Extraction of Pharmaceuticals

4.6.1.1 Water

A liquid/liquid extraction technique was used to extract the analytes from water samples. Water samples (1-5 ml) were initially fortified with 2.5, 5.0 or 10.0 ng labeled internal standard (ISTD) followed by the addition of dichloromethane (DCM) or (1+1) (v/v) hexane (HEX): ethyl acetate (EA) (5-10 ml). Contents were mixed thoroughly on a high speed vortex unit (Fisher Scientific, Houston, TX) for at least 2 min. Test tubes were allowed to settle for 5-10 min and organic fractions were separated into clean 10.0 ml glass vials (Fisher Scientific). Some samples required "salting out" with saturated potassium chloride (KCl), centrifugation or a combination of both for better separation of organic layer. Extraction was repeated, organic fractions combined, and then evaporated to dryness under gentle stream of nitrogen.

4.6.1.2 Plasma

Frozen plasma samples were brought to room temperature. In a clean 15 ml glass centrifuge tube (Kimble Chase, Vineland, NJ), 50-200 µl thawed plasma was fortified with 2.5, 5.0 or 10.0 ng labeled ISTD. Ice-cold acetone (8 ml) was added to precipitate protein. Contents in the test tube were mixed thoroughly for 1 min and then centrifuged at 2000 rpm to separate acetone layer. Care was exercised not to aspirate any of the settled precipitate. Extraction was repeated, acetone layers combined, and then evaporated under nitrogen. Residual contents after nitrogen evaporation were back extracted into 5 ml of 1+1 (v/v) HEX: EA (hitherto referred to as the "extraction solvent"). Extraction was repeated, organic layers combined, and then evaporated to dryness under nitrogen.

4.6.1.3 Tissues

Frozen tissues were thawed, blot dried (Kimberly-Clark, Irving, TX) and weighed directly into 5 ml polypropylene vials (USA Scientific, Ocala, FL). The tissue weights depended on fish species and tissue type. For catfish studies, the tissue weights were 100-200 mg for all tissue types. However, fresh wet weights for liver, kidney, brain or heart tissues of fatheads ranged between 20 and 50 mg. Weighed tissues were extracted for 3 min. in 4.0 ml of the extraction solvent using a Mini-Beadbeater™ (Biospec Products, Bartlesville, OK). Extraction was carried out with 1 mm glass beads (Biospec Products). After this step, tissue extracts were further processed using one of the following approaches.

Extracts were filtered into pre-weighed 10 ml glass vials (Fisher Scientific) through 0.45µm polytetrafluoroethylene (PTFE) syringelss filters (Whatman, Sanford, ME). In this method, the filter membrane was initially wetted thoroughly with the extraction solvent followed

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by loading all of the sample extract including the beads. Extraction vials were rinsed twice (2 ml each) with the extraction solvent. Filtration was allowed to occur without any external force. However, some samples (higher tissue mass loads) needed gentle forcing of the extracts through the filter with the aid of a plunger. Sufficient care was taken to avoid breakage of membranes. Accidental breakage of membrane, in some instances, required the sample back extracted into the sample solvent followed by filtration.

Alternatively, sample extract (including beads) was transferred into 15 ml glass centrifuge tubes (Kimble Chase) and rinsed twice with the extraction solvent followed by addition of 2-3 ml Milli-Q water. After adding an additional 5 ml extraction solvent, contents were mixed thoroughly on vortex unit (Fisher Scientific) for at least two minutes. Organic extract was later separated by centrifuging the mixture at 2000 rpm. Extraction was repeated, organic layers combined in a pre-weighed vial, and then evaporated to dryness under nitrogen.

Nitrogen dried vials were weighed and lipid content was determined gravimetrically. Dried extracts were resolubilized 1 ml acetonitrile (ACN) or DCM for further cleanup processes.

4.6.2   Cleanup Methods

Three kinds of cleanup strategies either alone or in combination were applied to tissue extracts to obtain a relatively "interference-free" (hereafter referred to as "clean" extract) sample prior to chromatographic analysis. Selection of clean-up technique depended on tissue type, mass, analyte of interest and finally on the analytical technique (GC/MS or LC/MS).

4.6.2.1 Acetonitrile (ACN) Lipid Removal

Tissue extracts in ACN were stored at 4°C overnight. If the extracted sample was in a different solvent, exchange of solvent to ACN preceded the overnight refrigeration method.

Samples were centrifuged and supernatants were carefully separated into clean vials, evaporated to dryness and finally reconstituted in 100 µl ACN or DCM. Although this method resulted in a relatively cleaner extract for smaller tissue masses (<100 mg), extracts obtained from larger tissues (>150 mg) required further cleanup.

4.6.2.2 Gel Permeation Chromatography (GPC) Cleanup

This clean up method involved injecting 100 µL tissue extract (in DCM) into a 350 mm x 12.2 mm EnviroSep-ABC column (Phenomenex, Torrance, CA) installed in an Agilent 1100 series high performance liquid chromatograph (HPLC) system equipped with a fraction collection system (Agilent, Palo Alto, CA). The fraction collection windows were based on the retention times (RTs) that were initially established with an EPA calibration standard consisting of corn oil, diethylhexphthalate and sulfur. This is the standard calibration mixture from which the elution profiles of above compounds are determined for collecting fractions of interest (Fig 2).
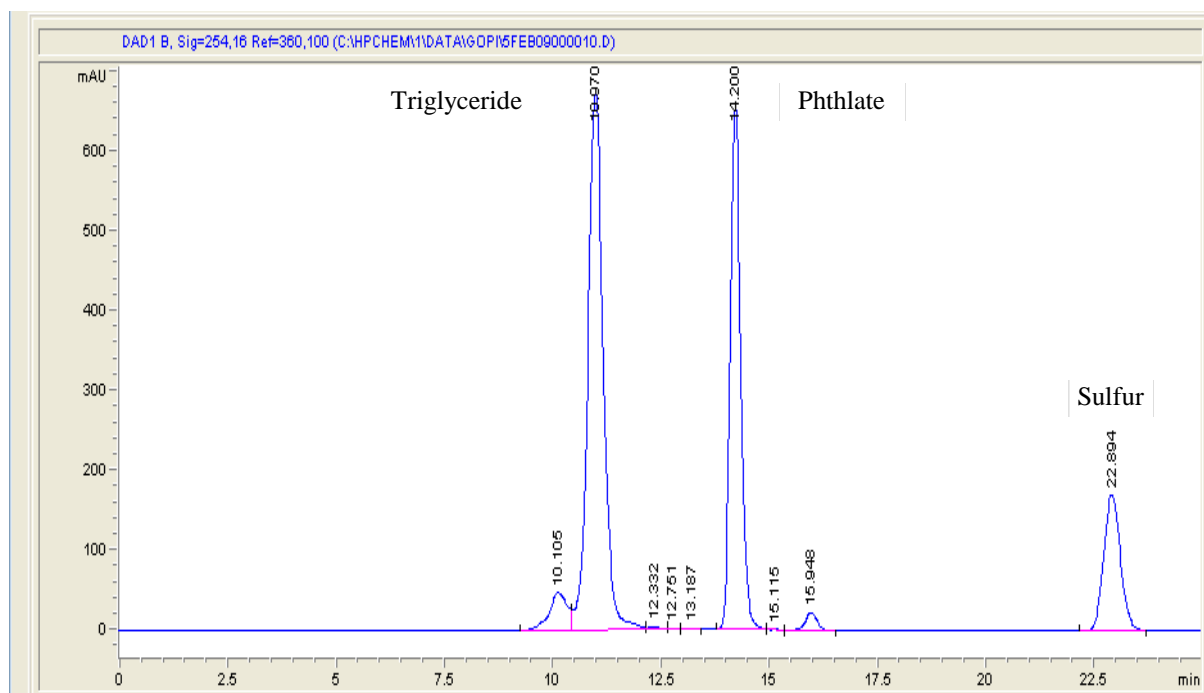
Fig. 2. Gel permeation chromatography (GPC) calibration mix standard run on EnviroSep GPC column. Column effluent of tissue extracts (12.3-22.8 min) was fractionated to remove lipid.

In this cleanup method, high molecular weight triglycerides were excluded using appropriate timed windows of the collection of the column effluent. Before their injection into HPLC, all samples were filtered through 0.22 µm polyvinylidene fluoride (PVDF) membrane filters (Millipore, Billerica, MA). HPLC was set up to run in an isocratic mode with DCM as mobile phase (4.5 ml min^{-1}) with a total run time of 25 min. Column effluent (12.3-22.8 min) was collected into 10 ml glass beakers in the fraction collection attached to the HPLC. Collected solvent fractions were evaporated under nitrogen and reconstituted in 100 µl final volume in DCM or ACN.

4.6.2.3 Solid Phase Extraction (SPE) Cleanup

In spite of the above mentioned cleanup methods, some tissue extracted samples posed challenges in obtaining cleaner chromatograms due to interference from fatty acids. To

overcome this difficulty, a clean-up with florisil (3 cc, 0.5 g) Resprep™ SPE cartridges (Restek, Bellefonte, PA) was conducted. Cartridges were initially conditioned with two tube-full volumes of the extraction/sample solvent (DCM or (1+1) (v/v) HEX: EA) to activate the sorbent before sample extraction. Since the sorbent was used to remove unwanted compounds from tissue extracts, effluent from SPE unit was collected during sample addition. In order to completely remove the desired material from the SPE column, two tube-full volumes of sample solvent was used in washing/elution step. Eluted extracts were evaporated to dryness under nitrogen using RapidVap® N2/48 (Labconco, Kansas City, MO) and finally reconstituted in 100 µl in DCM or ACN.

4.6.3   GC/MS and LC/MS/MS Analysis

The selected pharmaceuticals in this project were analyzed using gas chromatography/ mass spectrometry (GC/MS) and/or atmospheric pressure ionization-electrospray interface-liquid chromatography tandem mass spectrometry (API-ESI-LC/MS/MS). All analyses were conducted using isotopic dilution mass spectrometry (IDMS) technique using labeled ISTDs of each compound. As a first step in selecting the instrument of choice, neat standards of the compounds (10 μg ml^{-1} in ACN) were individually infused directly into mass selective detector (MSD) chamber of LC/MS system (SL Ion Trap, Agilent) using a syringe pump (600 μl hr^{-1}). To operate the MS in direct infusion mode, nebulizer pressure, dry gas (nitrogen) flow and temperature were set at 15 psi, 6 l min^{-1} and 325°C respectively. The direct infusion method served as a tool to determine the suitable ionization mode (positive or negative ion polarity) and to select the appropriate mass transitions. All compounds and labeled standards were infused into the MSD that was operated in either negative or positive ion polarity. The instrument was set to

scan masses (m/z) from 50 to 500 with 50 m sec accumulation time in the trap. The resulting

mass spectrum was used to select the strongest precursor ion signal. After isolating the precursor

ion of each analyte in the trap, collision induced dissociation (CID) energy (0.5-1.0 amplitude)

was applied to get the transition ions. Therefore, ESI-LC/MS/MS (Ion-Trap) had always been the

preferred analytical technique for analytes providing good signal to noise (S/N) ratios. However,

analytes with weak transition ion formation and /or poor S/N ratios were considered for GC/MS

analysis. Descriptions of detailed instrumental analytical methods for each compound are

provided in the corresponding chapters.

Norethindrone, ibuprofen and fenofibrate and their labeled ISTDs yielded poor responses

in LC/MS direct infusion analysis (data not presented) and hence were analyzed using GC/MS

technique. Norethindrone and ibuprofen needed to undergo derivatization (discussed in the

respective sections) to get more non-polar, thermally stable and volatile compounds amenable

for GC analysis. However, GC/MS analysis of fenofibrate did not require any derivatization. All

three analytes were initially run on GC/MS under total ion current (TIC) mode.

4.6.3.1 TIC Chromatograms

High concentration (10 µg ml^{-1}) standards (in DCM or HEX) of each analyte were

autoinjected (2 µl) in splitless mode at 265°C onto a 30 m Econo-Cap™ EC-5 (5% phenyl, 95%

dimethylpolysiloxane) capillary column (0.25 mm id, 0.25 µm film; Alltech, Deerfield, IL)

connected in Agilent 6890N GC (Agilent, Palo Alto, CA). Ultrapure helium (Air Liquide,

Houston, TX) was used as a carrier gas with an initial flow of 1.2 ml min^{-1} and with an average

velocity of 38 cm sec^{-1}. The instrument was operated under constant pressure mode. The generic

SCAN method had the following temperature program for GC oven: initially at 40°C for 3 min,

increased to 150°C at 15°C min^{-1} with no hold time followed by final ramp to 300°C at 6°C

min^{-1} and held at this temperature for 10 min. The total run time in the GC was 45.3 min. Column effluent was monitored with a mass selective detector, MSD 5973 (Agilent) that was operated to scan from 40-550 amu. The temperatures of transfer line, MS quadrupole and source were set at 280, 150 and 230°C respectively. The resultant TIC chromatograms of analytes and their corresponding ISTDs (Figs A1-A3) were used to obtain the mass spectra of each compound. The mass spectra were compared to those available in the standard mass spectral libraries (National Institute of Technology, NIST and Wiley). The MS data of each analyte was then used to select the best ions for identification and quantification of test compounds in selected ion monitoring (SIM). The chemical-specific instrumental analytical methods (GC/MS or LC/MS) are detailed in the respective chapters.

4.7   BCF Estimation

Tissue-specific proportional BCFs (BCF_ps) were estimated from the ratio of chemical concentration in tissue to the time-weighted average measured exposure concentration.

$$BCF_p \text{ (l kg}^{-1} \text{ wet wt.)} = [\text{chemical}]_{\text{tissue}} \div [\text{chemical}]_{\text{water}}$$

BCF_ps were also expressed as lipid normalized values. Lipid normalized BCFs were estimated only for the catfish tissues due to the availability of larger and more uniform tissue masses.

$$BCF_p \text{ (l kg}^{-1} \text{ lipid wt.)} = [\text{chemical}]_{\text{lipid norm tissue}} \div [\text{chemical}]_{\text{water}}$$

Kinetic BCFs (BCF_ks) were calculated from uptake (k_1) and depuration (k_2) rate constants determined from the plots of tissue concentration of a chemical at different time points during exposure (uptake) and depuration. Assuming the first order kinetics, k_1 and k_2 were calculated by kinetic parameter calculator (KPC) method (courtesy of TA Springer; Appendix

C), a sequential method that combines linear and non-linear regression models (Newman, 1995).

Estimations of k_1 and k_2 are based on the model:

$$C_f = C_w. (k_1/k_2). (1- e^{k2-t})$$

where C_f and C_w represent the chemical concentrations in fish and water respectively (OECD, 1996). Depuration rate constant, k_2 is first calculated from the slope of the depuration plot using the simple linear curve fit model: $\ln C_f = a (t) + b$. Uptake rate constant (k_1) is then calculated by substituting k_2 in the equation above. More frequently, depuration rate constant, k_2 is used to predict the time required for 50 % reduction in the concentration of a chemical in the organism using the following equation:

$$t_{1/2} = \ln (2) \div k_2 = 0.6931 / k_2$$

Using this approach, the time required for 50% reduction of the body burden was predicted, providing important information on how fast the chemical gets cleared from tissues.

4.7.1 Predicted BCFs

Tissue BCFs (1 kg^{-1} wet wt.) were estimated from US EPA's BCFWIN program (part of estimation program interface suite, EPISUITE). Plasma bioconcentration levels were predicted using the equation:

$$\text{Log P}_{b:w} = 0.73 \text{ x log K}_{ow} – 0.88 \text{ (Fitzsimmons et al., 2001)}$$

$P_{b:w}$ is the blood to water partition coefficient or plasma BCF. BCFs derived from the models were compared (refer to Data Analysis section) to the values obtained from the experiments.

4.7.2 Plasma Effect Ratio (ER)

Effect Ratios (ERs) for each pharmaceutical were calculated using the following equation:

$$ER = H_TPC \div F_{ss}PC$$

where H_TPC is the human therapeutic plasma concentration obtained from the maximum plasma

concentration(C_{max}) data (http://www.rxlist.com/script/main/hp.asp) while $F_{ss}PC$ is the

predicted plasma concentration, $F_{ss}PC = P_{b:w}$ x EC. Pb:w were computed using the

Fitzsimmon's equation. The reported median environmental concentrations of the

pharmaceuticals were used for ECs. The degree to which a chemical induces pharmacological

effect on fish was predicted from the ER values using certain criteria (Huggett et al., 2004).

4.7.3 Comparison of 28 and 7-day BCFs

Measured 28 day- BCFs (fathead minnow) for each test chemical were compared to those

of 7-day BCFs (catfish). This comparison provided an opportunity for examining the species

differences (fathead and catfish) in accumulating different test compounds. More importantly,

this comparison was used to test the usefulness of the reduced BCF test design (7 day).

4.8 Quality Control

4.8.1 Calibration Curves

For each of the five analytes (norethindrone, ibuprofen, verapamil, clozapine and

fenofibrate), an eight-point calibration curve (1000-8 µg l^{-1}) was generated with linear curve fit

between response ratio and the concentration. Coefficient of determination (r^2) was estimated for

each and curves with $r^2 \geq 0.9$ only were used for quantification.   In all the sample analyses, a

continuing calibration (CONCAL) standard was run after every 10 samples in a sequence. New

calibration standards were prepared if the response of a CONCAL was not within ±15%

acceptable range of the concentration based on the original standard curve. In GC/MS analyses,

poor response of the CONCAL standard warranted cleaning the injector liner that included

changing the septum and inlet sleeve. The column was also cut (ca. 5 cm) and baked for 10 min

at 300° C. Finally the instrument was auto tuned for the mass axis calibration for

perfluorotributylamine (PFTBA) peaks m/z 69, 219 and 502. The frequency of this GC/MS

maintenance depended on sample load as well as the expected absolute responses for the

standards.

4.8.2   Method Detection Limits

Method detection limits (MDLs) for each analyte were determined following the US EPA

guidelines. Briefly, seven replicates of the control fathead minnow muscle tissue (referred to as

"clean tissue") spiked with the lowest concentration in the calibration or at the level that was

readily differentiated from the background measurements of the clean tissue were run and the

MDL was calculated using the formula: MDL = 3.14 x SD, where SD is the standard deviation

of seven replicate measurements and 3.14 is the student's t-value at 99% confidence level.

4.8.3   Accuracy and Precision

As part of the QC approach, every sample batch included method blanks, method blank

spikes, control (unexposed) tissues and tissue spikes. For each of the test compounds, water,

plasma and tissues (n= 2-10) from the control group were fortified with native compounds at

concentrations that represented the middle concentration for each of the corresponding

calibration curves. The accuracy of the method was estimated in terms of percent recoveries

calculated by comparing the measured concentrations with the nominal spike concentrations.

The precision of the analytical method was estimated in terms of relative standard deviation

(RSD). The results from CONCAL standard runs were used to estimate the percent instrument accuracy as well as the precision of the performance of the instrument.

4.9   Data Analysis

Data analyses were conducted using SAS® (version 9.1, SAS Institute Inc., Cary, NC). In all statistical tests, a probability (P) value less than 0.05 was considered significant. Data (if sample size, n ≥ 5) were initially subjected to Shapiro-Wilk normality testing. If data were not normal or sample size was less than 5, non-parametric statistics were applied. Condition factor between control and exposed fish was analyzed with an independent t-test (or Mann Whitney U test) to examine the difference between the two groups. One-way ANOVA (or Kruskal-Wallis test) was conducted on tissue indices (liver, kidney, heart and brain) to determine differences in these metrics between the control and exposed fish. Differences (if any) in tissue-specific uptake for each chemical were also analyzed using One-way ANOVA. Significant results ($P \leq 0.05$) were subjected to Dunnett's and/or Student Newman Keul (SNK) post hoc multiple comparison tests. Data that were not amenable to statistical analysis (for example, comparison of predicted and measured BCFs, n=1) were plotted for a gross numerical comparison of the results. All other data (measured concentrations and percent recoveries) were expressed as means ± SD.

CHAPTER 5

BIOCONCENTRATION OF NORETHINDRONE IN FISH

5.1   Abstract

The environmental presence of an oral contraceptive, norethindrone (NET) has been reported and shown to have reproductive effects in fish at environmentally realistic exposure levels. The current study examined bioconcentration potential of NET in two freshwater fishes, fathead minnow (*Pimephales promelas*) and channel catfish (*Ictalurus punctatus*). Fathead minnow were exposed to 50 µg l^{-1} for 28 days and allowed to depurate in clean water for 14 days. In a reduced 14-day test design, catfish were exposed to 100 µg l^{-1} for 7 days followed by depuration for 7 days. In the fathead test, tissues (muscle, liver and kidneys) were sampled during the uptake (days 1, 3, 7, 14 and 28) and depuration (days 35 and 42) phases. Similarly, during the uptake (days 1, 3, and 7) and depuration stages (days 10 and 14) in the catfish test, muscle, liver, gill, brain and plasma were collected. NET exposure resulted in a significant (P=0.01) increase in the hepatic somatic indices (HSIs) of the catfish while higher but non-significant liver weights were noticed for the fathead minnows. NET tissue uptake levels were determined by gas chromatography/mass spectrometry (GC/MS). The accumulation levels had the following order: liver > plasma > gill > brain > muscle. The tissue-specific BCFs ranged between 2.6 and 40.8, suggesting a low bioconcentration potential of NET in the two fish species. The BCF levels are also of little concern in view of the current regulatory guidelines (BCF ≥ 2000). Nevertheless, NET may still need chronic risk assessments due to its reported reproductive effects in fish.

5.2   Introduction

Steroidal sex hormones (estrogens, progestins and androgens) have been detected in the effluents from sewage treatment plants (STPs) and surface waters, often at low ng l^{-1} range (Desbrow et al., 1998; Ternes et al., 1999; Labadie and Budzinski, 2005). Environmental presence, though at low levels, of these compounds is of great concern due to their endocrine disruptive effects on the non-target organisms (Purdom et al., 1994; Sumpter and Jobling, 1995; Kidd et al., 2007). For example, 17-α-ethinylestradiol (EE2) exposure at 100 pg l^{-1} in male rainbow trout induced the synthesis of vitellogenin, an egg yolk precursor protein that normally occurs in female fish (Purdom et al., 1994). In addition to this well-documented biomarker of estrogen exposure in wild fish (Tyler et al., 1996), other reproductive effects such as changes in sexual maturation, reduction in sperm counts, intersexual condition or altered sex ratios have been reported in fish exposed to estrogenic chemicals (Jobling et al., 1998; Tyler et al., 1998). Due to high sorption efficiencies (log P ≥ 3.0), most steroid hormones have a tendency to partition to sludge and sediments and hence are predicted to bioaccumulate in the aquatic and terrestrial species (Ternes et al., 2002; Kuster et al., 2004; Markman et al., 2007).

Few studies have reported bioaccumulative potential of estrogenic compounds by algae and aquatic invertebrates (Lai et al., 2002; Gomes et al., 2004; Dussault et al., 2009). However, the potential bioaccumulation of sex hormones in aquatic vertebrates and the resultant altered sexual functions, is more alarming because of greater homology of steroid receptors between fish and mammals (Gunnarsson et al., 2008; Al-Ansari et al., 2010). For example, estrone, 17 β-estradiol and EE2 were reported to concentrate in the bile of rainbow trout exposed to an STP effluent. The accumulation levels were at least 4-6 orders of magnitude higher than the water concentrations (Larsson et al., 1999). In another study, fathead minnows exposed (158-245 days)

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to EE2 at environmentally relevant concentrations (12 and 47 ng l^{-1}) had whole-body BCFs of

660 and 610 respectively (Länge et al., 2001). Bioconcentration of androgens in fish has also

been previously reported. Testosterone exposure at a nominal concentration of 1 µg l^{-1} for 6 days

in three-spined stickleback resulted in the plasma BCF of 200 (Maunder et al., 2007). Although

studies exist to confirm the bioaccumulation of estrogens and androgens in fish, current

understanding on the potential toxicological effects including the bioaccumulative potential of

progesterone and/or its synthetic analogues in the aquatic environment is limited (Besse and

Garric, 2009).

Norethindrone (19 nor-17-alpha-ethinyltestosterone) is a synthetic progestational

hormone widely used as an oral contraceptive and for treating endometriosis, various types of

menstrual disorders, pre/post-menopausal syndromes, and breast and ovarian cancers. Although

the therapeutic doses of NET for contraceptive use are in "µg" to low "mg" range, higher

dosages are prescribed for the treating the conditions listed above. NET is a potent inhibitor of

ovulation which exerts its effects by binding to the progesterone receptor in the target cells (e.g.

reproductive) leading to altered responses in gonadotropin releasing hormone (GnRH) from

hypothalamus. In addition, NET has weak androgenic and estrogenic properties. The usage of

NET has been extensive due to its availability as a non-prescription drug. For example, in 2002,

34 products containing NET were approved in Sweden and around 30 million drugs (containing

this ingredient) sold in the same year. Based on these sales volumes, NET was prioritized as

"dangerous for the environment (R 51/53)" (Carlsson et al., 2006).

The physicochemical properties of NET (Table 5) indicate its potential to partition into

organic rich fractions. Previous studies have reported accumulation of NET in the river

sediments (Alda et al., 2002). Synthetic hormones including NET are designed to resist structural

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changes in order to enhance their persistence in the body. Once excreted, this pharmacologically desirable drug design becomes deleterious to the environment, as they resist natural degradation processes. Although NET undergoes considerable metabolism in mammals (only about 5% is excreted unchanged), the excreted glucuronidated products could be converted back (through action of β-glucuronidase or aromatase) to active hormonal substances by microorganisms (Labadie and Budzinski, 2005; Carlsson et al., 2006). These findings underscore the need for understanding the bioaccumulative potential of NET in aquatic organisms, fish in particular due to the highly conserved progesterone receptors (Huggett et al., 2004).

A recent study from our laboratory indicated complete shutdown of egg production in fathead minnow and Japanese medaka exposed to NET at environmentally relevant concentrations (Paulos et al., 2010). Occurrence of reproductive or other chronic effects in aquatic species are frequently attributed to an organism's ability to accumulate xenobiotics. This is because bioaccumulation over a period of time results in the critical tissue concentrations that may trigger certain toxicological responses. Therefore, the current study is aimed at determining the tissue-specific uptake and bioconcentration of NET in two fresh water fishes, fathead minnow and channel catfish. The studies on bioconcentration of pharmaceuticals are significant, in view of the regulatory importance of assessing bioconcentration potential in prioritizing pharmaceuticals for environmental risk assessments (ERAs).

5.3   Experimental

5.3.1   Chemicals and Reagents

Norethindrone (NET, (17α)-17-hydroxy-19-norpreg-4-en-20-yn-3-one, CAS#68-22-4), norethindrone-d6 (NET-d6, 4-estren-17α-ethynyl-17β-ol-3-one-d6) were obtained from Toronto

Research Chemicals (TRC; North York, ON, Canada). HPLC grade solvents acetonitrile, methanol, n-hexane, ethyl acetate, dichloromethane, dimethyl formamide and acetone were procured from Fisher Scientific (Houston, TX). N-methyl-N-(trimethyhlsilyl) trilfluoroacetamide (MSTFA, CAS# 24589-74-4) derivatization reagent was purchased from Thermo Scientific (Rockford, IL). Tricaine mesylate (MS-222, ethyl 3-aminobenzoate methanesulfonic acid, CAS# 886-86-2) was obtained from Sigma-Aldrich (St. Louis, MO). Milli-Q water (18.3 M Ω. cm) was obtained in the laboratory using Milli-Q Water System (Millipore, Billerica, MA).

5.3.2   Fish Exposures

After a week's acclimation to the standard laboratory conditions of $25 \pm 2°$ C and 16:8 hr. light: dark cycle, adult fathead minnow (source: UNT aquatic toxicology lab) and juvenile channel catfish (source: Pond King Inc., Gainesville, TX) of either sex were exposed to NET using the following experimental designs. In the fathead minnow test, fish (n=50) were randomly distributed into two tanks (20 lit) and exposed to 50 µg NET l^{-1} l (in dimethyl formamide, DMF < 0.003% in exposure tanks) for 28 days in a continuous flow-through system. The fish were then allowed to depurate in clean water for 14 days. For the catfish study, 20 fish were randomly distributed into two 60 l glass tanks and exposed to 100 µg NET l^{-1} under similar flow-through conditions. This test consisted of 7 days of exposure and a one week depuration in dilution water. To account for possible effects carrier solvent (DMF), a solvent control (n=10) was included in both the tests. Fish were fed (brine shrimp flakes or trout chow) to satiation twice daily during the course of the experiments.

5.3.3 Sample Collection

For the fathead study, at the end of days 1, 3, 7, 14, 28, 35 and 42, fish (n=4-6) were

anaesthetized in a solution of MS-222 (100 mg l^{-1}) to record the wet weight and length. Muscle,

liver and kidney tissues were excised to record weights (liver and kidney) and later frozen for

analysis. In the catfish test, fish (n=4) were sampled after days 1, 3, 7 and 14, anaesthetized to

collect blood and tissues (muscle, brain, gill, and liver). Blood was collected from caudal vein

using heparinized syringes into test tubes with heparin and plasma was later separated by

centrifugation (12,000 x g). Liver and brain weights were recorded and all the collected tissues

were stored at - 20° C for further processing. Water samples (n=3-6) in the exposure tanks were

collected during the uptake phases of both the tests to determine the measured exposure

concentration.

5.3.4 NET Extraction and Cleanup

All extraction procedures on the test samples were preceded by the addition of 5 ng of d^6-

NET. NET was extracted from water samples (1-2 ml) following liquid/liquid extraction with

1+1 (v/v) hexane/ethyl acetate (ca. 5 ml), hitherto referred to as the "sample solvent." Plasma

samples (~ 200 µl) were treated with 5 ml ice-cold acetone, evaporated and back extracted into

the sample solvent. The extracted contents from water and plasma samples were dried under

nitrogen for further processing. Approximately 0.1-0.2 g of blot-dried tissue was homogenized in

4 ml sample solvent using a Mini-Beadbeater™ (Biospec products, Bartlesville, OK). The

extracted contents were filtered into pre-weighed vials using 0.45 µm polytetrafluoroethylene

(PTFE) filters (Whatman, Sanford, ME). The tissue extracts needed a cleanup method. Briefly,

organic extracts were dried and lipid weights were determined gravimetrically. The dried

residues were resolubilized in acetonitrile (1 ml) and stored overnight at 4˚C followed by centrifugation and separation of the supernatant. The resultant "clean" extract was later evaporated to dryness.

5.3.5   GC/MS Analysis

Dried organic extracts were subjected derivatization with MSTFA following the methods described elsewhere (Shareef et al., 2006). The derivatized residues were resolubilized in 100 µl DCM and analyzed using GC/MS. An Agilent GC 6890N (Agilent Technologies, Palo Alto, CA) connected to a mass selective detector (MSD 5973, Agilent) was used for quantification. Briefly, samples were auto-injected (2 µl) in pulsed splitless mode at 260° C onto a 30 m x 0.25 mm x 0.25 µm EC™ - 5 capillary column (Alltech, Deerfiled, IL). Ultrapure helium served as a carrier gas and the separation was achieved in the column using the following temperature program for the GC oven: initially at 40°C for 3 min, increased to 200°C at 15°C min^{-1} with no hold time followed by final ramp to 300°C at 15°C min^{-1} and held at this temperature for 10 min, with a total run time of 36.17 min. The MS quadrupole, source and transfer line temperatures were set at 150, 230 and 280°C respectively. MSD was operated under selected ion monitoring (SIM) with dwell time of 50 m sec for the following ions: NET-d^6-TMS: 309, 361 and 376; NET-TMS: 303, 355, and 370 (underlined ions used in quantification) (Fig A4). NET quantification was achieved using an eight-point calibration curve (4000-31 pg µ l^{-1}) (Fig A9).

5.3.6   Condition Factor and Somatic Indices

Fish weight (g) and caudal length (cm) were used to calculate Fulton's condition factor (K= weight/ (length) ^3 x 100). Liver, kidney and brain weights were used in determining the

corresponding somatic indices (tissue weights expressed as % body weights) viz., hepatic somatic index (HSI) and nephritic somatic index (NSI) and brain somatic index (BSI) respectively.

5.3.7   BCF Estimation

Tissue-specific BCFs were estimated using two approaches: a) A proportional BCF (BCF_p) calculated as the ratio between the mean concentration of NET in a tissue and the time-weighted measured exposure concentration and b) kinetic BCF (BCF_k) determined as the ratio between uptake and depuration rate constants. Assuming the first order kinetics, the uptake (k_1) and depuration (k_2) rate constants were determined following the methods described by Newman (1995).

5.3.8   Data Analysis

Data analysis was performed using SAS® (version 9.1, SAS Institute Inc, Cary, NC). In all the statistical tests, a *P* value less than 0.05 was considered significant. The differences in the condition factor and somatic indices of the control and NET exposed fish were determined using t-test and Kruskal-Wallis ANOVA respectively. One-way ANOVA followed by SNK multiple range testing revealed the differences in the tissue uptake levels in the catfish. All the results were expressed as means ± SEM (for measured concentration and QC results, SD was used to indicate the variation).

5.4   Results

5.4.1   Water Quality and Measured Test Concentrations

Weekly measured water quality parameters (mean ± SD, n=5) temperature, pH and dissolved oxygen (DO) in exposure tanks were 21.3 ± 0.6, 7.4 ± 0.3 and 7.9 ± 0.7 respectively. The data indicate fairly constant levels of oxygen saturation and pH in the continuous flow-through exposure system. During the 28-day exposure, the mean (±SD) time-weighted measured NET water concentration ($\mu g\ l^{-1}$) was 35.4 (± 8.78, n=7) which is about 71% nominal exposure level, 50.0 $\mu g\ l^{-1}$. In the catfish test, the average measured exposure concentration over the 7-day period was about 81% of the nominal exposure level (82.5 ± 8.5 (n=4)). NET was not detected ($< 8\ \mu g\ l^{-1}$) in the solvent control.

5.4.2   Condition Factor and Tissue Somatic Indices

The mean condition factors of the NET exposed fathead minnow and catfish were not significantly different (*P*=0.45 and 0.16) from that of the control fish (Figs 3 and 7), indicating the absence of chemical stress in the exposed fish. In addition, the mean K values (> 1.0) for the control and exposed fish suggest that the fish were well nourished. Additional information on the physiological status of the fishes was obtained from tissue somatic indices. For the fathead minnows, relative liver (except for fish sampled at day 1) and kidney weights of NET exposed fish were larger than those of the control fish (Figs 4 and 5). The differences, however, were not statistically significant (*P*=0.32 and 0.06). Brain somatic indices (BSIs) of catfish in the control and exposed groups (both uptake and depuration) did not differ significantly (*P*= 0.18) (Fig 8). However, significantly higher (*P*=0.01) liver weights were observed in the NET exposed catfish

compared to those measured for the control fish (Fig 9). Interestingly, the relative liver weights of the depurated fish reverted to the weights comparable to those of the control fish.

5.4.3   Tissue-Specific NET Concentrations and BCFs

5.4.3.1 Fathead Minnows

NET concentration (ng g^{-1} wet wt.) in the muscle, liver and kidney tissues of fathead minnow sampled during exposure and depuration are presented in Fig 6. The uptake levels by the muscle, liver and kidneys ranged from 47-167, 75-411 and 576-1445 ng g^{-1} wet wt respectively. In the muscle tissue, except for the initial high concentration at day 1, NET accumulation steadily increased during the exposure. Similar trend was noticed for the concentrations in the liver tissue, but with a reduction in the concentration at 28 days post exposure. NET accumulation in the kidney, however, decreased during the course of exposure. Overall, the accumulation levels in various tissues had the following trend: muscle < liver < kidney. There were no detectable levels (< 25 ng g^{-1}) of NET in the tissues of fish from the solvent control as well as those from the depurated fish.

Tissue-specific NET kinetic and 28-day proportional BCFs for fathead minnow ranged from 2.9-26.8 (Table 6). In accordance with the tissue uptake trend, kidney and muscle tissues had the highest and lowest bioconcentration respectively, while the BCFs for the liver tissue were intermediate. The measured BCFs were well below (< 2%) the regulatory trigger value (BCF=2000), suggesting a very less bioconcentration potential of NET.

5.4.3.2 Channel Catfish

NET concentration (ng g^{-1} wet wt) in the muscle, brain, gill, liver and plasma (µg l^{-1}) of

catfish are presented in Fig 10. The accumulation in the muscle tissue decreased from days 1

through 7 with about 47% reduction in the uptake level. In all the other tissues, there was an

increase in the concentration with the highest accumulation noticed at day 3 post-exposure.

While the increase in NET concentration from day1 through 3 was approximately 4X for the

liver tissue, the corresponding increases were smaller (ca. 1.5X) for the plasma, gill and brain

tissues.

The 7-day NET uptake levels were significantly different (P=0.01) among tissues (Fig

11). SNK post-hoc test indicated that the accumulation levels in the liver, gill and plasma did

not differ significantly, but were significantly higher than those for the muscle and brain tissues.

NET was not detected ($<$ 25 ng g^{-1}) in the tissues of the control and depurated fish.

5.4.4   BCFs

Tissue-specific kinetic and 7-day proportional NET BCFs for the catfish are presented in

Table 7. The BCFs (wet wt.) ranged from 4.5 and 40 with the following trend: liver > plasma >

gill > brain > muscle. The 7day- lipid normalized BCFs were approximately an order of

magnitude higher than the corresponding wet wt BCFs. Results from the BCF estimates

indicated that NET has less tendency (i.e. BCF<2000) to bioconcentrate in catfish.

5.4.5   QC Results

The accuracy of the extraction methods was expressed in terms of the percent recoveries

obtained by spiking a known concentration of NET (500 µg l^{-1}) to the dilution water, method

blank (extraction solvent), control fish plasma and tissues (Fig A14) and the results are presented

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in Table 8. The precision (% RSD) of the instrument was estimated at 2.1% (n=11) using a continuing calibration standard (CONCAL).

5.5 Discussion

To the best of our knowledge, this is the first report on the experimentally determined bioconcentration of NET in fish. Therefore, the results are compared/ contrasted with the available data on the estrogenic/ (anti)-androgenic compounds. We think this is important as most progestins interact with estrogen and androgen receptors, in addition to their primary biological targets, progesterone receptors (PRs). Also, synthetic progestins including NET have estrogenic and/or (anti)-androgenic effects (Sitruk-Ware, 2008). NET effects of on the fish condition factor and somatic indices obtained from this study are compared to the existing data on fish and/or mammals. The significance of the bioconcentration results is discussed in the context of current regulatory guidelines for ERA of pharmaceuticals. Finally, the potential usefulness of QSAR based models in predicting the BCFs of pharmaceuticals in fish is also included in the discussion.

5.5.1 Condition Factor and Somatic Indices

NET exposure did not affect the overall well-being of the fish compared to the unexposed group, suggesting that the exposure levels used in this study are not overtly toxic for the two fish species. This may not be surprising, as the levels are about 10% of the no observed effect concentrations (NOECs, survival) obtained from the early life stage (ELS) experiments conducted on the fathead minnow larvae (data not shown). The 7-day ELS chronic tests are sensitive because the fish larval stage is considered the most vulnerable to the toxic insults.

Therefore, the exposure concentrations used in this study may be considered "conservative" in terms of effects on gross parameters, such as fish shape.

In our study, NET exposure resulted in enlargement of livers in the catfish and discontinuation of exposure for a week resulted in "normal" liver weights as compared to those in the control group. Enlargement livers due to NET exposure may be explained by two possible interconnected responses in the fish. First, liver hypertrophy is a natural response to a chemical exposure and is an indication of increased hepatic metabolic activity. The second and probably more relevant explanation could be that NET exposure has induced vitellogenin synthesis. Although no previous studies exist to support the latter plausibility, increased liver weighs have previously been reported in the fish exposed to estrogenic compounds (Allen et al., 1999; Al-Ansari et al., 2010). NET has also estrogenic effects, and hence is likely to cause elevated HSIs. In a study (not included in this dissertation), adult rainbow trout exposed to $100 \, \mu g \, l^{-1}$ NET for 7 days had decreased gonadal somatic index (GSI) and increased HSI (Fig B1 and B2). NET has also shown to induce strong androgenic effects in fish (Paulos et al., 2010). Although it is clear from these studies that NET has reproductive effects in fish, it is not quite obvious whether NET's effects are manifested by its progestogenic, estrogenic or androgenic characteristics. Nevertheless, the increased fish HSIs in our study has further bolstered the previous findings by Paulos et al., (2010) on NET's reproductive effects. Two other indices, NSI (fathead study) and BSI (catfish test) were not affected by the NET exposures. The increase in the kidney weights (though non-significant) in the NET exposed fathead minnow could be due to the possible nephritic hypertrophy. Similar effects were previously reported in mammals exposed to xenoestrogens (Son et al., 2000). Reproductive effects of steroidal hormones are modulated by the brain hypothalamus-pituitary-gonad (HPG) chain in mammals and this mechanism is also

conserved in aquatic vertebrates. In mammals, testosterone's exposure was shown to result in decreased brain weights (Whaling et al., 1990). Since NET has been shown to possess potent androgenic effects in fish (Paulos et al., 2010), we hypothesized that it influences the brain weights of the fish. Although not significant, exposed catfish (after one day exposure) had smaller brain weights.

5.5.2   NET Accumulation in Tissues and BCFs

Overall, NET uptake by various tissues had the following rank order: (kidney > liver) > plasma > gill > brain > muscle. High accumulation in the kidneys suggests that renal excretion could be a major elimination pathway. A similar trend in tissue uptake was noticed in a study when rainbow trout exposed to100 µg l^{-1} NET for 7 days (Fig B3). Mammalian pharmacology of NET indicates that it is subjected to the first-pass metabolism, about 61% bound to albumin and after extensive biotransformation more than 50 % is eliminated via urine (http://www.rxlist.com) and the reminder through biliary excretion. Taking clues from this information, higher accumulation of NET is expected in the tissues with greater perfusion rates. This may explain the lowest accumulation in the muscle tissue. However, in addition to the perfusion rates, tissue-specific uptake of xenobiotics depends on the relative diffusion and/or affinity to the tissue components such as proteins. NET was also detected in the gills indicating that this could be an important site for the uptake and clearance. Although NET was not determined in the fish bile in our study, such analysis could be useful in predicting the magnitude of exposure (in terms of bioaccumulation) in wild animals (Larsson et al., 1999; Pettersson et al., 2006) as well as in metabolite profiling (Kallio et al., 2009).

The tissue-specific wet weight BCFs ranged between 2.6 and 40.8, suggesting that NET has a very less tendency to bioconcentrate in the two fishes. Also, the BCF levels are well below the current regulatory trigger value of 2000 used in pharmaceutical prioritization approaches (EMEA, 2006). On the other hand, Environment Canada (EC) has no separate ERAs for pharmaceuticals and hence the medicinal products are also subjected persistent (P), bioaccumulative (B) and toxic (T) assessments. Under U.S. EPA's PBT policy, a compound with BCF value between 100-1000 is of "medium concern" in terms of its environmental effects. Based on the aforementioned regulatory guidelines, no assessments are necessary for NET. However, it should be noted that the BCF criteria in prioritization schemes apply only when other potential concerns like endocrine disruption, genotoxicity etc. are absent. NET has recently been shown to induce reproductive effects in fish even at concentrations as low as 25 ng l^{-1} and hence may require complete ERA assessments.

It is important to emphasize the need for tissue-specific BCF assessments here. For example, in our study, the highest (liver) and lowest (white muscle) NET wet weight BCFs differ by an order of magnitude. Although this difference is not large in regulatory view point, fish tissue BCFs have been reported to differ by several orders of magnitude (Schwaiger et al., 2004). In the absence of potential concerns like endocrine disruption, bioconcentration becomes an important regulatory end point in prioritizing pharmaceuticals for ERAs. Most bioconcentration studies focus on the plasma concentrations or uptake by white muscle. There are few reports on the whole fish BCF assessments in which the authors suggested the need for conducting tissue-specific BCFs (Paterson and Metcalfe, 2008). Therefore, it is essential to generate tissue-specific BCF data, at least on few selected compounds. It may not be necessary to analyze every single

tissue, but the selection could be based on the available mammalian drug target information. This ensures the selection of optimum BCFs in screening approaches.

In summary, NET has very less potential to bioaccumulate in fish. However, NET exposure results in the potential reproductive effects and hence further assessments on chronic risks on non-target species are desirable. Tissue-specific BCF assessments are useful in order to correctly prioritize pharmaceuticals for chronic risk assessments.

5.6   Chapter References

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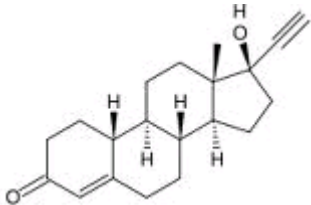
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Table 5

Physicochemical characteristics of norethindrone (retrieved from EPISUITE (U.S. EPA, 2009

Property	Description
CAS #	68-22-4
Molecular weight	298.43
Partition Coefficient, log K_{ow}	2.97
log D_{ow} (Log P @ pH 7.0)	3.15£
Water solubility @ 25° C, mg l^{-1}	7.04
Vapor pressure @ 25° C, mm Hg	7.31E-09
Henry's Law Constant @ 25°C, atm-m3 mol-1	5.8E-10
Estimated half-lives (hrs) : water	1.44E+3
soil	2.88E+3
sediment	1.3E+4
Environmental persistence# (hr)	2.1E+3
Structure	

£ www.cerep.com; # using emission rates of 1000 kg/hr

Table 6

Tissue-specific kinetic and proportional norethindrone BCFs for fathead minnow exposed to 50 $\mu g \, l^{-1}$.

Tissue	BCF_k	28 d BCF
Muscle	2.6	4.7
Liver	9.3	7.4
Kidney	26.8	16.3

Table 7

Tissue-specific kinetic and 7-day proportional norethindrone BCFs for channel catfish exposed to 100 $\mu g \, l^{-1}$.

Tissue	BCFk	7 d BCF	
		Wet wt. basis	Lipid normalized
Muscle	7.1	4.5	186
Brain	7.4	4.9	39.5
Gill	11.1	7.5	73.9
Plasma	13.4	10.6	--
Liver	40.8	24.5	251.7

Table 8

Norethindrone percent recovery (mean ± SD) for different sample matrices.

Test	Matrix	n	% Recovery
Fathead minnow	Water	1	98.7
	Tissue#	6	115 ± 21
Channel catfish	Method blank*	2	112 ± 13.4
	Plasma	3	112 ± 24
	Tissue	11	120 ± 14.4

* sample solvent; #muscle tissue from control fish
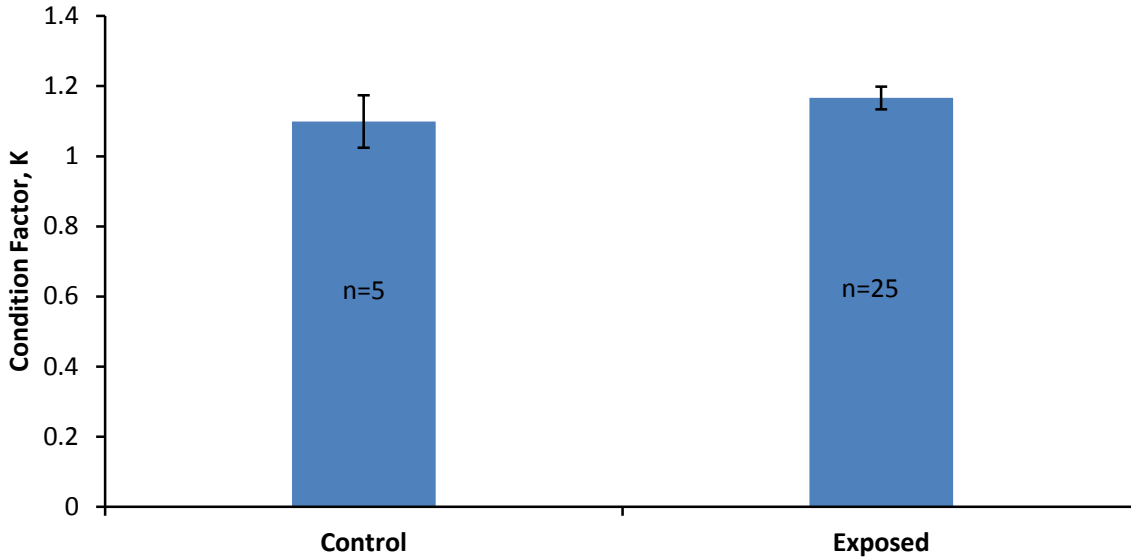
Fig. 3. Condition factor, K (mean ± SEM) of control and norethindrone exposed (50 µg l^{-1}) fathead minnow. Mean K value of exposed fish was not significantly different (P=0.45, independent t-test) from that of the control fish.
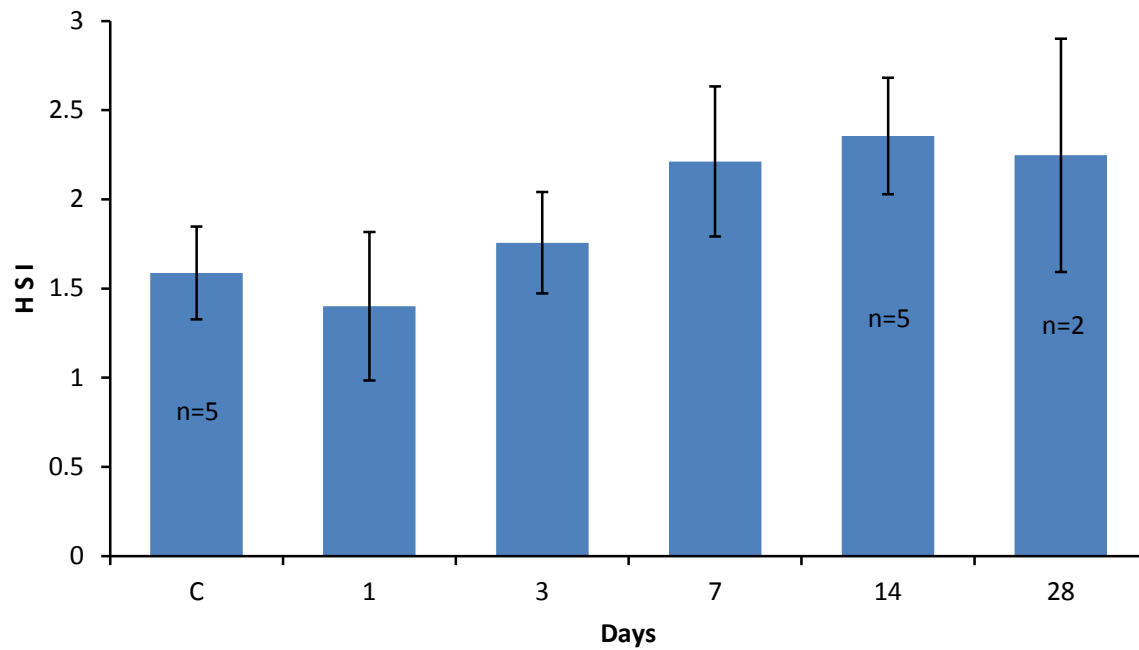
Fig. 4. Hepatic somatic index, HSI (mean ± SEM, n=6) of control and norethidnrone exposed (50 µg l^{-1}) fathead minnow. Mean HSIs did not differ significantly (P=0.32, Kruskal-Wallis) between the control and exposed fish.
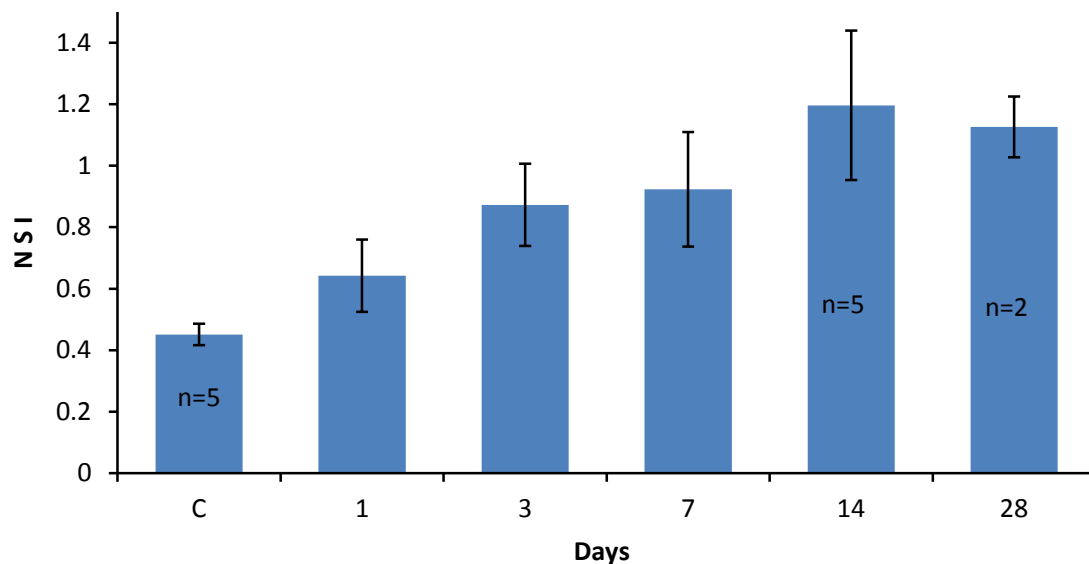
Fig. 5. Nephritic somatic index, NSI (mean ± SEM, n=6) of control and norethidnrone exposed (50 µg l^{-1}) fathead minnow. Mean NSIs did not differ significantly (*P*=0.06, Kruskal-Wallis test) between the control and exposed fish.
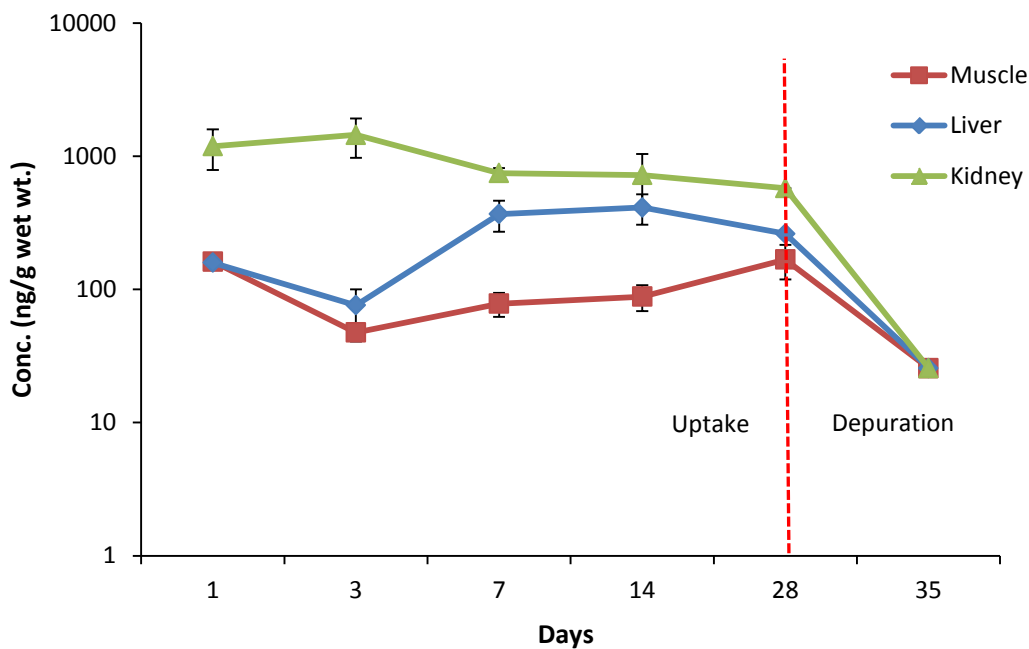
Fig. 6. Norethindrone (NET) concentration (mean ± SEM, n=1-5) in muscle, liver and kidney tissues of fathead minnow exposed to 50 µg l^{-1}. NET concentration was below the lower limit of calibration, LLC (25 µg l^{-1}) in the tissues of depurated fish.
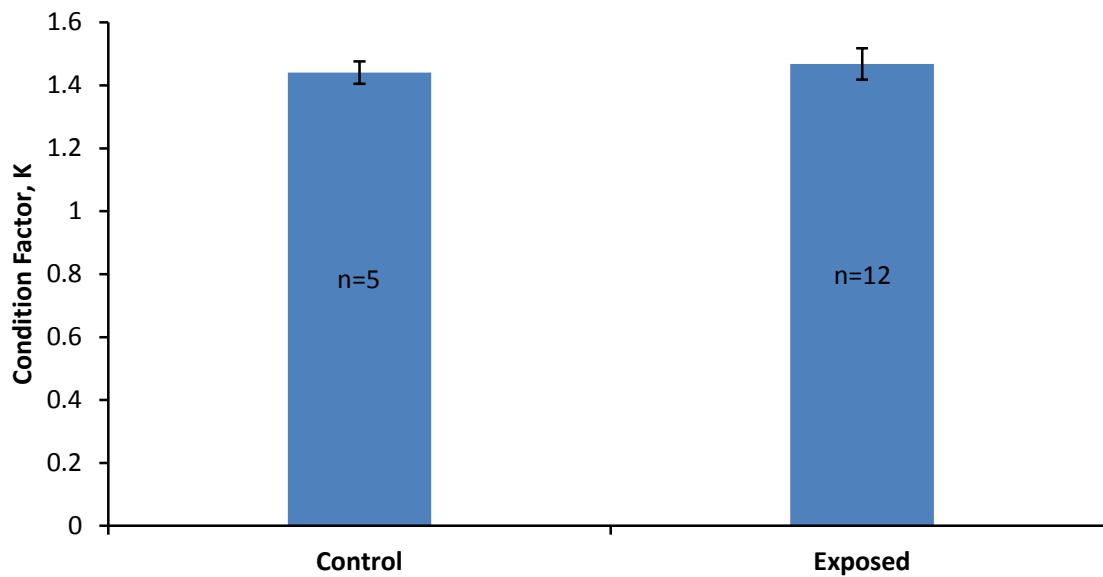
Fig. 7. Condition factor, K (mean ± SEM) of control and norethindrone exposed (100 µg l^{-1}) channel catfish. Mean K did not differ significantly (*P*=0.16, independent t-test) between the groups.
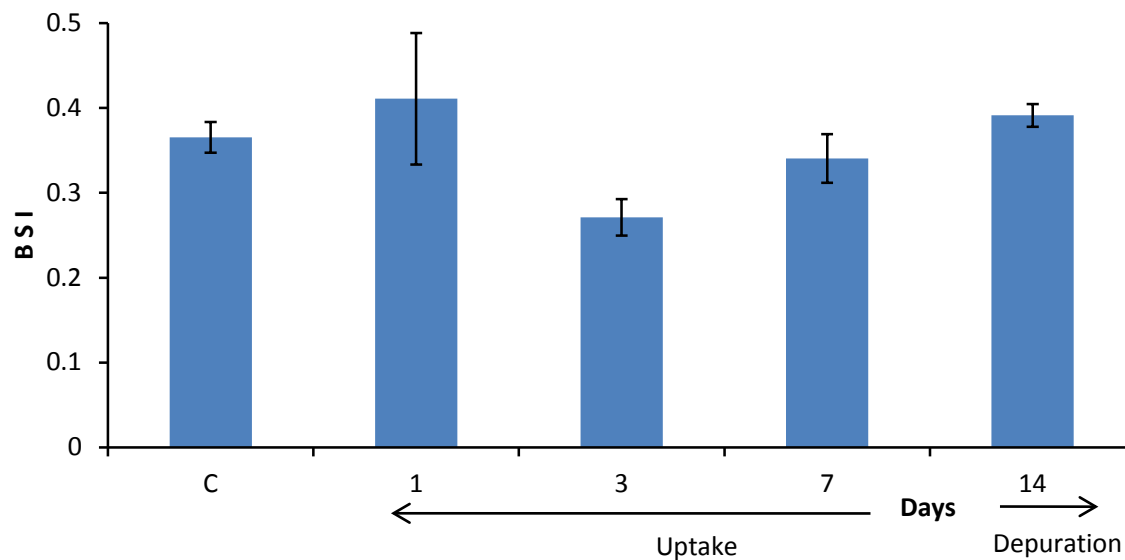
Fig. 8. Brain somatic index, BSI (mean ± SEM, n=3-5) of control and norethindrone exposed (100 µg l^{-1}) channel catfish. Mean relative brain weights were not significantly different (*P*=0.18, Kruskal-Wallis test) between the control and exposed fish.
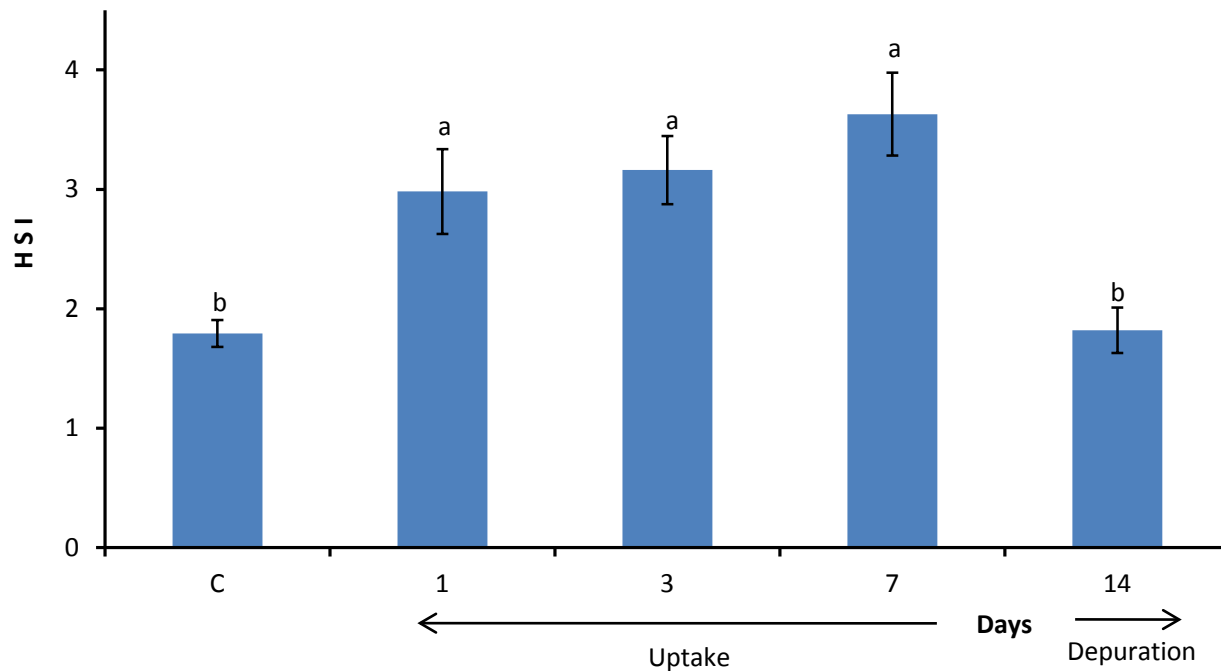
Fig. 9. Hepatic somatic index, HSI (mean ± SEM, n=3-5) of control and norethindrone exposed (100 µg l^{-1}) channel catfish. Mean relative liver weights of exposed fish were significantly higher (*P*=0.01, Kruskal-Wallis, SNK post-hoc test) that of the control and depurated fish. Means with the same letter are not significantly different.
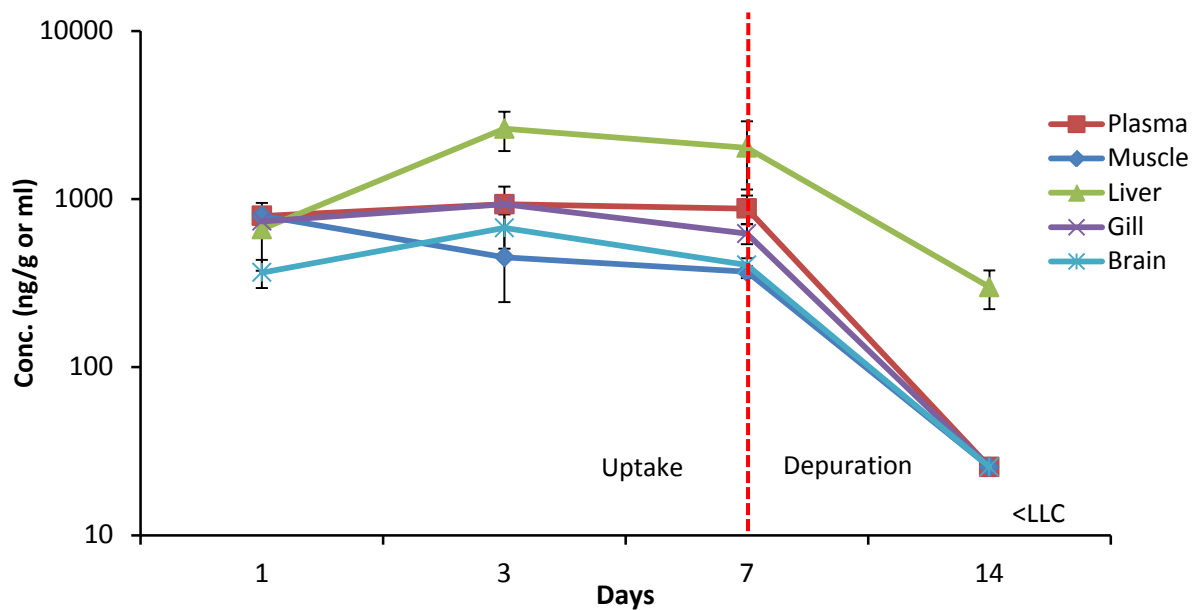
Fig. 10. Norethindrone (NET) concentration (ng g^{-1} wet wt or µg l^{-1} plasma, n=2-5) in different tissues of channel catfish exposed to 100 µg l^{-1}. NET concentrtion was below the lower limit of calibration (LLC, 25 µg l^{-1}) in the tissues of depurated fish.

Fig 11. Norethindrone (NET) concentration (mean ± SEM, n=3-4) in different tissues of channel catfish exposed to 100 µg l^{-1} for 7 days. NET accumulation levels in gill, plasma and liver tissues were significantly higher (P=0.01, Kruskal-Wallis, SNK post hoc test) than those for the muscle and brain tissues. Means with the same letter are not significantly different.

CHAPTER 6

BIOCONCENTRATION OF IBUPROFEN IN FISH

6.1   Abstract

Pharmaceutical products and their metabolites are being widely detected in aquatic

environments and there is a growing interest in assessing potential risks to fish and other non-

target species. Ibuprofen is one of the most commonly used analgesic drugs and aquatic exposure

to it may result in chronic toxicity in fish. While it is commonly detected in the environment, no

laboratory studies have evaluated the tissue specific bioconcentration of ibuprofen in fish. In the

current study, fathead minnow (*Pimephales promelas)* were exposed to 250 µg ibuprofen l^{-1} for

28 days followed by a 14 day depuration phase. In a reduced bioconcentration factor (BCF) test

design, channel catfish (*Ictalurus punctatus*) were exposed to 250µg ibuprofen l^{-1} for a week and

allowed to depurate for 7 days. Tissues were collected during uptake and depuration phases of

each test and the corresponding proportional and kinetic BCFs were estimated. The results

indicated that the BCF levels were very low (0.08–1.4) implying the lack of bioconcentration

potential for ibuprofen in the two species. The highest accumulation of ibuprofen was observed

in the catfish plasma as opposed to individual tissues. The minimized BCF test design yielded

similar bioconcentration results as those of the standard test and has potential for its use in

screening approaches for pharmaceuticals.

6.2   Introduction

The use of advanced analytical techniques coupled with monitoring studies (Daughton

and Ternes, 1999; Kolpin et al., 2002; Barnes et al., 2008; Focazio et al., 2008) have increased

the awareness of the widespread presence of pharmaceuticals and their transformation products

in the environment. In a national pilot study on the occurrence of pharmaceuticals and personal care products (PPCPs), Ramirez et al. (2009) reported the presence of several pharmaceuticals in fish collected from the effluent dominated rivers in the U.S. More recently, Fick et al., (2010) measured multiple pharmaceuticals in the plasma of fish exposed to waste water treatment plant (WWTP) effluent.

Pharmaceuticals are designed to interact with specific biological pathways in mammals and these enzyme/receptor systems are conserved across species (Gunnarson et al. 2008, Huggett et al., 2003). Aquatic exposure to human and veterinary pharmaceuticals or their transformation products could therefore elicit similar pharmacodynamic effects in fish (Ankley et al. 2009). Due to the growing concerns over the potential chronic risks to fish and other aquatic species, the requirements for a thorough environmental risk assessment (ERA) of new and the existing pharmaceuticals has been recognized (EMEA, 2006).

Assessment of the bioconcentration potential for chemicals is an important regulatory end point (Arnot and Gobas, 2006) and often considered useful in prioritizing chemicals to evaluate their chronic risks to non-target organisms (Nordberg and Rudén, 2007). Fish BCFs are usually determined using the Organization for Economic Cooperation and Development (OECD) Test Guideline 305 (OECD, 1996). However, the test requires large numbers of animals with long duration of exposure. Hence generating empirical BCF data for thousands of compounds is not practical due to cost, time and animal use constraints. Therefore, a minimized BCF test design that would more rapidly screen chemicals and is less demanding on resources has been suggested as a viable way to prioritize chemicals for ERAs (Springer et al., 2008).

Ibuprofen is a non-steroidal anti-inflammatory (NSAID) drug widely used as an analgesic and in the treatment of fever and rheumatic disorders (Table 9). Due to its availability as an over-

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the-counter (OTC) medicine often with high therapeutic doses (500 mg day^{-1}), ibuprofen is frequently detected in rivers and streams across the North America (Kolpin et al., 2002) and the Europe (Buser et al., 1999). Maximum ibuprofen concentrations in the surface waters in the UK and the US were reported at 5 and 25 µg l^{-1} respectively (Ashton et al. 2004; Metcalfe et al. 2003). Previous research has indicated that chronic exposure to ibuprofen may alter reproduction (Flippin et al., 2007) and development (Pancharatna & David, 2009) in fish. In general, a xenobiotic with a bioaccumulative potential in an organism may also result in subtle long-term effects. This is because bioconcentration leads to an increase in the internal tissue concentrations that subsequently could cause toxic effects. Because of this and due to the fact that BCF assessments are part of the current tiered risk assessments, the present study was aimed at determining the bioconcentration potential of ibuprofen in fathead minnow and channel catfish. The fathead minnow (FHM) is widely used in aquatic toxicology and bioconcentration studies (Ankley and Villeneuve 2006). The channel catfish (CC) was also used in this study as it is an important aquaculture species and has been used in BCF testing (Weisbrod et al., 2009). More importantly, catfish are large enough to evaluate bioconcentration of ibuprofen into fish plasma, which has recently been reported in fish exposed to WWTP effluent (Fick et al., 2010).

6.3   Experimental

6.3.1   Chemicals and Reagents

Ibuprofen (2-(4-isobutylphenyl)-propionic acid) and ibuprofen-methyl-d3 were obtained from Toronto Research Chemicals Inc. (ON, Canada). Boron trifluoride methanol solution, 14% (BF3-MeOH, CAS # 373-57-9) and MS222 (3-aminobenzoicacid ethyl ester methane sulfonate, CAS # 886-86-2) were purchased from Sigma Aldrich (St. Louis, MO). N, N-

dimethylformamide (DMF), hexane (HEX), ethyl acetate (EA), acetonitrile (ACN), acetone, methanol (MeOH) and dichloromethane (DCM) were > 90% pure and procured from Fisher Scientific (Houston, TX).

6.3.2 Animals and Housing

Adult fathead minnow (~ 3.0 g) and juvenile catfish (~50.0 g) of either sex were obtained from the University of North Texas (UNT) Aquatic Toxicology facility and Pond King Inc. (Gainesville, TX) respectively. All fish were allowed to acclimatize in tanks with carbon filtered dechlorinated tap water and to the standard laboratory conditions of 20º C and 16:8 hr light: dark cycle for at least a week before the start of exposure studies. Minnows and catfish were fed twice daily with brine shrimp flakes (Ocean Start International (OSI), Snowville, UT) and pellets of AquaMax Grower 600 diet (PMI International LLC, Brentwood, MO) respectively. The food residues in the tanks were siphoned off approximately two hours after feeding.

6.3.3 Fish Exposure

6.3.3.1 Ibuprofen Stocks

Stock solutions of ibuprofen were prepared in DMF targeting an exposure concentration of 250 μg l^{-1}. The stock solutions were stored in 250 ml amber bottles (Fisher Scientific, Houston, TX) and refrigerated at all times. The same stock solutions were used over the entire experimental period.

6.3.3.2 Flow-Through System

A continuous flow-through system was used for the fish exposures. Briefly, a peristaltic

pump (Masterflex® L/S®, Cole-Parmer®, Veron Hills, IL) delivered a constant volume of 150

ml City of Denton, TX dechlorinated tap water per minute into mixing chambers where stock

solutions of the analyte or DMF (solvent control) in 30 ml polycarbonate syringes (Becton

Dickinson, Franklin Lakes, NJ) were infused at 5 µl min^{-1} using a syringe pump (PHD 2000;

Warner Instruments, Hamden, CT). From the mixing boxes, the targeted concentration of

ibuprofen was gravity fed into the exposure tanks. Ibuprofen was not detected in the

dechlorinated tap water used in this study.

6.3.4   BCF Tests

In two separate BCF tests, fathead minnow and catfish were exposed to the test chemical

using the following experimental design. Fish were exposed to 250µg ibuprofen l^{-1}, which is

approximately one-tenth of the 7 day-no observed effect concentration (NOEC) level obtained

from an early life stage (ELS) toxicity test conducted in our laboratory. A solvent control (DMF,

<0.003% in tanks) was also included to account for any possible side effects. Minnows were

randomly distributed into exposure (n=36) and control tanks (n=10). The exposure tanks were of

20 liters with an estimated ten volume replacements per day. Following the OECD 305 BCF test

guidelines (OECD, 1996), the fish were exposed for 28 days, followed by depuration in clean

tanks for 14 days. Fish (n=4) were sampled after days 1, 3, 7, 28, 35 and 42 and were

anaesthetized using 100 mg l^{-1} MS222 to collect tissue samples of muscle, gill and liver. In the

second test, catfish (n=20) were exposed to 250 µg ibuprofen l^{-1} in 60 l tanks with at least four

volume replacements per day. This study involved a minimized BCF test design in which the fish

were exposed to the test chemical for 7 d followed by depuration for an additional 7d. At the end

of days 1, 7, 10 and 14, fish (n=4) were sampled to collect blood, muscle, gill, liver and kidney

tissues. The blood samples were collected from caudal vein using heparinized syringes and plasma was separated later by centrifugation at 12,000 g. Tissues and plasma were stored at - 20º C until analysis. In both the tests, wet weight and total length of the fish were recorded to calculate the condition factor (K = (weight, g *100) ÷ (standard length, cm)^3). Liver and kidney weights of catfish were used to calculate hepatic somatic index [HSI; (liver wt*100) ÷ body wt] and nephritic somatic index [NSI; (kidney wt*100) ÷ body wt], respectively. All fish handling methods were in accordance with the UNT Institutional Animal Care and Use Committee (ACUC) guidelines.

6.3.5   Ibuprofen Extraction and Analysis

Isotopic dilution (ID) gas chromatography/mass Spectrometry (GC/MS) was used to quantify ibuprofen concentration in water, plasma and tissue samples. Prior to conducting any extraction, 5 ng ibuprofen-methyl-d^3 was included in all the samples that served as an internal standard (ISTD).

6.3.5.1 Water

Four to five ml water samples from exposure tanks were collected in glass sample vials (National Scientific, Rockwood, TN) on days 1, 7, 14, 28 (fathead test) and 1, 7 (catfish test) during the uptake phase and refrigerated at 4° C for subsequent measurements of test concentrations. The results are expressed as time-weighted average concentrations of ibuprofen. A liquid/liquid extraction with 5.0 ml (1+1) (v/v) hexane/ ethyl acetate (hitherto referred to as the "extraction solvent") was conducted to extract ibuprofen in 1.0 ml water samples. The extraction

was repeated, organic layers combined and evaporated to dryness under gentle stream of nitrogen.

6.3.5.2 Plasma

Frozen plasma samples were brought to room temperature followed by the addition of 5 ml of ice-cold acetone. After thorough mixing on a vortex mixer (Fisher Scientific), contents were centrifuged at 2000 rpm for 10 min. Supernatant was separated, evaporated to 0.5 ml, back extracted into the extraction solvent and finally evaporated to dryness. Samples were analyzed for ibuprofen by GC/MS analysis, while the metabolites were analyzed using liquid chromatography/ mass spectrometry (LC/MS).

6.3.5.3 Tissues

Blot-dried tissue (0.1-0.2 g) was extracted in 4 ml extraction solvent for 3 min in a Mini-Beadbeater™ (Biospec Products, Bartlesville, OK) using 2.5 mm glass beads (Biospec Products). Extracts were transferred into 15 ml glass centrifuge tubes (Kimble; Vineland, NJ). Milli-Q water (2 ml; Millipore, Billerica, MA) was added, thoroughly mixed, followed by centrifugation for 10 min. The top organic layer was carefully separated into clean 10 ml glass vial. The extraction process was repeated and the combined organic extracts were evaporated to dryness. Lipid weights were estimated gravimetrically. Dried residues were dissolved in 10 ml ACN and refrigerated until cleanup.

6.3.5.4 Solid Phase Extraction (SPE) Cleanup and Derivatization

The tissue sample extracts were cleaned-up using ResPrep™ Florisil SPE (3 ml, 500 mg;

Restek, Bellefonte, PA). Briefly, the cartridges were conditioned with two tube-full volumes of hexane followed by acetonitrile. The ten ml tissue extracts were passed through the conditioned cartridges with no vacuum and eluates were collected in clean glass test tubes (Fisher Scientific). The florisil cleaned extracts were evaporated to dryness under nitrogen. Dried samples were derivatized with BF_3/MeOH following the method adopted by Metcalfe et al., (2003) with some modifications. The derivatization was accomplished by the addition of 50 µl each of the reagent and acetonitrile and incubating the mixture at 85ºC for 2 hrs. After cooling the sample to room temperature, the derivatization reaction was stopped by addition of 0.5 ml Milli-Q water. The contents were then extracted back into the extraction solvent, evaporated to dryness and finally reconstituted in 100 µl DCM for GC/MS analysis.

6.3.5.5 GC/MS Analysis

Derivatized extracts were separated using GC 6890N (Agilent, Palo Alto, CA) fitted with a 30 m x 0.25 mm x 0.25 µm EC™- 5 capillary column (Alltech, Deerfield, IL). Ultrapure helium (Air Liquide, Houston, TX) was used as carrier gas and the instrument was operated in constant pressure (9.5 psi) mode with an initial flow of 1.2 ml min^{-1} and an average velocity of 40 cm sec^{-1}. Samples were auto- injected (2.0 µL) in pulsed splitless mode at 260º C and the separation was achieved using the following temperature program for the GC oven: initially at 40º C for 3 min, increased to 200º C at 10º C min^{-1} with no hold time followed by final ramp to 300º C at 15º C min^{-1} and held at this temperature for 5 min. After separation in the GC column, ibuprofen was identified from the mass spectra obtained by electronic impact ionization (70ev; positive ion polarity) in a mass selective detector, MSD 5973 (Agilent) operated under selected ion monitoring (SIM) with a dwell time of 50 ms and at transfer line temp of 280º C. The MS

quadrupole and source temperatures were maintained at 150º C and 230º C respectively.

Quantification of ibuprofen in the test samples was achieved from an eight-point calibration

curve generated by plotting (linear curve fit) the relative response factors (derived from the ratio

of response (area) between native and mass labeled ibuprofen) and the concentration (pg μl^{-1})

(Fig A10) The following characteristic ions (m z^{-1}) are used in the quantitative analysis: *161*,

177, 220 for native IBU and *163*, 223 for ISTD (italicized ions used for quantification) (Fig A5).

6.3.5.6 LC/MS Metabolite Analysis

Catfish plasma samples were analyzed for the presence of known human metabolites.

Qualitative metabolite analysis was conducted via LC/MS. The high performance liquid

chromatography (HPLC) system consisted of an Agilent solvent delivery system and an Agilent

autosampler (Agilent Technologies, Inc., Santa Clara, CA, 95051). Chromatography was

performed on a Phenomenex Synergi Hydro RP-C18 (4.6 mm x 150 mm, 4 µm) column. The

mobile phase was comprised of water (solvent A) and acetonitrile (solvent B) each with 10 mM

ammonium acetate (pH=7) with flow rate of 1.0 ml min^{-1}. Metabolite identification was

performed using a Thermo linear trap quadrupole (LTQ) mass spectrometer (MS) equipped with

an electrospray interface (ESI) (Thermo Electron, CA). The HPLC column effluent was split so

approximately 50 µl min^{-1} was introduced into the ESI interface. The electrospray voltage

operated at 4000 V as the MS collected data in the negative ion mode. The analysis investigated

2- and 3-hydroxy ibuprofen (2- and 3-OH-IBU; m z^{-1} = 221) metabolites as well as the parent

compound (m z^{-1} = 205) (Fig 12).

6.3.6 BCF Measurements

BCFs in tissues and plasma were expressed as proportional BCF (BCF_p) and kinetic BCF (BCF_k). BCF_p was determined from the ratio of ibuprofen concentration in tissue/plasma to the measured exposure concentration. BCF_k was estimated by computing the ratio between the calculated uptake (k_1) and depuration (k_2) rate constants. Assuming the first order kinetics, the rate constants were determined using the methods described in Newman (1995).

6.3.7 Method Detection Limits (MDLs), Percent Recoveries and Precision

Following the standard EPA protocols, ibuprofen MDLs were determined by analyzing seven replicates of the unexposed fish tissues/plasma samples spiked with the lowest calibration standard that was clearly differentiated from the background concentration. MDL was calculated using the equation: $MDL = SD \times 3.14$ where SD is the standard deviation of seven replicate measurements and 3.14 represents the Student's t-value at 99% confidence level. Accuracy was determined by percent recovery calculated by comparing the measured and spiked (known) concentrations. Reproducibility of the analytical method was evaluated in terms of % relative standard deviation (RSD) calculated using the formula: $RSD = (SD \div mean) \times 100$.

6.3.8 Data Analysis

Data analysis was performed using GraphPad Prism® (version 5.02, La Jolla, CA) and SAS® (version 9.1, SAS Institute Inc., Cary, NC). In all the statistical tests, a P value less than 0.05 was considered significant. Kruskal-Wallis one-way ANOVA was conducted to examine the differences in condition factor, HSI and NSI between the control and ibuprofen exposed fish. Tissue specific ibuprofen levels and the corresponding proportional BCF estimates were

expressed as means ± SEM. Kruskal-Wallis one-way ANOVA was conducted to evaluate

differences in tissue and time-specific ibuprofen accumulation levels. Significant results were

subjected to Student-Newman-Keuls (SNK) multiple comparison test for mean separation.

6.4   Results

6.4.1   Efficiency of the Flow-Through System

Ibuprofen levels in the exposure tanks were compared to the nominal test concentrations.

The results (Table 10) indicated that the time-weighted average analyte concentration in water

samples were approximately 63 and 90 % the nominal exposure levels in the fathead minnow

and catfish tests respectively. The measured test concentrations were used to calculate BCF

values.

6.4.2   Toxicological Effects of Ibuprofen on Fish

Condition factor (K), an indicative of overall well-being of fish, of minnows and catfish

did not differ significantly (P=0.16, 0.93) from that of the control fish (data not shown).

Additional information on the physiological status of catfish was obtained by determining the

somatic indices; HSI and NSI that did not differ significantly (P=0.80, 0.71) when compared to

those obtained for the control fish (data not shown).

6.4.3   Ibuprofen Concentration in Tissues

6.4.3.1 Fathead Minnow Test

Fathead minnow in the solvent control group had tissue concentrations below the MDL

(<14.0 ng g^{-1} wet wt). The uptake of ibuprofen in the muscle, gill and liver tissues of exposed

fish ranged from 53-129, 115-173 and 85-173 ng g^{-1} wet wt., respectively (Table 11). Ibuprofen

levels in the gill and liver tissues did not differ significantly (P=0.10, 0.11) over the entire

exposure period, but a significant difference (P=0.01) was found in muscle tissue uptake, with

the highest concentration observed at day 3 post exposure. Although the uptake by different

tissues at 3 and 28 days of exposure did not differ significantly, liver and gill tissues collected at

days 1 and 7 accumulated significantly higher (P=0.02) ibuprofen compared to the

corresponding muscle tissue concentrations. Ibuprofen was not detected (< 14 ng g^{-1} wet wt) in

any tissue sampled during the depuration period.

6.4.3.2 Channel Catfish Study

      Ibuprofen concentrations (Mean $\pm$ SEM) in various tissues of catfish are presented in

Table 12. The results indicate significant differences in uptake by the tissues of fish collected at

days 1 and 7 (P= 0.004, 0.003) with the highest concentration (314.85 ± 55.19 µg l^{-1}) observed in

the plasma. Plasma samples that were analyzed for metabolites contained hydroxylated products

of ibuprofen viz., 2- and 3-OH-ibuprofen (Fig 13). Muscle tissue had the lowest ibuprofen

accumulation (18.74 ± 0.88 ng g^{-1} wet wt), while the concentrations were similar in the gill and

kidney tissues (98-142 ng g^{-1} wet wt). Although there was no detectable ibuprofen by the liver

tissue at day 1, tissue accumulation at day 7 reached levels that were similar to those observed in

the gill and kidney tissues. Ibuprofen was not detected in tissues (<14 ng g^{-1} wet wt) and plasma

(<8 µg l^{-1}) of fish from the solvent control group or from those of fish depurated in clean tanks.

6.4.4   BCF Estimates

      In the present study, BCFs were estimated on tissue basis (Table 13). The 28-day $BCF_{p}s$

for the muscle, gill and liver tissues of FHM were 0.7, 1.0 and 0.7, respectively. The

corresponding BCF_ks for muscle, gill and liver were 0.6, 0.7 and 1.0, respectively. For the

reduced BCF test conducted on the catfish, the 7-day BCF_ps for the muscle, gill, liver, kidney

and plasma were 0.08, 0.4, 0.5, 0.6 and 1.4, respectively. The corresponding BCF_ks were

estimated at 0.2, 0.5, 0.5, 0.7 and 1.4 respectively. Overall, BCF estimates in different tissues of

minnows and catfish were ≤ 1.4.

6.4.5   Quality Control (QC)

Quality control (QC) data for the analytical method used in quantifying ibuprofen in

tissues (Fig A15) and plasma are presented in Table 14. The percent recoveries in different

matrices ranged from 90 to 104. The reproducibility (% RSD) of the method varied from 0.6 –

12. These data indicate good recovery and repeatability of the method.

6.5   Discussion

The study presented in this paper is the first full 42-day laboratory bioconcentration study

focusing on ibuprofen in fathead minnow, as well as an abbreviated study design with channel

catfish. The results obtained for the tissue-specific BCF levels ranged between 0.08 and 1.4

indicating that ibuprofen may not bioconcentrate above the levels fish are exposed to in the

water. Studies have reported that other NSAIDs may bioconcentrate in fish (Swaiger et al. 2004).

Rainbow trout (*Oncorhynchus mykiss*) exposed to diclofenac at environmentally relevant

concentrations (1 $\mu g\ l^{-1}$) for 28 days had significant bioconcentration (BCF = 69-2732) in their

tissues (Schwaiger et al., 2004). Diclofenac is more lipophilic (log K_{ow}= 4.02; U.S. EPA, 2009)

and hence may have greater tendency to accumulate in tissues. Ibuprofen is a weak acid

amenable to ionization at environmental pH levels and hence may not be absorbed well into the organism. The absence of bioconcentration of ibuprofen in minnows and catfish could also be explained on possible clearance mechanisms. *In vitro* experiments on ibuprofen clearance from hepatic and gill S-9 fractions of the trout and catfish showed rapid depletion of the substrate over the 120-hr incubation period (Gomez et al., 2010, unpublished). The presence, while low in comparison to parent ibuprofen, of known human metabolites in catfish plasma suggests that metabolism is an elimination mechanism once ibuprofen is absorbed in fish. This identification of metabolites suggests that fish are able to metabolize ibuprofen in a manner similar to mammals (Davies, 1998).

In the standard OECD bioconcentration test, BCF is expressed as a function of total wet weight of fish. Estimation of bioconcentration in plasma is typically not included in this test. However, plasma BCF measurements are useful for calculating a blood: water partitioning coefficient ($P_{B:W}$) that can be used to help predict potential chronic risks of pharmaceuticals to fish (Huggett et al. 2004; Huggett et al., 2003). In the current study, the analytical method for determining ibuprofen concentration in plasma required 500 µl blood samples. A pilot study with fathead minnow (data not shown) required pooling blood from 7 or 8 fish to achieve this analytical requirement. Such exercise would necessitate the use of large numbers of fish for the exposure tests. To overcome this limitation, a parallel BCF test with reduced sampling design was conducted with catfish. This proved useful as the highest concentration of ibuprofen was observed in the plasma. This is an important finding as future field studies should consider measurement of pharmaceuticals in fish plasma as an indicator of exposure, as opposed to focusing solely on tissue levels (Ramirez et al., 2009). Fick et al. (2010) recently measured the levels of 25 pharmaceuticals in the plasma of rainbow trout exposed to effluent from WWTPs.

In addition, the sample preparation of plasma for ibuprofen analysis is less time consuming when compared to that of a tissue. Therefore, rapid screening for exposure to pharmaceuticals in biota may be accomplished through plasma analysis. The downside to simply measuring plasma would be the potential for rapid elimination of compounds such as ibuprofen, so tissue analysis would still be beneficial if initial plasma samples were below analytical detection.

In the present study, Fulton's condition factor (K), HSI and NSI of the ibuprofen-exposed fish were similar to those in the unexposed group indicating the lack of the chemical stress on the organisms. However, female Japanese medaka (*Oryzias latipes*) exposed to ibuprofen (1-100 $\mu g \, l^{-1}$) for six weeks had reduced spawning activity and elevated HSIs (Flippin et al., 2007). David and Pancharatna (2009) exposed zebrafish to ibuprofen for 7 d (10-100 $\mu g \, l^{-1}$) that resulted in reducing hatching success, growth and other developmental abnormalities. Han et al. (2010) reported a 120-d survival NOEC of 0.0001 mg l^{-1} for Japanese medaka, as well as altering steroid hormones and vitellogenin. The exposure concentrations at which these effects were observed in these studies are less than the exposure level considered in our study, however no changes in survival or parameters measured in this experiment were significantly altered. The slightly higher BCF levels observed in fathead minnow may be attributed to the absence of growth dilution.

In summary, the BCF levels obtained in the present study were well below the currently available regulatory criteria for a chemical to be considered bioaccumulative (UNEP 2001; EC 2001; U.S. EPA 2003). The 28 and 7-day BCF levels of fathead minnow and catfish were comparable indicating the usefulness of the reduced test design (7day) in potential screening approaches for pharmaceuticals. Overall, the test results indicated that ibuprofen may not significantly bioconcentrate, per regulatory criteria, (i.e. BCF $\geq$ 2000) in fish.

6.6   Chapter References

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Table 9

Physical/chemical properties* of ibuprofen.

Property	Description
CAS #	15687-27-1
Molecular weight	206.29
Partition Coefficient, Log P	3.97
Log D (Log P @ pH 6.5)	1.68£
Water solubility @ 25°C, mg L-1	21.0
Henry's Law Constant @ 25°C, atm-m3 mol-1	1.52 x10-7
Dissociation Constant, pKa	4.91¥

* EPISUITE (USEPA, 2009); £ACD/PhysChem Suite (ACD, 2006); ¥ SRC PhysProp Database (SRC, 2009)

Table 10

Time-weighted measured concentrations of ibuprofen ($\mu g\ l^{-1}$; means $\pm$ SD) in test water during the exposure period of the fathead minnow and channel catfish tests.

	Fathead Test	Catfish test
Nominal concentration	250.0	250.0
Measured concentration	152.6 $\pm$ 25.38	223.9 $\pm$ 6.2
% Nominal	61.0	89.6
n	6	4

n: # samples analyzed

Table 11

Tissue and time-specific ibuprofen uptake (ng g^{-1} wet weight; mean $\pm$ SEM, (n)) by fathead minnow exposed to 250 $\mu g\ l^{-1}$ for 28 days.

Exposure time (days)	Muscle	Gill	Liver
1	53.07 $\pm$ 8.78 (4)	115.39 $\pm$ 19.12 (4)	140.55 $\pm$ 33.11(3)
3	129.94 $\pm$ 14.38 (4)	157.66 $\pm$ 62.99 (3)	85.28 $\pm$ 6.82 (2)
7	45.99 $\pm$ 5.39 (4)	173.08 $\pm$ 15.53 (4)	172.97 $\pm$ 8.42 (4)
28	104.81 $\pm$ 19.72(4)	167.11 $\pm$ 37.36 (4)	105.43 $\pm$ 14.07 (3)

n: # samples analyzed; ND-non detect (< 14 ng/g wet wt.)

Table 12

Ibuprofen uptake by tissues (mean ± SEM ng g^{-1} wet weight; n=4) and plasma (mean ± SEM µg l^{-1}; n=4) of catfish exposed to 250 µg l^{-1} for 7 days.

Exposure (days)	Muscle	Gill	Liver	Kidney	Plasma
1	21.14 ± 1.14*	100.72 ± 8.73	ND#	93.96 ± 11.85	227.19 ± 35.09
7	18.74 ± 0.88	98.55 ± 4.1	114.22 ± 21.3	142.32 ± 26.43	314.85 ± 55.19

* n = 2;   ND-non detect (<14 ng g^{-1} wet wt.)

Table 13

Tissue-specific proportional (p) and kinetic (k) BCF values for the two fishes: fathead minnow and channel catfish exposed to 250 µg ibuprofen l^{-1}.

		Muscle	Liver	Gill	Kidney*	Plasma*
Fathead test	BCF$_p$	0.69	0.69	1.09	--	--
	BCF$_k$	0.63	1.04	0.72	--	--
Catfish test	BCF$_p$	0.08	0.51	0.44	0.63	1.40
	BCF$_k$	0.22	0.47	0.55	0.68	1.43

* sampled only for the catfish test

Table 14

Ibuprofen percent recovery, precision and accuracy calculated for different sample matrices.

Matrix	n	Recovery (%) (Mean ± SD)	Precision (% RSD)	% Accuracy
Method Blank*	11	96.8 ± 4.2	4.4	96.8
Water	2	101.9 ± 12.3	12.0	101.9
Plasma	2	90.6 ± 0.53	0.6	90.6
Tissue	17	104.2 ± 12.91	12.38	104.2

* 50/50 (v/v) Hexane : Ethylacetate extraction solvent; n: # samples analyzed; % RSD = SD/Mean x 100
% Accuracy = avg. measured concentration/ spiked concentration x 100
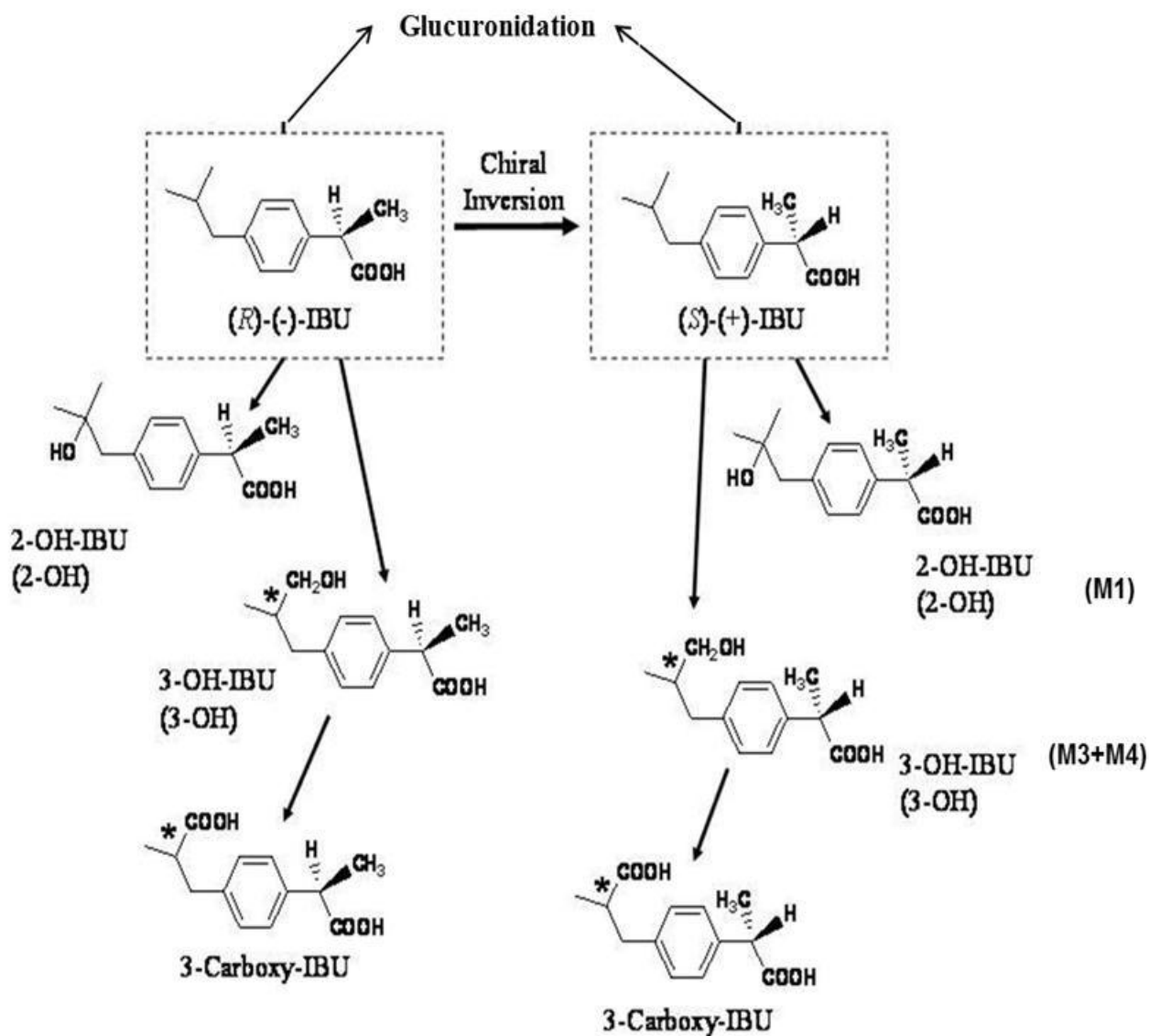
Fig. 12. Biotransformation of ibuprofen in mammals. Hydroxylation is the major metabolic pathway resulting in 2- and 3- OH-IBU metabolites. 3-OH-IBU gets converted to 3-carboxy-IBU. These metabolites plus the parent compound are subjected to phase II glucuronid glucuronidation. Adapted from Chang et al. (2008).

A)
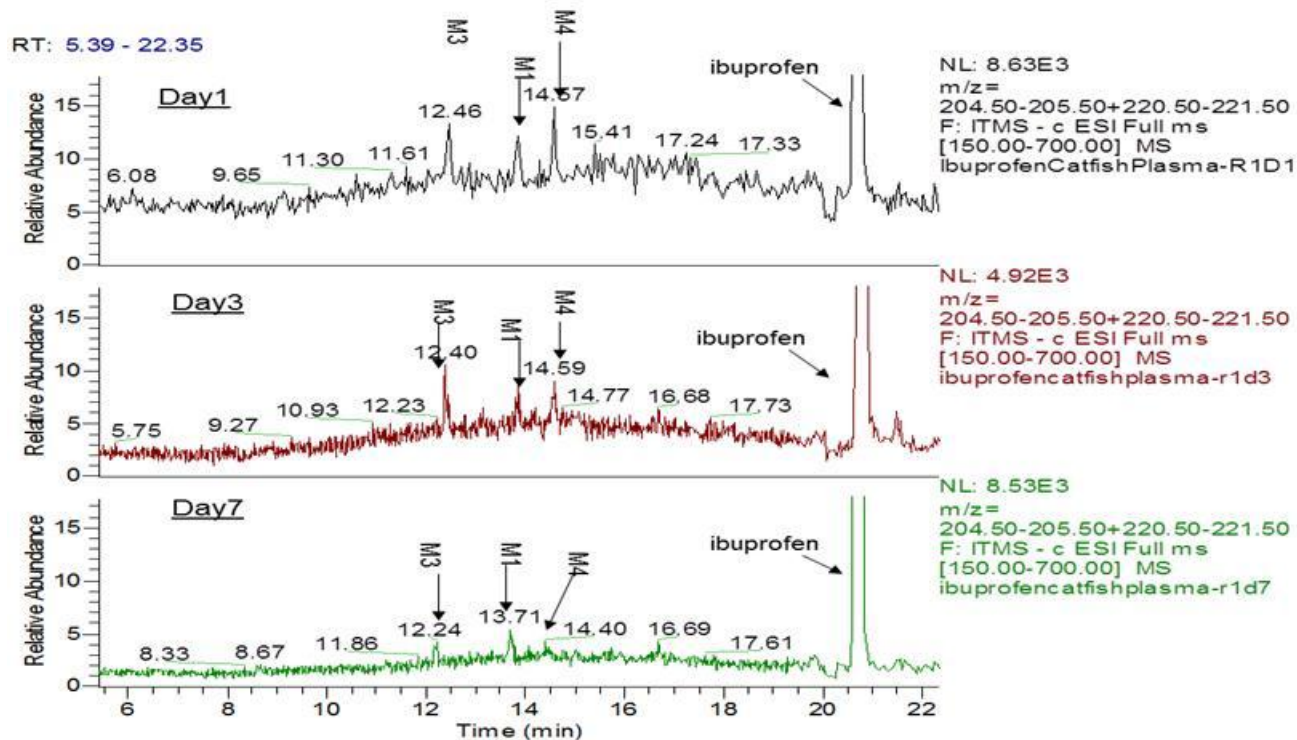
B)
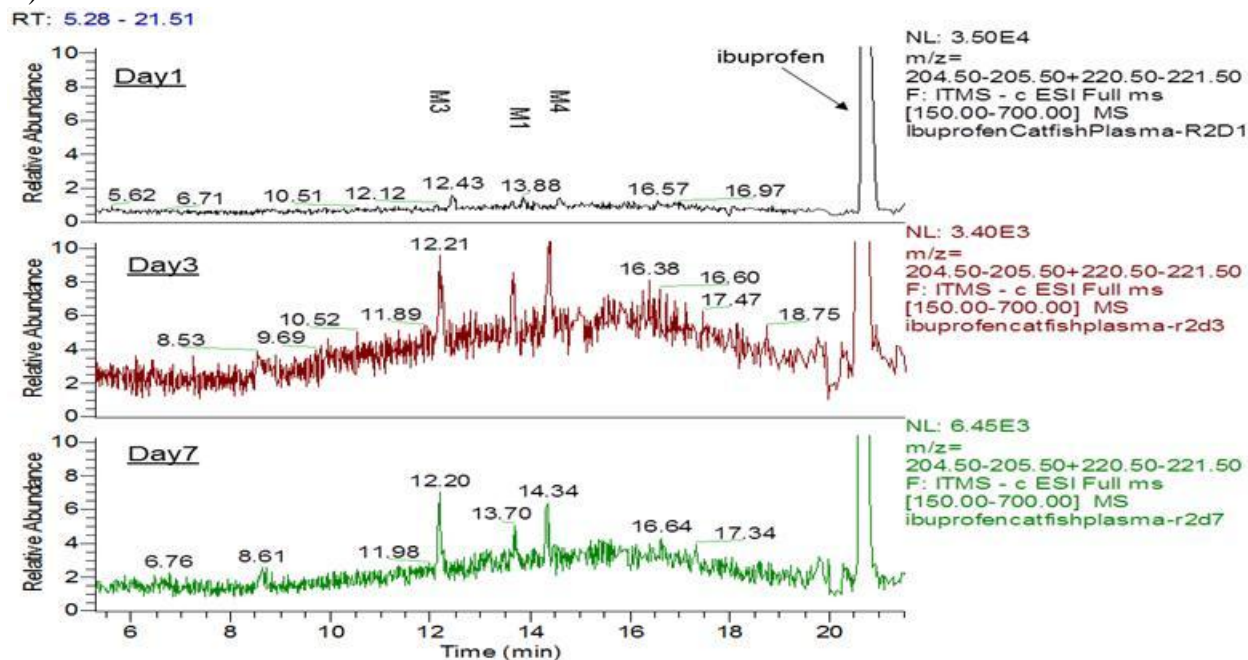
Fig. 13. Identification of ibuprofen metabolites: 2 and 3-OH-IBU in the plasma A) replicate 1 and B) replicate 2 of the channel catfish exposed 250μg l^{-1} for 7 days.

CHAPTER 7

BIOCONCENTRATION OF VERAPAMIL AND CLOZAPINE IN FISH

7.1   Introduction

After animal use, residual pharmaceuticals end up in the environment through waste water treatment plant (WWTP) effluents or via land application of biosolids. The knowledge on the consequences (i.e. possible risks to non-target organisms) of the environmental presence of these bioactive compounds has been emerging. Several reports on the prioritization approaches and/or ranking of pharmaceuticals that pose potential threat to the environment are available in the literature (Stuer-Lauridsen et al., 2000; Jones et al., 2002; Sanderson et al., 2004b; Schwab et al., 2005; Carlsson et al., 2006; Besse and Garric, 2008; Grung et al., 2008; Kostich and Lazorchak, 2008; Madden et al., 2009). In a recent study, a preliminary environmental risk assessment (ERA) database consisting of top 200 prescription drugs identified cardiovascular and neuroactive compounds (in addition to antibiotics) as high risk therapeutic classes (Cooper et al., 2008). The ecotoxicological effects of both central nervous system (CNS) and cardiovascular drugs have thoroughly been reviewed (Fent et al., 2006; Khetan and Collins, 2007; Santos et al., 2009). Examination of the available literature indicated that among the several classes of cardiovascular drugs, calcium channel blockers received less attention in terms of ecotoxicological effects. Similarly, in the CNS drugs category, the current knowledge on the potential environmental risks associated with antipsychotics is limited.

Calcium channel blockers are commonly prescribed for the treatment of hypertension, angina pectoris and cardiac arrhythmia. They act by inhibiting the voltage gated calcium channels in the smooth muscle and prevent the constriction of blood vessels. Verapamil (VER) represents an important drug in this group and its usage has been very extensive (Pascoe et al.,

2003). For instance, in 2005, the defined daily dose (DDD) of the prescribed VER accounted for the total usage of about 41 tons in Germany (Trautwein et al., 2008) . Due to the incomplete mammalian metabolization, VER has been detected in the effluent discharges and rivers (Hummel et al., 2006). Nevertheless, very few studies exist on its potential toxicological effects on aquatic organisms (Villegas-Navarro et al., 2003; Sanderson et al., 2004a). In a very recent study on the acute toxicity effects of VER on rainbow trout, an LC-50 (96 hr) of 2.72 mg l^{-1} was reported (Li et al., 2010). From the physiological and biochemical responses of the fish measured in this study, the authors felt the need for long-term exposure studies. In addition to its own toxic effects, VER is a potent inhibitor of the drug efflux pump proteins such as P-glycoprotein and hence may enhance an organism's susceptibility to toxic effects by other xenobiotics (Kurelec, 1995). Therefore, it is necessary to understand the potential accumulation of VER in aquatic organisms, fish in particular, due to the presence of highly conserved (> 90%) biological targets (i.e. calcium channels) (Gunnarsson et al., 2008).

Antipsychotics (typical and atypical) are widely used to treat schizophrenia. Due to the absence of Parkinson disease- type movements, various conditions of psychosis are often treated with atypical antipsychotics. Few drugs in this category (e.g. quetiapine, resperidone) were blockbusters in 2005 in terms of their global sales (Khetan and Collins, 2007) . Clozapine (CLZ) was the first atypical antipsychotic developed in 1970s and due to its side effects (agranulocytosis), its current usage is restricted only for treatment-resistant schizophrenia (Alvir et al., 1993). In spite of its restricted usage, CLZ has been prescribed in large quantities. For example, in 2006, about 600 kg was of this drug accounted for the total prescription usage in Sweden. However, to the best of our knowledge, peer-reviewed data on the environmental occurrence, fate and toxicity of CLZ is lacking. The 2006 Swedish screening program on

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pharmaceuticals, a solitary report on clozapine's environmental occurrence, indicated that it was

not detected in surface water, sludge, sediment or biota (Woldegiorgis et al., 2007). The

antipsychotic action of CLZ is triggered by its binding with the dopamine and serotonin

receptors. Dopamine receptors have also been identified in aquatic vertebrates (Huggett et al.,

2004) and few studies have reported adverse effects of waterborne antipsychotics in fish

(Giacomini et al., 2006).

VER and CLZ are basic pharmaceutical compounds. The physicochemical properties

(Table 15) indicate that at neutral pH, some proportion of these compounds may exist in

unionized forms. In general, basic pharmaceuticals tend to partition into sludge and sediment and

hence have higher environmental persistence (Fent et al., 2006). In view of this and due to the

presence of highly conserved biological targets in aquatic vertebrates, information on the uptake

and depuration kinetics of the two compounds in fish may be useful in predicting the potential

chronic effects. Accordingly, the aim of the current study was to characterize the

bioconcentration potential of verapamil and clozapine in two fresh water fishes, fathead minnow

and channel catfish. To achieve this, the uptake and depuration kinetics of the two compounds

were experimentally determined through waterborne exposure studies.

7.2   Experimental

7.2.1   Chemicals

Clozapine (CLZ, 8-chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo[b,e][1,4]diazepine,

CAS#5786-21-0), clozapine-d8 (CLZ-d8, 8-chloro-11-(4-methyl-1-piperazinyl-d8)-5H-

dibenzo[b,e][1,4]diazepine) were purchased from Toronto Research Chemicals (TRC; North

York, ON, Canada). HPLC grade solvents acetonitrile (ACN), methanol (MeOH), n-hexane

(HEX), ethyl acetate (EA), dimethyl formamide (DMF) and acetone were obtained from Fisher Scientific (Houston, TX). Formic acid (CAS# 64-18-6), ammonium acetate (CAS# 631-61-8) and tricaine mesylate (MS-222, Ethyl 3-aminobenzoate methanesulfonic acid, CAS# 886-86-2) were purchased from Sigma-Aldrich (St. Louis, MO).

7.2.2   Fish

Juvenile channel catfish (ca. 50 g; not sex differentiated) were obtained from a local commercial fish hatchery (Pond King Inc., Gainesville, TX). Adult fathead minnow (ca. 3 g) of either sex were obtained from the stock of UNT's aquatic toxicology facility. Before the actual exposure tests, fish were allowed to adjust for a week to 18:6 hr light/dark photoperiod at 20° C in glass aquaria with continuously aerated carbon filtered dechlorinated tap water under flow-through conditions. All fish were fed commercial fish food twice daily.

7.2.3   BCF Experiments

7.2.3.1 Fathead Minnow Tests

BCF tests on the fathead minnow followed the OECD 305 test guidelines with few modifications. Briefly, in two separate tests, fish (n=50-55) were randomly distributed into aquaria (n=3) and exposed to 500 and 50 $\mu g\, l^{-1}$ of verapamil and clozapine for 28 days. VER stocks were prepared in Milli-Q water and DMF (<0.003% in tanks) was used as carrier solvent for CLZ. A solvent control (n=10; for clozapine test) was also included. During the uptake phase, fish (n=5-6) were sampled at the end of days 1, 3, 7, 14 and 28 and subsequently placed in clean tanks and depurated for two weeks. During the 14 day-depuration, fish (n=5-6) were sampled twice (days 35 and 42).

7.2.3.2 Catfish Tests

The uptake and depuration of verapamil and clozapine in catfish were determined using a reduced BCF test design. Briefly, fish (n=25) in two separate tests, were exposed to VER and CLZ (the same test concentrations as used in the fathead studies) for 7 days and allowed to depurate in clean tanks for 7 days. Fish (n=5) were sampled thrice (days 1, 3 and 7) during the uptake and twice during the depuration period (days 10 and 14).

7.2.4   Samples and Data Collection

Water quality was monitored weekly and the mean ($\pm$ SD, n=8) temperature (°C), pH, dissolved oxygen (mg/L) and conductivity (μS cm-1) of water in the exposure tanks were 20 ± 0.5, 7.7 ± 0.3, 7.9 ± 0.4 and 300 ± 26 respectively. Water samples from exposure tanks were sampled (n=5-13) periodically to determine the measured exposure concentrations. At each sampling event, fish were sacrificed in a solution of MS-222 (100 mg/l) and wet weight (g) and fork length (cm) were recorded to determine the condition factor [(weight x100) $\div$ (length^3)]. Blood was collected (catfish only) from caudal vein-artery complex of individual fish and placed in heparinized tubes and plasma was separated. Muscle, gill, liver and kidney tissues were collected in both VER and CLZ tests. Additionally, while VER test included collection of heart, brain tissues were collected in CLZ test. Individual tissue weights (except muscle and gill) were recorded to calculate the corresponding somatic indices [(tissue weight (g) x100) $\div$ body weight (g)]. The tissue and plasma samples were frozen (-20°C) for further analysis. Throughout the experiments, fish were handled in accordance with the Institute's Animal Care and Use Committee (ACUC) guidelines.

7.2.5   Extraction

All the extraction procedures included the addition of 5 ng labeled standard (d6-VER or d8-CLZ; hereafter referred to as internal standards, ISTDs). Water samples (1-2 ml) were liquid/liquid extracted using 50/50 (v/v) n-hexane/ethyl acetate (hitherto referred to as the "extraction solvent"), evaporated and resolubilized in 100 µl acetonitrile (ACN). After treating initially with 10 ml ice-cold acetone, plasma samples (100-200 µl) were extracted using the same method used in water extraction. Tissue samples (0.1-0.2 g) were extracted (in 4 ml extraction solvent) using a Mini-Beadbeater™ (Biospec Products, Bartlesville, OK), filtered through 0.45µm polytetrafluoroethylene (PTFE) syringelss filters (Whatman, Sanford, ME), evaporated to dryness and lipid weights (catfish tests only) were determined gravimetrically. The dried residues were resolubilized in 100 µl ACN. The samples were stored at 4° C overnight and subjected later to either centrifugation or filtration through 0.22 µm polyvinylidene fluoride (PVDF) membrane filters (Millipore, Billerica, MA) before their injection into the HPLC.

7.2.6   LC/MS/MS Analysis

7.2.6.1 Verapamil

VER was analyzed by atmospheric pressure ionization-electro spray interface (API- ESI) liquid chromatography/mass spectrometry (LC/MS/MS) technique (Agilent 1100 Series; Agilent Technologies, Palo Alto, CA). All samples and calibration standards were auto injected (2 µl) into 5µm x 2.1 mm x 150 mm Agilent Extend- C18 column installed in the HPLC system. The mobile phase was a binary gradient consisting of Milli-Q water with 5 mM ammonium acetate (Solvent A); 95% acetonitrile and 5% Milli-Q water with 5 mM ammonium acetate (Solvent B)

run at 300 µl min^{-1} with a total run time of 18 min. VER eluted at ~8.0 min and the separation in the column was achieved with the following gradient program (Table 16).

Effluent from the column was monitored using LC/MSD Trap (Agilent) using appropriate time-windows established with the help of the divert valve that directs effluent to either MS source or waste. Appropriate MS operating conditions were set for isolating and monitoring the selected ions (Table 17). The chromatograms and mass spectra of VER and its labeled standard are presented in Fig A7. Quantification of VER was conducted using a ten-point (4000-8 pg µl^{-1}) calibration curve (Fig A12).

7.2.6.2 Clozapine

Clozapine was also analyzed by API- ESI LC/MS technique. All samples were auto injected (2 µl) into the C-18 column installed in the HPLC system. The mobile phase was a binary gradient consisting of 0.1% (v/v) formic acid in Milli-Q water (Solvent A) and acetonitrile (Solvent B) run at 300 µl min^{-1} with a total run time of 18 min. CLZ eluted at ~8.5 min and the separation in the column was achieved with the following gradient program (Table 18).

Effluent from the column was monitored using LC/MSD Trap using appropriate time-windows established with the help of divert valve that directs effluent to either MS source or waste. The operating conditions for MSD were set appropriately for isolating and monitoring the selected ions (Table 19). The chromatograms and mass spectra of CLZ and its labeled standard are presented in Fig A8. Quantification of CLZ was conducted using a ten-point (4000-8 pg/µl) calibration curve (Fig 13).

7.2.7 Uptake and Depuration Rate Constants and BCFs

Tissue-specific proportional BCFs (BCF_ps) were estimated from the ratio of chemical concentration in tissue to the time-weighted average measured exposure concentration.

$$BCF_p \ (l \ kg^{-1} \ wet \ wt.) = [chemical]_{tissue} \div [chemical]_{water}$$

BCF_ps were also expressed as lipid normalized values. Lipid normalized BCFs were estimated only for the catfish tissues due to the availability of larger tissue mass.

$$BCF_p \ (l \ kg^{-1} \ lipid \ wt) = [chemical]_{lipid \ norm \ tissue} \div [chemical]_{water}$$

Kinetic BCFs (BCF_ks) were calculated from the uptake (k_1) and depuration (k_2) rate constants determined from the plots of tissue concentration at different time points during exposure (uptake) and depuration. Assuming the first order kinetics, k_1 and k_2 were calculated by kinetic parameter calculator (KPC) method, a sequential method that combines linear and non-linear regression models (Newman, 1995). Estimation of k_1 and k_2 was based on the model:

$$C_f = C_w. \ (k_1 \div k_2). \ (1- e^{\ k2-t})$$

where C_f and C_w represent the chemical concentrations in fish and water respectively (OECD, 1996). Depuration rate constant, k_2 was first determined from the slope of the depuration plot using the simple linear curve fit model: $ln \ C_f = a \ (t) + b$. Uptake rate constant (k_1) was then calculated by substituting k_2 in the equation above. More frequently, $k2$ is used to predict the time required for 50 % reduction in the concentration of a chemical in the organism using the equation: $t_{1/2} = ln \ (2) \div k2$ or $0.6931 \div k2$.   In the present study, elimination half-lives were determined that provided important information chemical clearance from tissues.

7.2.8   Quality Control

7.2.8.1 Method Detection Limits

Method detection limits (MDLs) were determined following the EPA guidelines (U.S. EPA, 2000). Briefly, seven replicates of the unexposed fish tissue(s) or plasma were spiked with the lowest concentration in the calibration or at the level that was readily differentiated from the background measurements of clean tissue were run and the MDL was calculated using the formula: $MDL = 3.14 \times SD$, where SD is the standard deviation of seven replicate measurements and 3.14 is the student's t-value at 99% confidence level.

7.2.8.2 Accuracy

As part of the QC approach, each sample batch included method blanks, method blank spikes, control (unexposed) tissues and tissue spikes. For each of the test compounds, water, plasma and tissues (n= 2-4) from control group were fortified with native compounds and the accuracy of the extraction method was estimated in terms of percent recoveries calculated by comparing the measured concentrations with the nominal spike concentrations.

7.2.9   Data Analyses

Data analyses were conducted using SAS® (version 9.1, SAS Institute Inc., Cary, NC). In all statistical tests, a probability (P) value less than 0.05 was considered significant. All data were initially subjected to normality testing. Differences in the condition factor between the control and exposed fish were analyzed with an independent t-test (or Mann Whitney U test). One-way ANOVA (or Kruskal-Wallis test) was conducted on the tissue indices (liver, kidney, heart and brain) of the control and exposed fish. Tissue-specific uptake levels were analyzed

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using One-way ANOVA. Significant results ($P \leq 0.05$) were subjected to Dunnett's and/or Student Newman Keul's (SNK's) post hoc multiple comparison tests. All the data (measured concentrations and percent recoveries) were presented as Means ± SEM (or SD).

7.3   Results

7.3.1   Verapamil Results

7.3.1.1 Fathead Minnow Test

Measured Test Concentrations

The time-weighted average (±SD, n=13) measured test water concentration ($\mu g \, l^{-1}$) was 190 (± 64.4) which is only 38 % of the nominal exposure concentration 500 ($\mu g \, l^{-1}$). VER was not detected ($<5.8 \, \mu g \, l^{-1}$) in the control tanks.

Condition Factor and Somatic Indices

Fulton's condition factor, K of VER exposed fathead minnow was significantly higher ($P=0.003$) than that of the control fish (Fig 14) suggesting that VER exposure did not result in any undue stress. HSIs of exposed fish did not differ significantly ($P>0.05$) from that of the control fish (Fig 15). However, SNK post hoc test revealed significantly lower ($P=0.002$) liver weights for the fish sampled at days 1 and 3 compared to those for the fish at days 7, 14 and 35. Interestingly, the trend in the NSI data was somewhat similar to that observed in the HSI results. No significant differences ($P>0.05$) were found in NSIs between the control and exposed fish, but a significantly lower ($P=0.03$) kidney weights were noticed for the fish sampled at day1 compared to those for the fish collected at other sampling points (Fig 16). The data on HSI and NSI showed that VER exposure resulted in smaller liver and kidneys until 1 or 3 days post exposure followed by an increase in the organ weights comparable to that of the control fish. The

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relative weights of liver and kidneys were maximum (though not significant) at 7 and (or) 14 days post exposure. Heart weights (as % body weight) were not significantly different (P=0.27) between the control and exposed fish (Fig 17).

Verapamil Uptake, Depuration and BCFs

VER uptake and depuration plots for different tissues of fathead minnows are presented in Fig 18. VER concentration (ng g^{-1} wet wt) in the liver, gill and heart tissues had decreased initially from day 1 to 3, followed by an increase in the uptake at 14 days and was reduced towards the end of the exposure. The chemical uptake by the kidneys had the similar trend except the absence of reduction in the concentration at day 3. The uptake by the muscle, unlike other tissues, had the maximum VER concentration at 28 days. After 7 days of depuration in clean tanks, there was 83-97% reduction in the tissue concentrations. Using the depuration rate constants, the time required for 50% elimination was estimated (Table 20). From the data, it is evident that VER had higher residence times in the muscle and gill tissues compared to those in the liver and kidneys. However, fish depurated for another week had their tissue VER concentrations below or slightly above the MDL (35 ng g^{-1}).

Comparison of VER accumulation in the muscle, liver and gill tissues after 28 days of exposure showed no significant (P=0.34) differences (Fig 19). Accumulation levels in the heart and kidneys were not included in the comparison due to small sample size (n=1-2). Overall, VER uptake by different tissues of fathead minnow exposed for 28 days had the following trend: heart < muscle < gill < kidney < liver.

The 28 day- proportional VER BCFs for different tissues ranged between 14.6 and 40 while the kinetic BCFs were 17.3-75 (Table 20). As six individual hearts needed pooling to get one sample (n=1), BCF$_k$ was not computed for the heart tissue. From the BCF data, VER

appeared to bioconcentrate to a greater extent in the liver and kidneys than in the muscle, gill and heart tissues.

7.3.1.2 Catfish Test

Measured Test Concentrations

The time-weighted mean measured concentration ($\mu g\ l^{-1}$) of VER within the tanks during the 7 day exposure was $419\pm106\ \mu g\ l^{-1}$, which is about 84% nominal exposure concentration 500 $\mu g\ l^{-1}$. VER concentration was below detection (5.8 $\mu g\ l^{-1}$) for the dilution water in the control tanks.

Condition Factor and Somatic Indices

There was no significant difference (P=0.72) in the mean condition factors between the control and exposed fish (Fig 20) indicating the absence of chemical stress. Liver weights (as % body mass) were slightly higher for the exposed fish (except day 1) than that for the control fish. However, the differences were not statistically significant (P=0.07) (Fig 21). Although no significant differences (P>0.05) in NSIs between the control and exposed fish were found, fish depurated in clean tanks had larger kidney weights compared to those of the fish sampled at days 1 and 3 post exposure (Fig 22). There were no significant differences (P=0.13, 0.36) in brain and cardio somatic indices of the control and VER exposed fish (Figs 23 and 24).

Verapamil Uptake, Depuration and BCFs

VER was not detected in the plasma ($< 6\ \mu g\ l^{-1}$) and tissues ($<36\ ng\ g^{-1}$ wet wt) of the control fish. VER accumulation in various tissues of catfish is presented in Fig 25. The uptake in the plasma was fairly constant throughout the exposure with only 39% increase after 7 days of

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exposure. Muscle tissue had approximately twice the accumulation levels as those observed for the plasma. The highest concentration (528 ng g^{-1} wet wt) in the muscle tissue was noticed for the fish sampled at 7 days post exposure. For the gill and kidneys, VER accumulation was also peaked after 7 days of exposure. However, the accumulation trend was different for the heart and liver tissues. While the accumulation in the heart tissues was highest soon after the exposure (at day 1), liver tissues had highest VER concentration after 3 days of exposure, followed by a slight (ca. 9%) decrease in the concentration after 7 days. Overall, VER levels in different tissues had the following trend: plasma < muscle < heart < gill < liver < kidney. Interestingly, VER was found above the detection limits in the liver, gill, heart and kidney tissues of the fish depurated for 14 days. VER concentration in the heart tissue of the depurated fish was about half of that noticed for the liver and gill tissues. Kidneys had the highest VER concentration at the end of the depuration phase. The detection of measurable levels of the test compound in the tissues of fish resided in clean tanks for 14 days indicates that VER may not get rapidly cleared from the tissues as was evident from the times taken for 50% reduction of the test chemical concentration (Table 21). The tissue-specific half-life data indicates that VER persists longer in the muscle compared to the other tissues.

The 7day-VER uptake levels by the tissues differed significantly (P=0.002) (Fig 26), approximately with an order of magnitude of higher accumulation levels in the kidneys than those for the liver and gill tissues. The accumulation levels in the liver and gill, in turn, were about an order of magnitude higher than those observed for the plasma and muscle tissue.

After 7 days of exposure, VER BCFs for different tissues of catfish ranged between 0.6 and 50 with lowest and highest bioconcentration observed for the plasma and kidneys

respectively. The tissue-specific VER uptake and depuration rate constants, half-lives and the BCFs are presented in Table 21.

7.3.1.3 QC Results

Verapamil calibration curve (1000-2 µg/l) between relative responses and concentrations was linear with r^2 of 0.9977 (Fig A12). VER concentration in the control tanks (dilution water), tissues of the unexposed fish were below the respective MDLs. The mean percent recoveries for the VER spiked control water, plasma and tissue samples (Fig A17) and the MDLs are presented in Table 22. In addition, continuing calibration standard (CONCAL) runs were used to estimate the precision (% RSD) of the instrument.

7.3.2 Clozapine Results
7.3.2.1 Fathead Minnow Test
Measured Concentrations of CLZ

During the 28-day exposure, the average time-weighted measured concentration of CLZ within the exposure tanks was 28.5 ± 5.5 µg l^{-1} (n=5). The measured test concentration was about 57% nominal exposure concentration 50 µg l^{-1}. CLZ was not detected in the dilution water (< 14.4 µg l^{-1}) used for the control group.

Condition Factor and Somatic Indices

Fulton's condition factors (Ks) of the control and CLZ exposed fathead minnow were not significantly different (*P*=0.16) (Fig 27). The condition factor results conveyed two findings: a) the fish were well nourished and b) the test chemical did not cause any stress in the exposed fish. HSIs of the exposed fish (except for days 3 and 7) were smaller in comparison with those of the

control fish. Similarly, fish in the exposed group had smaller kidney weights than those observed for the control fish. However, both liver and kidney weights (as % body mass) of the exposed fish were not significantly (P=0.06, 0.2) different from that of the control fish (Figs 28 and 29). Brain somatic indices of the fish sampled at end of exposure (28 days) and after depuration for a week (day 35) were significantly higher (P=0.004) than the BSIs of the control fish (Fig 30).

Tissue-Specific Clozapine Uptake, Depuration and BCFs

CLZ was not detected in the plasma ($< 14 \ \mu g \ l^{-1}$) and tissues ($<58 \ ng \ g^{-1}$ wet wt) of the control fish. CLZ tissue concentrations (ng g^{-1} wet wt) are presented in Fig 31. During the exposure from days 1 through 28, CLZ levels in the muscle (2022-2672 ng g^{-1}) were fairly stable with the maximum accumulation at 3 days post exposure. A week depuration in clean tanks, however, resulted in 96% reduction in the concentration. Clozapine uptake by the gill and kidneys had somewhat similar trend, though the magnitude of accumulation levels were different. In both the tissues, CLZ concentration increased gradually during the exposure with 107 (gill) and 577 % (kidney) increase in the concentrations from day 1 through 28. Liver had highest accumulation compared to all other tissues, at least until 14 days of exposure. From days 1 through 7, the concentration decreased from 25024 to 19184 ng g^{-1} followed by 68% increase after 14 days. At the end of the exposure (28 days), CLZ concentration in the liver was reduced again by 47%. In the brain tissue, there was a steady increase (9371-15011 ng g^{-1}) in the uptake until 14 days of exposure followed by a 29 % reduction in the accumulation level at 28 days. Interestingly, the brain tissues of the fish sampled after 7 days of depuration (day 35) had the highest CLZ concentration compared to the other tissues sampled during the same time period. Fish depurated for another week still had residual amounts of CLZ in all the tissues. These findings indicate that CLZ may have long residence times in the tissues as evident from the

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elimination half-lives presented in Table 23. Incidentally, CLZ persisted longest in the brain in comparison with the other tissues. The 28-day CLZ uptake by various tissues differed significantly (P=0.001) (Fig 32). Post hoc test results indicated the following accumulation pattern: muscle < brain < kidney/liver/gill.

Tissue-specific uptake and depuration kinetics, time taken for 50% elimination, kinetic and 28-day proportional BCFs are outlined in Table 23. The BCF_k data indicated that CLZ had the maximum bioconcentration in the liver followed by brain, kidney, gill and muscle tissues (in that order). However, BCFs at 28 days were highest for the gill followed by liver, kidney, brain and muscle. CLZ bioconcentration levels in the tissues (except the muscle that had uptake levels close to the border line) ranged between 475-939.

7.3.2.2 Catfish Test

Measured Test Concentrations

The average (±SD) time-weighted measured concentration of CLZ in water during the 28 day exposure was 40.0 ± 6.9 (n=8), which is approximately 80% of the nominal exposure level of 50 µg l^{-1}. There were no detectable levels (<14.4 µg l^{-1}) of CLZ in the control water.

Condition Factor and Somatic Indices

No significant differences (P=0.9) were found in the condition factors of control and CLZ exposed catfish (Fig 33) indicating possible absence of chemical stress. Liver weights were significantly (P=0.001) higher for the fish in the exposed group (except for the fish at days 1 and 7) than those observed for the control group (Fig 34). Relative kidney weights of the exposed fish were significantly higher (P=0.001) than those observed for the control fish (Fig 35). In addition, SNK post-hoc test revealed an interesting trend in the kidney weights. Fish depurated in

clean tanks for a week had higher kidney weights than those observed for the exposed fish. CLZ exposure also resulted in the enlargement of brains and the BSIs of the exposed fish (except for day3) were significantly higher (P=0.0003) than that of the control fish (Fig 36).

Clozapine Uptake, Depuration in Tissues and BCFs

Tissue-specific CLZ accumulation is presented in Fig 37. CLZ concentration in the plasma increased from 704 to 1221 µg l^{-1} during the exposure. Plasma levels were reduced by 97% after the fish were depurated for one week. Further depuration resulted in no detectable levels of CLZ in the plasma. CLZ accumulation was the lowest in the plasma compared to all other tissues. The uptake levels in the muscle tissues were fairly stable (2354-3240 ng g^{-1}) with only a 27% increase in the concentration over a week of exposure. However, the accumulation levels in the muscle tissue were approximately 2 to 2.5 times higher than those observed for the plasma. CLZ concentrations in brain, gill, liver, and kidney tissues were at least an order of magnitude higher than those noticed in the muscle. The uptake levels steadily increased in the brain from day 1(11562 ng g^{-1}) through 7 (15680 ng g^{-1}) with approximately 26% increase. In the gill tissue, there was about 12% increase in the concentration from day1 through 3 followed by an increase in the concentration by 54% at day 7. CLZ concentrations in the liver and kidneys were much higher soon after the exposure started (i.e. at day 1). While an increase in the concentration was observed for the kidneys during the course of exposure, the uptake levels in the liver decreased initially at day 3 followed by an increase at day 7. Nevertheless, liver had the highest accumulation when compared to the rest of the tissues. Interestingly, except for the plasma, significant amount of CLZ was noticed in the tissues of the fish depurated for a week, indicating that clearance rates for this compound from the depository organs are slower as indicated by the half-lives in Table 24. Although not a wide difference in the elimination rates,

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CLZ depurated faster from the plasma compared to that in other tissues. Overall, 7day- CLZ accumulation in the tissues differed significantly (P=0.0002) with the following trend: plasma < muscle < brain/muscle < kidney/liver (Fig 38).

Tissue-specific uptake, depuration kinetics and the BCFs are presented in Table 24. CLZ BCFs in the catfish ranged from 30 to 1200 with lowest and highest bioconcentration in the plasma and liver respectively. BCFs in the plasma and muscle were lowest, brain and gill had intermediate accumulation levels, but substantially higher BCFs were estimated for the liver and kidney tissues. The BCF data indicates that CLZ has high potential to bioconcentrate in the tissues. The 7day tissue-specific lipid normalized BCFs were very high that ranged from 1741 to 23260.

7.3.2.3 QC Results

Clozapine calibration curve (1000-4 µg l^{-1}) was linear with r^2 value of 0.9987 (Fig A13). The percent recoveries for CLZ spiked control water, plasma and fish tissues (Fig A18) are presented in Table 25.

7.4   Discussion

7.4.1   Verapamil Results

7.4.1.1 Condition Factor and Somatic Indices

Condition factor (K) reflects the overall well-being of fish in terms of optimum allocation of energy resources for growth. However, it is known that environmental stressors (e.g. chemical exposure) result in the compromised condition (i.e. decline in K values) in fish. In our study, verapamil exposure did not negatively impact the Ks of fathead minnow and channel catfish.

This finding is in agreement with a recent study in which VER exposure (0.5 -10 mg l^{-1}) did not change the weight and fork length of juvenile rainbow trout (Li et al., 2010).

Liver and kidney weights of the fish were reduced initially after 1 to 3 days of exposure and had subsequently recovered during the rest of the exposure period. Calcium is known to play a vital role in the proliferation of differ types of cells and blockade of calcium channels by VER could result in the attenuation of organ growth. Although such effects have previously been reported in mammals (Broulik, 1998), no clear explanation may be provided on the subsequent recovery of the organ weights except that tissue hypertrophy is a common adaptive strategy to deal with xenobiotic exposure, especially in metabolically active organs.

7.4.1.2 Tissue-Specific Uptake, Depuration and BCFs

Verapamil uptake by different tissues of fathead minnow and catfish had the following trend: plasma < muscle < (gill ≤ heart) < (kidney ≤ liver). It is evident from this ranking that tissues that receive relatively higher blood supply had greater VER uptake, while the metabolically less demanding white muscle had lower concentration. Similar trends (in terms of accumulation order) in the fish tissue-uptake levels of pharmaceuticals (e.g. NSAIDs) have previously been reported (Schwaiger et al., 2004). On the other hand, plasma had the lowest VER concentration compared to the other tissues. However, mammalian pharmacology of VER indicates that it is 90% bound to plasma proteins (www.rxlist.com). It is not known whether VER has similar affinity for fish plasma albumins. Lower uptake levels in plasma could also be due to the greater affinity for tissue partitioning, which is supported by the physicochemical characteristics (Table 15). The uptake levels in all the tissues (except muscle) appeared to have reached the peak concentrations after two weeks of exposure as further exposure (2 weeks) had

resulted in the reduction in tissue concentrations suggesting that there was a possible disposition of VER from these tissues. On the contrary, uptake levels in the muscle continued to increase during the course of the exposure. From these observations, we hypothesize that prolonged exposure of VER in fish could result in the higher accumulation in muscle tissue.

The depuration profiles of VER enabled a direct comparison of the half-lives of this compound in the fish to that in the mammals. On average, VER has a half-life of 8 hr in mammals while the corresponding time for the fish was estimated to be 46 hrs. Assuming similar pharmacological receptor responses between mammals and fish, higher residence in the plasma and tissues of the fish could imply greater pharmacodynamic activity and hence greater potential for adverse effects in the non-target species. This finding is significant in view of a recent study indicating that sublethal levels of VER exposure in fish results in increased oxidative stress as well as altered behavioral responses (Li et al., 2010).

The tissue-specific VER BCFs varied widely among the tissues which can be attributed to their preferential uptake. The estimated BCFs also differed among the species with relatively lower bioconcentration potential in the catfish. The most obvious reason for the species-specific differences in BCFs is the fish size and hence the possibility of growth dilution effect. In addition, the measured VER exposure concentration in fathead study was lower (ca. 2X) than that for the catfish. It may not be significant enough but bioconcentration levels, in general, are inversely related to the exposure concentration.

VER BCFs among different tissues ranged between 0.7 – 75. The reported BCFs may not be of great concern in terms of current regulatory criterion (BCF $\geq$ 2000). However, as evident from this study, bioconcentration assessments relying on the plasma or muscle tissues could underestimate VER's bioconcentration potential in fish. Also, VER is metabolized extensively in

mammals resulting in various phase I and II metabolites including norverapamil that are shown

to have possible toxicological properties (Trautwein et al., 2008). However, it is not clear

whether a similar potential for biotransformation exist in fish that could cause undesirable effects

in aquatic vertebrates.

7.4.2 Clozapine Results

7.4.2.1 Condition Factor and Somatic Indices

At 50 μg l^{-1} exposure level, clozapine did not affect the weight-to-length relationship in

fathead minnow and channel catfish. However, psychoactive compounds like CLZ are dopamine

and serotonin agonists and the receptor responses are known to play an important role in the

regulation of hypothalamic-pituitary-gonadal (HPG) axis which in turn regulates various

reproductive functions (Van Der Ven et al., 2006; Villeneuve et al., 2010). Therefore, it is

possible to have the reproductive fitness of fish challenged, if the target concentrations of the

antipsychotics are large and specific enough to interact with the neurotransmitters.

The results on the liver and kidney somatic indices were mixed. While CLZ exposure did

not cause changes in the HSIs and NSIs of fathead minnow, increases in the liver and kidney

weights of the catfish were noticed. The reasons for this species-specific trend are not very clear

except that the two fishes may have different sensitivities towards CLZ. In addition, there was

30% difference between the measured concentrations of CLZ in the catfish (higher) and fatheads.

On the other hand, the results on the brain somatic indices are more convincing as CLZ exposure

resulted in significantly higher brain weights in both fishes. Considering of pharmacological

activity of antipsychotics, this finding may not be a surprise. In general, the increase tissue

weights are often correlated with higher accumulation of the chemical (discussed in the next

paragraph). Also, these compounds are known to interact with dopamine receptors and have been shown to disrupt the locomotors activity in fish (Giacomini et al., 2006).

7.4.2.2 Tissue Specific Uptake, Depuration and BCFs

Clozapine accumulation in different tissues echoed that of verapamil and had the following general uptake trend for both fishes: plasma < muscle < (gill ≤ brain) < (kidney ≤ liver). However, CLZ is more lipophilic compared to VER, and hence had higher BCF levels. Again, in addition to the characteristics such as log D_{ow} (see Table 5.3.1), the exposure concentrations in VER and CLZ tests differed exactly by an order of magnitude and hence it is expected that CLZ would bioconcentrate higher compared to VER. Accordingly, the estimated BCFs for the liver, kidney and gill for the CLZ exposed fish differ by one to two orders of magnitude from the corresponding levels in VER exposed fish.

CLZ uptake by gill tissue increased steadily through the exposure suggesting that gills could be the primary site of uptake, depuration or both. Similarly, increased accumulation in the kidneys during exposure indicates possible reabsorption. On the other hand, CLZ concentrations in liver and brain had opposite trend in accumulation at least during the initial 2 week-exposure period. When CLZ concentrations in the brain increased, there was a decrease in the liver accumulation. This suggests that liver could have been actively metabolizing CLZ while brain acted as a major depot for storing it. Of all the tissues, brain had the longest elimination half-life, further supporting its storage potential. Similar findings have previously been reported for another class of neuroactive compounds, selective serotonin reuptake inhibitors (SSRIs). Fish captured from an effluent dominated stream had the highest accumulation levels of SSRIs in the

brain followed by liver and muscle tissues (Brooks et al., 2005). In a similar study, SSRIs were shown to accumulate in the brain tissue (Schultz et al., 2010).

The tissue-specific BCFs (wet wt) varied between 31 and 1226. Thus the accumulation potential of CLZ by various tissues differs by at least two-orders of magnitude. The BCF levels for liver, kidney, gill and brain approach the current BCF criterion of 2000 and far exceed the U.S. EPA's BCF criterion (100-1000) for a compound to be designated as persistent, bioaccumulative and toxic (PBT). The tissue-specific analysis of CLZ provided important information regarding the relative accumulative potential.

In conclusion, the present study on the bioconcentration potential of two basic pharmaceuticals, verapamil and clozapine, in fish has provided useful information in terms of differential tissue uptake by these compounds. Liver and kidneys were shown to have the highest potential to accumulate these pharmaceuticals. Also, the collection of tissues based on the compound-specific pharmacological action for example, heart in VER test and brain in CLZ test, proved beneficial as their accumulation levels sometime paralleled that of liver and kidneys. Finally, consideration of relevant tissues for conducting bioconcentration potential of pharmaceuticals may be beneficial in terms of avoiding potential underestimation of the true BCFs. This is key as most risk assessment approaches incorporate BCF as an important end point.

7.5   Chapter References

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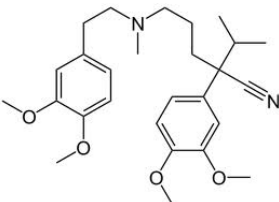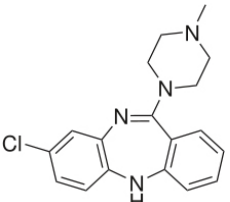
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Table 15

Physicochemical characteristics of verapamil and clozapine (retrieved from SRC PhysProp database; http://www.syrres.com and EPISUITE, U.S. EPA).

Property	Verapamil	Clozapine
CAS #	52-53-9	5786-21-0
Molecular weight	454.61	326.83
Partition Coefficient, log K_{ow}	3.79	3.23
log D_{ow} (Log P @ pH 7.0)	£2.41	#3.23
Water solubility @ 25° C, mg l^{-1}	4.47	11.8
Vapor pressure @ 25° C, mm Hg	2.79E-11	9.48E-09
Henry's Law Constant @ 25°C, atm-m^3 mol^{-1}	8.79E-15	9.29E-15
Dissociation constant, pK_a	8.92	7.5
Estimated half-lives (hrs) : water	4.32E+03	1.4E+03
soil	8.64E+03	2.8E+03
sediment	3.89E+04	1.3E+04
Environmental persistence# (hr)	1.4E+04	3.1E+03
Structure		

£(www.cerep.com); #(Sköld et al., 2006)

Table 16

HPLC binary gradient program for verapamil.

Time (min)	Mobile Phase Composition (%)	
	Solvent A	Solvent B
0.0	70	30
2.0	70	30
12.0	0	100
15.0	0	100
15.1	70	30
18.0	70	30

Solvent A: Milli-Q water with 5 mM ammonium acetate, solvent B: 95% acetonitrile and 5% Milli-Q water with 5 mM ammonium acetate

Table 17

LC/MSD Trap operating conditions required for monitoring verapamil.

Parameter	Description
Polarity	Positive ion
ESI nebulizer pressure	20 psi
Dry gas flow & temperature	8 L/min & 350°C
Scan range & maximum accumulation time	300-470 m/z & 50 m sec
Fragmentation mode	MRM
Mass Transitions	VER: EIC:303.2, +MS/MS:455.3
	VER-d6: EIC:309.2, +MS/MS:461.3
CID	1.0

MRM: multiple reaction monitoring, VER: verapamil, EIC: extracted ion chromatogram, CID: collision induced dissociation (amplitude)

Table 18

HPLC binary gradient program for clozapine.

Time (min)	Mobile Phase Composition (%)	
	Solvent A	Solvent B
0.0	90	10
5.0	70	30
10.0	40	60
12.0	0	100
14.0	0	100
14.1	90	10
18.0	90	10

Solvent A: Milli-Q water with 0.1 %(v/v) formic acid, solvent B: acetonitrile with 0.1%(v/v) formic acid

Table 19

LC/MSD Trap operating conditions required for monitoring clozapine.

Parameter	Description
Polarity	Positive ion
ESI nebulizer pressure	20 psi
Dry gas flow & temperature	8 L/min and 350°C
Scan range & maximum accumulation time	300-470 m/z and 50 m sec
Fragmentation mode	MRM
Mass Transitions	CLZ: EIC:270, +MS/MS:237
	CLZ-d8: EIC:275, +MS/MS:335
CID	0.8

Table 20

Tissue-specific verapamil uptake and depuration rate constants, half-lives and BCFs for fathead minnow exposed to 500 µg l^{-1} for 28 days.

Tissue	Rate constant (day^{-1})		t $_{1/2}$ (days)	BCF$_k$	28 d BCF
	k$_1$	k$_2$			
Heart#	--	--	--	---	14.6
Muscle	5.2	0.30	2.3	17.3	23.1
Gill	12.1	0.30	2.3	40.3	29.3
Kidney	26.8	0.36	1.9	74.0	34.4
Liver	27.0	0.36	1.9	75.0	40.0

BCF$_k$ not calculated as 6 individual tissues pooled to get one replicate

Table 21

Tissue-specific verapamil uptake and depuration kinetics and BCFs for channel catfish exposed to 500 µg l^{-1} for 7 days.

Tissue	Rate constant (d^{-1})		t $_{1/2}$ (d)	BCF$_k$	7 d BCF	
	k$_1$	k$_2$			Wet wt.	Lipid based
Plasma	0.51	0.37	1.9	0.7	0.7	--
Muscle	0.54	0.35	2.0	1.5	1.3	58.3
Heart	3.3	0.49	1.4	6.7	5.2	9.3
Gill	3.0	0.46	1.5	6.5	6.7	27.1
Liver	8.3	0.47	1.5	17.6	13.4	224
Kidney	18.9	0.45	1.5	42.1	46.5	296

Table 22

Verapamil percent recovery (mean $\pm$ SD), precision (% RSD), and MDLs (μg l^{-1} or ng g^{-1}) for water, plasma and tissues.

Test	Matrix	n	Recovery (%)	Precision (% RSD)	MDL
Fathead	Water	2	101 $\pm$ 4.4		5.8
	Tissue	5	92 $\pm$ 13.8		35.9
	CONCAL#	13		4.6	
Catfish	Plasma	4	112 $\pm$ 2.0		
	Muscle	3	103 $\pm$ 2.4		
	Liver	2	120 $\pm$ 4.3		
	Gill	2	116 $\pm$ 2.7		
	Kidney	2	113 $\pm$ 3.8		
	Heart	2	99 $\pm$ 5.9		
	CONCAL	15		9.5	

continuing calibration standard

Table 23

Tissue-specific clozapine BCFs for fathead minnow exposed to 50 μg l^{-1} for 28 days.

Tissue	Rate constant (d^{-1})		t $_{1/2}$ (days)	BCFk	28 d BCF
	k_1	k_2			
Muscle	31.5	0.34	2.0	92.8	71
Brain	140	0.26	2.7	538	375
Kidney	150	0.29	2.4	520	556
Liver	432	0.46	1.5	939	605
Gill	195	0.41	1.7	475	830

Table 24

Tissue-specific clozapine BCFs for channel catfish exposed to 50 μg l^{-1} for 7 days.

Tissue	Rate constant (d^{-1})		t $_{1/2}$ (days)	BCF$_k$	7 d BCF	
	k$_1$	k$_2$			Wet wt.	Lipid based
Plasma	22.5	0.7	1.0	31.2	30.5	--
Muscle	47.9	0.6	1.1	80.0	81	1741
Brain	252	0.6	1.1	420	392	4057
Gill	259	0.6	1.1	432	501	6104
Kidney	610	0.6	1.1	1016	958	12156
Liver	736	0.6	1.1	1226	1048	23259

Table 25

Clozapine percent recovery (mean $\pm$ SD) for water, plasma and tissues and MDLs (μg l^{-1} or ng g^{-1}).

Test	Matrix	n	Recovery (%)	MDL
Fathead	Water	1	101	14.4
	Tissue	10	93.5 $\pm$ 8.2	58.2
Catfish	Plasma	3	92 $\pm$ 5.3	
	Muscle	2	101 $\pm$ 15.3	
	Liver	2	97.8 $\pm$ 0.5	
	Gill	2	106 $\pm$ 5.1	
	Kidney	2	88.6 $\pm$ 8.4	
	Brain	2	99.2 $\pm$ 2.8	
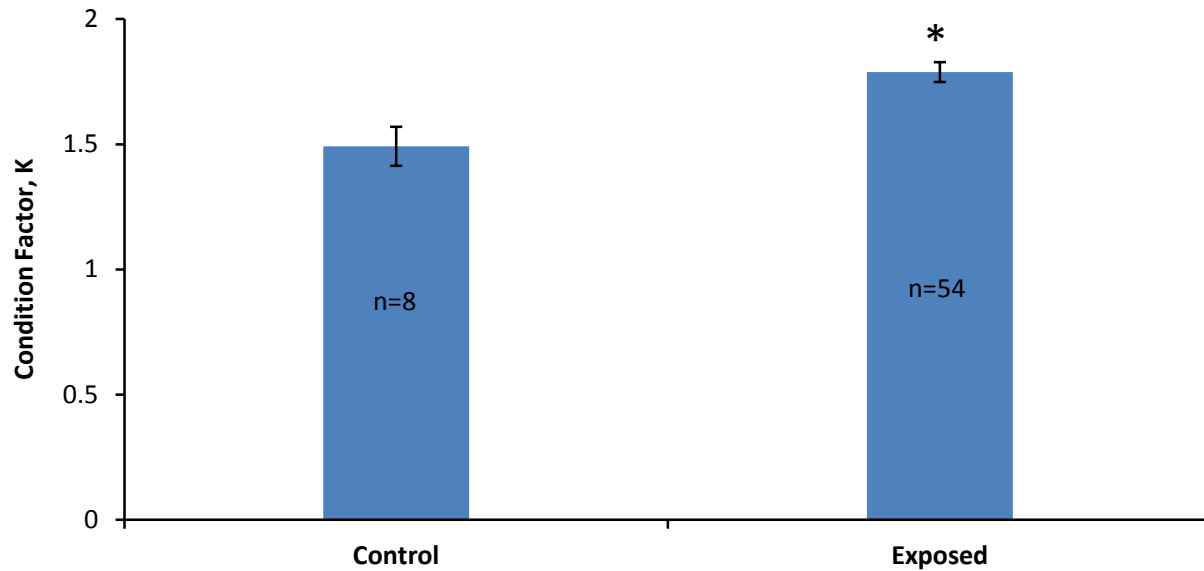
Fig. 14. Condition factor, K (mean ±SEM) of control and verapamil exposed (500 µg l^{-1}) fathead minnow. Mean K of exposed fish was significantly higher (P=0.003; independent t-test) than that of control fish.
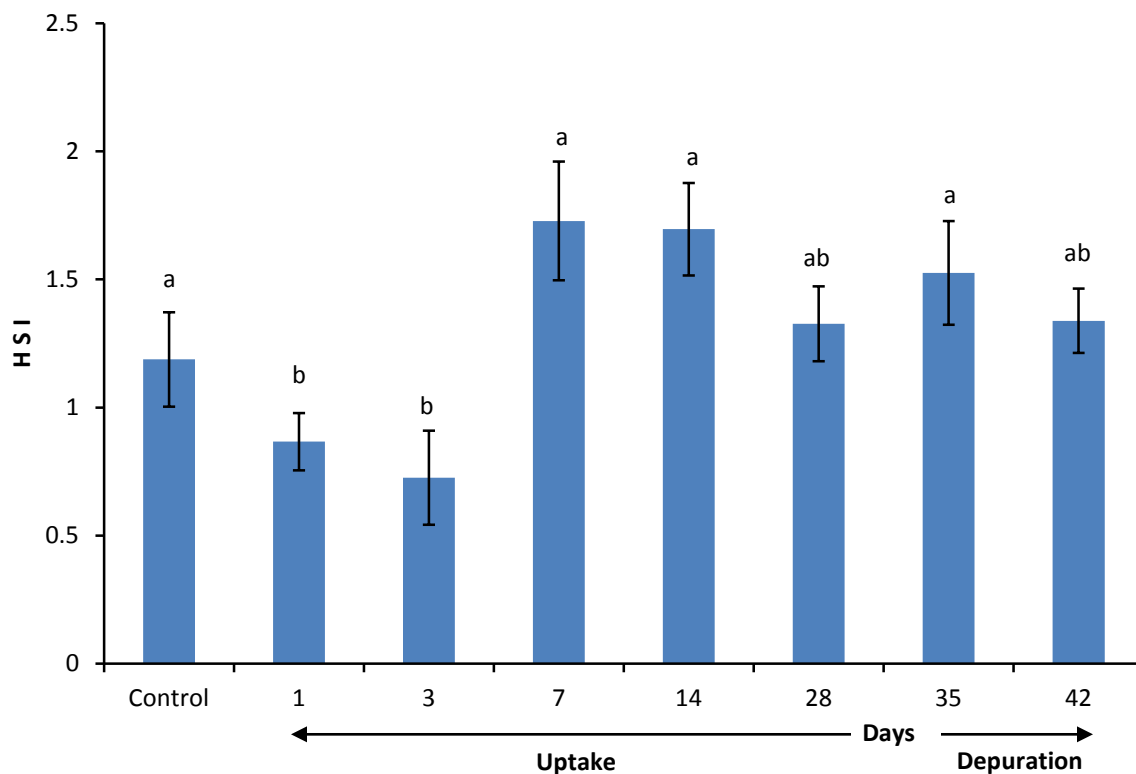
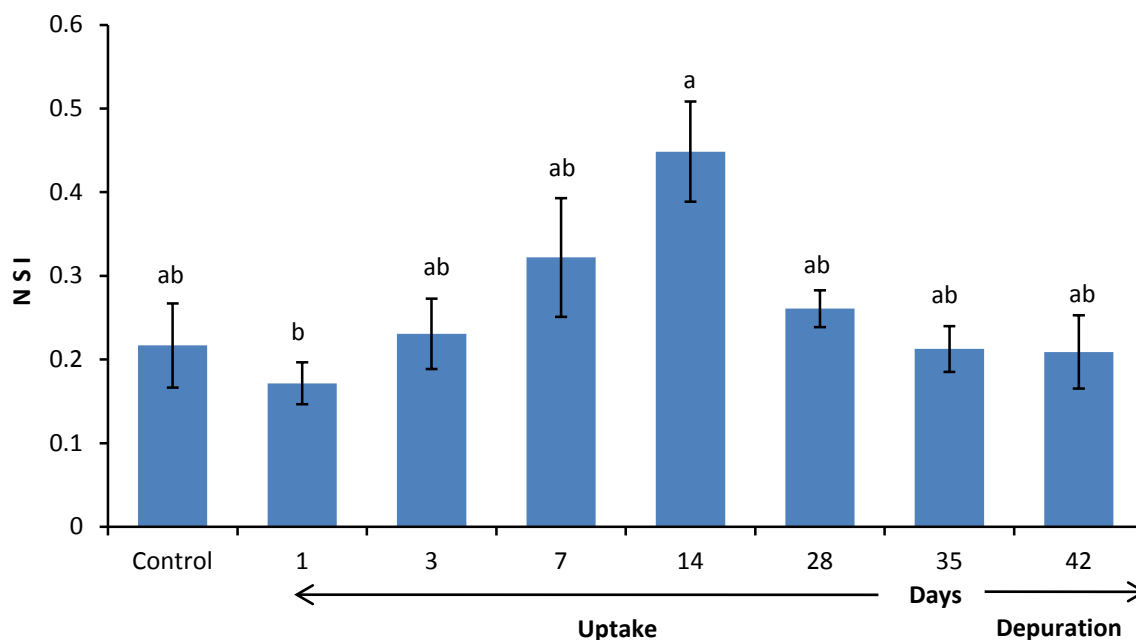**Fig. 15**. Hepatic somatic index, HSI (mean ± SEM, n=6) of control and verapamil exposed (500 µg l^{-1}) fathead minnow. Mean HSIs did not differ significantly (P>0.05, Kruskal Wallis, Dunnett's post hoc test) between the control and exposed fish. However, mean HSIs of exposed fish at days 1 and 3 were significantly lower (*P*=0.002, SNK post-hoc test) than those for fish at days 7, 14 and 35. Means with the same letter do not differ significantly.

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Fig. 16. Nephritic somatic index, NSI (mean ± SEM, n=6) of control and verapamil exposed (500 µg l^{-1}) fathead minnow. Mean NSIs did not differ significantly (P>0.05, Kruskal-Wallis, Dunnett post hoc test) between the control and exposed fish. However, mean NSI of fish at day 14 was significantly (*P*=0.03, SNK post-hoc test) higher than that for the fish at day1. Means with same letter are not significantly different.
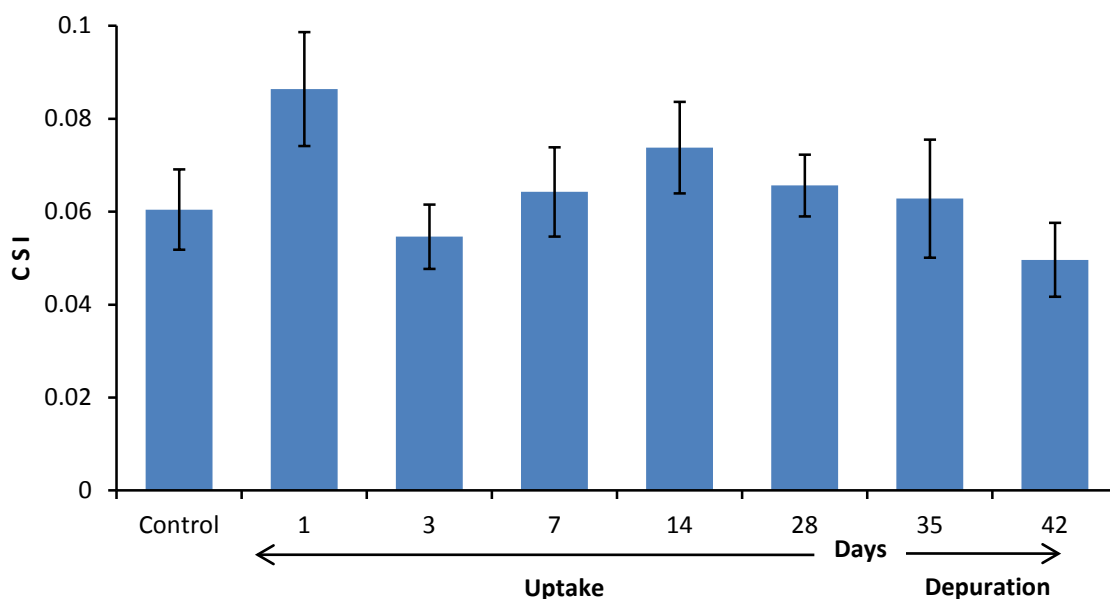
Fig. 17. Cardio somatic index, CSI (mean ± SEM, n=6) of control and verapamil exposed (500 µg l^{-1}) fathead minnow. Mean relative heart weights did not differ significantly (*P*=0.27, Kruskal-Wallis test) between the control and exposed fish.
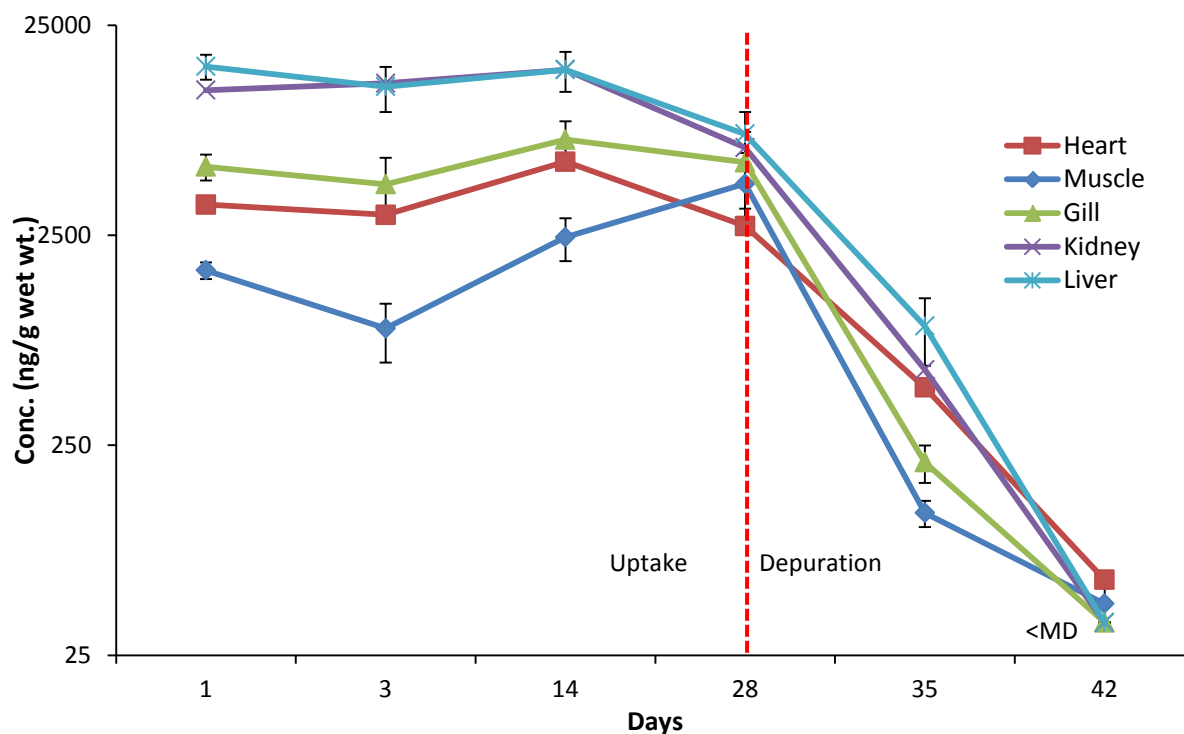
Fig. 18. Verapamil concentration (mean ± SEM ng g^{-1} wet wt., n=4-6) in muscle, gill, liver, heart and kidney tissues of fathead minnow exposed to 500 µg l^{-1}. Kidney (n=1-3) and heart (n=1) tissue results represent only the mean or a single value. There were no detectable levels (<MDL, 35 ng g^{-1}) of verapamil concentrations in gill, kidney and liver tissues of the fish depurated for 14 days.
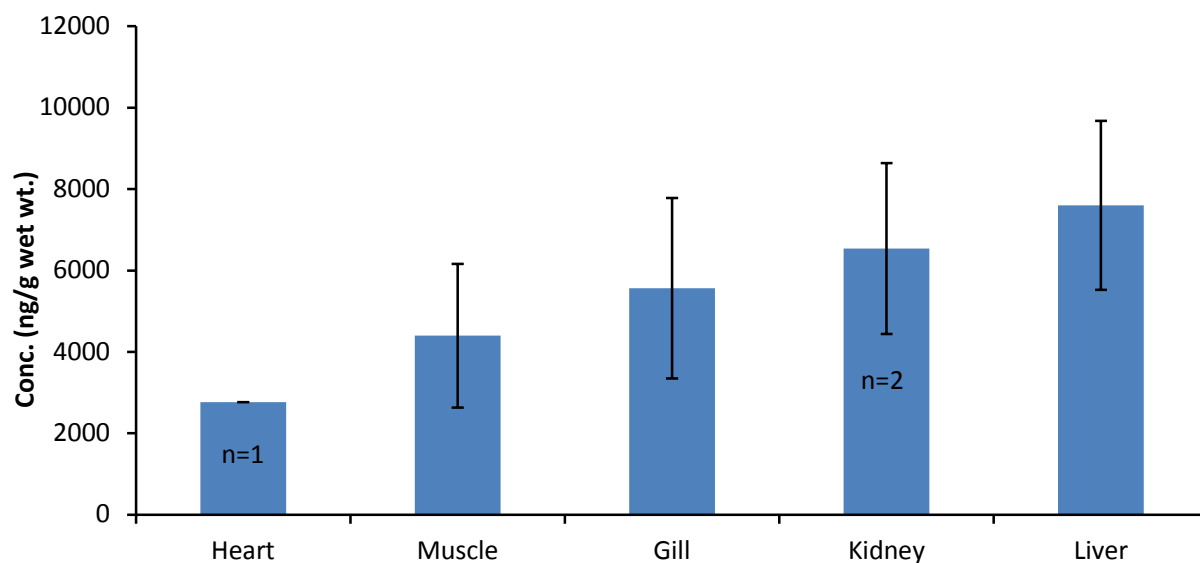
Fig. 19. Verapamil concentration (mean ± SEM, n=6) in tissues (ng g^{-1} wet wt.) of fathead minnow exposed to 500 µg l^{-1} for 28 days. The accumulation in the muscle, gill and liver tissues did not differ significantly (*P*=0.34, Kruskal-Wallis). Heart and kidney results were not included in the statistical analysis.
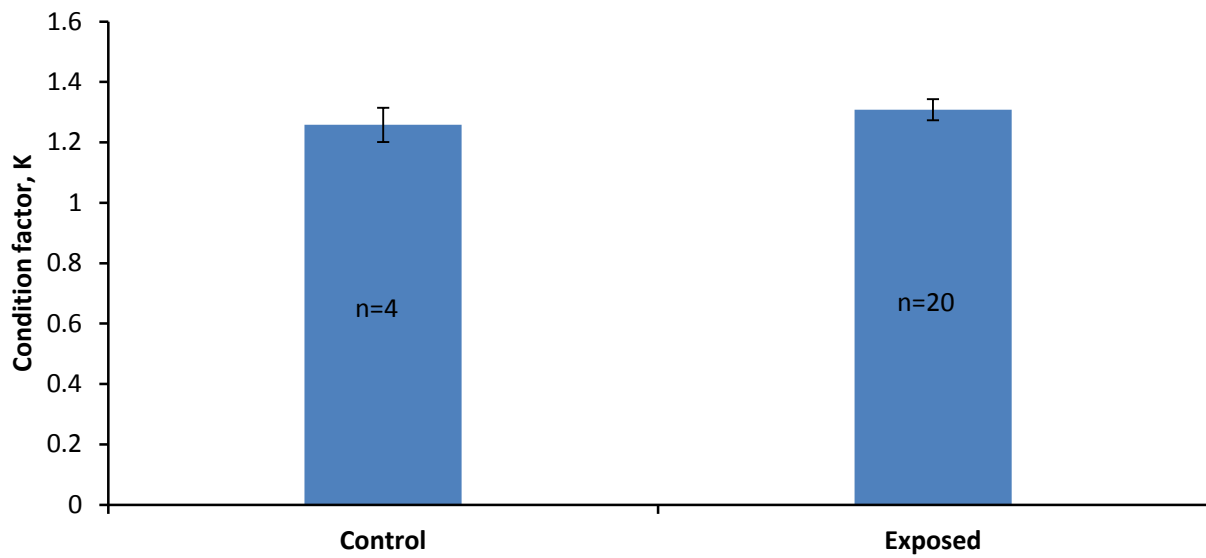
Fig. 20. Condition factor, K (mean ± SEM) of control and verapamil exposed (500 µg l^{-1}) channel catfish. Mean Ks did not differ significantly (*P*=0.72, independent t-test) between the groups.
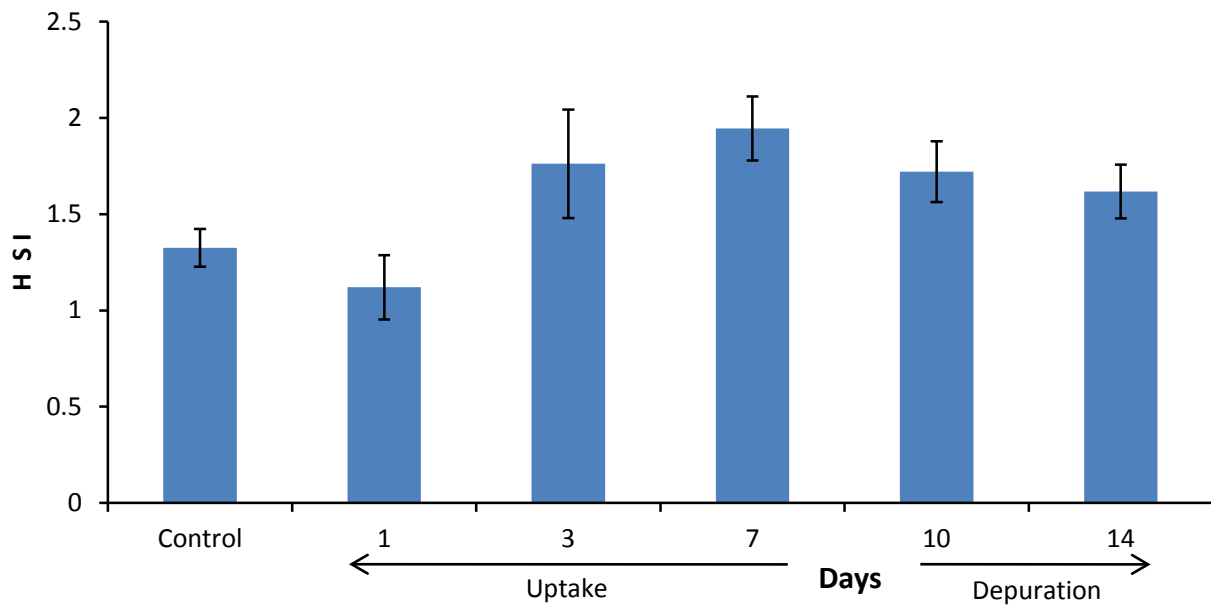

Fig 21. Hepatic somatic index, HSI (mean ± SEM, n=4) of control and verapamil exposed (500 µg l^{-1}) channel catfish. Mean HSIs did not differ significantly (P=0.07, Kruskal-Wallis test) between the control and exposed fish.
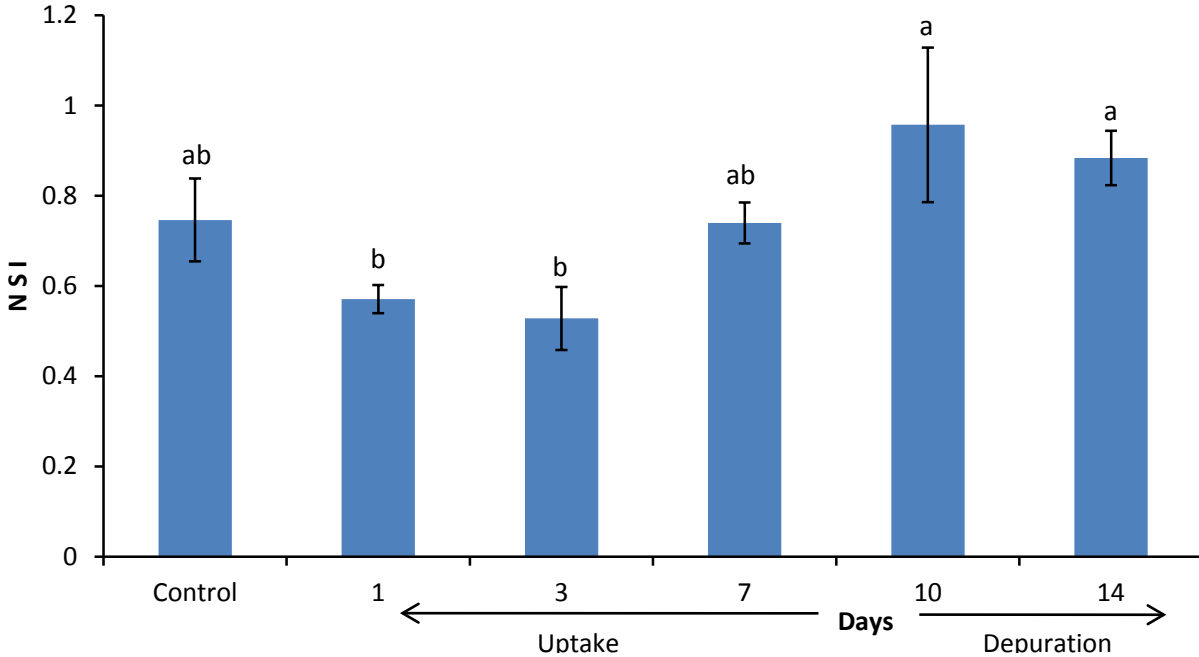
Fig 22. Nephritic somatic index, NSI (mean ± SEM, n=4) of control and verapamil exposed (500 µg l^{-1}) channel catfish. Mean NSIs of fish at days 1 and 3 were significantly lower (P=0.01, Kruskal-Wallis, SNK test) than those for the fish at days 10 and 14. However, mean NSI levels in the control and exposed fish were not significantly different. Means with the same letter are not significantly different.
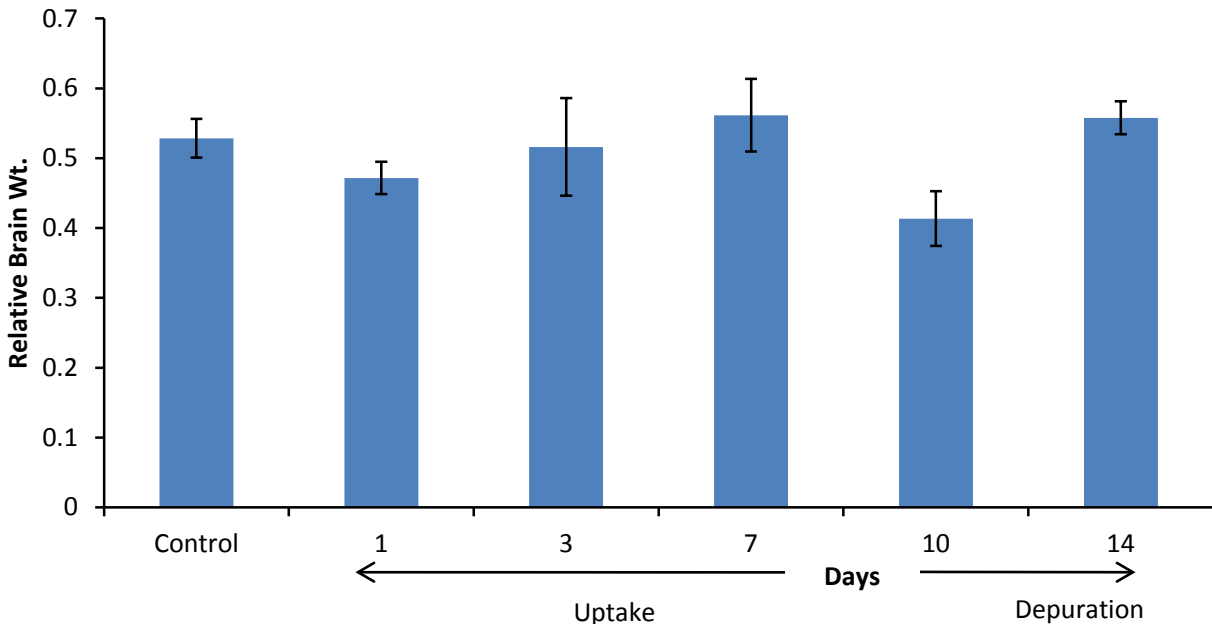

Fig 23. Relative brain weight (mean ± SEM, n=4) of control and verapamil exposed (500 µg l^{-1}) channel catfish. Mean brain weights did not differ significantly (P=0.13, Kruskal-Wallis test) between the control and exposed fish.
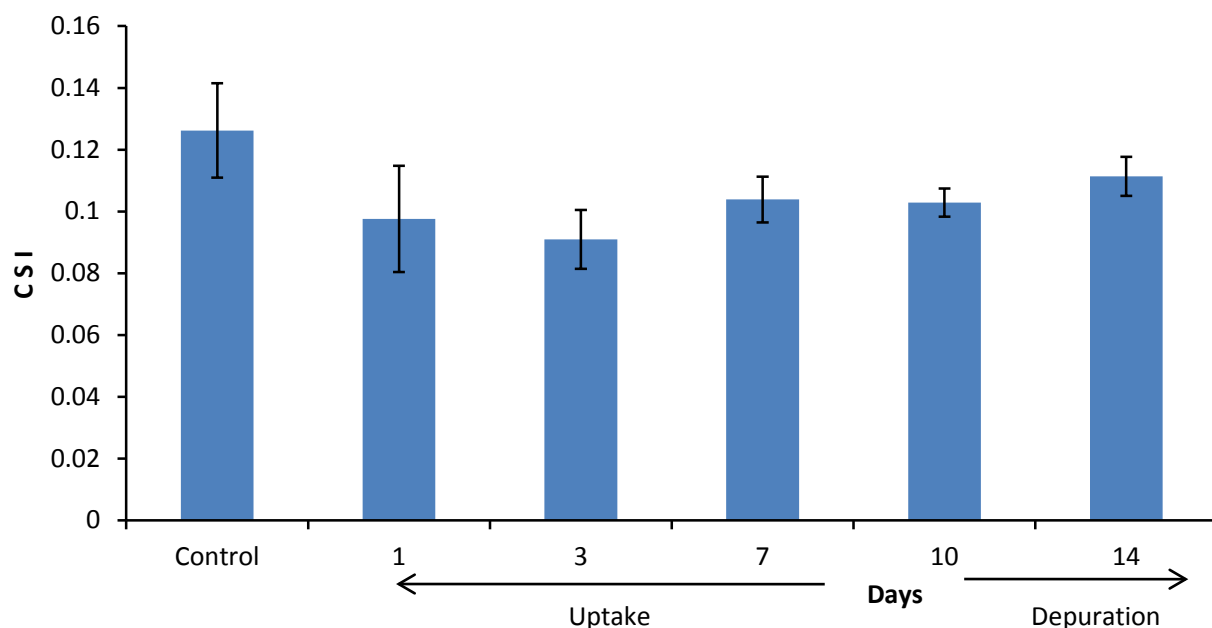
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Fig. 24. Cardio somatic index, CSI (mean ± SEM, n=4) of control and verapamil exposed (500 µg l^{-1}) channel catfish. Mean heart weights did not differ significantly (*P*=0.36, Kruskal-Wallis test) between the control and exposed fish.
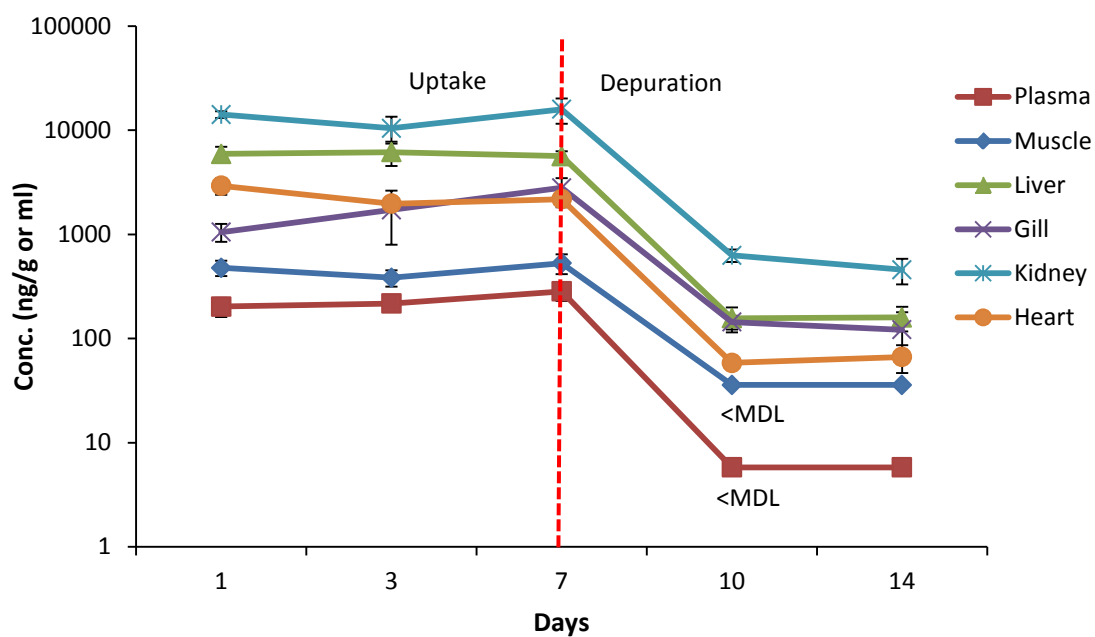

Fig. 25. Verapamil concentration (ng g^{-1} wet wt or ml plasma) in different tissues of channel catfish exposed to 500 µg l^{-1} for 7 days. There were no detectable levels of verapamil in plasma (<MDL, 6 µg l^{-1}) and muscle tissue (<MDL, 35 ng g^{-1}) of depurated fish.
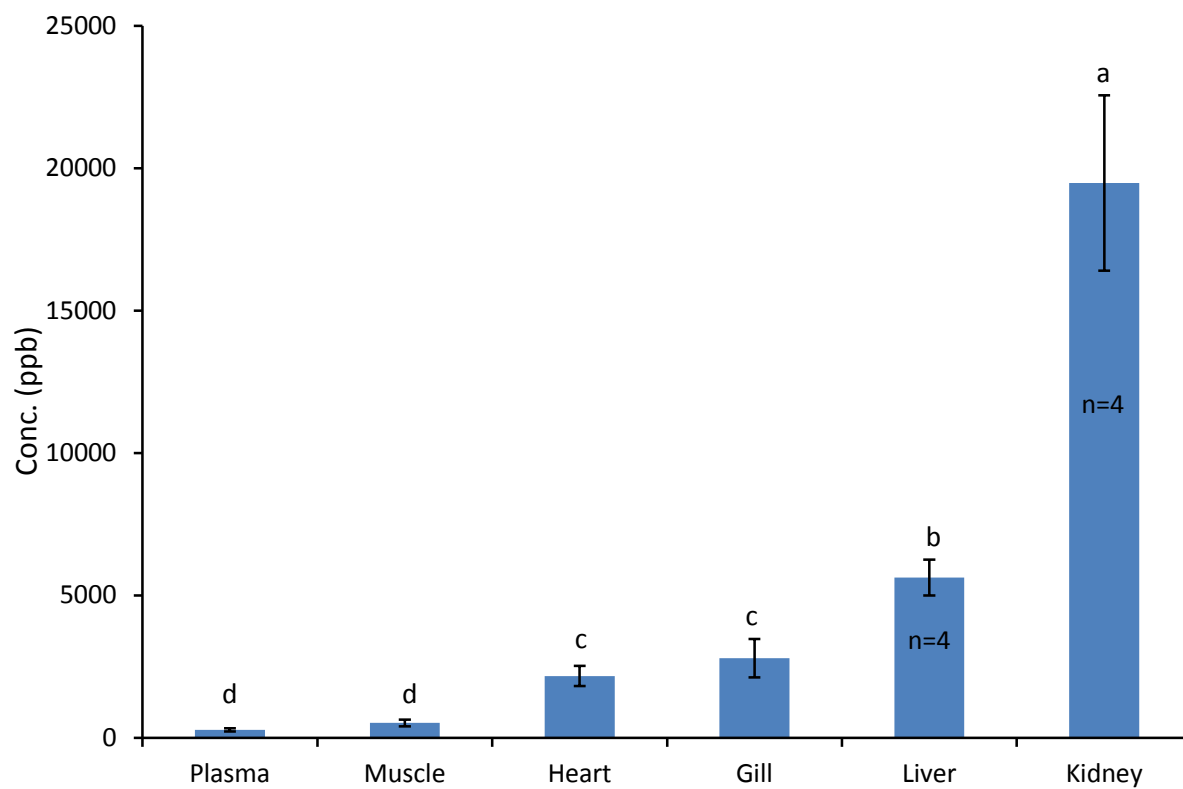
.
Fig. 26. Verapamil concentration (mean ± SEM, n=5) in plasma (µg l^{-1}) and tissues (ng g^{-1} wet wt) of channel catfish exposed to 500 µg l^{-1} for 7 days. Verapamil accumulation was significantly different (P=0.0002, Kruskal-Wallis, SNK test) among tissues. Means with the same letter are not significantly different.
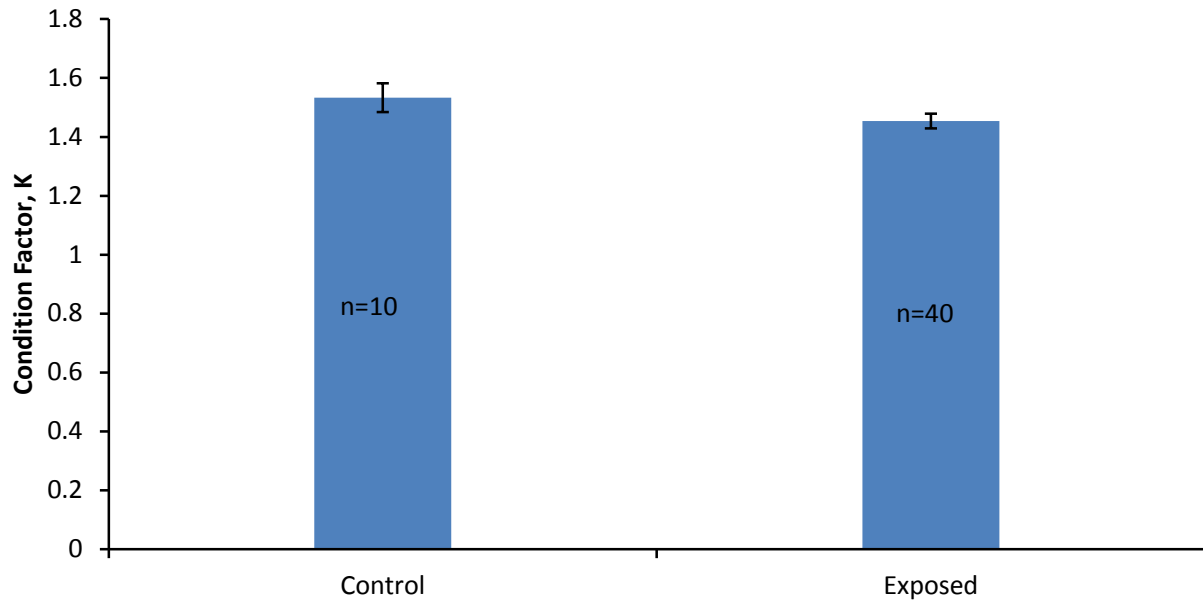
Fig. 27. Condition factor, K (Mean ± SEM) of control and clozapine exposed (50 µg l^{-1}) fathead minnow. Mean K values of the two groups were not significantly different (P=0.16, independent t-test).
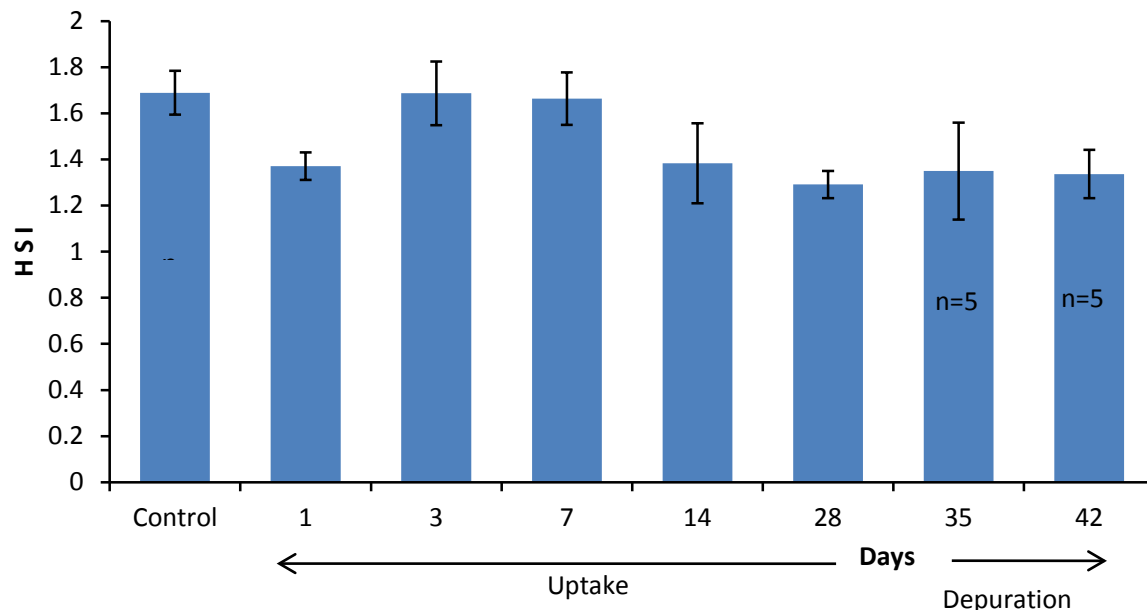

Fig. 28. Hepatic somatic index, HSI (mean ± SEM, n=6) of control and clozapine exposed (50 µg l^{-1}) fathead minnow. Mean HSIs did not differ significantly (P=0.059, 1-way ANOVA) between the control and exposed fish.
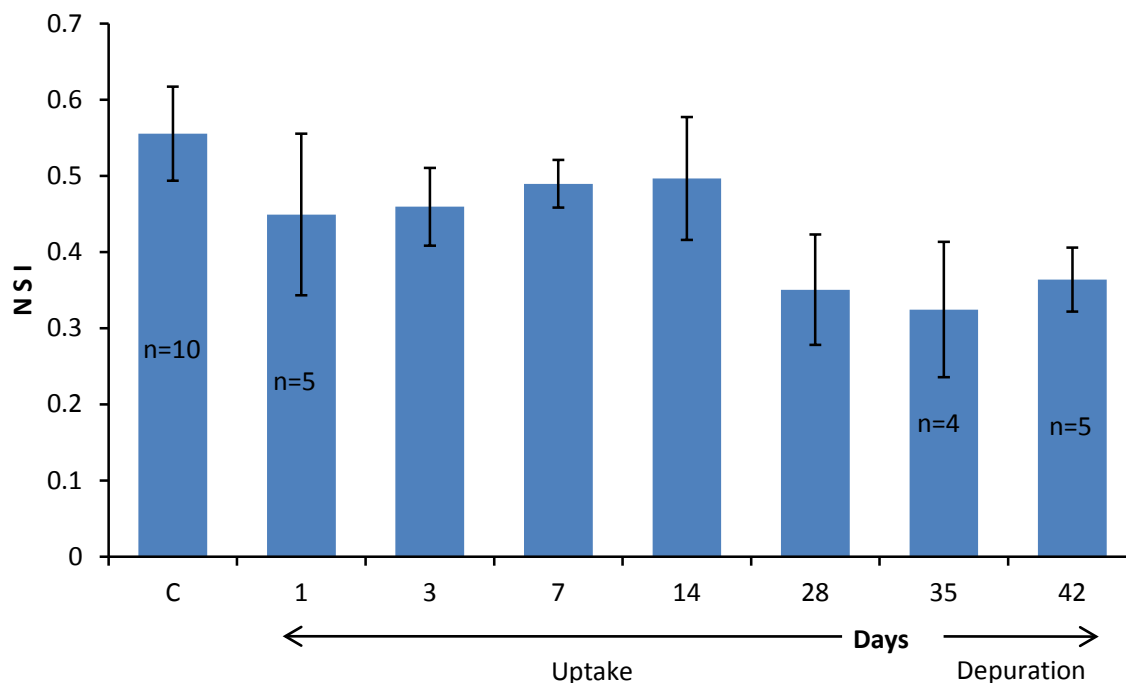
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Fig. 29. Nephritic somatic index, NSI (mean ± SEM, n=6) of control and clozapine exposed (50 µg l^{-1}) fathead minnow. Mean NSIs did not differ significantly (*P*=0.20, Kruskal-Wallis test) between the control and exposed fish.

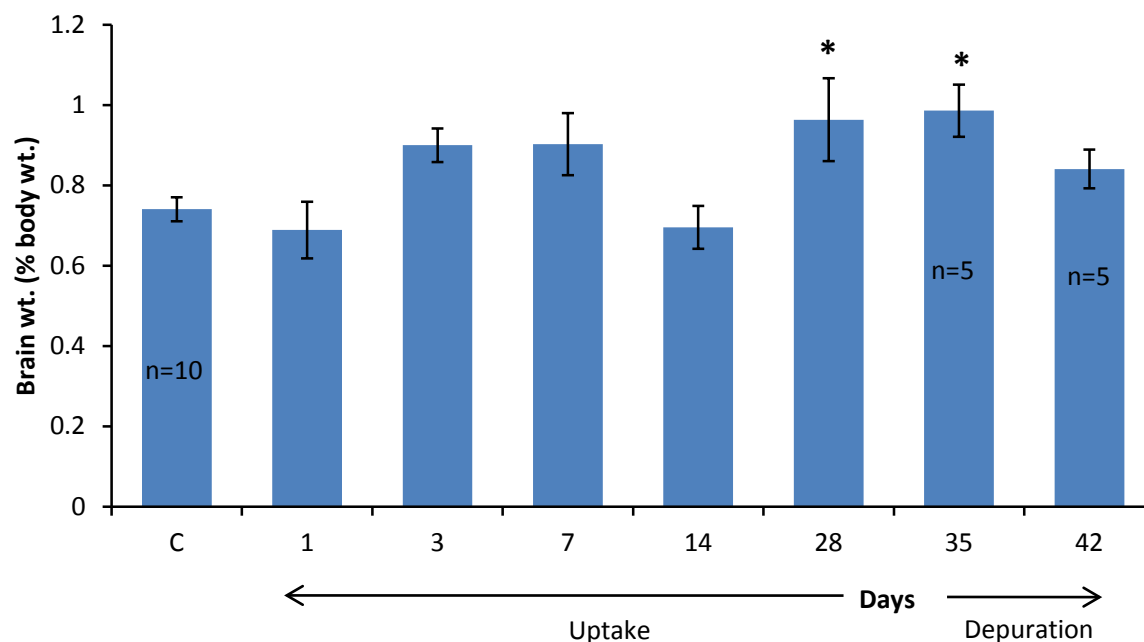**Fig. 30**. Relative brain weight (mean ± SEM, n=6) of control and clozapine exposed (50µg l^{-1}) fathead minnow. Mean brain weights were significantly higher (*P*=0.004, 1-way ANOVA, Dunnett's post-hoc test) in the exposed fish at days 28 and 35 than those of the control fish.
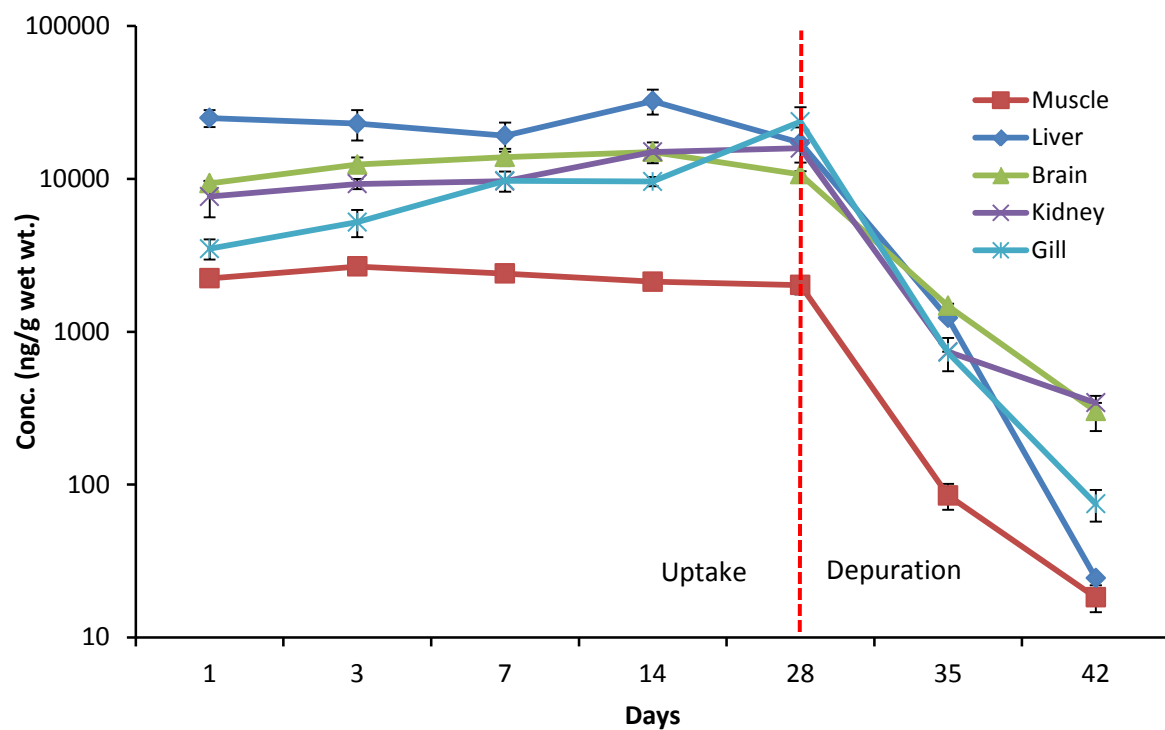
158

Fig. 31. Clozapine concentrations (ng g^{-1} wet wt) in different tissues of fathead minnow exposed to 50 µg l^{-1} for 28 days.
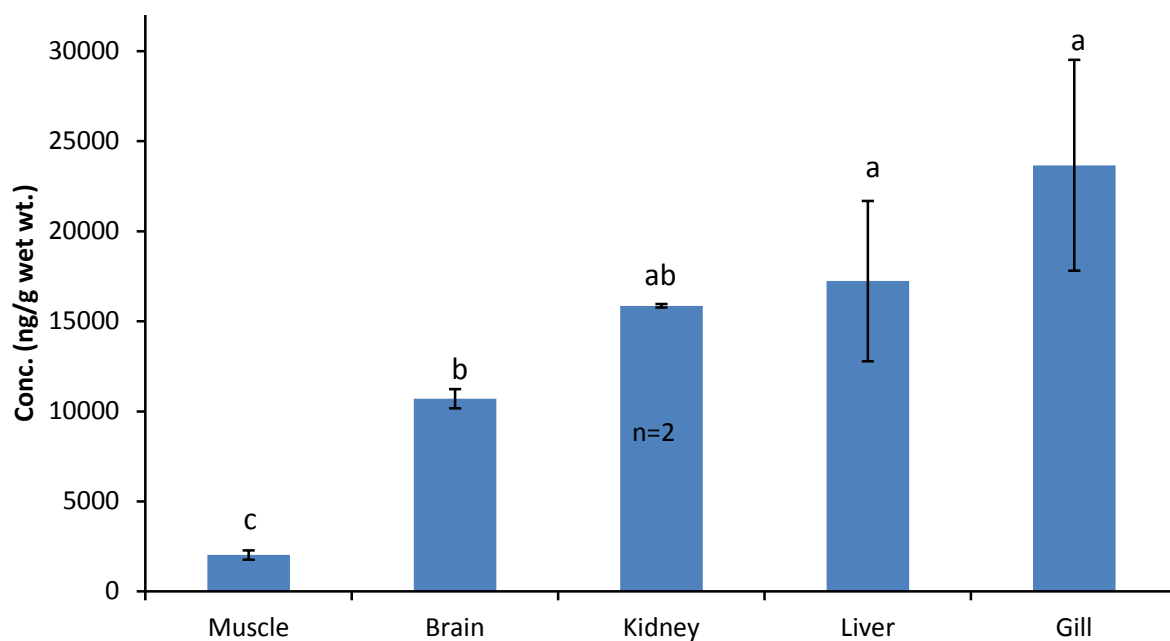

Fig. 32. Tissue-specific concentrations of clozapine (mean ± SEM, n=6) in fathead minnow exposed to 50 µg l^{-1} for 28 days. Mean concentrations differ significantly (P=0.001, Kruskal-Wallis, SNK post-hoc test) among tissues. Means with the same letter are not significantly different.

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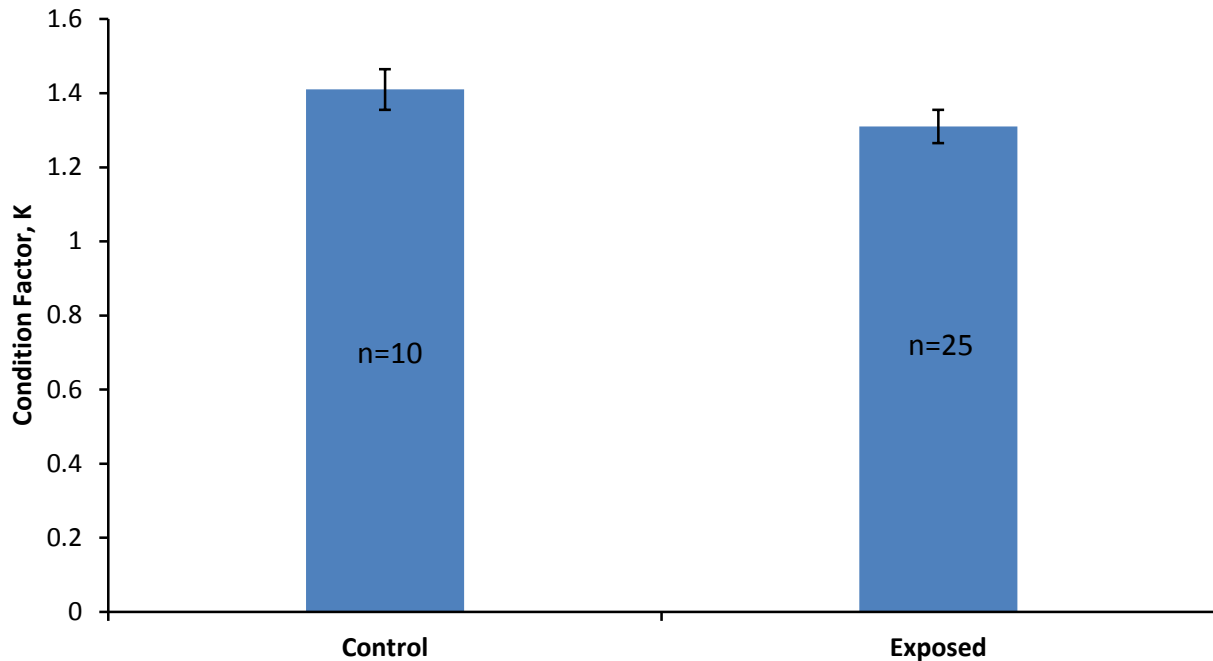
Fig. 33. Condition factor, K (mean ± SEM) of control and clozapine exposed (50 µg l^{-1}) channel catfish. Mean K values were not significantly different (P=0.19, independent t-test) in the two groups.

Fig. 34. Hepatic somatic index, HSI (mean ± SEM, n=5) of control and clozapine exposed (50 µg l^{-1}) channel catfish. Mean HSIs of the exposed fish at days 3, 10 and 14 were significantly higher (P=0.001, Krusal-Wallis, Dunnett's post-hoc test) than that of the control fish.
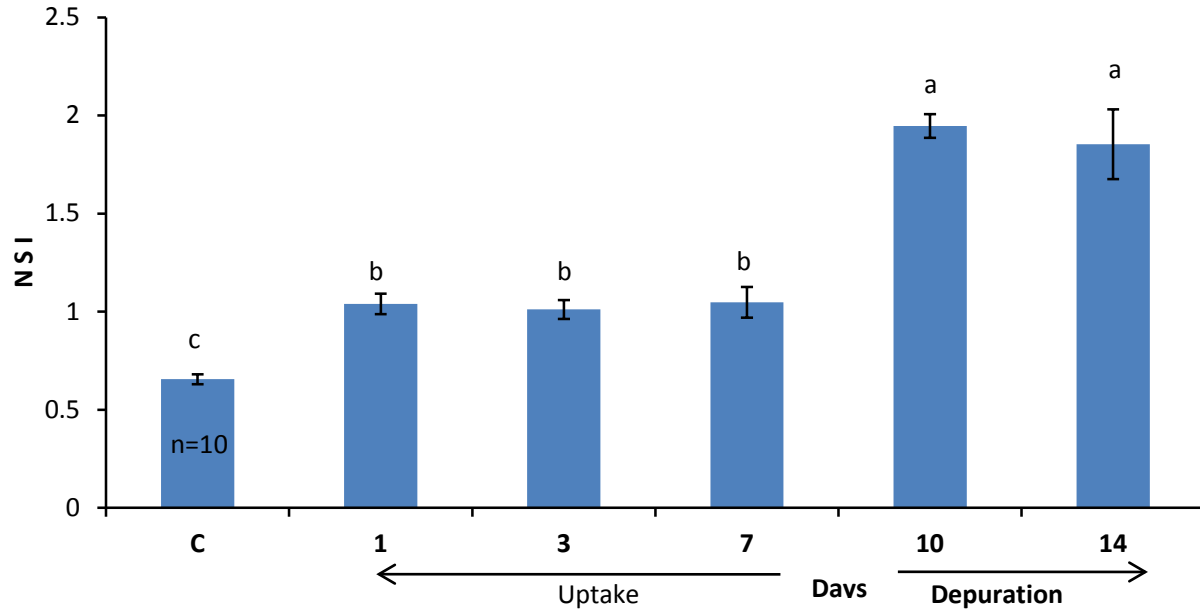
Fig. 35. Nephritic somatic index, NSI (mean ± SEM, n=5) of control and clozapine exposed (50 µg l^{-1}) channel catfish. Mean NSIs of exposed fish were significantly higher (*P*<0.001, 1-way ANOVA, SNK post-hoc Test) than that of the control fish. Means with same letter are not significantly different.
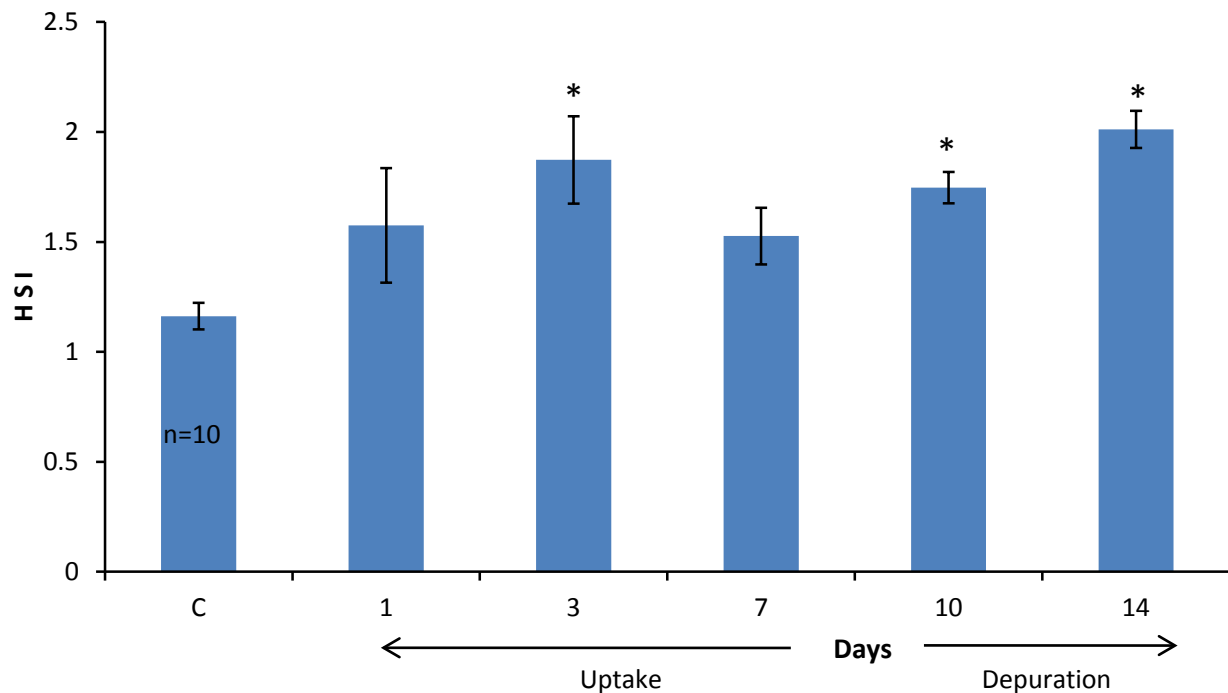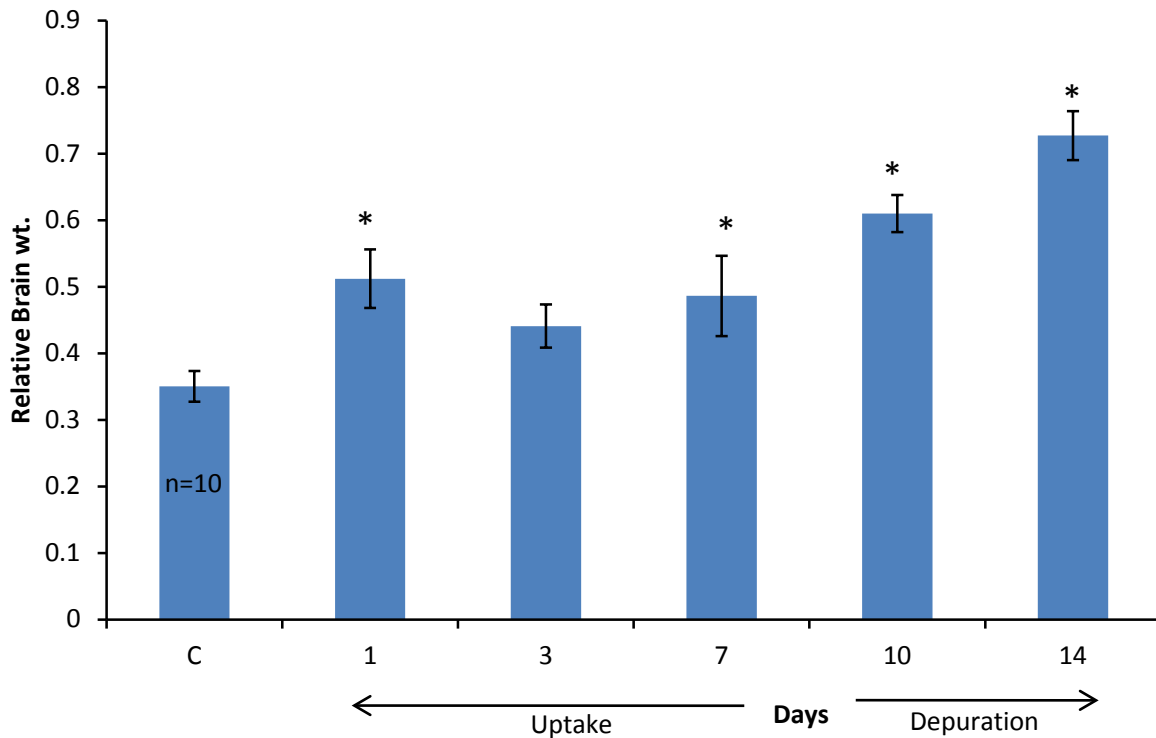

Fig. 36. Brain somatic indices, BSI (mean ± SEM, n=5) of the control and clozapine exposed (50 µg l^{-1}) channel catfish. Mean relative brain weights of the exposed fish (except for day3) were significantly higher (*P*=0.0003, Krusal-Wallis, Dunnett's post-hoc Test) than that of control fish.
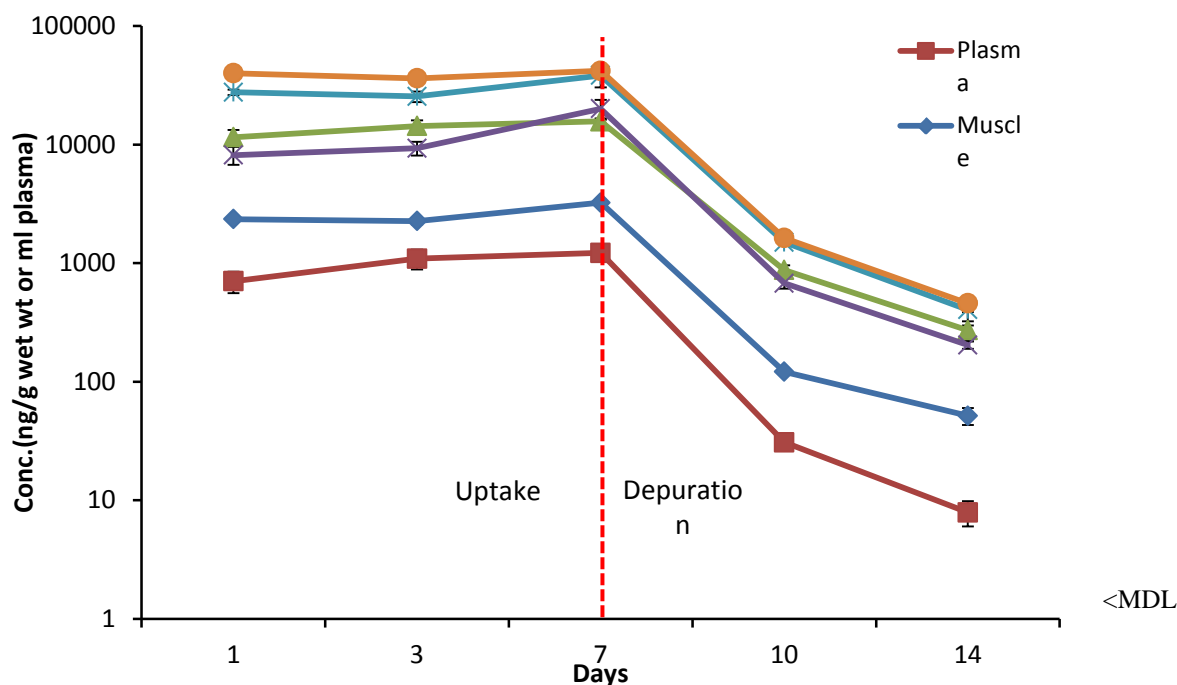
Fig. 37. Clozapine concentration (mean ± SEM, n=4-5) in different tissues of channel catfish exposed to 50 µg l^{-1} for 7 days. No detectable levels of clozapine (<MDL) was found in the plasma of the fish depurated for 14 days.
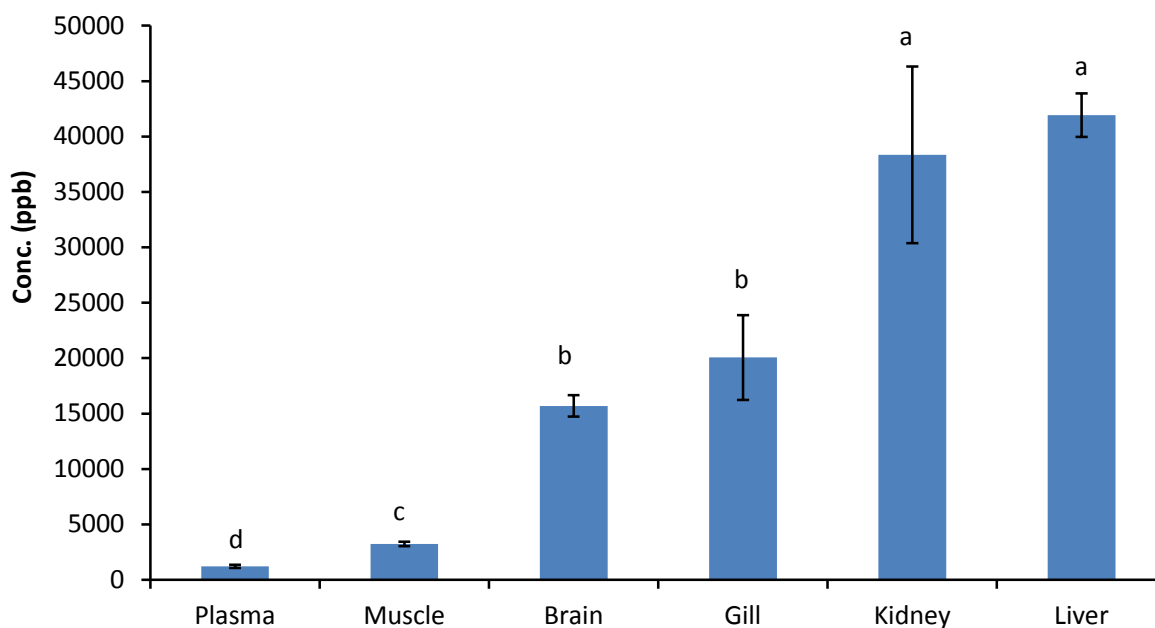
Fig. 38. Clozapine concentration (mean ± SEM, n=5) in plasma (µg l^{-1}) and tissues (ng g^{-1} wet wt) of channel catfish exposed to 50 µg l^{-1} for 7 days. Clozapine uptake was significantly different (*P*=0.0002, Kruskal-Wallis, SNK post-hoc Test) among tissues. Means with the same letter are not significantly different.

CHAPTER 8

BIOCONCENTRATION OF FENOFIBRATE IN FISH

8.1   Introduction

Lipid lowering agents constitute an important therapeutic option for reducing the risks

associated with coronary heart diseases. Statins and fibrates represent two major classes of drugs

used in treating dyslipidemia. Globally, these classes of drugs are among the heavily consumed

pharmaceuticals. For example, among the top 200 prescription drugs of 2009, atorvastatin

(Lipitor) was a blockbuster product with sales over \$7.5 billion (Lindsley, 2010). While statins

selectively inhibit cholesterol synthesis, fibrate class drugs are used to reduce the levels of both

cholesterol and triglycerides in the blood plasma. Fibrates (fibric acid derivatives) bind to

peroxisome proliferator-activated receptors (PPARs) and activation of these nuclear receptors

(especially, PPAR alpha type) induces lipolysis and regulates apoprotiens.

Among the fibrate class of drugs, clofibric acid, benzafibrate, gemfibrozil are the most

studied compounds in terms of their environmental occurrence, persistence and toxicity

(Mimeault et al., 2005; Emblidge and DeLorenzo, 2006; Hernando et al., 2007; Zurita et al.,

2007). However, such targeted studies are lacking for fenofibrate (FFB), another important

widely used fibrate drug. FFB has been detected in the sewage treatment plant (STP) effluents in

low ng l^{-1} levels (Andreozzi et al., 2003). Fenofibrate is readily hydrolyzed to its major

transformation product, fenofibric acid (FFA), which is also pharmacologically active. FFA is

also present in the environment and the levels often exceed that of the parent compound

(Daughton and Ternes, 1999). Fibrate drugs including FFB and their metabolites resist

biological degradation in STPs resulting in their increased environmental persistence (Ternes,

1998; Stumpf et al., 1999). FFB is a highly hydrophobic (log K_{ow} > 5) compound (Table 26) and

has been shown to be toxic to aquatic organisms. Much of the ecotoxicity data however is limited to algae, bacteria, rotifers or microcrustaceans (Hernando et al., 2007; Isidori et al., 2007; Rosal et al., 2010). In general, the aquatic toxicity levels of FFB ranged from few mg l^{-1} to sub µg l^{-1} levels with considerable variability among the species. Chronic tests on FFB have shown daphnia to be more sensitive organisms (Isidori et al., 2007). One study has reported toxic effects of FFB in an *in vitro* testing with fish hepatocytes (Laville et al., 2004). However, systematic long-term exposure studies on aquatic vertebrates are non-existent.

The environmental presence, physicochemical characteristics and toxicity in lower aquatic species increase concerns over FFB's ability to cause adverse effects in fish. This is significant because a) it has high bioaccumulative potential and b) PPAR receptors have been identified in fish (Andersen et al., 2000; Gunnarsson et al., 2008). Both these factors imply that FFB has a potential to cause pharmacodynamic activity in fish similar to that in mammals. The potential for adverse toxicity of FFB in fish could be predicted from the knowledge of its uptake kinetics. This is because uptake of a chemical leads to build-up of critical internal tissue concentrations that may lead to toxic effects. For this reason, a priori knowledge on bioaccumulative potential of pharmaceuticals is considered important in risk assessments.

The objective of the present study was to determine the tissue-specific bioconcentration potential of FFB in two fresh water species, fathead minnow (*Pimephales promelas*) and channel catfish (*Ictalurus punctatus*). Two bioconcentration factor (BCF) testing approaches were used: a long-term (28-day) study with fathead minnow and a reduced (14-day) test with catfish. The reduced testing was used 1) to obtain information on the bioconcentration potential of FFB in the plasma and 2) to determine the usefulness of reduced testing approach in BCF assessments.

8.2   Experimental

8.2.1   Chemicals

Fenofibrate (FFB, 2-[4-(4-Chlorobenzoyl)phenoxy]-2-methyl-propanoic acid 1-methylethyl ester, CAS#49562-28-9) and Fenofibrate-d6 (FFB-d6, 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl-propanoic acid-d6 1-methylethyl ester) were purchased from Toronto Research Chemicals (TRC; North York, ON, Canada). HPLC grade solvents hexane (HEX), ethyl acetate (EA), dichloromethane (DCM), dimethyl formamide (DMF) and acetone were obtained from Fisher Scientific (Houston, TX). Tricaine mesylate (MS-222, ethyl 3-aminobenzoate methanesulfonic acid, CAS# 886-86-2) was purchased from Sigma-Aldrich (St. Louis, MO).

8.2.2   Fish Exposures

Adult fathead minnow (~ 3 g) and juvenile channel catfish (~ 40 g) of either sex were obtained from UNT aquatic toxicology facility and Pond King Inc. (Gainesville, TX) respectively. After a week of acclimation to the standard laboratory conditions, fathead minnow (n=49) and catfish (n=35) were randomly distributed and exposed to 25 µg FFB l^{-1} for 28 and 7 days respectively using a continuous flow-through exposure. After the exposure period, fathead and catfish were depurated in clean tanks for 14 and 7 days respectively.

8.2.3   Sampling Plan

Muscle, liver, gill, kidneys and blood samples were collected at the end of defined sampling points. Frequency of fish sampling, tissue and blood collection, data collection (fish weights, fork length, tissue weights etc.), determination of condition factors and somatic indices

are provided elsewhere (Chapter 4). All fish handling methods followed the Institute's Animal Care and Use Committee (AUCC) guidelines.

8.2.4 FFB Extraction

Before extraction, plasma (200 µl) and tissues (0.2 g) were fortified with 5 ng d6-FFB internal standard (ISTD). Plasma samples were initially treated with ice-cold acetone (10 ml), centrifuged to separate the organic layer, evaporated, extracted the contents back into 1+1 (v/v) hexane/ethyl acetate (hitherto referred to as the "extraction solvent"), evaporated again and finally reconstituted in 100 µl DCM. Tissue samples were homogenized in 4 ml extraction solvent using a bead beater and the extract was filtered using 0.45 µm PTFE membrane syringeless filters. The extracts were evaporated to dryness, lipid weights were determined gravimetrically and the dried residues were solubilized in 100 µl DCM.

8.2.5 GC/MS Analysis

Fenofibrate extracted plasma and tissues were analyzed by GC/MS. The 100 µl sample extracts were auto-injected (2 µl) to separate FFB in Agilent 6890N GC fitted with a 30m x 0.25mm x 0.25µm EC™- 5 capillary column (Alltech, Deerfield, IL). Ultrapure helium (Air Liquide, Houston, TX) was used as carrier gas with an initial flow of 1.2 ml min^{-1} and an average velocity of 38 cm sec^{-1}. The sample injections were made in pulsed splitless mode at 265º C and the separation was achieved using the following temperature program for the GC oven: initially at 40ºC for 2 min, increased to 200ºC at 20º C min^{-1} with no hold time followed by final ramp to 300ºC at 8º C min^{-1} and held at this temperature for 10 min, with a total GC run time of 32.5 min. After separation in the GC column, FFB was identified from the mass spectra obtained by

electronic impact ionization (70ev; positive ion polarity) in a mass selective detector, MSD 5973

(Agilent, Palo Alto, CA) that was operated under selected ion monitoring (SIM) with a transfer

line temp of 280º C. The MS quadrupole and source temperatures were maintained at 150º C and

230º C respectively. Quantification of FFB in the test samples was achieved from an eight-point

calibration curve generated by plotting (linear curve fit) the relative response factors and the

concentrations (4000- 31pg/µl) (Fig A11). The following characteristic ions (m/z) each with

dwell time of 50 m sec. are used in the quantitative analysis: FFB- 232, 273, 360; FFB-d6-279,

366 (underlined ions used for quantification) (Fig A6).

8.2.6   BCF Estimation and Data Analysis

Tissue-specific FFB uptake levels (μg l^{-1} or ng g^{-1}) were used to compute the proportional

and kinetic BCFs. Nominal exposure concentration was used in the estimates. The procedures for

BCF determination and statistical approaches for the data analyses are detailed in Chapter 4.

8.3   Results

8.3.1   Fathead Minnow Test

8.3.1.1 Condition Factor and Somatic Indices

Condition factor (K) of FFB exposed fish was significantly lower (P=0.019) than that of

the control fish (Fig 39). Reduced condition factor for the exposed fish indicates possible

chemical stress in the fish. There were no significant differences (P>0.05) in the relative liver

and kidney weights of the control and exposed fish (Figs 40 and 41). However, SNK post hoc

analysis on the mean HSIs of the exposed fish indicated that liver weights of fish at 7 days,

however, were significantly (P=0.015) higher than those for the fish sampled on days 1 and 28.

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8.3.1.2 FFB Concentration in Tissues and BCFs

FFB uptake was measured at the end of the exposure (28 days) and significant differences (P=0.006) were noticed in the tissue-specific accumulation levels (Fig 42). Kidney and gill tissues had higher accumulation levels than that of liver, which in turn accumulated higher levels of the test compound compared to the muscle tissue. FFB uptake levels at 1, 3, 7 and 14 days post-exposure, as well as in the depuration phase results varied widely (data not shown). This inconsistency in the accumulation data prevented the estimation of kinetic BCFs and hence the BCFs (28 day) were determined at the end of the exposure. The results are presented in Table 27. The BCFs ranged between 7 and 57 with the following trend: muscle < liver < gill < kidneys.

8.3.2   Catfish Test

8.3.2.1 Condition Factor and Somatic Indices

Mean condition factor (K) of FFB exposed catfish was significantly lower (P=0.0004) than that of the control fish (Fig 43). Additional information on the physiological status was obtained from the relative liver and kidney weights that were significantly higher (P<0.0001) in the exposed fish compared to those of the unexposed fish (Figs 44 and 45).

8.3.2.2 FFB Concentration in Tissues

FFB uptake (ng ml^{-1} or g^{-1}) levels in plasma, muscle, liver, gill and kidney tissues are presented in Fig 46. FFB concentration in the plasma increased from 31 to 97 µg l^{-1} during the exposure, but no detectable levels were found in the plasma of depurated fish. The uptake level in the liver peaked at 3 days post exposure followed by 74% reduction in the concentration towards the end of exposure (7 days). While the uptake by the muscle tissue increased during

the 7d-exposure, gill and kidneys had their maximum uptake after 3 days of exposure. From days

1 through 3, FFB uptake levels in the kidney, gill and muscle tissues increased by 77, 81 and

87% for respectively. The 7-d FFB accumulation levels differed significantly (P=0.002) among

the tissues and SNK post hoc test showed the following trend in the uptake levels: plasma/liver <

gill/kidney/muscle (Fig 47). Accumulation was highest in the muscle tissue, though not

significantly different from gill and kidney uptake levels. FFB was not detected in the plasma (<

14 µg l^{-1}) and tissues (< 67 ng g^{-1}) of the control fish. Measurable analyte levels were observed in

the gill, kidney and muscle tissues of the fish depurated for 3 days (i.e. day 10) while liver and

plasma of these fish had no detectable levels of the chemical. However, at the end of the

depuration period, FFB was not detected in any of the tissues.

8.3.2.3 BCFs

Tissue-specific kinetic, 7-day proportional and lipid normalized BCFs are presented in

Table 28. BCFs for the muscle, gill and kidneys were approximately 5-18 times greater than

those estimated for the plasma and liver tissues. The BCF results imply that FFB has the highest

potential to bioconcentrate in the muscle compared to other tissues. The lipid based BCFs ranged

between 63 and 791.

8.3.3   QC Results

Unexposed fish plasma and tissues were spiked with 500 µg l^{-1} analyte (Fig A16) and the

extraction efficiencies of the analytical methods were determined from the percent recoveries

that ranged between 85 and 130 (Table 29).

8.4 Discussion

8.4.1 FFB Effects on Condition Factor and Somatic Indices

In our study, fenofibrate exposure reduced the condition factors of both the test species. No previous reports on FFB's effects on aquatic vertebrates exist to support this result. In one study, Isidori et al., (2007) reported a lowest observed effect concentration (LOEC) of 78 µg l^{-1}, which inhibited the growth in *C. dubia*. The authors also reported increased sensitivity of daphnia to fenofibric acid. Nevertheless, it is difficult to make direct comparison between FFB's adverse growth effects on invertebrate and fish, as the avenues to handle the toxicant exposure are different in each species.

FFB did not impact the liver and kidney weights of the fathead minnow while its exposure in the catfish resulted in the increased organ weights. This varied trend in the somatic indices in the two fish species could be attributed to their relative sensitivities towards FFB. Although there are no specific studies on FFB's effects on hypertrophy of fish organs (especially liver and kidney), one study has reported no significant changes in the gonadal somatic index (GSI) of gold fish exposed (1.5-1500 µg l^{-1}) to gemfibrozil, another common fibrate drug (Mimeault et al., 2005). On the contrary, FFB exposure at high concentrations (~ 100 ppm) in rats and mice resulted in significant increase (>300%) in the liver weights (Nishimura et al., 2007). Also, rodents treated with PPARα agonists developed hepatocarcinogenicity due to extensive cell proliferation (Cheung et al., 2004). A recent study has reported genotoxic and mutagenic effects of fibrate drugs on the aquatic organisms (Isidori et al., 2007).

8.4.2 Tissue Uptake and BCFs

The study demonstrated differential accumulation of FFB in tissues of fathead minnow

and catfish. The uptake in various tissues had the following trend: kidney > gill > liver > plasma. The results on the muscle uptake levels were unexpected. While the FFB uptake was lowest (compared to other tissues) in the muscle tissue of fathead minnow, the opposite was observed in the catfish. Although it is difficult to explain this unusual result (compared to the tissue uptake results of other pharmaceuticals studied in this dissertation), this was the only BCF test in which nominal exposure levels were used in BCF computations due to wide variability (2-580%) in the measured test concentrations. In spite of this, the uptake levels in the liver, gill and kidneys in both the fishes were close. The lowest accumulation potential was observed in the plasma. As FFB is a highly non-polar compound, low plasma concentrations can be expected. Also, the mammalian pharmacology of FFB indicates that it is rapidly hydrolyzed to FFA and no significant amounts of FFB are detected in the plasma (www.rxlist.com).

The tissue-specific BCFs ranged between 4 and 90. The physicochemical properties of FFB, (primarily hydrophobicity) suggest a high potential for its bioaccumulation. However, as noted previously, FFB is readily converted to its major metabolite FFA, which actually manifests the pharmacological actions of FFB. Since this study was not intended to conduct the metabolite profile of FFB, it is difficult to consider if FFB metabolism responsible for the reduced fish bioconcentration. The low bioaccumulative potential of a chemical may also be attributed to its propensity for uptake into an organism. Studies have shown that highly lipophlilic substances with large molecular mass (like FFB), may not easily permeate through the membranes limiting their ability for uptake (Jonker and Van der Heijden, 2007).

The tissue-specific BCF estimates for the fathead and catfish did not differ much. For example, the BCF levels for the liver, gill and kidneys of the two test species differed by absolute values of 11, 6 and 8 respectively. These differences are insignificant in terms of

bioaccumulation levels that are usually compared in orders of magnitude. Thus, the reduced BCF test has yielded similar test results as the long-term experiment, suggesting its potential for its use as a prioritization tool in environmental risk assessments.

In conclusion, although fenofibrate has shown potential to accumulate in the tissues of fish, the BCFs obtained in this study indicate less concern in terms of the current regulatory guidelines (i.e. BCF $\geq$ 2000). Also, the less resource-demanding short term BCF test has a potential for use in rapid screening methods for prioritizing pharmaceuticals for risk analysis.

8.5   Chapter References

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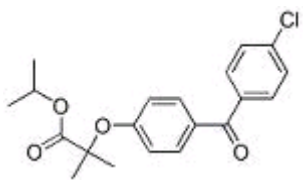
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Table 26

Physicochemical characteristics of fenofibrate (retrieved from EPISUITE (U.S. EPA, 2009).

Property	Description
CAS #	49562-28-9
Molecular weight	360.8
Partition Coefficient, log Kow	5.19
log Dow (Log P @ pH 7.0)	5.10£
Water solubility @ 25°C, mg l-1	0.19
Vapor pressure @ 25°C, mm Hg	6.2E-07
Henry's Law Constant @ 25°C, atm-m3 mol-1	1.5E-06
Structure	

£ (Yamashita et al., 2009)

Table 27

Tissue-specific fenofibrate BCFs ($l\,kg^{-1}$ wet wt) for fathead minnow exposed to 25 µg l^{-1} for 28 days.

Tissue	28 d BCF (wet wt.)
Muscle	7.0
Liver	15.9
Gill	45.0
Kidney	56.6

Table 28

Tissue-specific fenofibrate BCFs for channel catfish exposed to 25 µg l^{-1} for 7 days.

Tissue	BCFk	28 d BCF	
		Wet wt. basis	Lipid normalized
Muscle	90	90.8	791
Liver	17	4.7	63.3
Gill	86	51	613
Kidney	70	64.7	558
Plasma	4.7	3.9	---

Table 29

Fenofibrate MDLs (µg l^{-1} or ng g^{-1}) and percent recovery (mean ± SD) for different tissue matrices.

Test	Matrix	n	Recovery (%)	MDL
Fathead	Muscle	2	88.3 ± 3.2	
	Liver	2	101.7 ± 5.6	
	Gill	2	84.8 ± 0.6	
	Kidney	1	98.3	
Catfish	Plasma	2	95 ± 1.9	14.8
	Tissue	8	129 ± 34.4	67.7
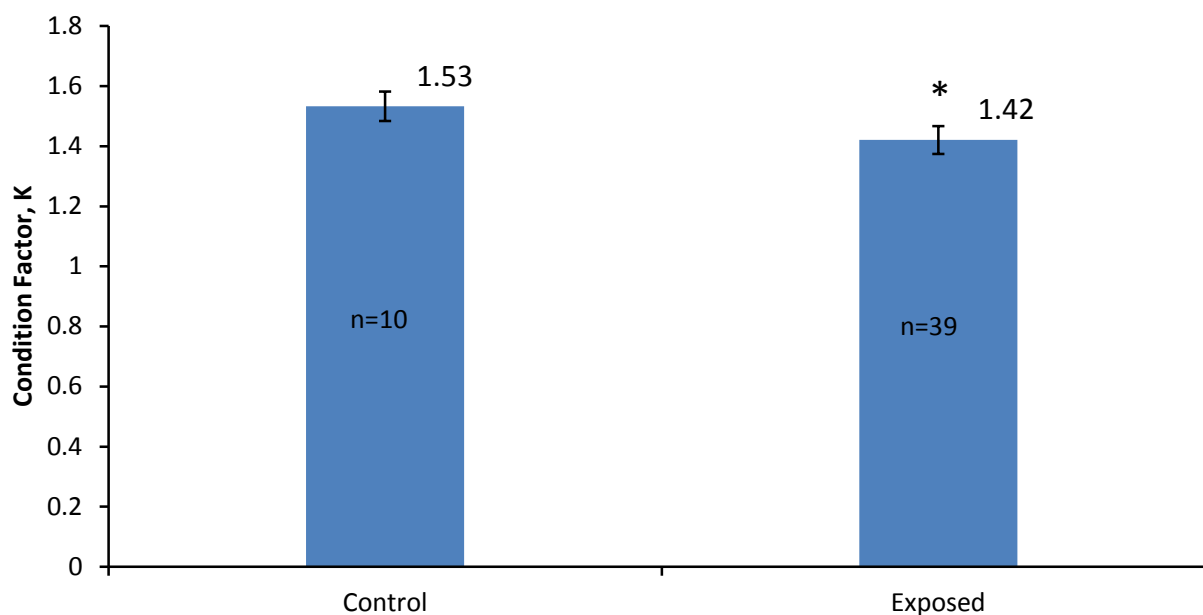
Fig. 39. Condition Factor, K (mean ± SEM) of control and fenofibrate exposed (25 µg l^{-1}) fathead minnow. Mean K of exposed fish was significantly lower (P =0.019, Mann Whitney U test) than that of the control fish.
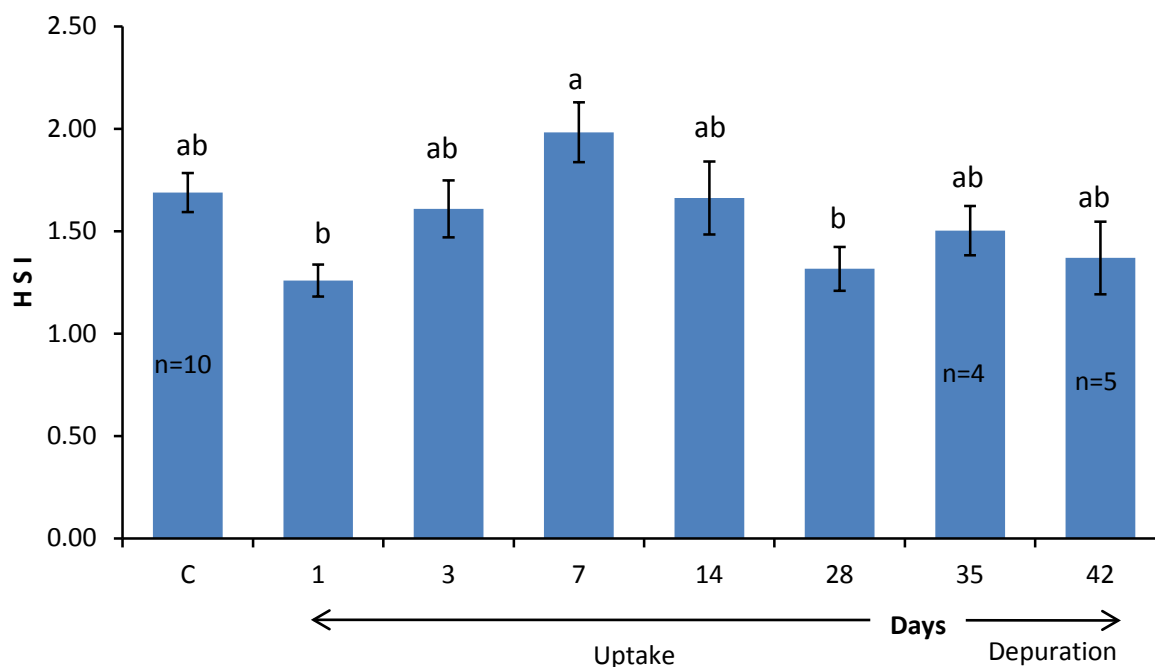

Fig. 40. Hepatic somatic index, HSI (mean ± SEM, n=6) of control and fenofibrate exposed (25 µg l^{-1}) fathead minnow. Mean HSIs did not differ significantly between the control and exposed fish (P>0.05, Kruskal Wallis, Dunnett's test). However, HSI levels for fish sampled at days 1 and 28 were significantly lower than that for the fish at day 7 (P=0.015, SNK post-hoc test). Means with the same letter do not differ significantly.
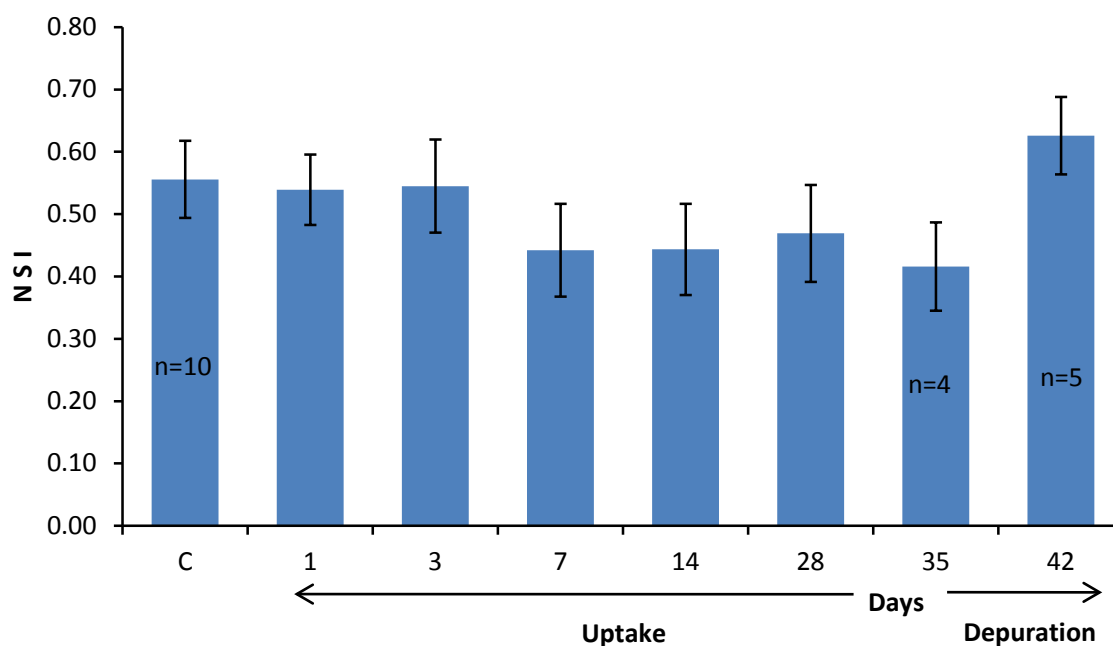
176

Fig. 41. Nephritic somatic index, NSI (mean ± SEM, n=6) of control and fenofibrate exposed (25 µg l^{-1}) fathead minnow. Mean NSIs did not differ significantly (*P*=0.42, Kruskal Wallis, Dunnett's test) between the control and exposed fish.
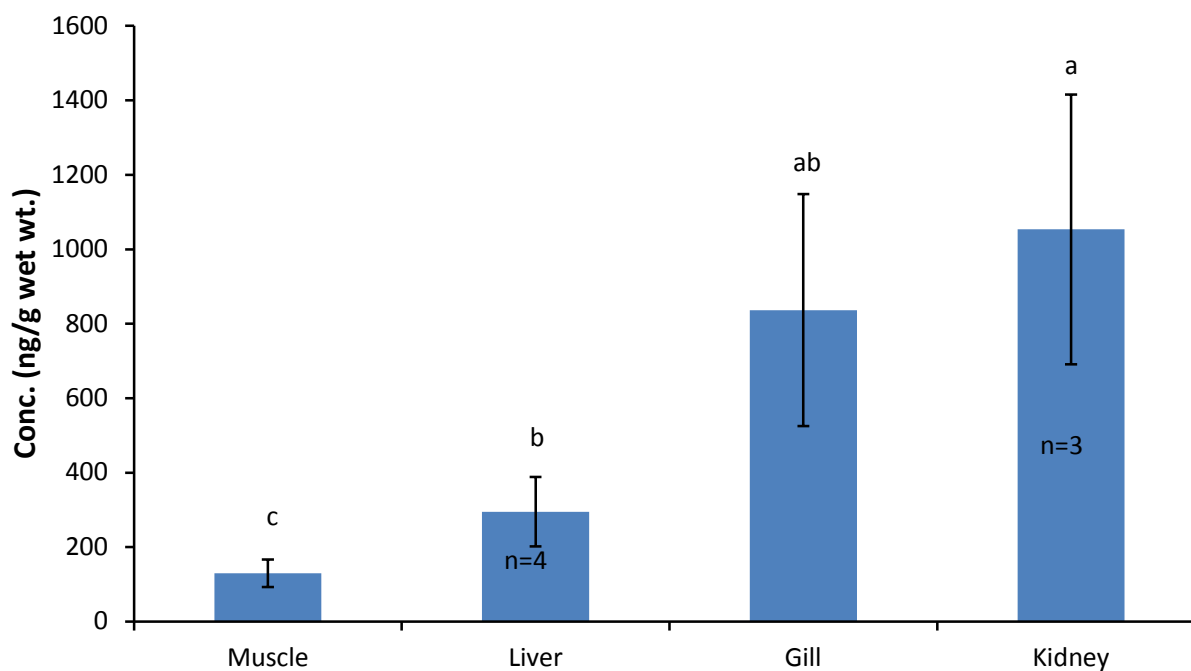

Fig. 42. Tissue-specific concentration (mean ± SEM, n=6) of fenofibrate in fathead minnow exposed to 25 µg l^{-1} for 28 days. Fenofibrate concentration differed significantly (*P*=0.006, Kruskal-Wallis; SNK test) among tissues. Means with the same letter do not differ significantly.
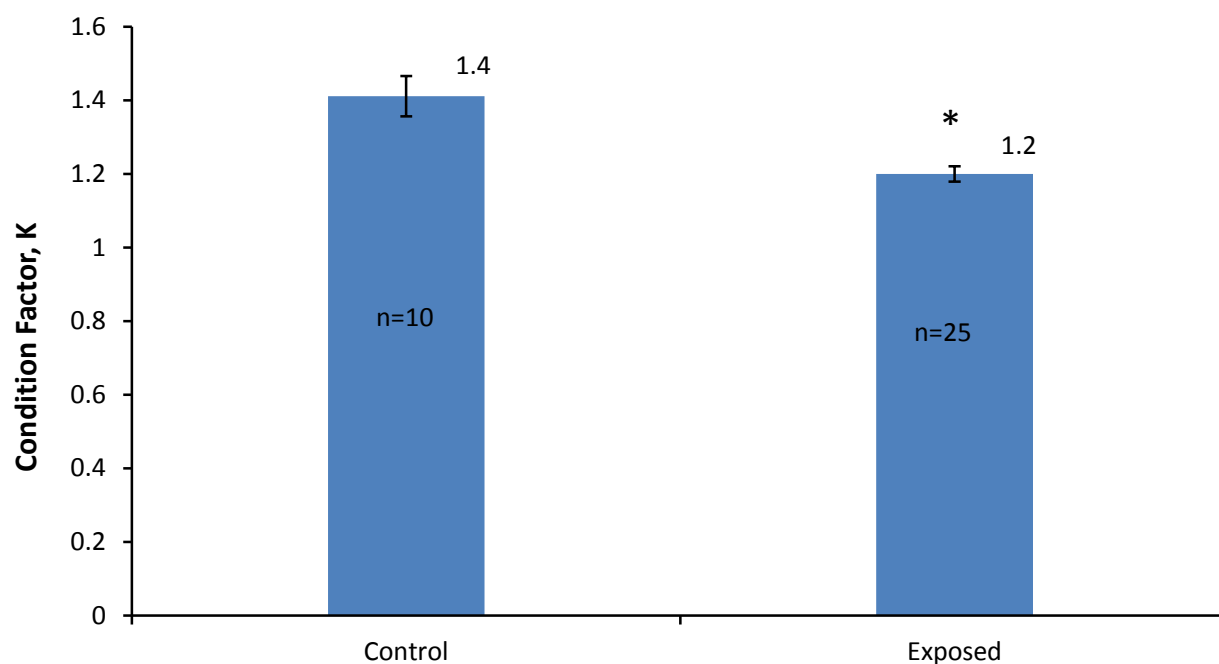
Fig. 43. Condition factor, K (mean ± SEM) of control and fenofibrate exposed (25 µg l^{-1}) channel catfish. Mean K value of exposed fish was significantly lower (*P*=0.0004, Mann-Whitney U test) than that of the control fish.
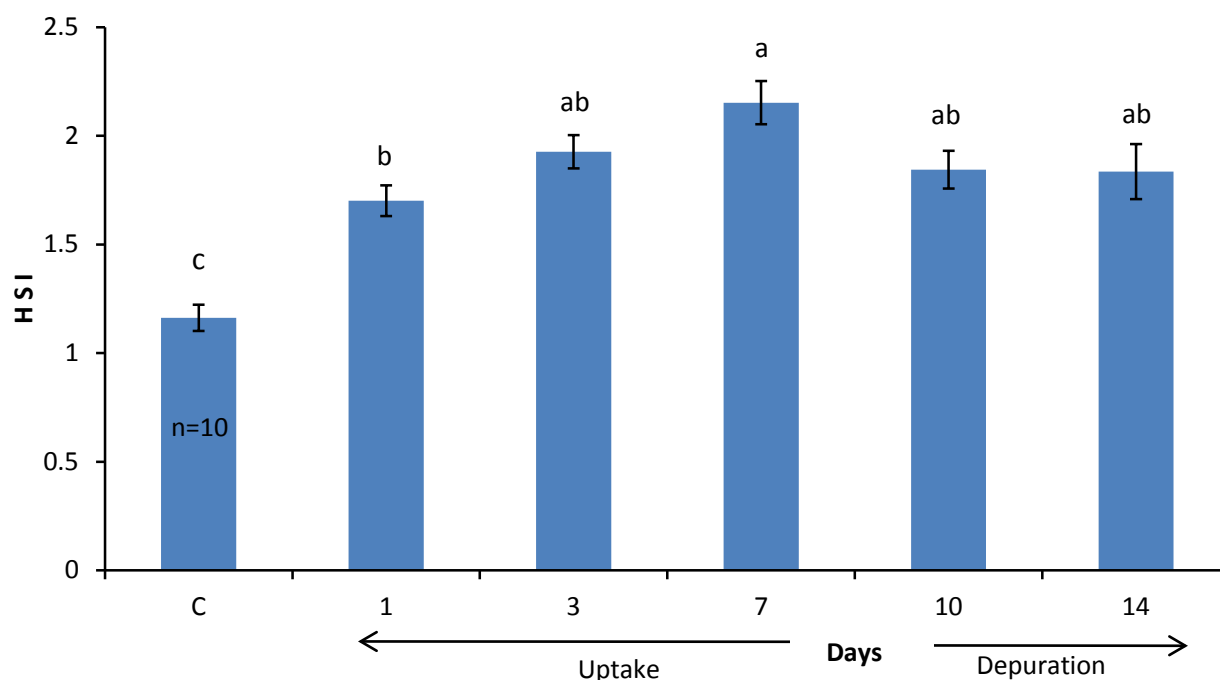

Fig. 44. Hepatic somatic index, HSI (Mean ± SEM, n=5) of control and fenofibrate exposed (25 µg l^{-1}) channel catfish. Mean HSIs of exposed fish were significantly higher (*P*<0.0001, 1-way ANOVA, SNK Test) than that of the control fish. Means with the same letter do not differ significantly.
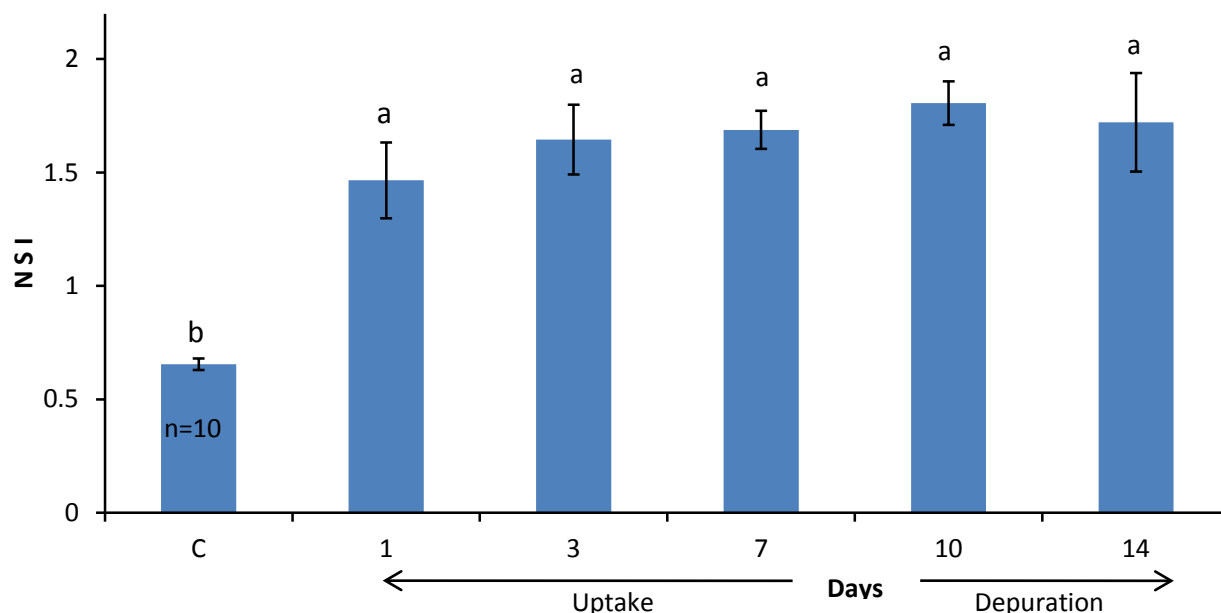
Fig. 45. Nephritic somatic index, NSI (mean ± SEM, n=5) of control and fenofibrate exposed (25 µg l^{-1}) channel catfish. Mean NSIs of exposed fish were significantly higher ($P<0.0001$, 1-way ANOVA, SNK test) than that of the control fish. Means with the same letter are not significantly different.
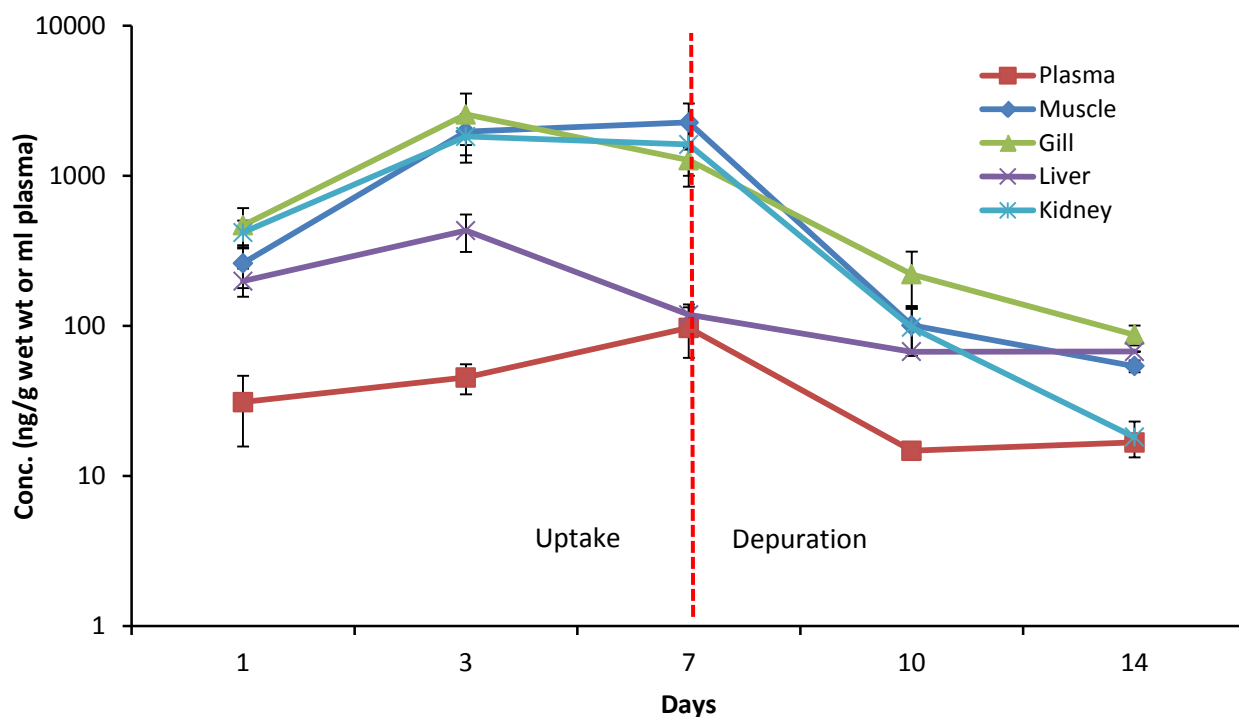

Fig. 46. Fenofibrate concentration (mean ± SEM, n = 3-5) in tissues (ng g^{-1} wet wt) and plasma (µg l^{-1}) of channel catfish exposed to 25 µg l^{-1} for 7 days. Fenofibrate was not detected in plasma (<15 µg l^{-1}) and liver tissues (<57 ng g^{-1}) of depurated fish. Muscle and kidney tissues had no detectable levels of fenofibrate only after 7 days of depuration.
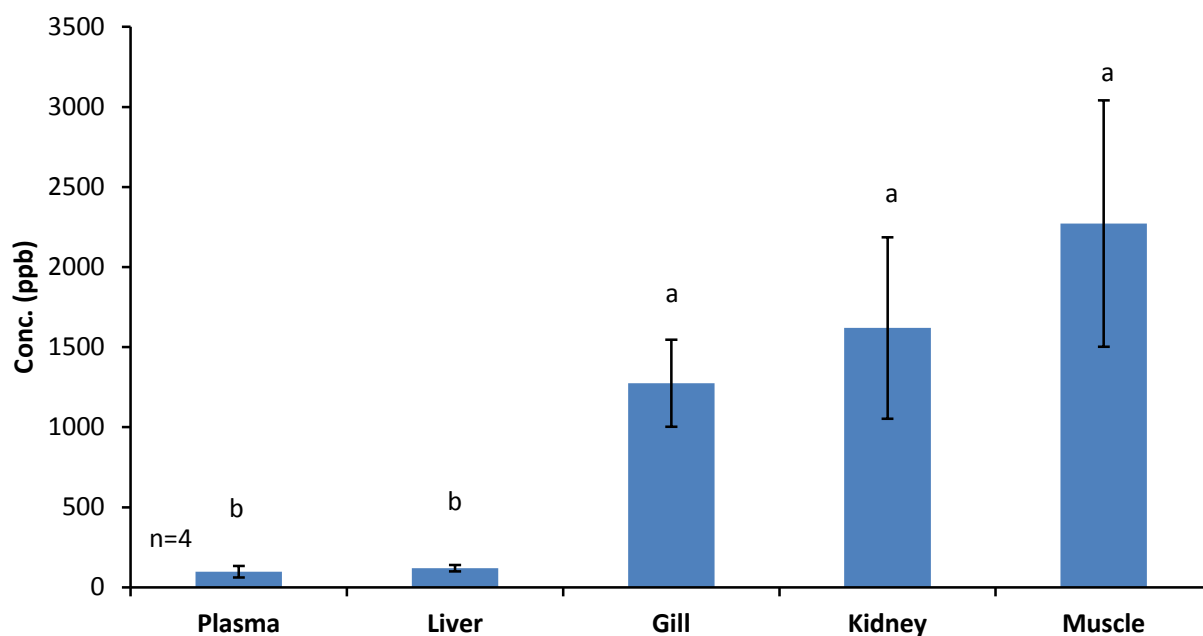
Fig. 47. Fenofibrate concentration (mean ± SEM, n=5) in plasma ($\mu g \ l^{-1}$) and tissues (ng g^{-1} wet wt) of channel catfish exposed to 25 $\mu g \ l^{-1}$ for 7 days. Mean concentrations were significantly different (P=0.002, Kruskal-Wallis, SNK test) among the tissues. Means with same letter are not significantly different.

CHAPTER 9

MEASURED AND PREDICTED BCFs AND PREDICTION OF CHRONIC RISK OF

PHARMACEUTICALS

This chapter has four sections. The first section covers the comparison of the measured

and predicted BCFs for the five pharmaceuticals: norethindrone (NET), ibuprofen (IBU),

verapamil (VER), clozapine (CLZ) and fenofibrate (FFB). The predicted BCFs of the

pharmaceuticals (using the log K_{ow} based models) are compared to the experimentally

determined tissue and plasma BCFs. The second section is devoted to examining the similarities/

differences in the measured BCFs obtained from the two testing approaches: Standard OECD

and a reduced test design. The third section focuses on prediction of chronic risks of the

pharmaceuticals in fish using the fish plasma model (FPM) derived effect ratios (ERs). In

essence, this chapter is intended to address the specific hypotheses framed in this dissertation

work.

9.1   Tissue BCFs: Measured Vs. BCFWIN Predicted

Comparison of the measured and predicted BCFs was the first objective of this work. It

was hypothesized that the BCFs of the selected pharmaceuticals can be predicted using the U.S.

EPA's BCFWIN model. Due to the design of the experiments, each pharmaceutical has four

measured BCFs (2 fish species + 2 ways of expressing BCF) and the highest BCF level was used

for uniform comparisons across the board. The measured and predicted BCFs utilized in the

comparisons are presented in Table 30.

Overall, the predicted BCFs did not differ largely (i.e. by orders of magnitude) from the

measured values. An exception to this trend was the antipsychotic compound, CLZ. The

empirical tissue (except the muscle) BCFs were 857-1860 % higher than the predicted value (63 l kg^{-1} wet wt) (Fig 49). However, the measured BCF level in the muscle tissue was close (131%) to that predicted by the model. This finding underscores the importance of conducting tissue-specific BCF estimates. For example, if the ERA of CLZ is depended entirely on its potential accumulation in the muscle, then this compound may not require further risk assessments, as per the current regulations. However, the BCF levels in other tissues (e.g. liver BCF=1168) exceed the U.S. EPA's current guidelines for classifying a compound as PBT and also are close to the current regulatory trigger level of 2000, the criterion used for prioritizing pharmaceuticals in risk assessments.

Contrary to the CLZ results, the predicted BCF levels for the rest of the pharmaceuticals were overestimated with the exception of the NET BCF in liver tissue (96% of the predicted level). The tissue-specific measured BCFs of the four pharmaceuticals were 5-63% of the corresponding predicted values (Fig 48). Although overestimation of BCFs is beneficial in terms of the "precautionary-principle," it leads to the unnecessary testing of compounds that in fact may pose very little threat to non-target species.

BCFWIN is a log K_{ow} based estimation program which is part of the U.S. EPA's EPISUITE estimation software (Meylan et al., 1999). Prediction of bioconcentration and hazard assessments of chemicals using QSAR based models has widely been reported in the literature (Jones et al., 2002; Sanderson et al., 2004; Hernando et al., 2007; Cooper et al., 2008; Sanderson and Thomsen, 2009). However, the applicability of this model to a wide range of pharmaceutical compounds is limited. This is because pharmaceuticals often exist as charged molecules and are subjected extensive metabolism. A recent study on the applicability of various BCF models for ionizable organic compounds reported that cell based models perform better in terms of BCF

prediction (Fu et al., 2009). Also, efforts are in place for developing approaches to incorporating metabolism as an important component in BCF estimations (Nichols et al., 2006; Arnot et al., 2008; Cowan-Ellsberry et al., 2008; Weisbrod et al., 2009). In summary, with few exceptions, the current work has demonstrated the usefulness of BCFWIN model in predicting BCFs of the selected pharmaceuticals. However, it should be noted that the BCFWIN is not designed for predicting the BCFs of pharmaceuticals. Models that directly address the aspects of ionization, persistence, mode of action or metabolism are needed for accurate predictions.

9.2   Plasma BCFs: Measured Vs. Predicted

Comparison of the measured fish plasma levels to those predicted by the log K_{ow} based model (Fitzsimmons et al., 2001) was the second objective of this dissertation. It was hypothesized that the model can be used to predict the blood to water partitioning coefficients ($P_{b:w}$ or BCFs) of the selected pharmaceuticals in fish. The regression equation in this model was slightly modified by replacing K_{ow} with D_{ow} to account for the ionization of pharmaceuticals. The details on the calculation of BCFs using the model are provided elsewhere (Chapter 3).

It should be mentioned here that catfish were not included in the research initially, but after a pilot trial with fathead minnow (not reported), it was realized that obtaining analytically demanding volumes of plasma from minnows was challenging. So, in order to achieve the important objective of comparing measured and predicted plasma BCFs, larger fish (catfish) were included. However, unlike fathead minnow experiments (28 days), tests on the catfish were short-term based (14 days). Inclusion of catfish in the dissertation work led to the formulation of

another objective (discussed next) in terms of testing the usefulness of reduced BCF testing approach.

The measured and predicted plasma BCFs are presented in Table 31. The results indicated good agreement (69-180%) between the measured and predicted values for NET, IBU and CLZ (Fig 50). On the other hand, the predicted plasma BCFs for VER and FFB were overestimated in comparison with the measured values. The measured BCFs for VER and FFB were only 9 and 1% of the predicted values. But, the magnitude of the difference between the measured and predicted VER BCF levels (0.7 and 7) may not be significant. In other words, in terms of bioconcentration, the BCFs of 0.7 and 7 essentially mean the same (less potential). However, the predicted (421) and measured (4.7) plasma BCFs of FFB differ by two-orders of magnitude. Here, it is reasonable to consider the predicted value as an overestimate. The low measured plasma BCF for FFB could be due to its inefficient permeation across the gills. Uptake of such large and highly lipophilic molecules in organisms, is limited (Jonker and Van der Heijden, 2007). In summary, the D_{ow} model has the potential to predict plasma BCFs of pharmaceuticals in fish. However, large sets of comparative data similar to the one shown in this work would enable incorporation of additional correction factors for better predictions.

9.3   Comparison of BCFs: Standard and Reduced Test Designs

This section compares the standard 28-day (fathead) and a reduced 14- day (catfish) BCF test results. The BCFs estimated using the standard and reduced tests were hypothesized to be similar. Bioaccumulative potential of pharmaceuticals are frequently monitored in plasma, white muscle and liver tissues (Brown et al., 2007; Ramirez et al., 2009; Fick et al., 2010a).Since plasma analysis was not part of the long-term exposure studies, BCF results on the muscle and

liver tissues obtained from the two testing approaches were compared (Fig 51 and 52).

Numerical comparison of the values indicate that the BCF values for the muscle and liver tissues

for the two test species are very close (again, as interpreted in bioconcentration terms). The only

exception was FFB, for which the bioconcentration levels in the two fishes differed. However,

the reasons for such variation in the FFB test results have already been discussed (Chapter 8).

Overall, the BCF results from the 42-day and 14-day studies are not widely divergent suggesting

that the reduced test design has a potential for application in rapid (relative to the long-term test)

screening of pharmaceuticals for prioritization. This finding is significant, in view of the

potential benefits (less resource-demanding) of the reduced test designs (Springer et al., 2008).

9.4   Prediction of Chronic Risk of Pharmaceuticals

9.4.1   Effect Ratios (ERs) of Pharmaceuticals

Utilizing the existing mammalian pharmacological information on the selected

pharmaceuticals, prediction of potential chronic risks in fish constituted the final objective of this

dissertation. The complete details on the approach are provided in Chapter 3. Briefly, the ratio

between the measured or predicted fish plasma levels of the pharmaceuticals and the

corresponding human therapeutic plasma concentrations was used to predict the likelihood of

pharmacological activity in fish. If the ratio, termed "effect ratio" (ER), is less than or equal to 1,

potential risk exists (Huggett et al., 2004). Assumption of functional equivalency of receptor

responses between mammals and fish requires incorporation of additional correction factors that

enhance the trigger level for ER (up to 1000).

The ERs for the four pharmaceuticals are summarized in Table 32. ER could not be

calculated for FFB as the C_{max} (maximum plasma concentration or H_TPC) is available only for its

major metabolite fenofibric acid (www.rxlist.com). The ERs of the pharmaceuticals ranged from 5 to 118125 (Fig 53). The results indicate that NET may have the greatest potential for chronic toxicity in fish. NET has previously been predicted to be "dangerous for the environment" (R 50/53) (Carlsson et al., 2006). VER and CLZ have ERs of 679 and 2080 respectively suggesting that these compounds may have relatively lower risk. CLZ has been predicted to persist in the environment and toxic to aquatic species with potential for bioaccumulation (Woldegiorgis et al., 2007). VER has recently been shown to induce adverse effects in fish at low mg l^{-1} levels (Li et al., 2010). Therefore, the FPM predictions on the potential chronic toxicity with NET, CLZ and VER are in concurrent with the reported/ predicted data. However, the ER value of IBU was 2-5 orders of magnitude higher than the rest of the pharmaceuticals implying very little or no risk to fish. But, studies have reported sublethal toxicity (reproduction) in fish exposed to IBU at sub $\mu g\ l^{-1}$ concentration (Flippin et al., 2007). The ERs of pharmaceuticals in this study were calculated from the predicted plasma levels with an assumption of the functional equivalency ratio (FER) of 1. Huggett et al., (2004) suggested that this model needs extensive validation and further refinements may enable more accurate predictions of chronic risks in fish. Nevertheless, this model has a potential for screening pharmaceuticals for further risk assessments. In fact, few recent studies in the Europe have used this model to prioritize pharmaceutical compounds for risk assessments (Fick et al., 2010a). Also, a recent study has used the model to predict the critical environmental concentrations (that would induce pharmacological effects in fish) of 500 pharmaceuticals (Fick et al., 2010b). Finally, although FFB's chronic risk could not be determined due to the absence of human therapeutic plasma levels, it has been identified as a priority compound for further risk assessments (Boxall, 2004).

9.4.2 Pharmaceuticals' Half-Lives: Fish vs. Humans

The rate at which a compound is eliminated from an organism severs as an index of its accumulative potential. The uptake and depuration kinetics determined for each of the five test pharmaceuticals were used to calculate the elimination half-lives. The fish half-lives were then compared to those available for humans (Fig 54). The elimination half-lives (hrs) of the pharmaceuticals in fish plasma are 2 to 7.5 times the corresponding values in humans. However, the exposure concentration, route of exposure, and differences in the extent of receptor responses could dictate the overall clearance rates of pharmaceuticals. Also, mammals may have better avenues for rapidly clearing the drugs from the body compared to the non-mammalian aquatic vertebrates. More importantly, fish are poikilothermic and have low metabolic rates and hence may be more susceptible to chronic effects.

Half-lives of the pharmaceuticals were also computed for other tissues like white muscle and liver. The fish plasma elimination half-lives were compared to those in the muscle and liver (Fig 55). In general, acidic pharmaceuticals (IBU and FFB) had longer residence times in the liver tissue, while the elimination of basic pharmaceuticals (CLZ and VER) was slower from the muscle tissue. For the neutral compound, the trend essentially followed that of acidic compounds. In summary, determination of uptake and depuration kinetics of the selected pharmaceuticals in fish was also helpful in computing the elimination half-lives. Overall, the data on half-lives indicated that pharmaceuticals may have longer residence times in the tissues in comparison to plasma.

9.5 Chapter References

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Table 30

Predicted[#] and measured BCFs (l kg^{-1} wet wt) of pharmaceuticals.

Tissue / Chemical ➜	NET	IBU	VER	CLZ	FFB
↓	**42.3**	**3.1**	**147**	**62.8**	**322**
Muscle	7.1	0.7	23.1	82.6	90.8
Heart	--	--	14.6	--	--
Brain	7.4	--	--	538	--
Gill	11.1	1.4	40.3	830	86
Kidney	26.8	0.7	46.5	958	70
Liver	40.8	1.1	75.0	1168	17

predicted BCFs (in bold) obtained from U.S. EPA's BCFWIN; -- not sampled.

Table 31

Predicted and measured plasma BCFs of pharmaceuticals.

Chemical	Measured	Predicted
Norethindrone	13.4	19.4
Ibuprofen	1.4	0.78
Verapamil	0.7	7.6
Clozapine	31.2	30.0
Fenofibrate	4.7	420

Table 32

Effect ratios (ERs) of the pharmaceuticals.

Chemical	H_TPC	Fish plasma data			ER
		MEC[#] [ ] µg l^{-1}	Predicted BCF	FPC	
Norethindrone	4.8	48	19	0.9	5
Ibuprofen	18500	200	1	0.1	118125
Verapamil	319	62	7	0.5	679
Clozapine	125	2	30	0.06	2080
Fenofibrate	--	95	420	40	--

H_TPC: human therapeutic plasma concentration (C_{max})-values retried from www.rxlist.com; #measured environmental conc.; FPC: fish plasma conc. (µg l^{-1}).
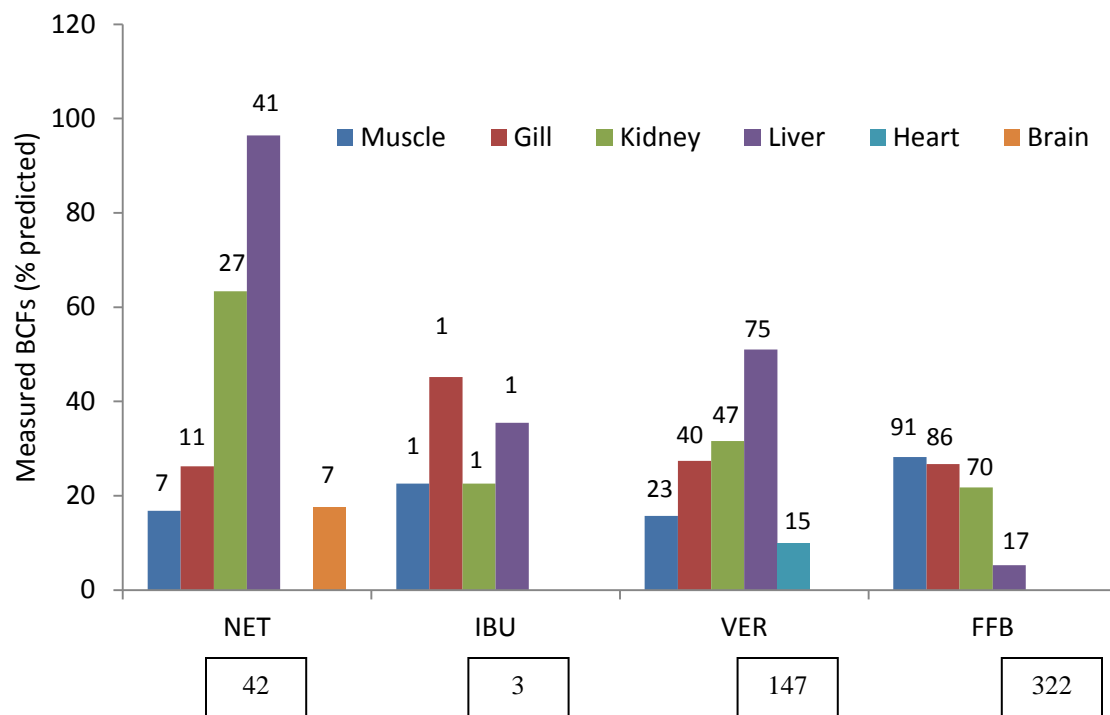
Fig. 48. Tissue-specific measured BCFs of pharmaceuticals expressed as % predicted BCF values (represented in the boxes). Values on the top of bars represent measured BCFs.
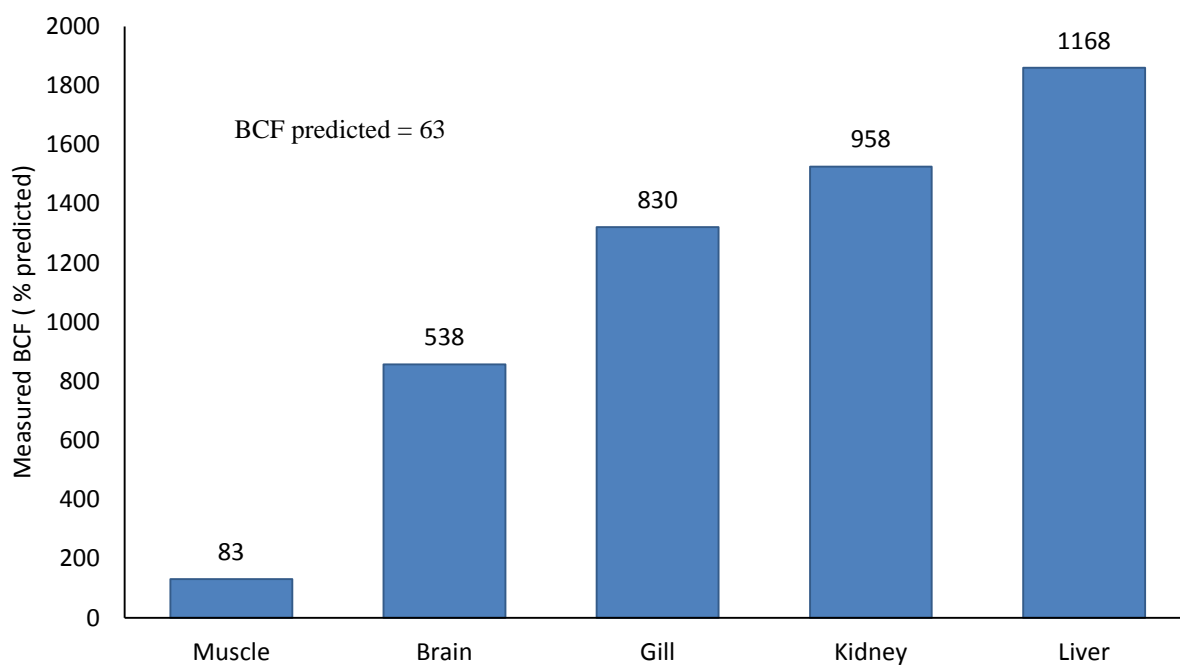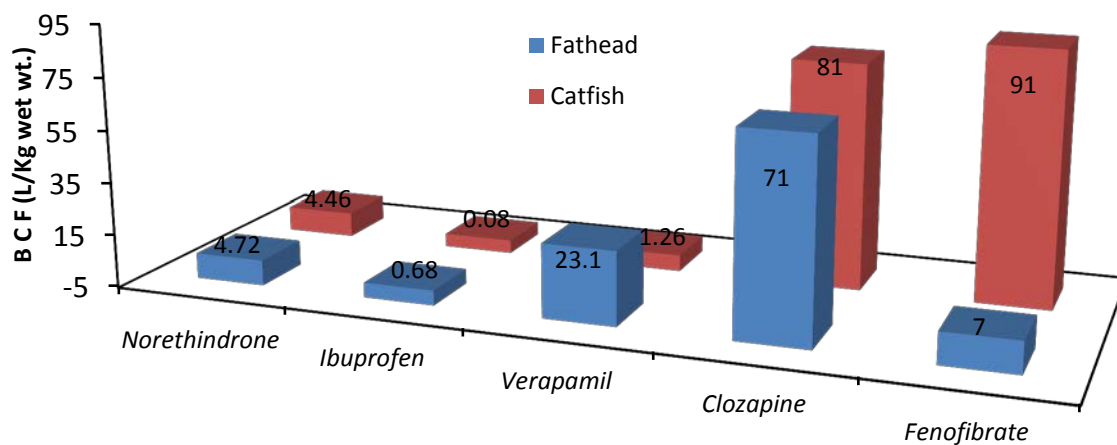

Fig. 49. Tissue-specific clozapine BCFs expressed as % predicted BCF value (63). Values on the top of bars represent measured BCFs.
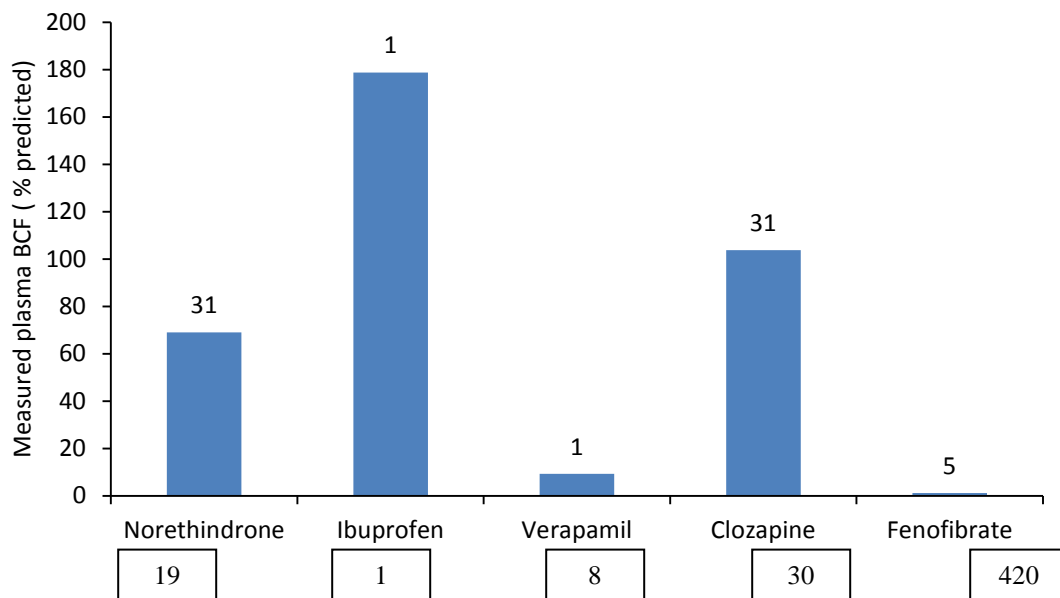
Fig. 50. Measured plasma BCFs of pharmaceuticals expressed as % predicted levels (represented in boxes). Values on the top of the bars represent measured BCFs.

 Fig. 51. Empirical BCFs of the select pharmaceuticals for the muscle tissues of fathead minnow (28 d test) and channel catfish (7 d test). 28 d test followed standard OECD BCF method and 7d test was conducted with a reduced sampling design.

192
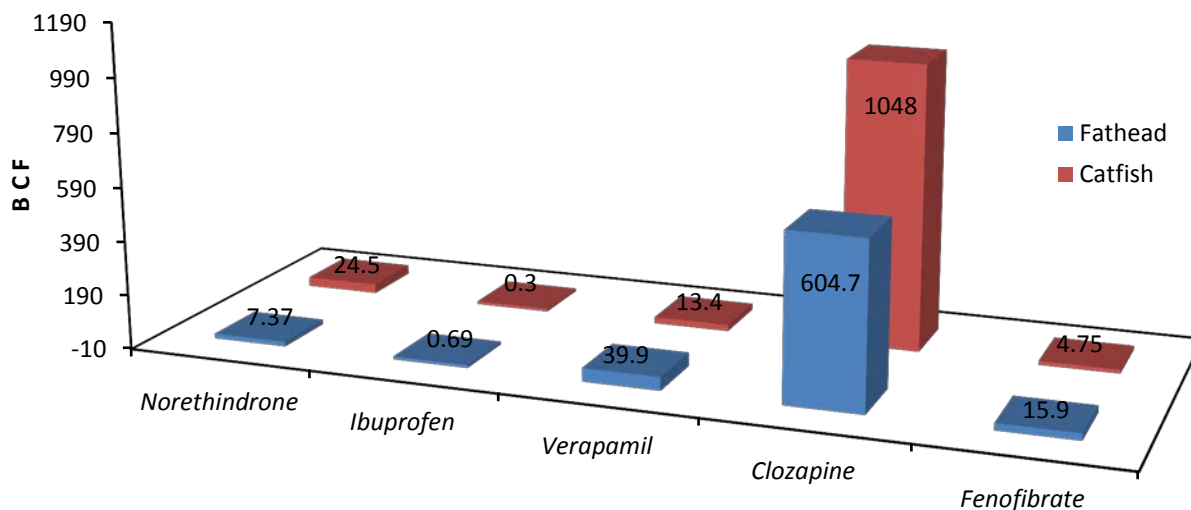
Fig. 52. Empirical BCFs of the select pharmaceuticals for the liver tissues of fathead minnow (28 d test) and channel catfish (7 d test). 28 d test followed standard OECD BCF method and 7 d test was conducted with a reduced sampling design.
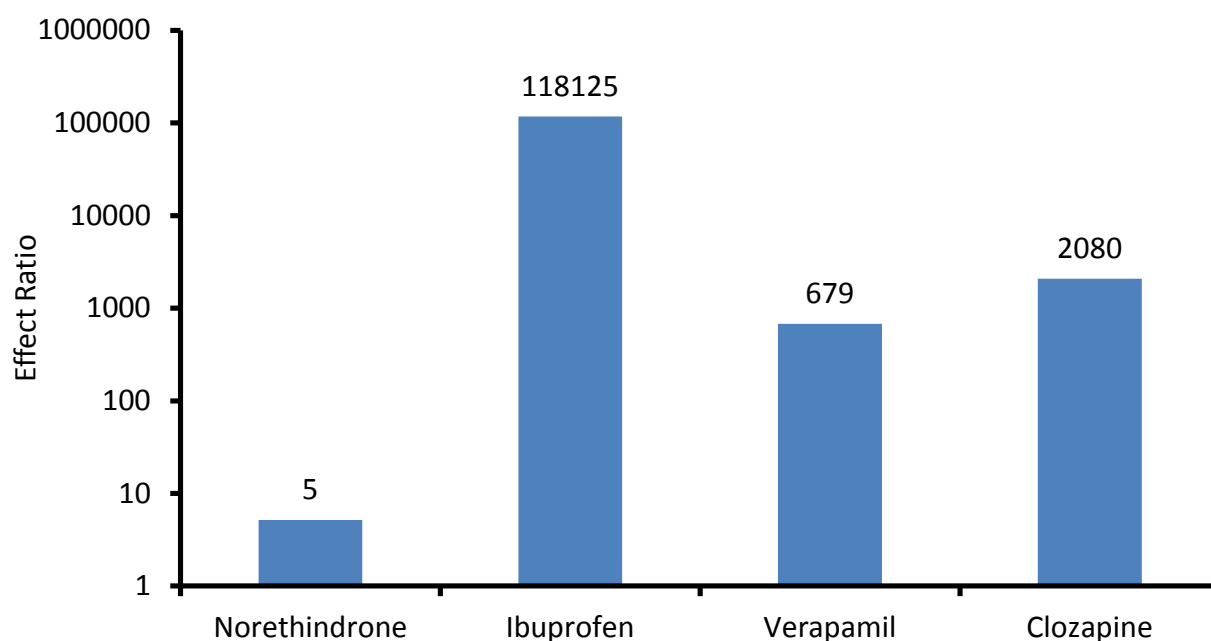
Fig. 53. Effect ratios (ERs) of the selected pharmaceuticals. ER is the ratio between human therapeutic plasma concentration (HTPC) and the fish plasma concentration (FPC). ER<1 indicates potential chronic risk to fish (Huggett et al. 2004).
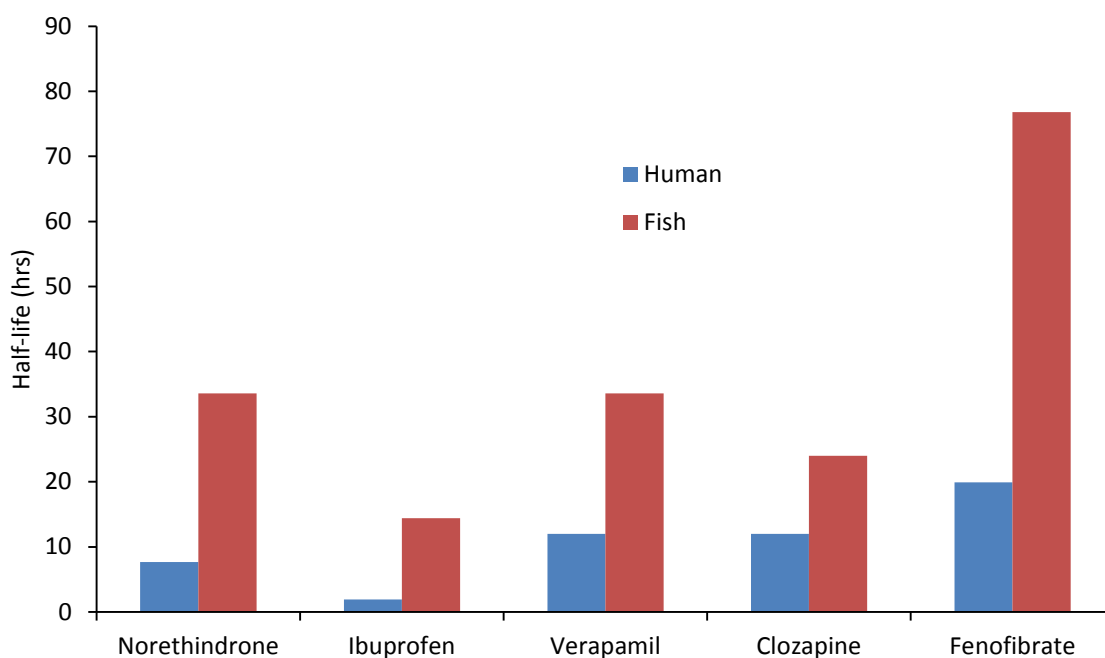
Fig. 54. Plasma elimination half-lives (hrs) of the pharmaceuticals in fish and humans.
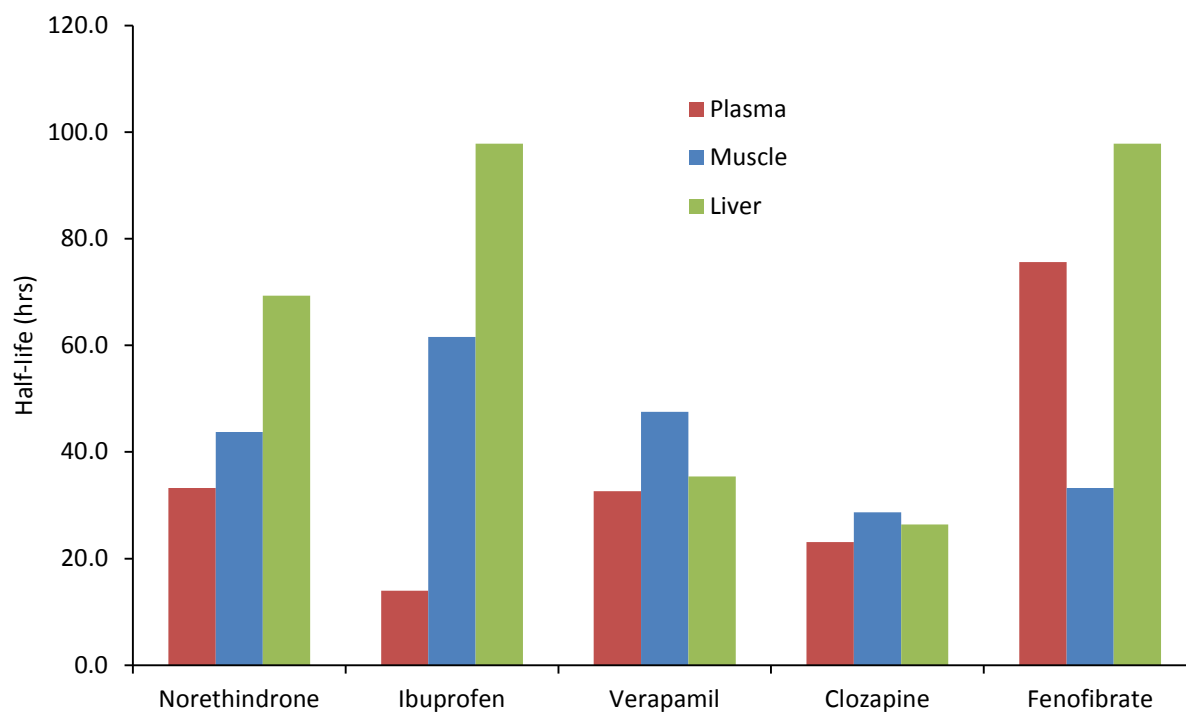
Fig. 55. Tissue-specific elimination half-lives (hrs) of the pharmaceuticals in channel catfish.

CHAPTER 10

CONCLUSIONS AND FUTURE DIRECTIONS

The overall goal of this dissertation work was the experimental determination of tissue-specific uptake and depuration kinetics of five selected pharmaceuticals in fish and compare the measured tissue BCFs to those predicted using the models. As the BCF tests were conducted on chemical by chemical basis, some of the salient outcomes from each study are presented here.

Norethindrone (NET) exposure in fish resulted in increased liver weights and reduced gonadal weights. NET differentially accumulated among the tissues and liver showed highest uptake potential. Although NET had shown potential for accumulation in fish, the measured BCF levels may not be a concern in terms of current regulations.

Ibuprofen (IBU) is the only chemical in the list that had shown very little or no tendency to bioconcentrate in fish. Lack of uptake and rapid clearance are the possible mechanisms for the very low bioconcentration. In the plasma, metabolites of IBU similar to those in human plasma have been identified. Unlike other test compounds, IBU had maximum uptake by plasma.

Verapamil (VER) results more or less were similar to those observed for NET. Liver and kidney showed highest potential for uptake, indicating high metabolic activity as well renal clearance and/ or reabsorption. The accumulation varied among tissues. Overall, VER has a potential for accumulation in fish, but the levels observed in this work are well below the current regulatory trigger level.

In terms of BCF results, clozapine (CLZ) was a stand-alone chemical. The BCFs obtained for this chemical were one to two orders of magnitude higher than the exposure concentration indicating a high potential for accumulation in fish. The BCF levels approached

the current regulatory trigger levels. Also, compared to other tissues, CLZ had longer residence time in the brain.

Results on fenofibrate (FFB) were mixed and unexpected. The physicochemical properties indicate very high accumulative potential but the BCFs were similar to those observed for VER or NET. The low bioconcentration for FFB could have resulted from lesser potential to permeate biological membranes and the BCF levels are not of a concern as per the current guidelines.

BCFWIN model for predicting tissue BCFs overestimated the values for NET, IBU, VER and FFB. However, the differences were not large. On the other hand, bioconcentration potential of CLZ was underestimated (except muscle tissue) as the measured BCFs were ranged between 800-1800% of the predicted values. Regarding the plasma BCFs, the D_{ow} model predicted reasonably well for NET, IBU and CLZ. However the predicted BCFs for VER and FFB were overestimated. The fish plasma model derived effect ratios (ERs) predicted chronic risk in fish from NET and relatively lower risk from VER and CLZ. As per the ER value for IBU, chronic risk in fish is unlikely. While the predictions may not completely be in conformity with the reported literature, more refinements in the model may enhance the accuracy of risk prediction.

In summary, the results from this dissertation indicated that the selected pharmaceuticals have tendency to accumulate in fish. The results emphasized the importance of conducting tissue-specific analysis in order to avoid under or over prediction of risk. Although the existing BCF models may not directly be applied to predict BCFs of pharmaceuticals, the accuracy of predictions could be increased if additional correction/assessment factors are included.

Future work may involve efforts to generate similar data with more sets of compounds representing several classes that cover wide range of physicochemical properties. Also, enough attention should be given to identify the uptake, clearance and metabolism characteristics in fish. Availability of such data on representative compounds would be useful for possible inclusion of correction factors in the existing models that facilitate better prediction of BCFs. This is significant, as it is imperative to rely on models to prioritize several thousand existing pharmaceuticals as well as those in the pipeline. Since the usage of pharmaceuticals is not expected to subside in the future, accurate prioritization is a key.

APPENDIX A

SUPPLEMENTAL FIGURES

A)
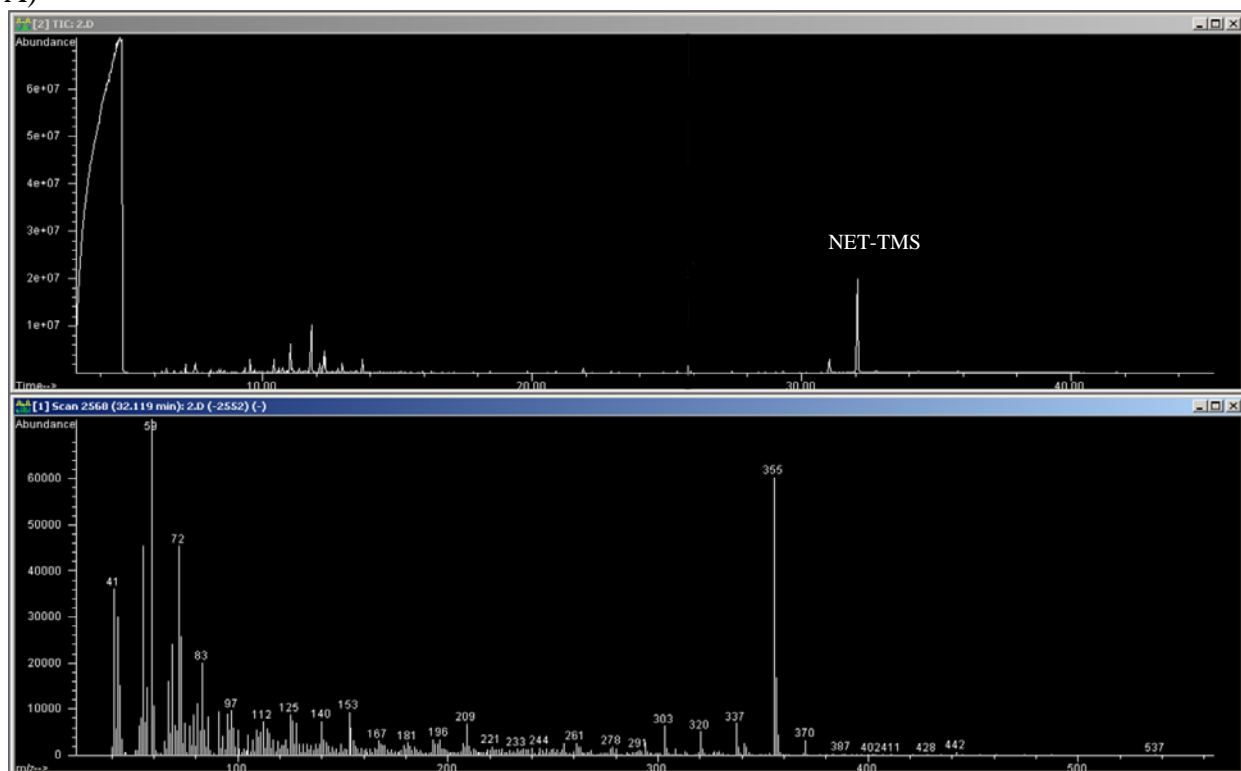
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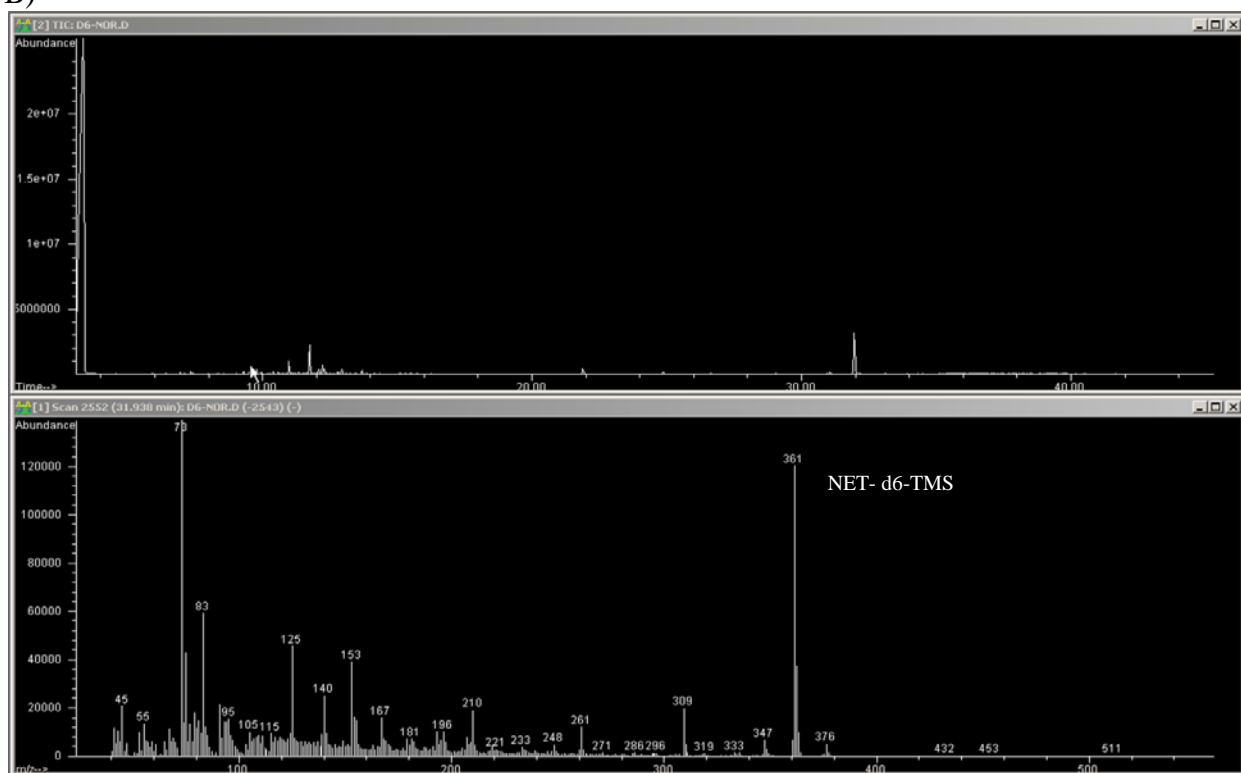
Fig. A.1. Total ion current (TIC) chromatograms and mass spectra of A) norethindrone (NET-TMS and B) NET-d6-TMS.
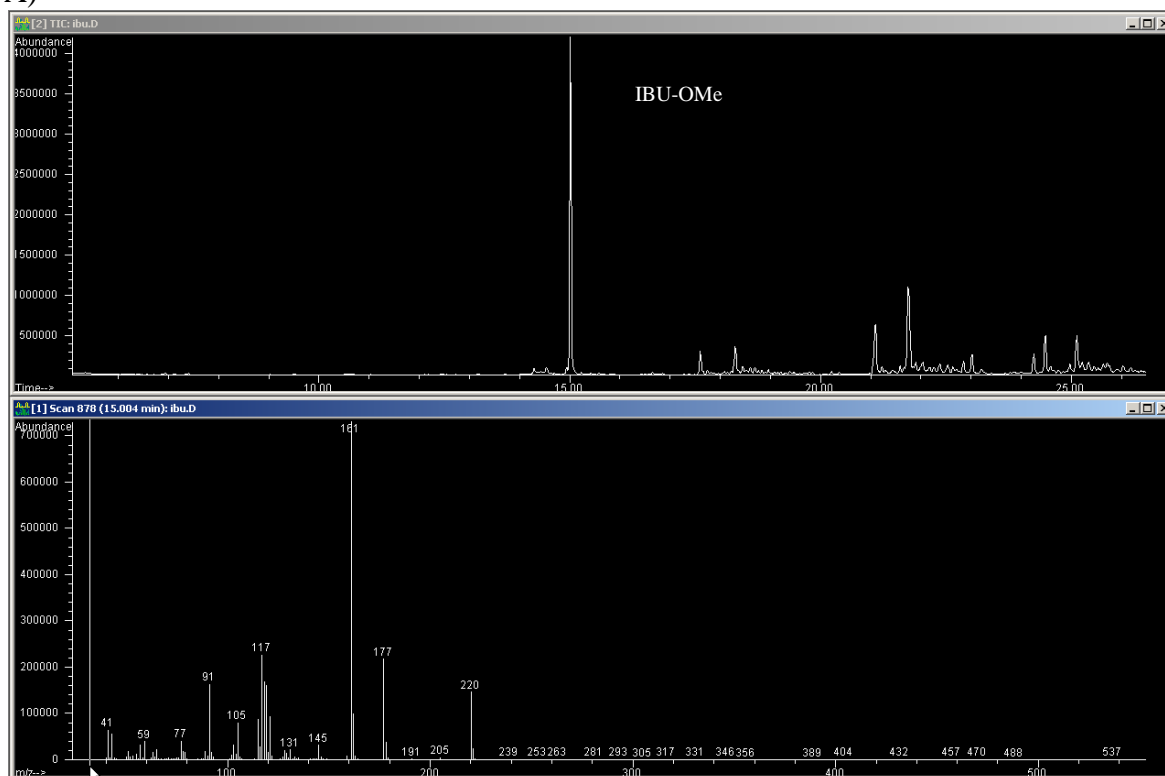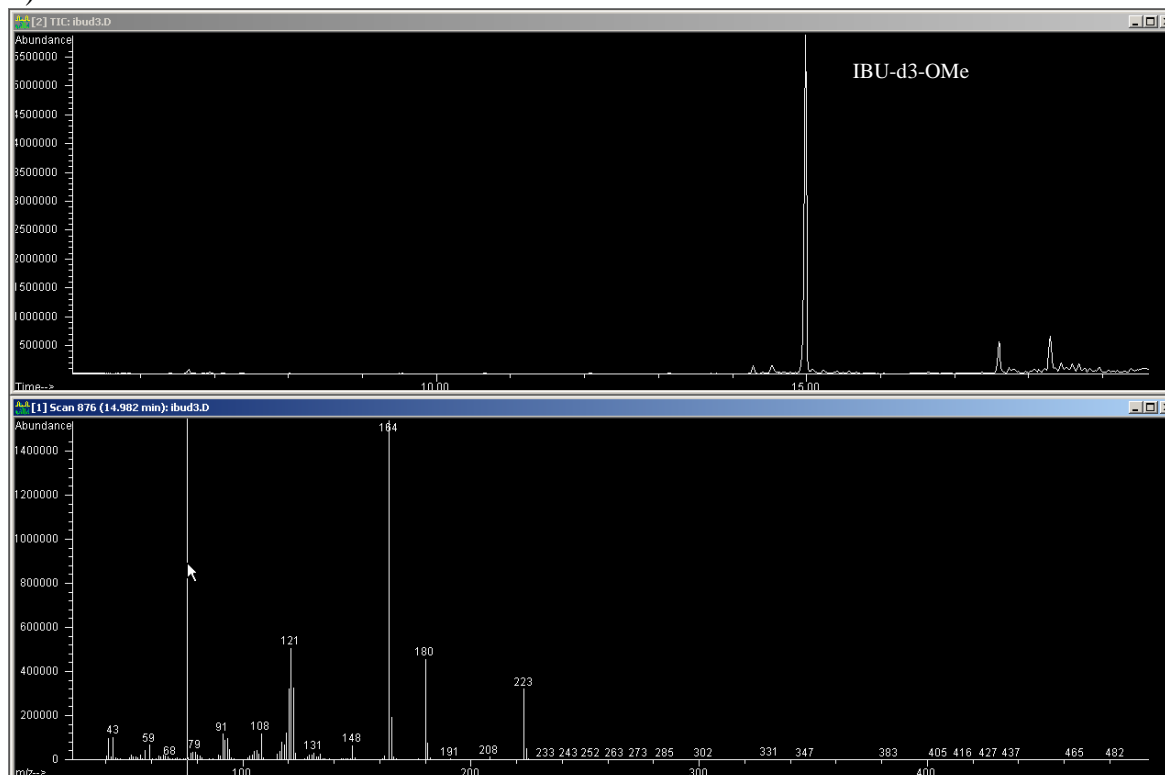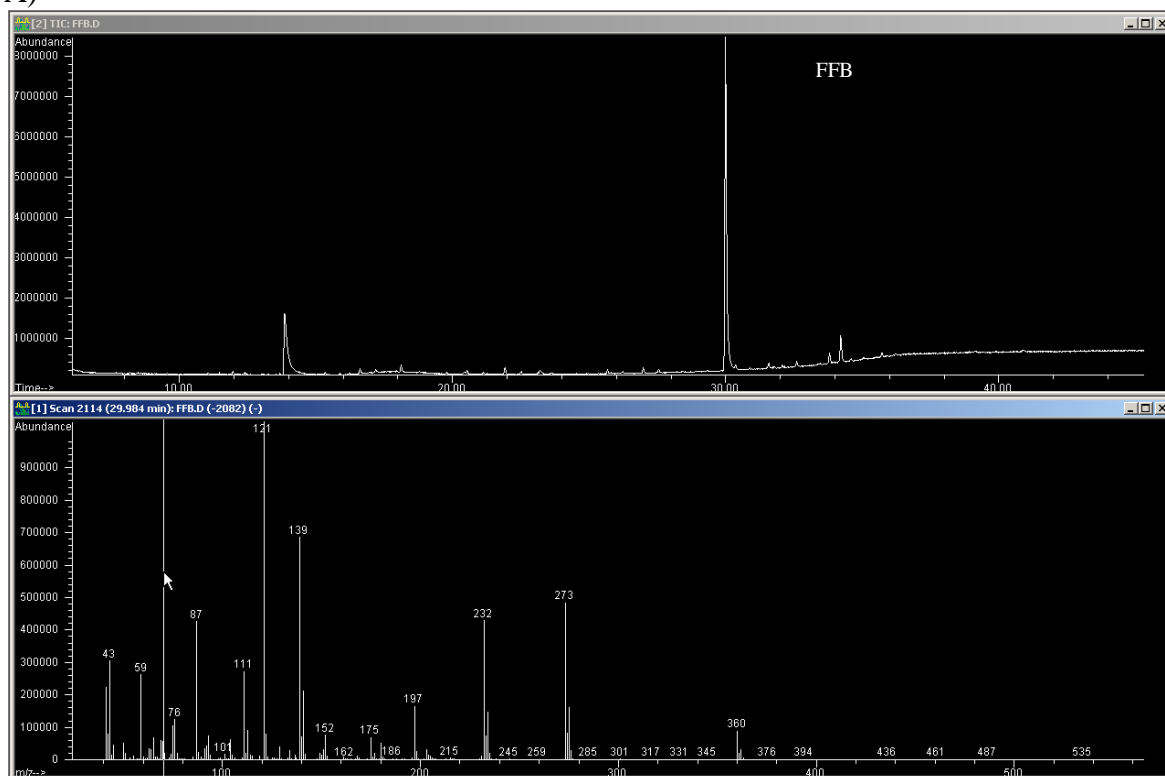
Fig. A.2. Total ion current (TIC) chromatograms and mass spectra of A) ibuprofen (IBU)-OMe and B) IBU-d3-OMe.
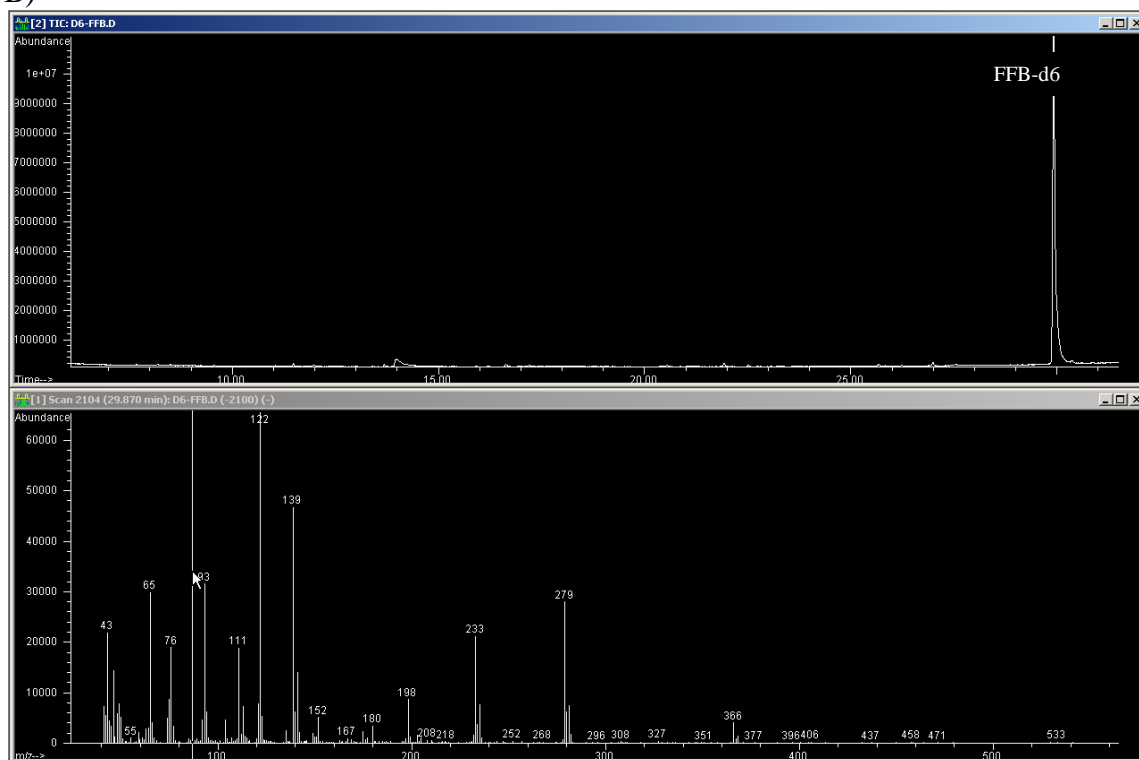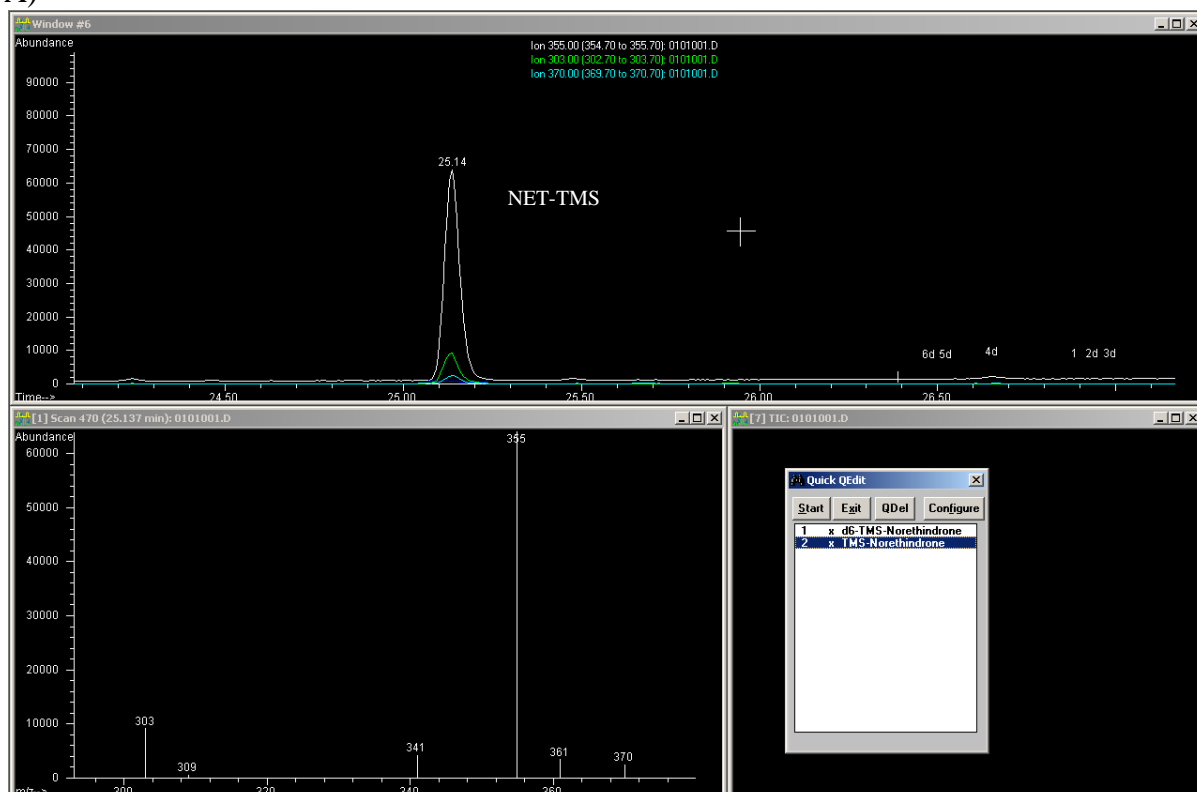
A)

B)

Fig. A.3. Total ion current (TIC) chromatograms and mass spectra of A) fenofibrate (FFB) and B) FFB-d6.
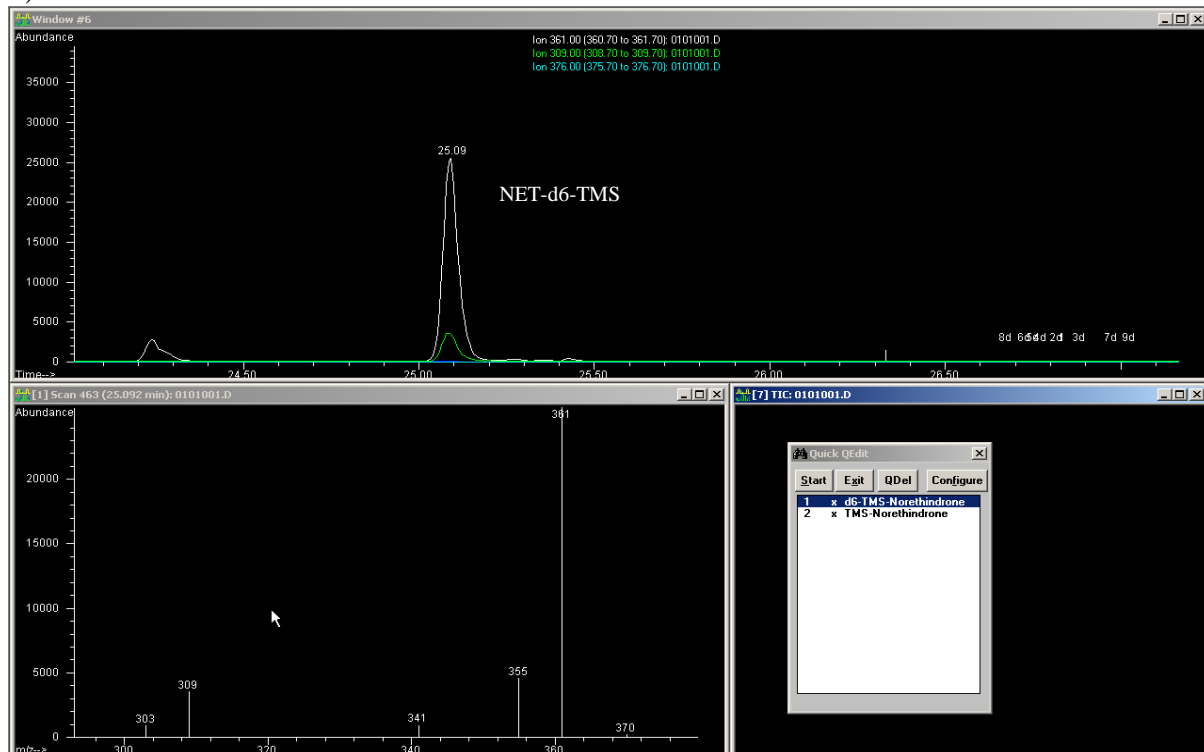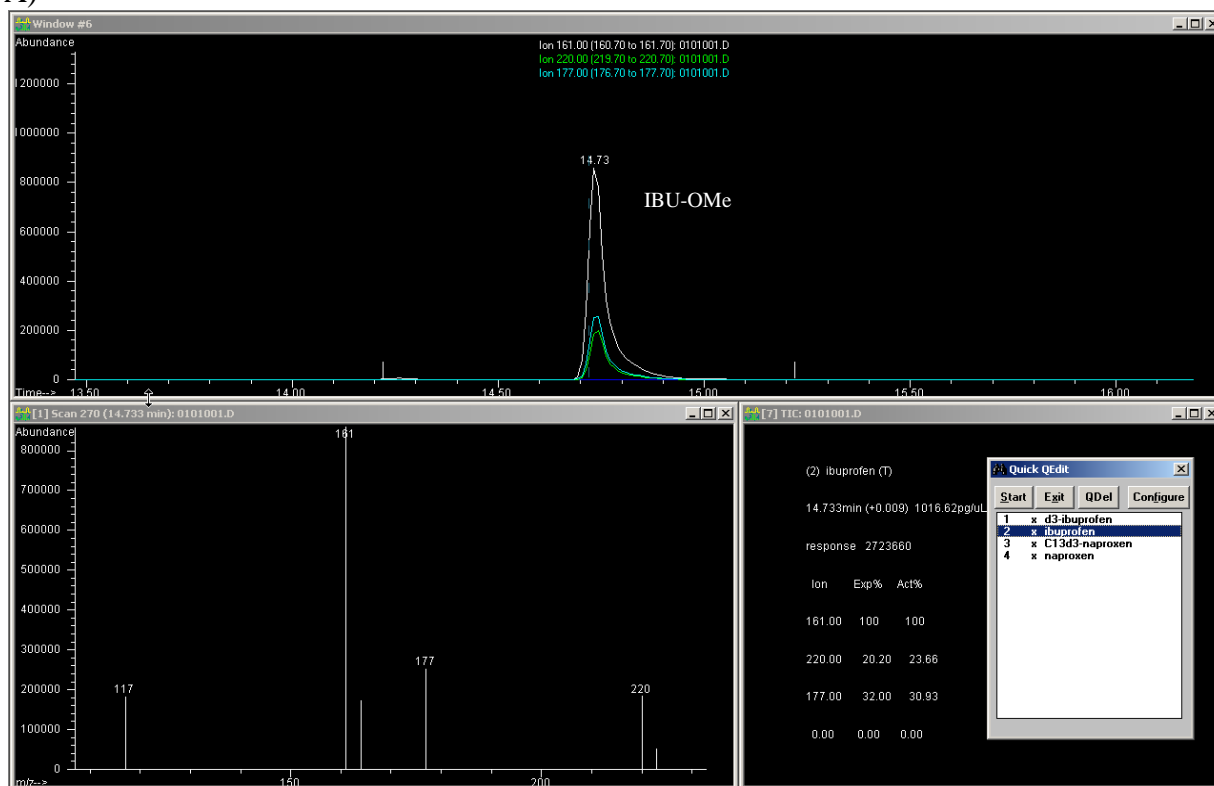
Fig. A.4. Selected ion monitoring (SIM) chromatograms and mass spectra of A) norethindrone (NET)-TMS and B) NET-d6-TMS.
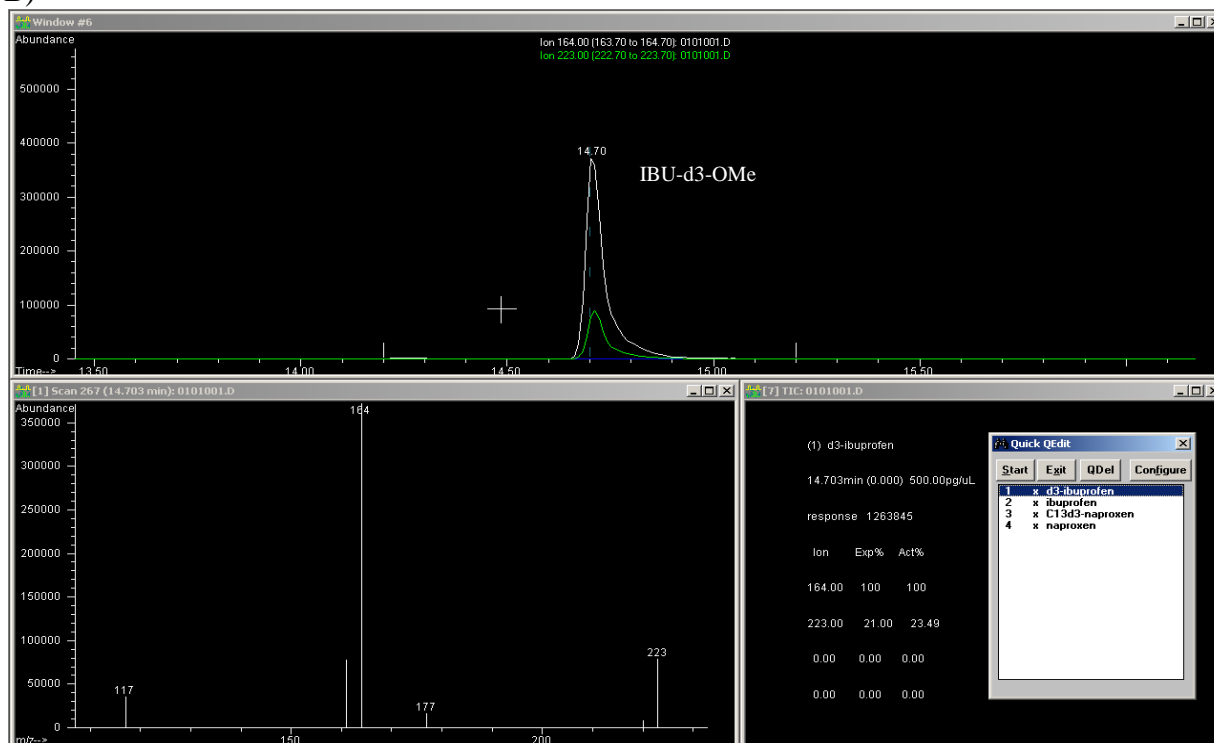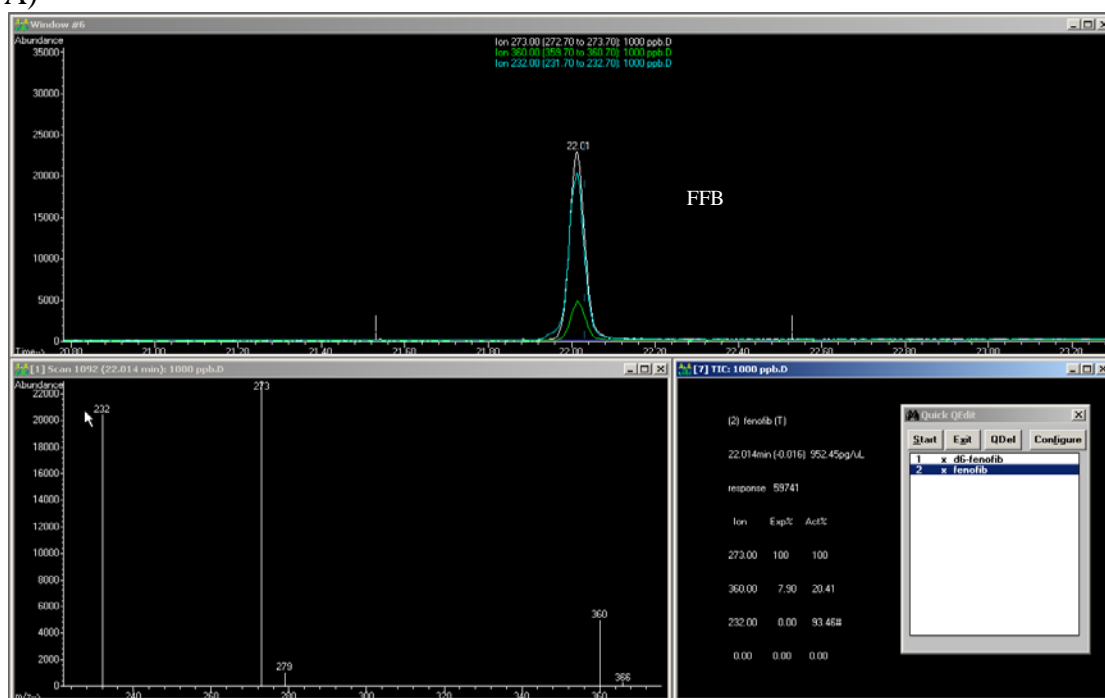
A)

B)

Fig. A.5. Selected ion monitoring (SIM) chromatograms and mass spectra of A) ibuprofen (IBU)-OMe and B) IBU-d3-OMe.
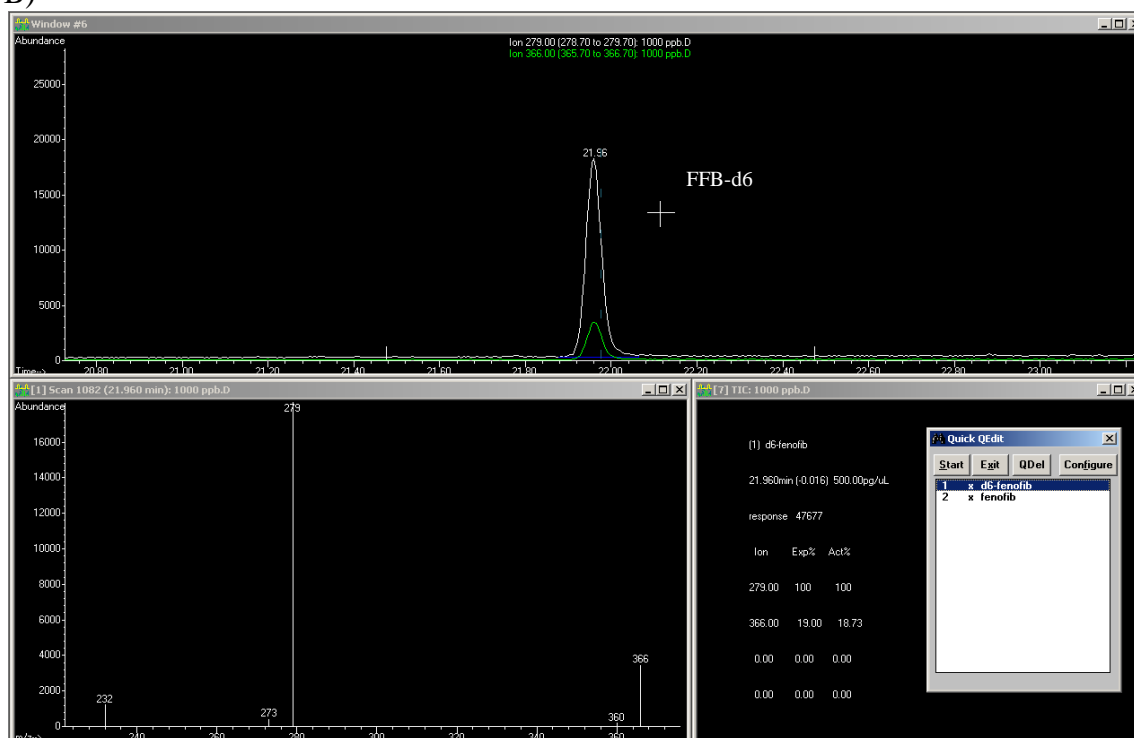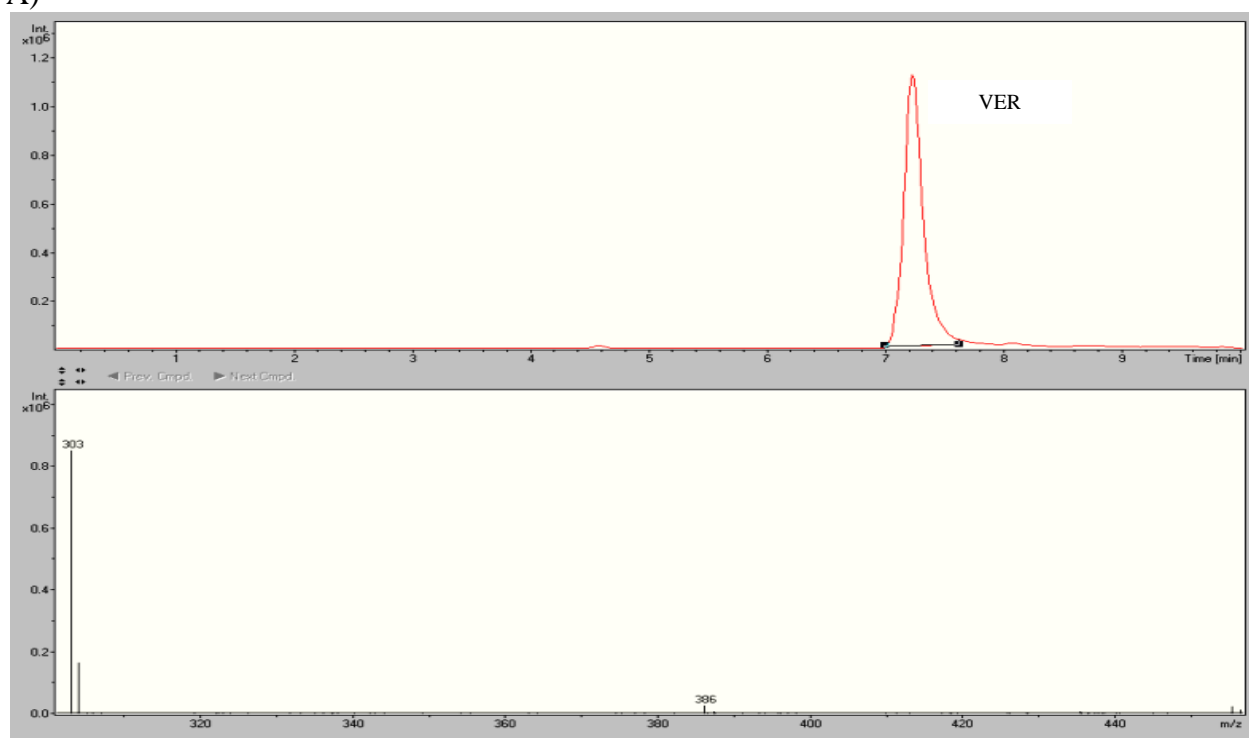
A)

B)

Fig. A.6. Selected ion monitoring (SIM) chromatograms and mass spectra of A) fenofibrate (FFB) and B) FFB-d6.
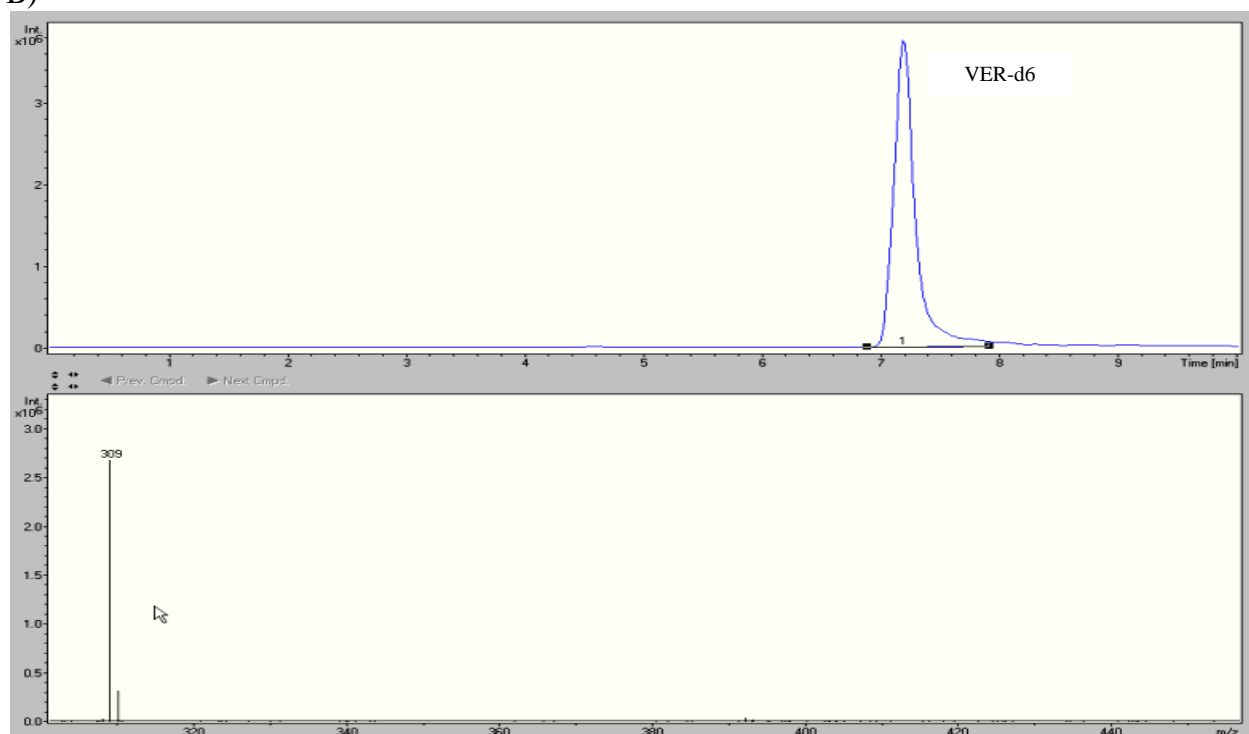
A)

B)

Fig. A.7. Chromatography and mass spectral profiles of A) verapamil (VER) & B) VER-d6.
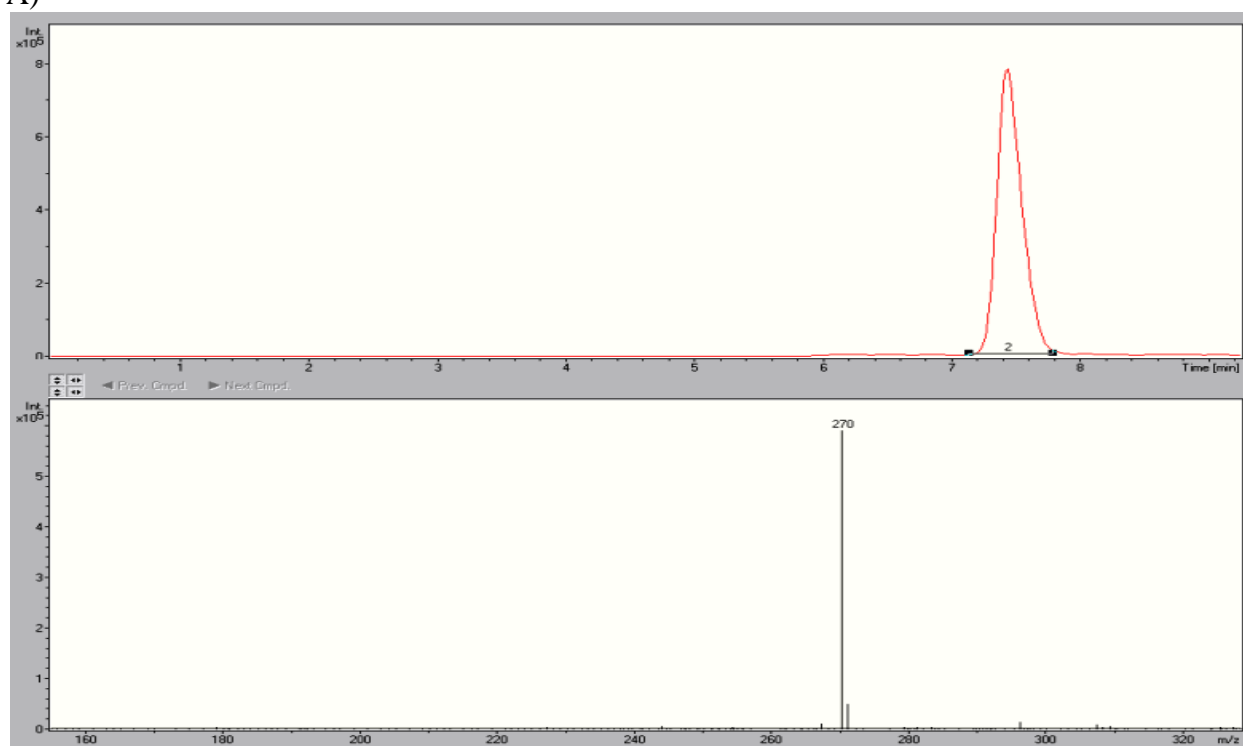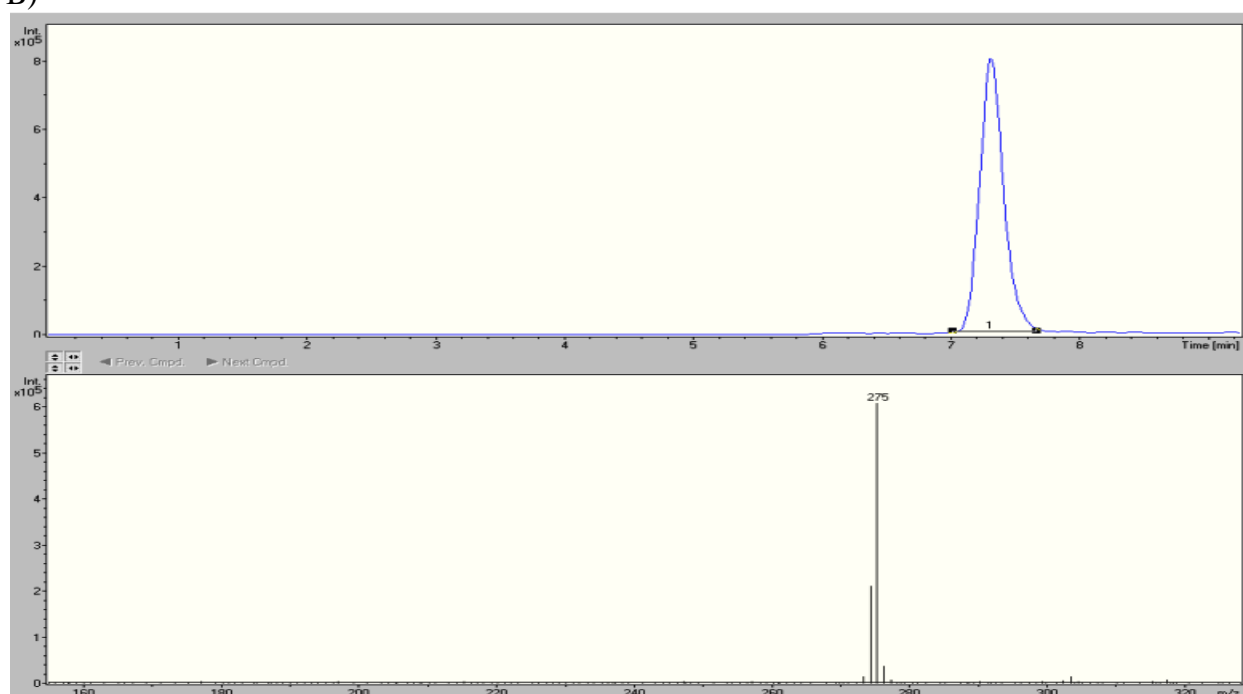
Fig. A. 8. Chromatography and mass spectral profiles of A) clozapine (CLZ) and B) CLZ-d8.
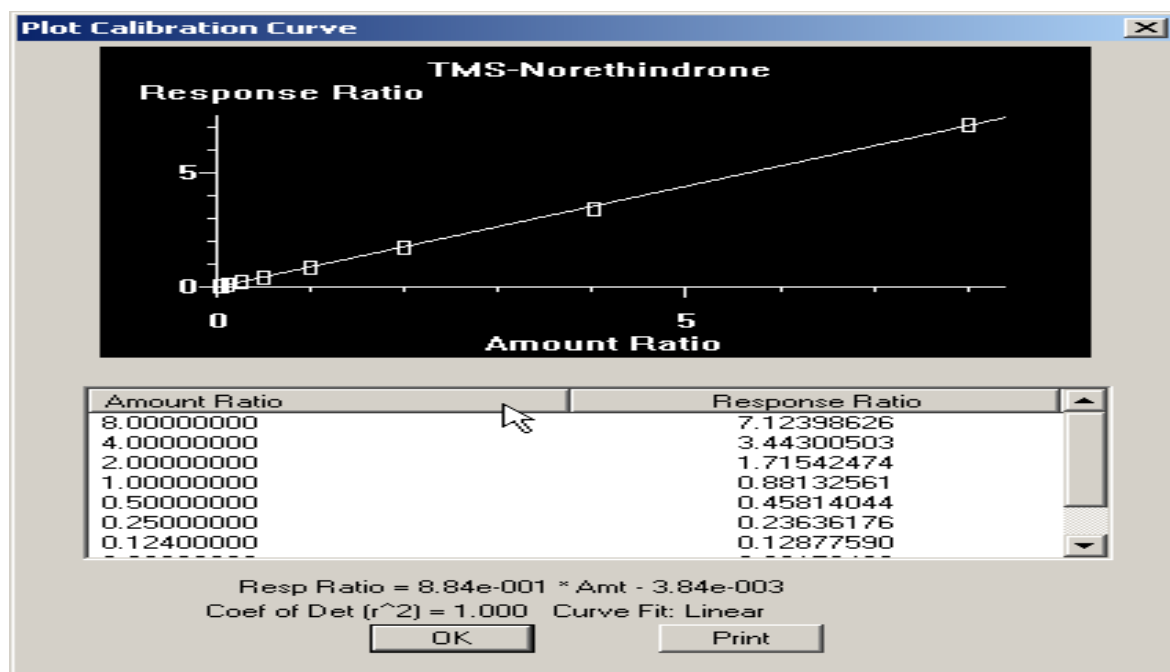
Fig. A.9. Norethindrone (NET)-TMS calibration plot between response and amount ratios calculated from the responses and concentrations of native and NET-d6.
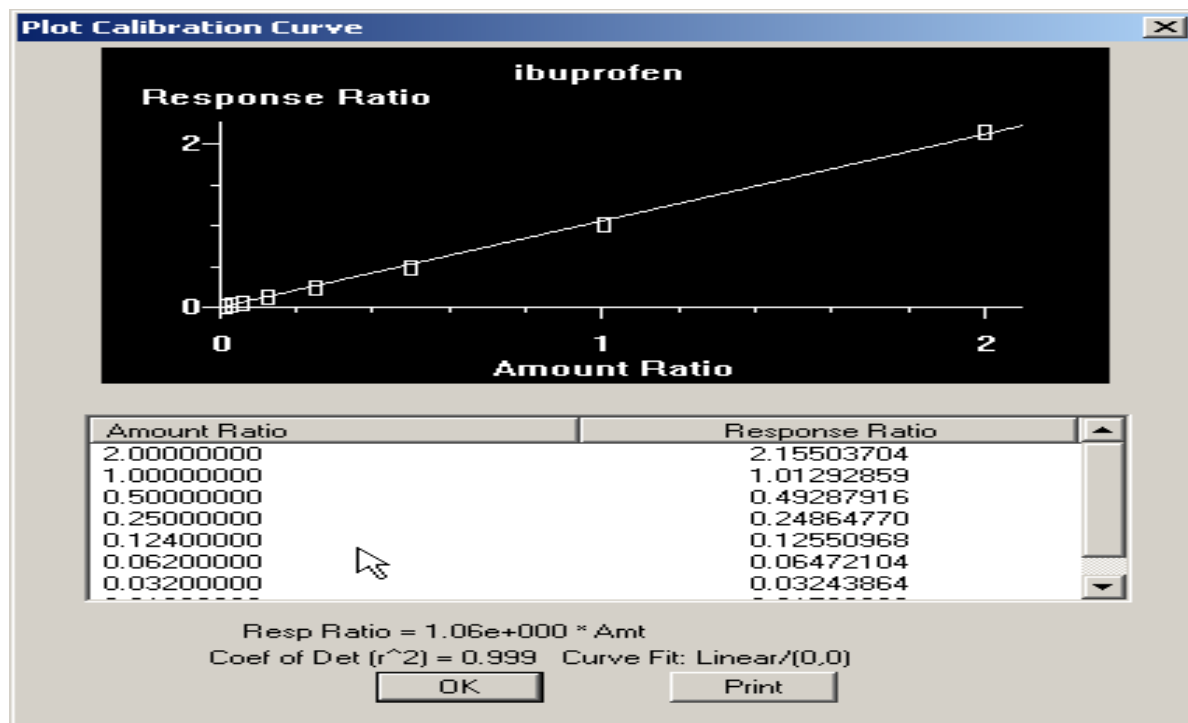
Fig. A.10. Ibuprofen (IBU)-OMe calibration plot between response and amount ratios calculated from the responses and concentrations of native and IBU-d3.
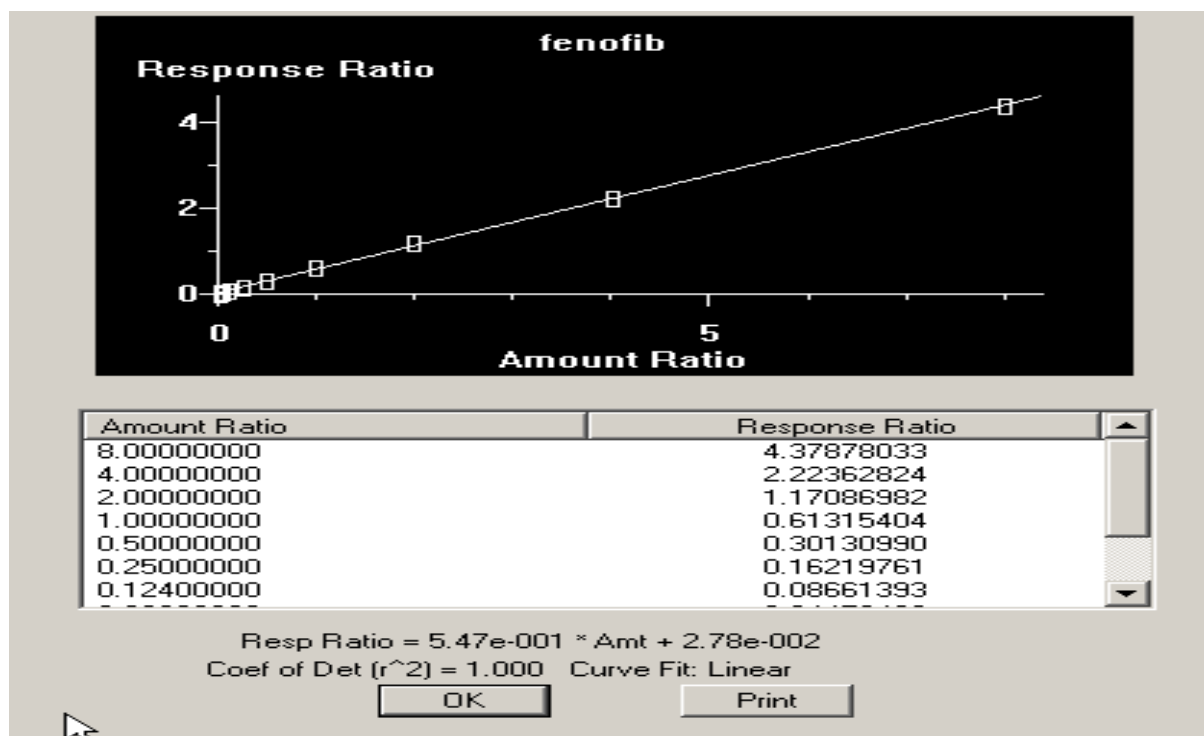
Fig. A.11. Fenofibrate (FFB) calibration plot between response and amount ratios calculated from the responses and concentrations of native and FFB-d6.
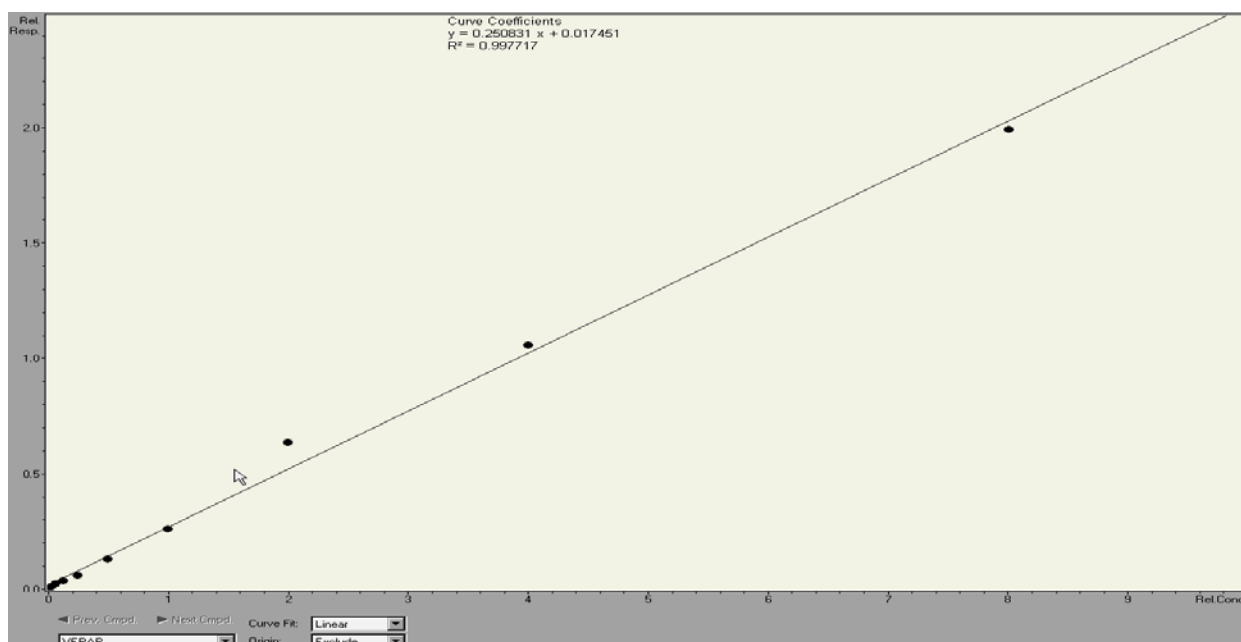
Fig. A.12. Verapamil calibration curve.
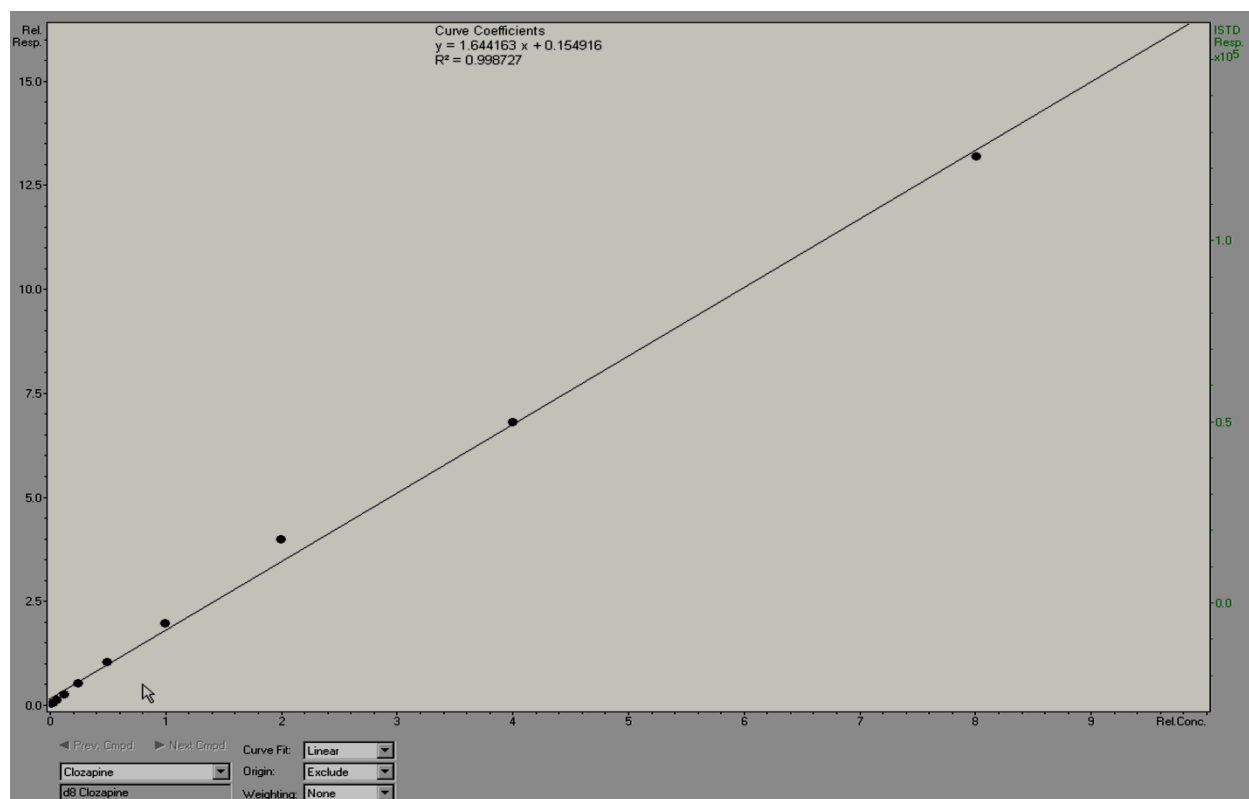
Fig. A.13. Clozapine calibration curve.

A)
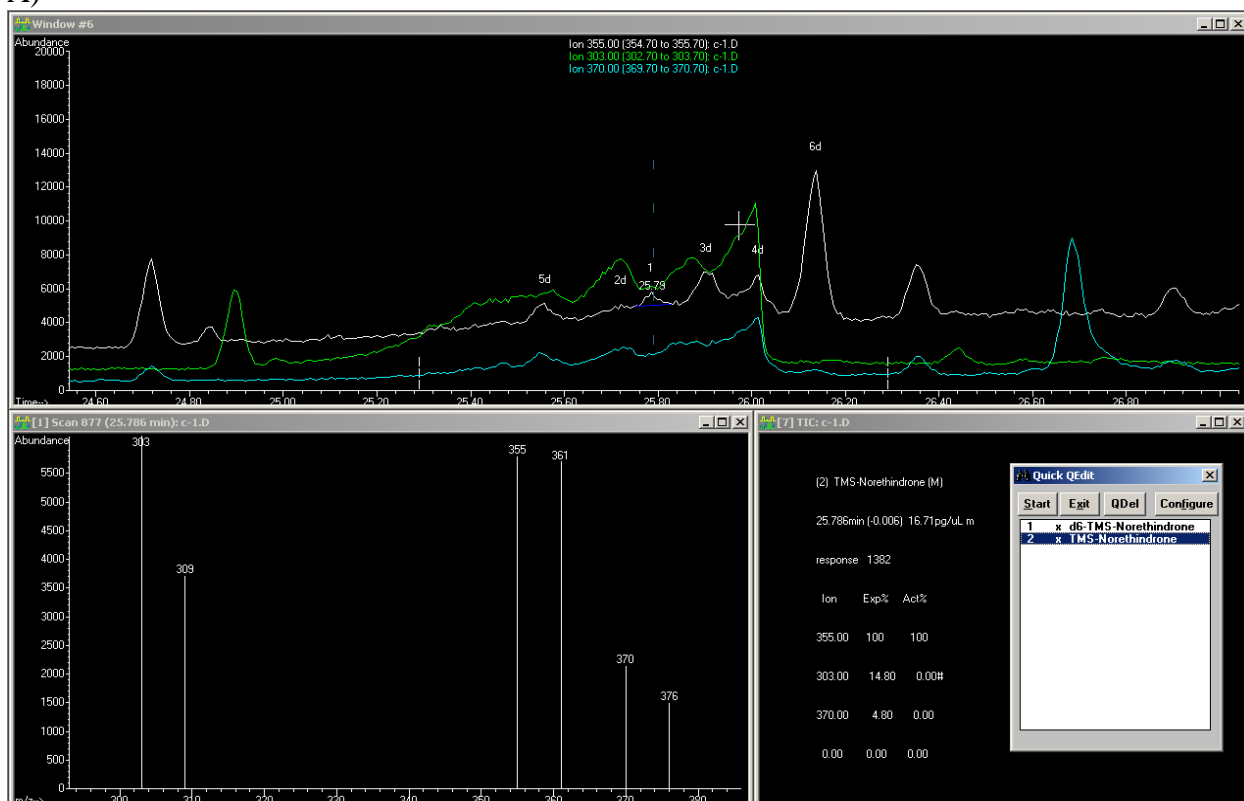
B)
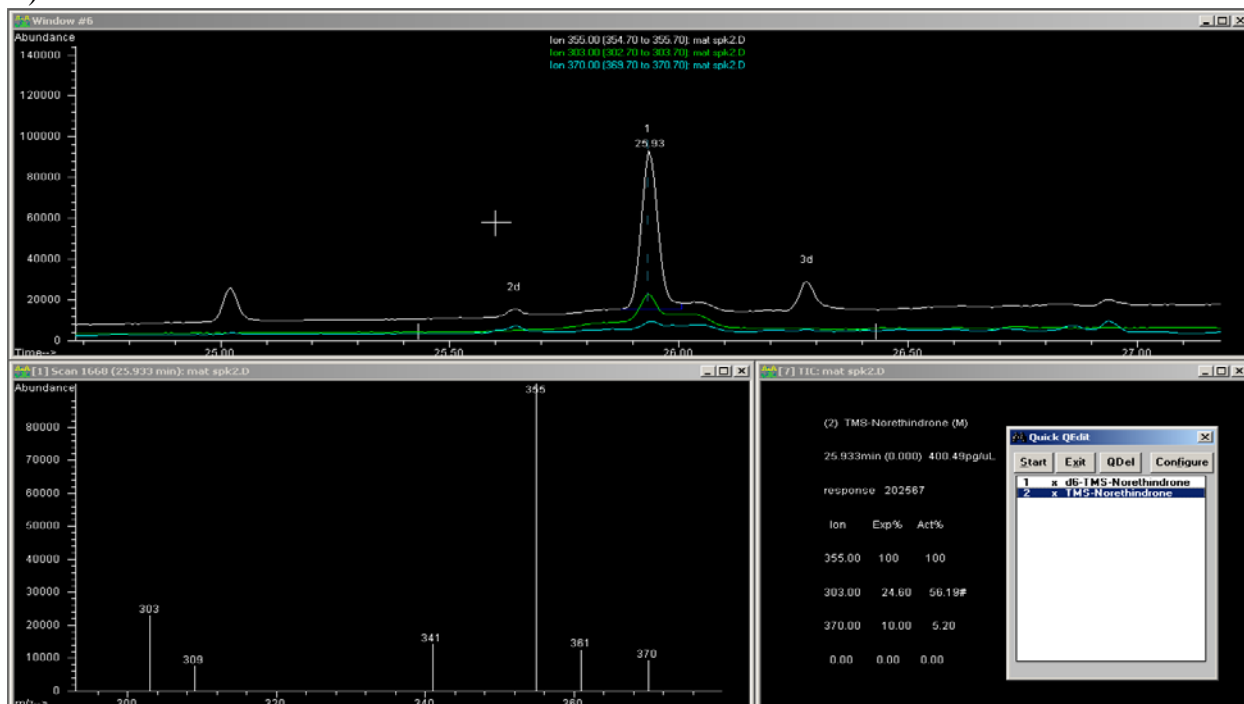
Fig. A.14. GC/MS chromatography and mass spectra of A) control muscle B) norethindrone spiked muscle at 500 pg µl^{-1}.

A)
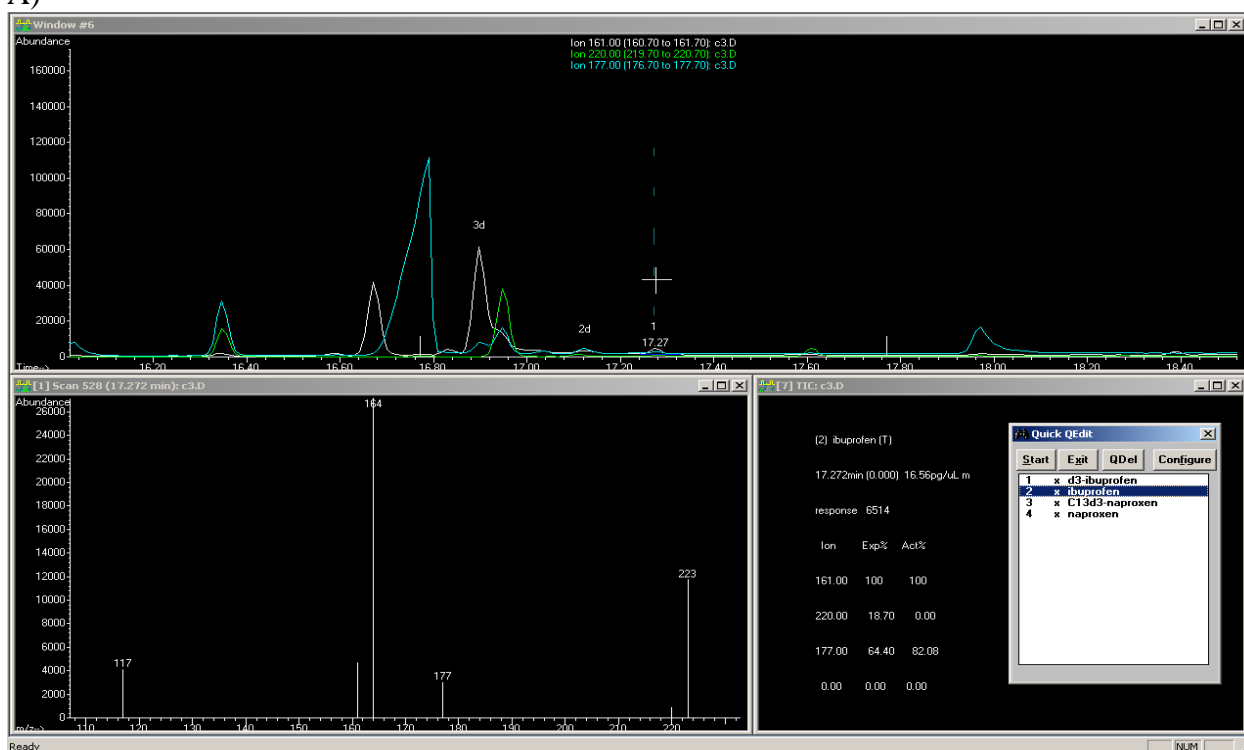
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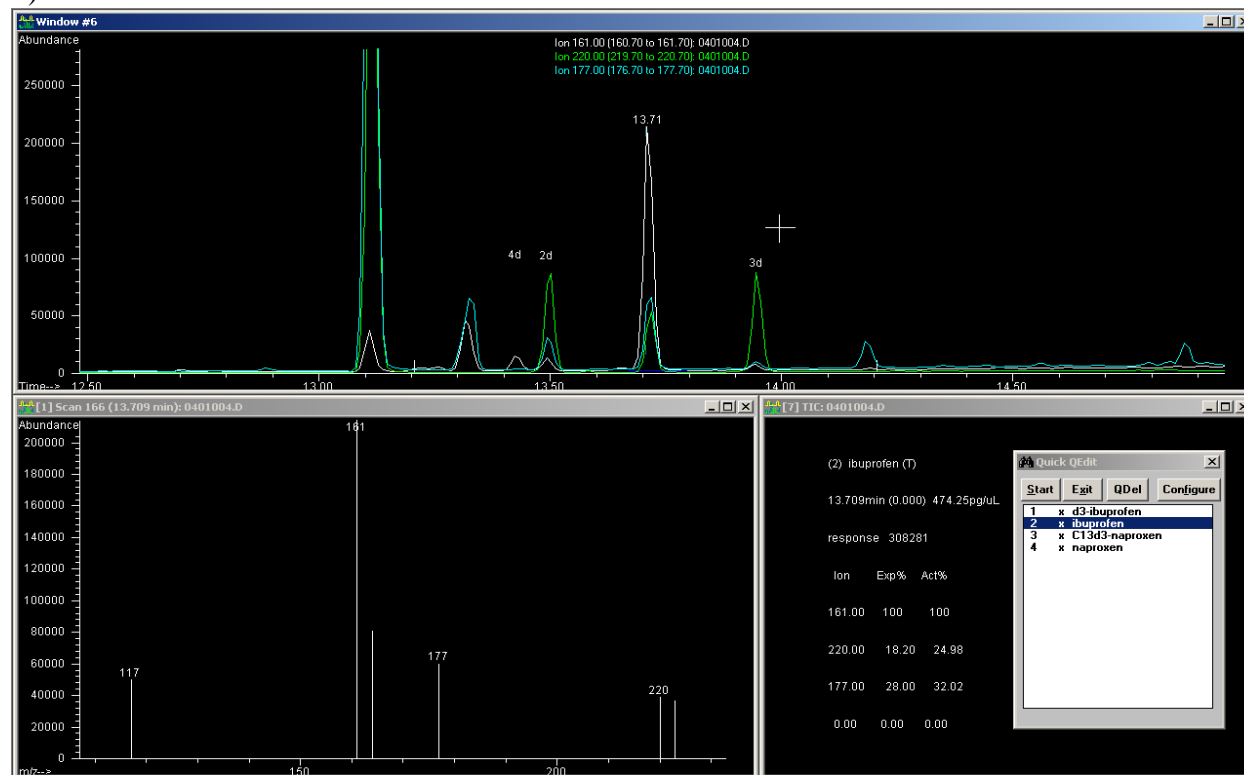
Fig. A.15. GC/MS chromatography and mass spectra of A) control muscle B) ibuprofen spiked muscle at 500 pg µl^{-1}.
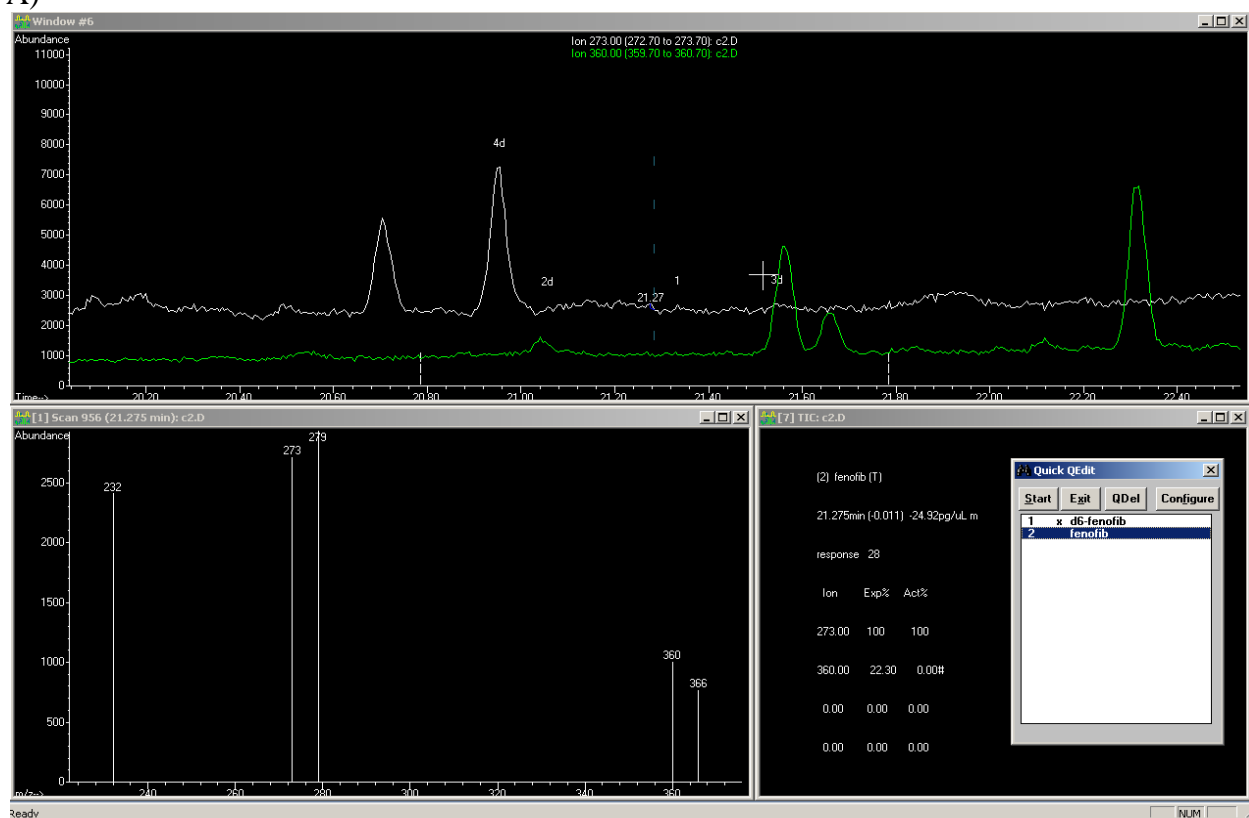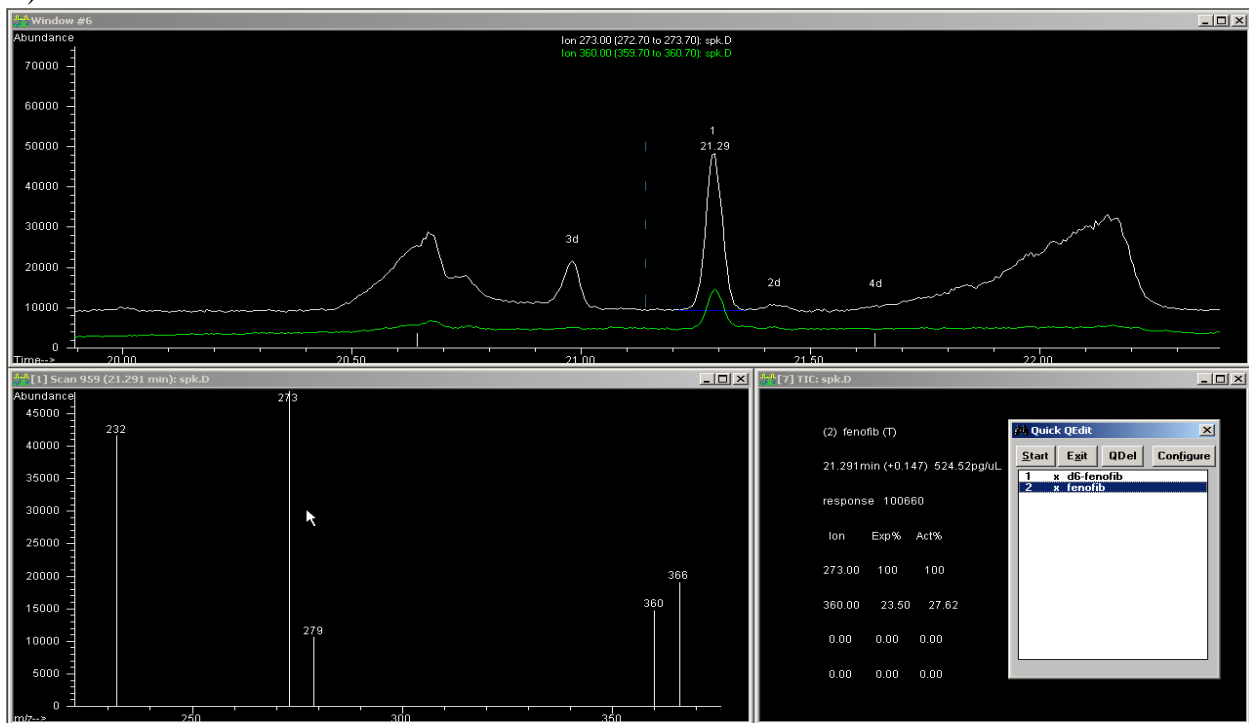
Fig. A.16. GC/MS chromatography and mass spectra of A) control muscle B) fenofibrate spiked muscle at 500 pg µl^{-1}.

A)
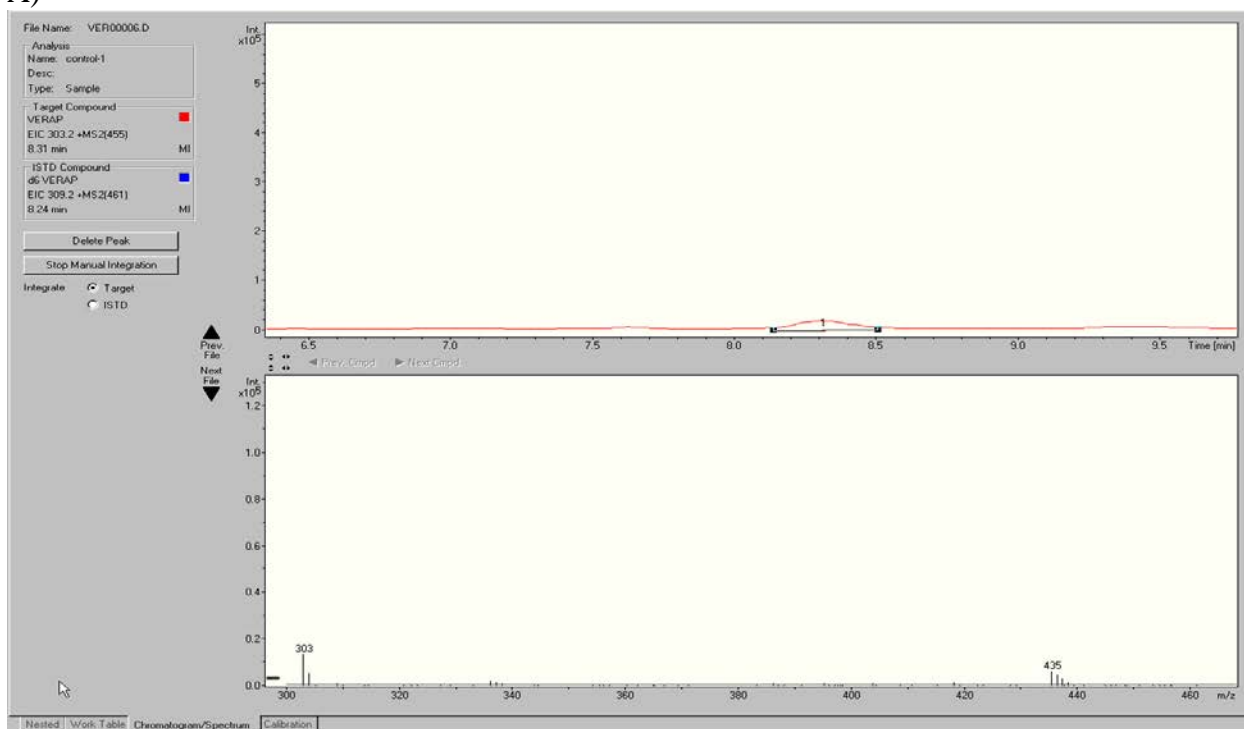
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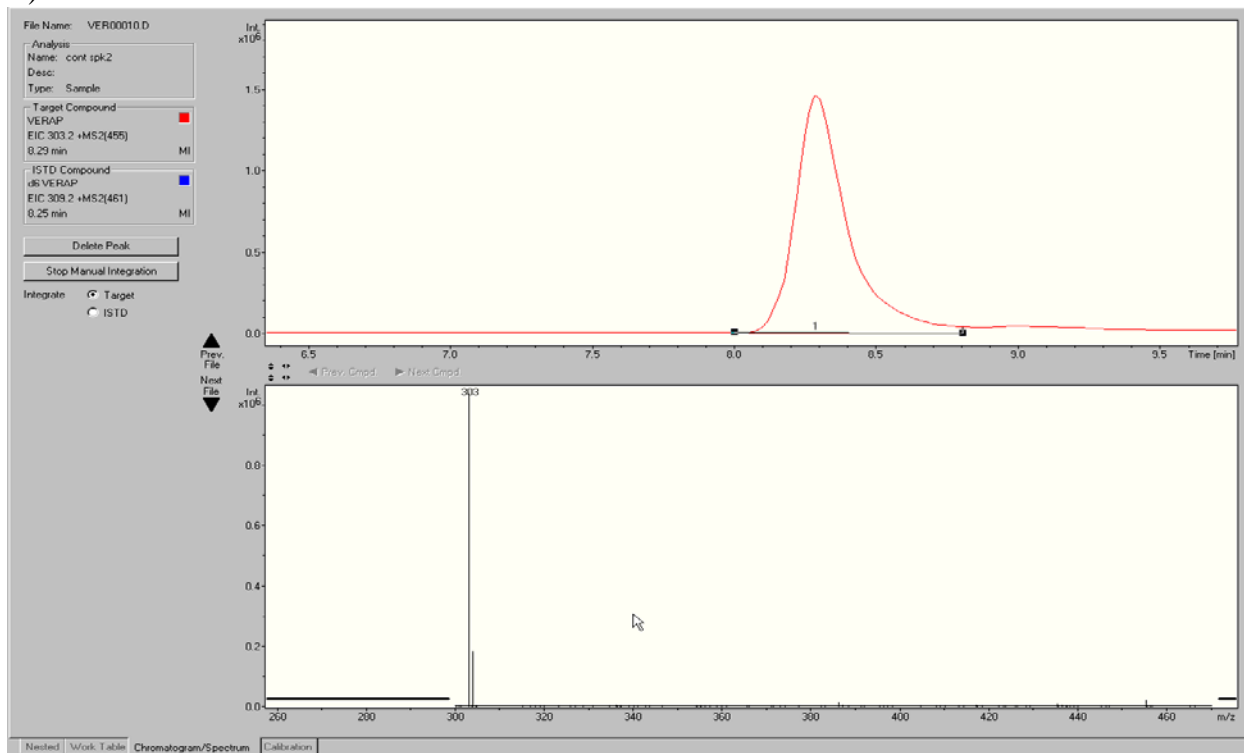
Fig. A.17. LC/MS/MS chromatography and mass spectra of for A) control muscle B) verapamil spiked muscle at 1000 pg μl^{-1}.

A)
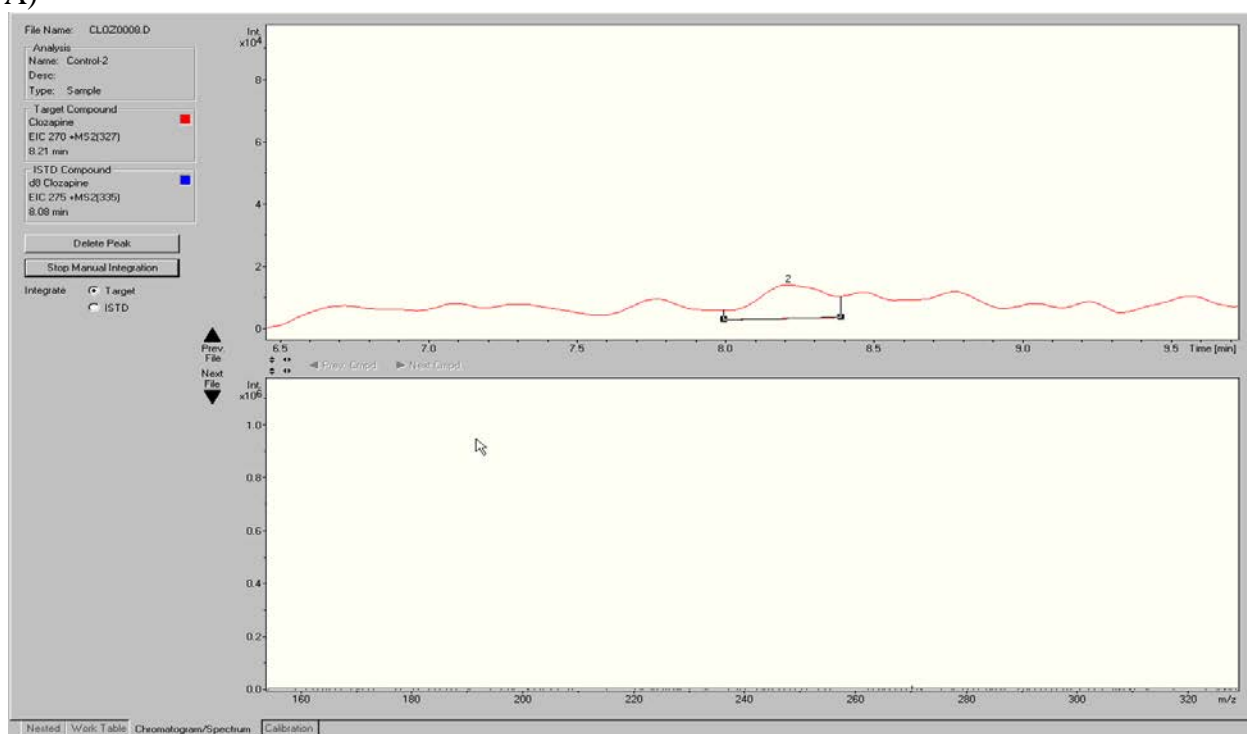
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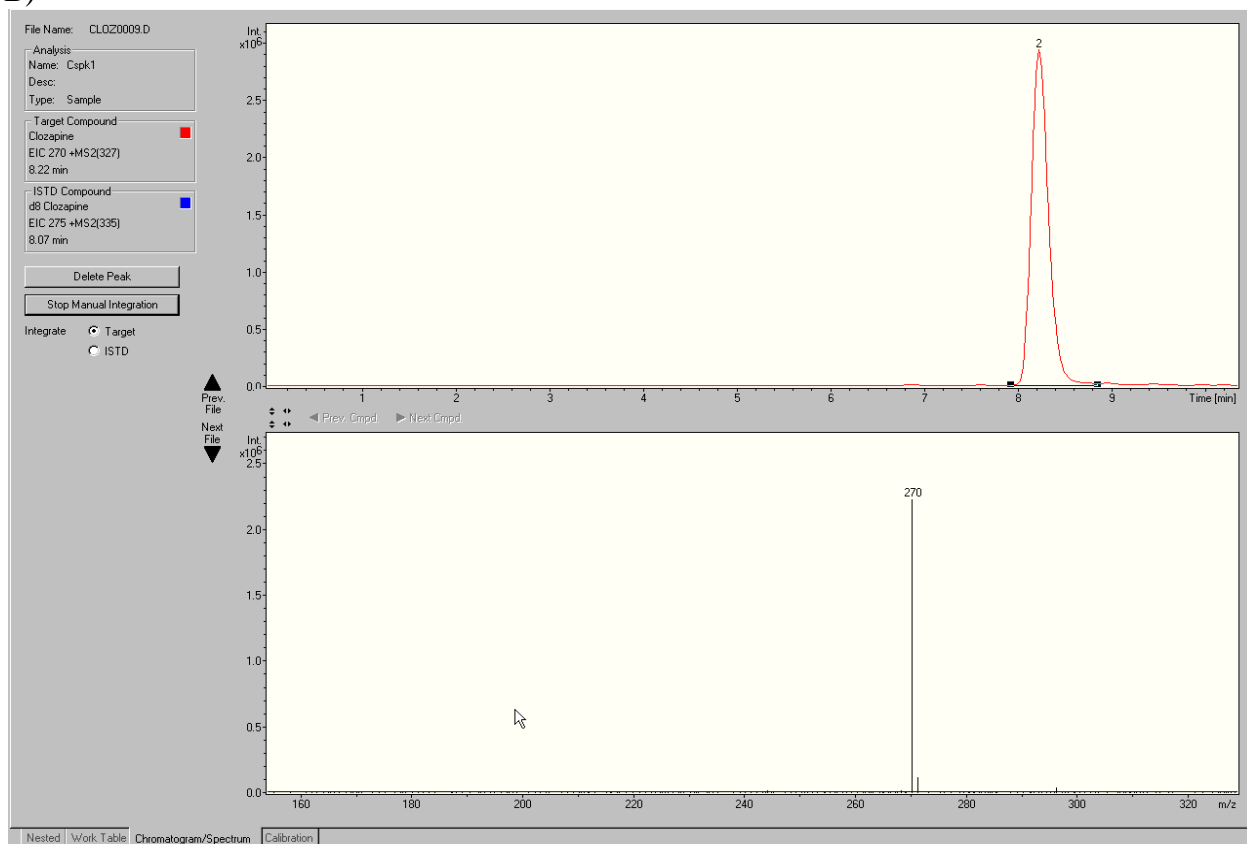
Fig. A.18. LC/MS/MS chromatography and mass spectra of for A) control muscle B) clozapine spiked muscle at 250 pg μl^{-1}.

APPENDIX B

NORETHINDRONE (NET) 7-DAY BCF TEST WITH RAINBOW TROUT
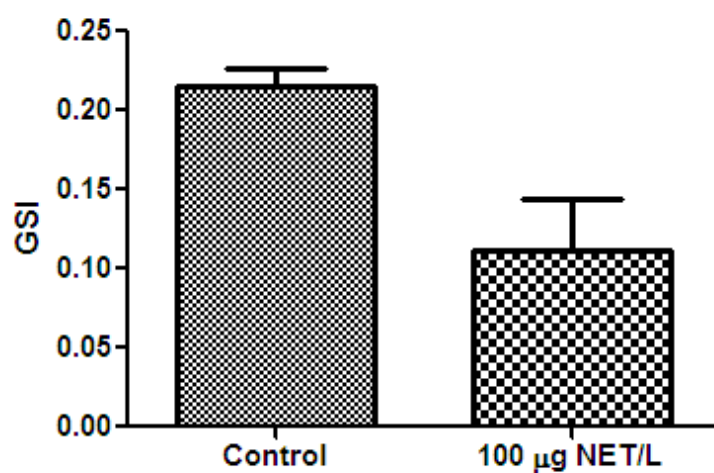
Fig. B.1.Gonadal somatic index (GSI) of rainbow trout exposed to norethindrone (100 µg l^{-1}) for 7 days.
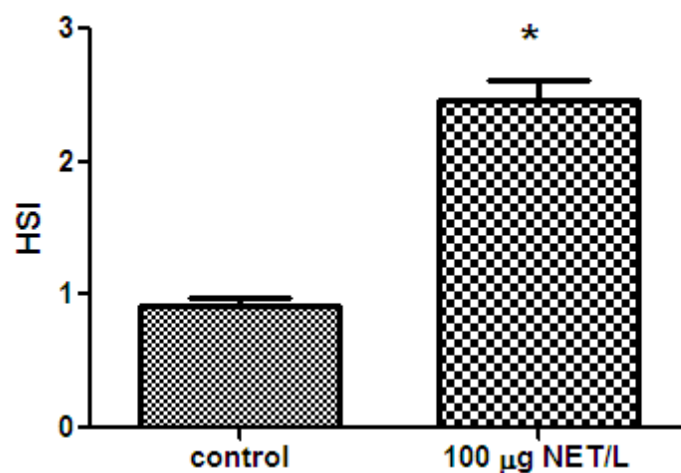
Fig. B.2. Hepatic somatic index (HSI) of rainbow trout exposed to norethindrone (100 µg l^{-1}) for 7 days. HSI of exposed fish is significantly higher (*P*=0.02) than that of control fish.
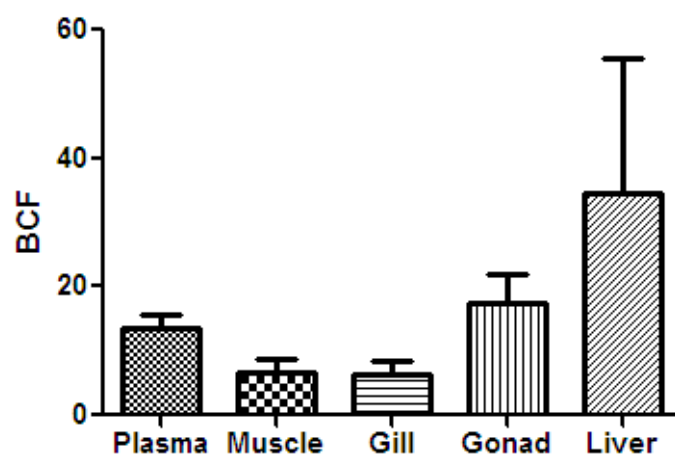
Fig. B.3. Norethindrone BCFs in plasma and tissues of rainbow trout exposed to100 µg l^{-1} for 7 days.

APPENDIX C

KINETIC PARAMETER CALCULATOR: DETERMINATION OF UPTAKE AND

DEPURATION RATE CONSTANTS

```
/**********************************************************************
*************/
/* PROGRAM: KPC.SAS (Kinetic Parameter Calculator for bioconcentration studies)       */
/* PURPOSE: A Kinetic Parameter Calculator for aquatic bioconcentration studies.       */
/*       Calculates first order rate constants K2 and K1 (depuration and uptake rate   */
/*       constants)using the sequential method (combining linear and nonlinear         */
/*       regression) to estimate the rates.                              */
/*       Based on methods in Newman, M.C. 1995. Quantitative Methods in Aquatic        */
/*       Toxicology. CRC Press, Boca Raton, as Modified by T.A. Springer. Provided     */
/*       courtesy of Wildlife International, Ltd., Easton MD       4/30/09       */
/**********************************************************************
*************/

/* INSTRUCTIONS:                                         */
/* TYPE IN THE EXPOSURE CONCENTRATION AS THE VALUE AFTER '%let
WATERCONC =' IN THE       */
/*  SAS CODE AND ENTER CONCENTRATIONS OF CHEMICAL AFTER 'CARDS'
*/
/* STATEMENTS IN THE SAS CODE BELOW. NOTE THAT DEPURATION AND
UPTAKE DATA ARE ENTERED     */
/* IN SEPARATE DATA BLOCKS. MODIFY TITLES AS NEEDED, USING DOUBLE
QUOTES TO ENCLOSE       */
/* THE TEXT. INCLUDE THE MACRO VARIABLE NAME &WATERCONC TO HAVE
THE EXPOSURE CONC.       */
/* APPEAR IN THE TITLE.                                      */

%let WATERCONC = 0.039;
Title1 "Project 100A-001 -- Test Concentration = &WATERCONC ug/L";
Title2 "Tissue: whole fish - total radioactivity";


/* ENTER DEPURATION DATA SO AS TO ALTERNATE DEPURATION DAY AND
MEASURED CONCENTRATIONS OF */
/* CHEMICAL IN FISH TISSUES.                                   */

DATA ELIMIN;
      INPUT DAY2 CONC2 @@;
      LCONC=LOG(CONC2);
CARDS;
3 137 3 212 3 165 3 165
6 113 6 72.0 6 83.7 6 73.7
10 48.8 10 51.3 10 58.7 10 70.6
14 39.9 14 29.5 14 41.2 14 35.0
;
RUN;
```

```
/* ESTIMATE K2 (DEPURATION RATE CONSTANT) = SLOPE OF REGRESSION OF
LOG CONCENTRATION OVER TIME */
/* DURING DEPURATION PERIOD. */

ODS OUTPUT ParameterEstimates=MyParameterEstimates FitStatistics=MyFitStatistics;

PROC GLM;
        MODEL LCONC=DAY2/intercept solution ;
        OUTPUT OUT=TSCONC2 P=Pred_Conc R=Residual;
RUN;

DATA STAGE_ONE;
  set myParameterEstimates;
  if parameter ='DAY2' then estimate = abs (estimate);
  if parameter ='DAY2' then call symput('DepSlope',estimate);
  if parameter ='DAY2' then parameter = 'K2';
run;

PROC PRINT; RUN;

/*PLOT THE DEPURATION GRAPH (SHOWING K2)  */
PROC PLOT DATA=TSCONC2;
        PLOT LCONC*DAY2 Pred_Conc*DAY2="*"/OVERLAY;
        PLOT Residual*DAY2="*"/VREF=0;
RUN;

/* ESTIMATE K1 (UPTAKE RATE CONSTANT) AFTER READING IN UPTAKE DATA
*/

/* ENTER UPTAKE DATA SO AS TO ALTERNATE DEPURATION DAY AND
MEASURED CONCENTRATIONS OF     */
/* CHEMICAL IN FISH TISSUES.                                   */

DATA ACCUM;
        INPUT DAY CONC @@;
        LCONC=LOG(CONC);
CARDS;
1 144 1 178 1 167 1 155
5 285 5 313 5 331 5 396
8 305 8 358 8 338 8 376
13 551 13 612 13 583 13 617
20 487 20 416 20 455 20 492
26 522 26 483 26 524 26 513
;
PROC PRINT;RUN;
```

```
/* NOW THE PROGRAM SUBSTITUTES THE ESTIMATED K2 (&DEPSLOPE) FROM
THE PREVIOUS PART OF    */
/* THE PROGRAM INTO THE UPTAKE PERIOD MODEL AND CALCULATES K1 USING
NONLINEAR REGRESSION.*/

PROC NLIN DATA = ACCUM;
      PARMS K1=0.001;
      BOUNDS 0<K1;
      MODEL CONC= &waterconc*(K1/&DEPSLOPE)*(1-EXP(-&DEPSLOPE*DAY));
      OUTPUT OUT=TSCONC3 P=Pred_Conc R=Residual;
RUN;

PROC PRINT; RUN;

PROC PLOT;
      PLOT CONC*DAY Pred_Conc*DAY="*"/OVERLAY;
      PLOT Residual*DAY="*"/VREF=0;
RUN;
```

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