DISSERTATION

3-CHLORO-P-TOLUIDINE HYDROCHLORIDE METABOLISM AND DETECTION OF EXPOSURE IN BIRDS

Submitted by

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ABSTRACT

3-CHLORO-P-TOLUIDINE HYDROCHLORIDE METABOLISM AND DETECTION OF EXPOSURE IN BIRDS

The avicide 3-chloro-4-methylanaline hydrochloride (chloro-*p*-toluidine hydrochloride, CPTH, DRC-1339) is used to control pest bird species that damage agricultural crops. While it is theorized that CPTH is a nephrotoxin, no definitive assessment of the mode of action has been performed. Additionally, the metabolic pathway of CPTH in birds has yet to be elucidated.

Radioactively labeled [^{14}C]-3-chloro-4-methylaniline hydrochloride (250 µg per bird) was delivered to 21 red-winged blackbirds (*Agelaius phoeniceus*) and 21 dark-eyed juncos (*Junco hyemalis*) via oral gavage, and the distribution and excretion of radioactivity were determined at 15 and 30 minutes and 1, 4, 8, 12, and 24 hours (n = 3 per time point). Direct measurement of radioactivity as well as measurement following combustion was accomplished using a liquid scintillation counter. Elimination from most tissues followed a two-compartment model, with very rapid elimination occurring between time 0 and 4 hours and a much slower elimination phase occurring after that. The average half-life of elimination for the initial phase in most tissues examined was 0.16 hours for juncos and 0.62 hours for blackbirds. The average for the slower second phase of elimination was 3.4 hours for juncos and 5.4 hours for blackbirds. The radioactivity in blackbird kidney tissues did not change significantly for the duration of the test, pointing toward the kidney as a possible site of action for this important agricultural chemical.

To further explore the mechanistic toxicology of CPTH, sub-cellular preparations were made from the liver and kidney of various avian species. *In-vitro* metabolism experiments were performed

using these preparations and the resulting metabolites were identified and quantified. Two metabolites were identified: 3-chloro-4-methylacetanilide (CAT) and N-[3-chloro-4-(hydroxymethyl)phenyl]acetamide (OH-CAT).

A comparison of two methods was made for the analysis of CPTH and its metabolites. Due primarily to the solubility and volatility of the three compounds, CPTH and CAT performed well on gas chromatography tandem mass spectrometry (GC/MS/MS) and adequately on liquid chromatography tandem mass spectrometry (LC/MS/MS). Conversely, OH-CAT performed optimally on LC/MS/MS. LC/MS/MS was chosen as the technique for analysis of exposure data. Both methods generated residue values that demonstrated a high degree of variability between individuals. Despite the variability issues, the data showed that the primary chemical species present in the tissues of exposed birds was OH-CAT, and that the concentration of observed residue was related to the dose administered.

In an effort to identify the target for tissue binding of CPTH or its metabolites in the kidney of exposed red-winged blackbirds, protein samples were extracted and digested with trypsin. Several chemical compounds were found to be significantly different between treated and control groups (α=0.05) and were subjected to tandem mass spectrometry to identify their chemical structure. Results from this analysis did not yield any identification of specific protein binding. Limitations of sensitivity and lack of sample enrichment likely led to this outcome.

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DEDICATION

This work would not have been possible without the support of my loving wife, Mary. Her steadfast belief in me and my ability to do this has been incredible. I love her with all my heart and could not have done it without her.

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CHAPTER 1 – INTRODUCTION

Agricultural crops suffer damage and losses from many sources. Among sources of damage are those due to birds feeding on the crops. The extent of this damage can vary dramatically from location to location. In some cases, the damage from these birds can be so extensive as to result in loss of an entire crop. Such effects can be devastating to the livelihoods of individual farmers (Kleingartner, 2003). Some tools that are available to control or minimize damage to crops include hazing, fumigants, shooting, habitat modification, and use of lethal agents.

During the post-World War II era, the United States experienced a rapid expansion in the chemical industry. New chemicals and products were being tested for any number of uses. Along with this increase in industrial chemical production came trials seeking new pesticides. Thousands of chemicals were screened for their bioactivity as pesticides (Eisemann et al., 2003). The results of this screening process were many new rodenticides and herbicides. Among those compounds identified for potential development was 3-chloro-*p*-toluidine hydrochloride (3-chloro-4-methylaniline hydrochloride; CPTH, DRC-1339), which was developed as an avicide. CPT (the non-hydrochloride free-base analog of CPTH) was a by-product of dye manufacture and of no commercial significance. It was found to have toxic effects at low exposure levels in blackbirds, starlings, rock doves, and corvids, all of which are species known or suspected to cause significant damage to agricultural crops as well as posing a risk to human health and safety.

CPTH is used for the control of pest bird species that damage agricultural crops, present hazards to aircraft, or have the potential to threaten human health or safety. Extensive research has been performed to determine its toxicity to many target and non-target bird species. It is a chemical agent that is a potent avicide to susceptible species and is capable of producing lethality following

ingestion of as little as one grain of rice containing 2% CPTH (Schafer, 1984). Integrated pest management (IPM) is a strategy for controlling pest species that incorporates multiple approaches to provide long term control of pests. IPM combines responsible pesticide use, habitat modification, use of pest-resistant crop strains, and changes to cultivation practices. It provides a holistic approach rather than focusing on one technique. As part of an IPM plan, use of CPTH has the potential to reduce crop damage caused by pest bird species.

Various products and approaches exist to minimize damage to crops from pest birds. An excellent review of the various approaches can be found in Linz et al. (2011). Hazing of the pest birds has been used since the first scarecrows were erected. Various scare devices such as air cannons have been used with limited effectiveness. The birds quickly become acclimated to the use of such devices and go back to feeding on crops. Chemical repellents containing a variety of compounds have been tested. Current products containing either methyl anthranilate or anthraquinone are the most popular. These products can be quite effective, but do not reduce populations merely pushing the over-populated birds to feed at another location. Habitat modification likewise discourages pest birds from feeding in certain regions, but does not reduce populations. Lethal control chemicals have also been tested over the years. Current products include Avitrol, which contains 4-aminopyridine, and DRC-1339 Concentrate, which contains CPTH. While Avitrol is a lethal control agent, the compound produces visible signs of distress in birds that consume the treated bait which has the added benefit of frightening other birds from the site of application. This can be a benefit to controlling bird-caused crop damage, but can cause public relations issues due to the obvious distress of the birds. CPTH produces lethality with very little evidence of distress and no vocalizations.

CPTH was initially registered with the U.S. Environmental Protection Agency (EPA) as an avicide in 1967 (Schafer, 1984), and is currently registered with the EPA under five labels: Compound DRC-1339 Concentrate – Feedlots, Compound DRC-1339 Concentrate – Gulls, Compound DRC-1339 Concentrate – Pigeons, Compound DRC-1339 Concentrate – Livestock, Nest & Fodder Depredations, Compound DRC-1339 Concentrate – Staging Areas. Under these five labels, CPTH is registered for controlling 22 bird species: Brewer's blackbird (*Euphagus cyanocephalus*), red-winged blackbird (*Agelaius phoeniceus*), yellow-headed blackbird (*Xanthocephalus xanthocephalus*), tri-colored blackbird (*Agelaius tricolor*), common grackle (*Quiscalus quiscula*), boat-tailed grackle (*Quiscalus major*), great-tailed grackle (*Quiscalus mexicanus*), brown-headed cowbird (*Molothrus ater*), European starling (*Sturnus vulgaris*), common raven (*Corvus corax*), Chihuahuan raven (*Corvus cryptoleucus*), American crow (*Corvus brachyrhynchos*), fish crow (*Corvus ossifragus*), black-billed magpie (*Pica hudsonia*), rock pigeon (*Columba livia*), eurasian collared dove (*Streptopelia decaocto*), herring gull (*Larus argentatus*), great black-backed gull (*Larus marinus*), ring-billed gull (*Larus delawarensis*), laughing gull (*Larus atricilla*), western gull (*Larus occidentalis*), and California gull (*Larus californicus*). It is registered for use in feedlots, at nesting areas, airports, dumps, landfills, rooftops, rangeland, pasture land, stubble fields, harvested dormant hay fields, open grassy or bare-ground noncrop areas, roads, roadsides, rooftops, industrial and commercial structures, and secured parking areas.

CPTH is a restricted use pesticide, meaning that it can only be used by licensed pesticide applicators under very tightly controlled conditions. Typically, these restrictions include observation of the proposed baiting site by the applicators to verify that there are no threatened or endangered species present. Use of CPTH involves a pre-baiting event in which a non-toxic form of the bait is placed at the intended bait site to verify that the target birds will accept the bait and that non-target

species are absent from the test area. It can be applied at between 0.2 and 2.0% (w/w) on brown rice, rolled barley, whole or cracked corn, whole or rolled milo, rolled whole corn, high nutrition animal feed, whole raisins, cull French fries or waste potatoes, dry dog or cat food, distiller's grain, bread slices, stale pastries, croutons or cubed bread, unpopped popcorn, dried peas, dried lentils, eggs, and meat cubes.

Detection of CPTH exposure in both target and non-target birds has been very problematic. Very early efforts to detect exposure were based on necropsies of the carcasses with examination to attempt to detect physiological indicators of exposure such as the accumulation of uric acid deposits (Johnston et al., 1999). Such deposits were thought to be indicative of CPTH exposure (DeCino et al., 1966). More recent advances have seen the use of deuterated standards and gas chromatography with mass spectrometry for the detection of CPTH residues at low levels in the tissues of exposed birds (Stahl et al., 2002). This newer technique has improved performance over older techniques with the added benefit of very good analytical sensitivity. The reported limit of detection was 12 ng/g for breast muscle and 25 ng/g for GI tract (Stahl et al., 2002). Despite these improvements, difficulties with noisy baselines and low extraction efficiencies led to very few verified exposures which were also prone to false positives.

CPTH underwent a reregistration eligibility process in 1995. In essence, the EPA evaluated the registration data and the respective registered uses and determined whether the product should remain available or be withdrawn as a bird control agent. In part, the EPA's decision read "The Agency has determined that Starlicide products, labeled and used as specified in this Reregistration Eligibility Decision, will not pose unreasonable risks or adverse effects to humans or the environment. Therefore, the Agency concludes that products containing Starlicide for all uses are eligible for reregistration." (EPA, 1995) Starlicide™ is the registered trademark for one formulated

product originally produced by Ralston-Purina (St. Louis, MO). It is also sold under the trade name DRC-1339 which was the designation given to it by the U.S. Fish and Wildlife Service's Denver Research Center when it was tested in the 1960s.

Despite the EPA's decision to reauthorize its use and the restrictions put upon its use, CPTH remains a very controversial chemical. Concerns among members of the public and environmental activists surround its continued use. Secondary hazards (in which birds that feed on the CPTH are then depredated by predators) and non-target hazards (in which birds other than the target pest birds consume the bait directly) are often cited as reasons to discontinue the use of CPTH as a bird control agent. Further investigation into the potential for secondary hazard, an understanding of the mechanism of toxicity, and the development of improved methods for the detection of CPTH exposure in non-target animals was needed.

1.1 Hypotheses

The present research efforts were undertaken in four phases. In Phase 1 we determined the distribution and excretion of CPTH when delivered to two species of birds via an oral gavage. The hypothesis for this phase was that CPTH will be rapidly excreted from most organs. Specifically, the questions we attempted to answer were:

- What is the excretion profile for CPTH and how does it differ between two different species of birds?
- Is there any evidence of strong tissue binding in either species?

In Phase 2 we determined what metabolites are formed by CPTH *in-vitro.* We also determined if an adduct could be formed in sub-cellular preparations from avian kidney. Our hypothesis for this

aim was that a reactive metabolite could be formed *in-vitro* and that it could attach to a target compound. Specifically, the questions we attempted to answer were:

- Can a reactive metabolite be formed *in-vitro*?
- Can glutathione or another suitable molecule be used as a substrate for this metabolite?
- Can we use mass spectrometry to identify the metabolites?

In Phase 3 we used the information generated in Phase 2 to look for metabolites of CPTH in the tissues of birds exposed to CPTH *in-vivo*. The specific questions related to this hypothesis were:

- What are the residue levels of CPTH and its metabolites in birds that were given a field-relevant dose of CPTH?
- Can the metabolites from Phase 2 be used as a diagnostic test for field exposures to CPTH?
- How does a newly developed liquid chromatography technique compare to a previously used gas chromatography technique?

In Phase 4 we used proteomics and metabolomics to look for two things: any protein adduct formed by CPTH or one of its metabolites, and previously unknown metabolites that were not detected in Phase 2. Our working hypothesis for this aim was that CPTH formed a reactive metabolite in the kidney of exposed birds that formed a covalent bond with the structural components of the kidney. We also believed that mass spectrometry could be used to detect this adduct or metabolite. The questions we attempted to answer were:

- Can a protein adduct be identified from a trypsin digest of whole kidney?

- Can additional metabolites be identified in the kidney of birds following an *in-vivo* exposure?
- Can tandem mass spectrometry be used to elucidate the structure of any metabolites?

CHAPTER 2 – PHASE 1 – RADIOISOTOPE EXPERIMENTS

2.1 Introduction[1]

Responsible use of any pesticide requires consideration of the potential effects to non-target species. Although specific baiting techniques and tools are employed to minimize potential exposures to secondary and non-target species (Pipas et al., 2003), inadvertent exposure is still possible. The potential risk for non-target species to be exposed should be investigated. The toxic effects of CPTH to birds have been known since the 1960s (Peoples and Henry, 1964; Schwab et al., 1964). Since that time, continued use has sparked extensive research of this chemical (Apostolou, 1969; DeCino et al., 1966; Mull, 1971; Westberg, 1969). A great deal is known about the acute toxicity of CPTH to various avian and mammalian species (Eisemann et al., 2003). Some investigations of the potential mode of action have been performed as well. These studies focused primarily on the pathological effects in exposed tissues and certain biochemical parameters such as blood pH (Apostolou, 1969; Mull, 1971; Mull et al., 1971). One thesis research project speculated which metabolites might be formed on the basis of likely metabolic pathways and then attempted to quantify these preselected metabolites in exposed bird species (Westberg, 1969). Packed-column gas-liquid chromatography was used to provide quantitation of the selected metabolites. However, no known attempt has been made to identify potential intermediate or unknown metabolites or to elucidate a mechanistic mode of action for this powerful toxicant.

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1 The majority of the research presented in this chapter was originally published in Goldade, et al. (2004), but has been added to and modified for this dissertation. Additional data and figures related to the binding of radioactivity have been added. Figure numbers have been modified to reflect that they are specific to this chapter, e.g. Figure 1 is now Figure 2.1. The material has been approved for inclusion in this dissertation (copyright clearance: Appendix I). The original article may be accessed at: http://pubs.acs.org/doi/abs/10.1021/jf0493977#ChemWorx_10.1021__jf0493977.

CPTH is a fairly selective pesticide with respect to the amount of chemical required to produce toxic effects in various species. It is somewhat common for differences in toxic response to exist between taxonomical classes or even orders. It is less common for large differences to exist within taxonomic families; CPTH possesses such a feature. An extensive review of the literature concerning CPTH toxicity has been performed (Eisemann et al., 2003). The data reviewed clearly demonstrate the differences in toxic response between varied avian families. Those whose acute oral LD_{50} values (LD_{50} is defined as the concentration of a chemical which is likely to cause mortality in 50% of exposed individuals) are >25 mg/kg could be referred to as resistant. Those possessing LD_{50} values of <25 mg/kg are characterized as sensitive to the toxic effects of CPTH. Understanding these differences could contribute to the more effective and responsible use of this important compound.

2.1.1 Hypothesis

Our hypothesis is that CPTH will be rapidly excreted from most organs. Specifically, the questions we are attempting to answer are:

- What is the excretion profile for CPTH and how does it differ between two different species of birds?
- What is the rate of elimination for CPTH and its metabolites in two species of birds?
- Is there any evidence of strong tissue binding in either species?

2.2 Materials and Methods

2.2.1 Animal Dosing

As a first step toward understanding these differences in toxic response, an aqueous solution (water from in-house filter system) containing ~9.25 µCi of [^{14}C]-CPTH (51.32 mCi/mmol; 98.77% radiochemical purity; Wizard Laboratories Inc., West Sacramento, CA) was administered orally to two species of birds: red-winged blackbirds (*Agelaius phoeniceus*), a CPTH sensitive species (LD_{50}=1.8-3.2 mg/kg), and dark-eyed juncos (*Junco hyemalis*), a CPTH resistant species (LD_{50}=162 mg/kg). The radioactive CPTH was isotopically diluted with non-radioactive CPTH (98.8% pure; Purina Mills) to produce the desired exposure level of ~250 µg per animal. The birds were housed in individual glass metabolism cages for a period of time ranging from 15 minutes to 24 hours until they were euthanized by carbon dioxide (CO_2).

Test animals were trapped from wild populations in Colorado and housed indoors with free access to a standard wild bird seed mixture and water prior to testing. Test animals were held in quarantine for no less than 14 days prior to initiation of the test. For each time point, three birds were selected at random and given a dose of 250 µg of [^{14}C]-CPTH in 100 µL of deionized water via oral gavage. The dosing solution was measured using a 100-µL Hamilton syringe and carefully transferred to a 1 cm^3 plastic syringe. Adequate headspace was left in the syringe to permit full delivery of the dose. The blackbirds had an average body weight of 70 g (SD=3.5 g), whereas the juncos averaged 18 g (SD=2.2 g). The resultant dose of CPTH for each species averaged 3.6 and 14 mg/kg for blackbirds and juncos, respectively. The birds were held in a supine position by an assistant while the dose was delivered using a 1 cm^3 syringe equipped with a 20 gauge animal feeding tube (Popper and Sons Inc., New Hyde Park, NY), that was inserted through the mouth

gently until the tip was just above the proventricular opening of the gizzard. Following administration of the dose, each bird was individually housed in a glass metabolism cage with a 10 L volume (Kent Scientific, Litchfield, CT) designed for large rodents (>300 g). The cages were large enough to permit the birds to stand upright without restricting their movement. The metabolism cages were set up as a closed system such that all excreted radioactivity could be collected. Air lines were connected to the cages to permit delivery of oxygen (300 mL/min) and collection of expired air. A coarse wire mesh was used as the floor of the cage, which permitted the collection of fecal-urate (birds excrete both urine and feces through the cloaca, so the term fecal-urate will be used to refer to all waste products). The birds had free access to food and water for the duration of the test period. All procedures involving animals were carried out with the approval of the NWRC Animal Care and Use Committee (NWRC protocol QA-771).

2.2.2 Sample Collection

For each treatment group, [^{14}C]-CPTH was administered to three birds of each species as described above. At the conclusion of each exposure time, the birds were removed from their cages and euthanized via exposure to CO_2. All birds were euthanized humanely in accordance with American Veterinary Medical Association standards and practices. Each carcass was removed from the euthanizing chamber, and a sample of whole blood was taken via cardiac puncture into a heparinized syringe. The carcass was then placed in a plastic bag and frozen (at -30 °C) until dissection could be performed. The food and water dishes were emptied into individual glass jars with Teflon lids and stored at 4 °C until analysis for ^{14}C content could be performed. Expired air (CO_2) and fecal-urate samples were placed in individual sample containers and stored at -30 °C until

analysis. The cage was then washed with 2 L of deionized water, and the wash water was analyzed for ^{14}C content as per the procedures for drinking water below.

2.2.3 Collection of Expired CO_2

Expired CO_2 was collected by using two glass trapping vessels in series. Each contained 30 mL of a basic scintillation trapping solution (Carbon-14 Cocktail; R. J. Harvey Instrument Corp., Hillsdale, NJ). Expired air was initially collected from the birds in the 24-hour exposure time period and found to contain no detectable amounts of ^{14}C. Therefore, the remainder of the treatment groups were housed with a wire mesh lid on the metabolism cage rather than a glass lid and no expired air was collected for those treatment groups. The behavior of the birds indicated that they were calmer when housed in the wire mesh topped cages.

2.2.4 Tissue Collection

Each carcass was allowed to thaw slightly before proceeding with the necropsy procedure. An incision was made in the skin covering the abdomen, and the birds were skinned completely. A lateral incision was then made and a pair of scissors used to cut the breast bone on each side. The breast was removed and a portion of the muscle tissue removed from it with a scalpel. The heart, lungs, liver, gastrointestinal (GI) tract (from esophagus to cloaca with contents included), and kidneys were then individually removed. Next the brain was removed by cutting the skull laterally between the orbital sockets with a pair of scissors. A cut was then made from one eye to the other around the circumference of the anterior skull and the top of the skull removed. The brain was cut free from the spinal cord and removed. Last, a portion of the leg muscle tissue was removed using a scalpel. A pair of scissors was used to mince each tissue before it was placed into a separate pre-weighed glass homogenization tube. Fecal-urate samples were allowed to thaw and placed in a pre-

weighed glass homogenization tube. Sample weights were recorded, and a measured volume of deionized water was added to each tube to produce approximately a 3:1 water-to-sample ratio. Each tissue was homogenized using a Teflon and glass Potter-Elvehjem homogenization tube and stored at -30 °C in individual glass vials until combustion analysis could be performed. Tissue and fecal-urate samples for individual birds were processed and stored separately and were not pooled. Feed samples were ground to a fine powder using a coffee grinder, weighed, and stored at -30 °C until analysis. Drinking water, cage wash water, and whole blood samples were directly analyzed as collected without further sample preparation steps.

2.2.5 Liquid Scintillation Counting Analysis

Depending on the nature of the sample matrix (liquid, solid, or homogenate), the samples were prepared for analysis and counted on the liquid scintillation counter (Table 2.1). Radioactivity was determined using a Packard Tri-Carb 1600TR liquid scintillation counter (LSC). Samples were counted in triplicate for 10 min (4-156 keV). Drinking water and cage wash water samples were analyzed for ^{14}C content by pipetting 1 mL into a 20-mL scintillation vial containing 20 mL of Scintiverse BD scintillation cocktail (Fisher Chemicals, Fair Lawn, NJ). The response of a background blank of deionized water was subtracted from each response and the total disintegrations per minute (DPM) determined for each sample.

Subsamples of each homogenate, feed, and whole blood sample were weighed in duplicate in porcelain combustion boats containing 0.5 g of mannitol (reagent grade; EM Science, Gibbstown, NJ). The samples were combusted in an R. J. Harvey model OX-600 biological oxidizer. Oxygen and nitrogen flows were 350 mL/min. The combustion and catalyst zone temperatures were held at 900 and 680 °C, respectively, and samples were combusted for 4 min. The CO_2 produced was

Table 2.1: Sample Analysis Techniques Employed To Determine Total Radioactive Residue Levels in Various Matrices

Matrix	Direct Scintillation Analysis	Liquid Sample	Combustion Analysis	Homogenate
Expired Air	yes	no	no	N/A
Drinking Water	yes	yes	no	N/A
Cage Wash	yes	yes	no	N/A
Feed	no	no	yes	no
Fecal-Urate	no	no	yes	yes
Brain	no	no	yes	yes
Breast Muscle	no	no	yes	yes
Leg Muscle	no	no	yes	yes
GI Tract	no	no	yes	yes
Heart	no	no	yes	yes
Kidney	no	no	yes	yes
Liver	no	no	yes	yes
Lung	no	no	yes	yes
Whole Blood	no	no	yes	no

N/A = Samples of this type could not be analyzed using this method.

trapped in a ^{14}C cocktail (R. J. Harvey). The cocktail was transferred to a glass vial and counted on the LSC using the method described above.

To investigate potential tissue binding, samples were subjected to serial extraction with 20:1 (v/v) heptane:chloroform (Solvent A), 20% (w/v) trichloroacetic acid (Solvent B), 10% (w/v) trichloroacetic acid (Solvent C), methanol (Solvent D), 2:2:1 (v/v) ethanol:diethyl ether:chloroform (Solvent E), and acetone (Solvent F). Following extraction with acetone, the tissue pellet was placed in a round-bottom flask and refluxed with a solution of 1 N hydrochloric acid for one hour. Following the acid reflux step, the pellet was weighed and subjected to combustion analysis as detailed above.

2.2.6 Data Collection

For data obtained from direct measurement of aliquots of aqueous samples, the raw counts were corrected for counting efficiency by the LSC through the use of a standard of known activity and reported as disintegrations per minute (DPM). The DPM of a background sample of deionized water was subtracted from all results including control samples. The data were further corrected by subtracting the average DPM of control samples from the results obtained for test samples from the same matrix. This yielded a background-corrected value (BCV). The BCV was divided by sample mass or volume to give DPM per unit mass or volume for each sample.

Data obtained from combustion analyses were treated in a similar manner, with the following exceptions. The efficiency of the combustion apparatus was determined by combusting an aliquot of mannitol fortified with a known quantity of [^{14}C]-CPTH at the beginning and end of each day's analyses. Recovered radioactivity was determined by counting on the LSC, and the percent recovery of DPM was calculated for each fortified sample. The average recovery of the two fortified samples was used as a correction factor for that day's analyses. All results obtained on that particular day were corrected for this efficiency value. A BCV was also obtained for each tissue type by subtracting the mean DPM value of the samples obtained from control animals from the observed DPM values for treated animals. Finally, a calculation was performed to relate the obtained result back to initial tissue weight instead of homogenate weight. This was accomplished through simple ratio calculation of unit mass of tissue per unit mass of homogenate.

2.3 Data Analysis

The data were analyzed on the basis of total radioactivity in each tissue type or "compartment". This value represents an estimate of the total DPM based on the average response for duplicate

analyses of each subsample. This value incorporates any dilutions of the sample resulting from sample preparation. In most cases, the entire organ was processed as a tissue homogenate in water. Subsamples of breast muscle, leg muscle, and blood were analyzed and the resulting values used to estimate the total DPM for the entire tissue. In the case of blood samples, it was assumed that blood volume was ~10% of the body weight of the bird (Phillips, 2002). For breast muscle and leg muscle, values of 26.9 and 8.9% of body weight were chosen for blackbird breast and leg; values of 22.7 and 10.5% were chosen for junco breast and leg, respectively (Hartman, 1961). These values were used to calculate the percentage of administered dose found in each tissue compartment at each time point (Table 2.2).

The data were also evaluated on the basis of concentration of CPTH and metabolites in parts per billion of CPTH equivalents. CPTH equivalents are defined as un-metabolized CPTH and all metabolites of CPTH containing ^{14}C. During statistical analysis of the data, it was determined that a log transformation of the data produced a better linear regression result. This determination was based on inspection of the residuals and an evaluation of the R^2 values. Using non-transformed data, plots of the residuals demonstrated that the variability was not uniform, with the data points closer to time 0 having much larger residuals than those at later time points (data not shown). Residual plots of the log-transformed data produced much more uniform variability. In addition, using log-transformed data improved the R^2 values significantly. The data demonstrated a two-phase elimination profile, with a very rapid early elimination phase and a much slower secondary elimination phase. A nonlinear two-compartment model could have been used to describe the data very well. However, calculation of the half-life of elimination was a prime goal of the research. Therefore, a linear model using log of concentration as the response versus time was employed, and the data were evaluated from 0 to 4 hours and from 4 to 24 hours as two separate linear regressions.

Table 2.2: Mean Concentration (CPTH Equivalents) and Percent of Recovered Dose Values for Dark-eyed Juncos and Red-winged Blackbirds

Tissue	Time (h)	Residue Levels in Juncos[a]		Residue Levels in Blackbirds[a]	
		ppb CPTH (equiv)	% of Dose	ppb CPTH (equiv)	% of Dose
Brain	0.25	7500	1.51	1900	1.94
	0.5	2600	0.56	1400	0.91
	1	730	0.17	1300	0.75
	4	39	<0.01	55	0.03
	8	7.2	<0.01	20	0.01
	12	9.1	<0.01	23	0.01
	24	11	<0.01	15	<0.01
Breast muscle	0.25	8000	15.05	2100	35.42
	0.5	3200	6.72	1900	22.58
	1	960	2.25	1700	18.13
	4	83	0.17	100	1.05
	8	7.8	0.02	32	0.34
	12	8.4	0.01	39	0.39
	24	7.3	0.01	26	0.22
GI Tract	0.25	58000	46.99	6300	18.24
	0.5	53000	45.08	8600	19.93
	1	47000	39.54	11000	22.48
	4	8800	7.17	4500	7.98
	8	1200	0.84	2200	3.91
	12	2100	1.19	1500	2.05
	24	360	0.24	640	1.01
Heart	0.25	11000	1.20	2000	1.48
	0.5	4600	0.53	1900	1.07
	1	2000	0.24	1600	0.74
	4	230	0.03	160	0.08
	8	64	<0.01	86	0.04
	12	79	<0.01	100	0.04
	24	48	<0.01	70	0.03
Kidney	0.25	35000	3.03	6900	2.41
	0.5	20000	1.35	22000	5.40
	1	17000	1.31	16000	3.59
	4	13000	1.00	12000	2.59
	8	1400	0.09	1100	2.45
	12	5100	0.31	18000	4.13
	24	490	0.04	11000	2.20

Table 2.2: Mean Concentration (CPTH Equivalents) and Percent of Recovered Dose Values for Dark-eyed Juncos and Red-winged Blackbirds

Tissue	Time (h)	Residue Levels in Juncos[a]		Residue Levels in Blackbirds[a]	
		ppb CPTH (equiv)	% of Dose	ppb CPTH (equiv)	% of Dose
Leg Muscle	0.25	8500	7.34	1500	8.66
	0.5	3500	3.32	1500	6.05
	1	1600	1.67	1300	4.46
	4	95	0.10	98	0.33
	8	24	0.02	58	0.20
	12	22	0.02	49	0.16
	24	19	0.02	41	0.12
Liver	0.25	19000	4.58	3600	4.08
	0.5	9600	2.35	4400	3.09
	1	4500	1.15	4900	3.19
	4	950	0.25	1700	1.10
	8	400	0.08	1900	1.05
	12	220	0.04	1300	0.68
	24	170	0.04	470	0.27
Lung	0.25	12000	1.64	2300	2.42
	0.5	7400	1.03	2100	1.51
	1	3700	0.57	2100	1.19
	4	570	0.11	400	0.22
	8	230	0.03	410	0.23
	12	240	0.03	260	0.16
	24	160	0.03	270	0.14
Blood	0.25	8600	7.32	1900	12.18
	0.5	3300	3.07	2100	9.19
	1	2200	2.22	2400	9.27
	4	*b*	*b*	300	1.18
	8	*b*	*b*	100	0.40
	12	*b*	*b*	190	0.66
	24	*b*	*b*	160	0.49

[a] Each number is the average of three observations.

[b] Observed values were below values for control samples.

The results of this transformation were also used to calculate elimination constants (K_{El}) and half-life of elimination ($t_{½}$) values for both elimination phases in each tissue or bodily fluid as well as combined values for whole body elimination. The slope of this line yielded the K_{El}. From this value,

the half-life of elimination was easily determined. Because a half-life is commonly defined as the amount of time for half of the remaining radioactivity to be excreted from the body, 100% elimination can never be mathematically achieved. A close approximation can be found using 7 times the value of $t_{½}$. This accounts for elimination of 99.2% of the radioactivity. The values for each statistic as well as the p value for the slope of the regression lines are given (Tables 2.3 and 2.4).

Table 2.3: Log DPM Linear Regression Results, K_{El}, and $t_{½}$ Values for Time 15 min to 4 h (n = 12 per Species)

Tissue	R^2	Slope	p	SE	K_{El}	$t_{½}$ (h)	95% CI of $t_{½}$	99.2% Elimi-nation
				Dark-eyed Junco				
Brain	0.840	-3.0129	0.0003	0.4598	6.939	0.10	0.08, 0.15	0.70
Breast Muscle	0.830	-2.7576	0.0004	0.4360	6.351	0.11	0.08, 0.16	0.77
GI Tract	*a*	-0.3237	0.4549	0.4092	*a*	*a*	*a*	*a*
Heart	0.804	-2.1478	0.0007	0.3698	4.946	0.14	0.1, 0.22	0.98
Kidney	*a*	-0.9618	0.1650	0.6204	*a*	*a*	*a*	*a*
Leg Muscle	0.646	-2.4066	0.0055	0.6096	5.542	0.13	0.08, 0.27	0.91
Liver	0.794	-1.9284	0.0008	0.3413	4.441	0.16	0.11, 0.25	1.12
Lung	0.661	-1.4220	0.0047	0.3489	3.275	0.21	0.14, 0.44	1.47
Blood	0.547	-2.3436	0.0137	0.7176	5.397	0.13	0.08, 0.36	0.91
Carcass	0.501	-1.0753	0.0199	0.3581	2.476	0.28	0.16, 0.92	1.96
				Red-winged Blackbird				
Brain	0.949	-1.0049	<0.0001	0.0777	2.314	0.30	0.26, 0.36	2.10
Breast Muscle	0.958	-0.8850	<0.0001	0.0614	2.038	0.34	0.3, 0.4	2.38
GI Tract	0.387	-0.2169	0.0323	0.0839	0.500	1.39	0.77, 7.27	9.73
Heart	0.929	-0.7642	<0.0001	0.0704	1.760	0.39	0.33, 0.49	2.73
Kidney	*a*	-0.1257	0.2182	0.0941	*a*	*a*	*a*	*a*
Leg Muscle	0.958	-0.8233	<0.0001	0.0570	1.896	0.37	0.32, 0.43	2.59
Liver	0.803	-0.3019	0.0003	0.0492	0.693	1.00	0.74, 1.51	7.00
Lung	0.962	-0.5173	<0.0001	0.0344	1.191	0.58	0.51, 0.68	4.06
Blood	0.927	-0.6039	<0.0001	0.0564	1.391	0.50	0.42, 0.62	3.50
Carcass	0.945	-0.4467	<0.0001	0.0360	1.029	0.67	0.58, 0.81	4.69

[a] Slope not significantly different from zero; no values can be calculated.

Table 2.4: Log DPM Linear Regression Results, K_{El}, and $t_{½}$ Values for Time 4 to 24 h (n = 12 per Species)

Tissue	R^2	Slope	p	SE	K_{El}	$t_{½}$ (h)	95% CI of $t_{½}$	99.2% Elimi-nation
				Dark-eyed Junco				
Brain	*a*	-0.0466	0.2073	0.0336	*a*	*a*	*a*	*a*
Breast Muscle	0.337	-0.0879	0.0592	0.0391	0.202	3.43	1.77, 49.12	24.01
GI Tract	0.678	-0.1587	0.0039	0.0376	0.365	1.90	1.27, 3.76	13.30
Heart	0.398	-0.0963	0.0406	0.0384	0.222	3.12	1.7, 18.98	21.84
Kidney	0.414	-0.1310	0.0365	0.0508	0.302	2.29	1.27, 12.17	16.03
Leg Muscle	0.327	-0.0681	0.0626	0.0308	0.157	4.41	2.27, 81.81	30.87
Liver	0.610	-0.0770	0.0079	0.0209	0.177	3.92	2.49, 9.08	27.44
Lung	0.614	0.0508	0.0076	0.0137	0.117	5.92	3.78, 13.6	41.44
Blood	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*a*
Carcass	0.698	-0.1425	0.0031	0.0323	0.328	2.11	1.43, 4.01	14.77
				Red-winged Blackbird				
Brain	0.430	-0.0539	0.0122	0.0177	0.124	5.59	3.31, 17.8	39.13
Breast Muscle	0.342	-0.0772	0.0269	0.0298	0.178	3.89	2.16, 20.27	27.23
GI Tract	0.559	-0.0868	0.0031	0.0224	0.200	3.47	2.25, 7.55	24.29
Heart	0.234	-0.0469	0.0635	0.0225	0.108	6.42	3.22, 139.59	44.94
Kidney	*b*	-0.0049	0.8655	0.0280	*b*	*b*	*b*	*b*
Leg Muscle	0.363	-0.0424	0.0225	0.0157	0.098	7.07	3.99, 31.75	49.49
Liver	0.387	-0.0750	0.0182	0.0266	0.173	4.01	2.3, 15.59	28.07
Lung	0.257	-0.0456	0.0533	0.0208	0.105	6.60	3.38, 147.76	46.20
Blood	*b*	-0.0217	0.3724	0.0233	*b*	*b*	*b*	*b*
Carcass	0.463	-0.0500	0.0089	0.0154	0.115	6.03	3.66, 17.02	42.21

[b] Values less than background; data not used. [b] Slope not significantly different from zero; no values can be calculated.

2.4 Results and Discussion

Red-winged blackbirds and dark-eyed juncos were chosen to represent species that exhibit varied sensitivities to CPTH. Red-winged blackbirds, a target species, are highly sensitive to CPTH, having an LD_{50} of 1.8-3.2 mg/kg (Schafer, 1972 and 1994), whereas dark-eyed juncos, a non-target species, are much less susceptible, with an LD_{50} of 162 mg/kg (Eisemann et al., 2003). Several variables

were considered in the design of this experiment: CPTH dose level, ^{14}C activity in dose, route and method of exposure, and length of exposure time.

Selection of dose level, that was representative of a real-world exposure, was one parameter considered. The selection of an appropriate dose level was based upon ingestion of a single grain of 2% CPTH-treated rice bait. Previous research with CPTH indicated that a single treated rice grain would typically be sufficient to induce acute toxicity in sensitive species, and that by diluting treated rice 1:25 with untreated rice, the desired exposure could be achieved (Schafer, 1984). This is equivalent to a dose of ~4 mg/kg for a 100-g bird. During study design, we assumed that the weight of the more sensitive blackbirds would be ~60 g. Therefore, a final dose of ~250 µg of CPTH was selected. The dose was uniform for all birds and was not adjusted for differences in body weight. Another key consideration was the desire to produce no mortality during the course of the study. The dose level chosen was below the published LD_{50} values for juncos and below a level expected to produce mortality in blackbirds in <24 hours. No mortalities occurred during the study as a result of CPTH toxicity: therefore, the dose seemed to be appropriate for the intended purpose.

The level of ^{14}C activity administered to each bird was based on the limit of detection (DL) for the LSC and biological oxidizer. The limit of detection for the biological oxidizer was ~50 DPM. Using this value, a method limit of detection for the samples was found to be ~2000 DPM for a 10-g tissue sample. If the bird retained as little as 1% of the administered dose in its body and a small portion of that dose was contained in the tissue being analyzed, the response for that sample should be ~10-fold above the method DL.

The route and method of exposure were chosen to permit a reasonable assurance that the entire dose was ingested by each test animal. The predominant route of exposure in a field application is via ingestion; therefore, an oral gavage of CPTH in 100 µL of deionized water was used.

Sampling times were selected to provide a cross-section of exposure. Previously published data indicated that most of the CPTH would be excreted in <24 hours (Giri et al., 1976; Westberg, 1969). Information on rate of distribution would also be important; therefore, the sampling times were focused toward shorter time periods rather than being evenly spaced over the 24-hour test period. The times selected were 15 and 30 minutes and 1, 4, 8, 12, and 24 hours post dose. The results for the 15- and 30-minute and 1- and 4-hour time points are found in Figure 2.1. No further time points are shown as greater than 90% of the radioactivity was excreted by the 4-hour time point.

In most of the tissues and fluids collected from both bird species tested, a similar pattern was observed in plots of the radioactive residue (RR) as a function of time post dose. In the case of every tissue except blackbird kidney, greater than 80-90% of the administered dose was excreted from the body of the test animal by 4 hours after exposure. This supports the conclusion that CPTH is not retained in the carcass of exposed birds in significant quantities, with the exception of the RR in liver and kidney of exposed birds.

In the case of blackbird kidney (Figure 2.2), RR levels significantly higher than background levels were observed for the duration of the test. This level of radioactivity is approximately equivalent to a mean kidney concentration of 15 mg/kg of CPTH equivalents. A slight elevation of RR level was also observed in junco kidney (Figure 2.2) and in blackbird liver (Figure 2.3). However, the results were not as pronounced as those for blackbird kidney. Possible reasons for this difference in elimination relate to potential binding of CPTH metabolites to these sensitive tissues.

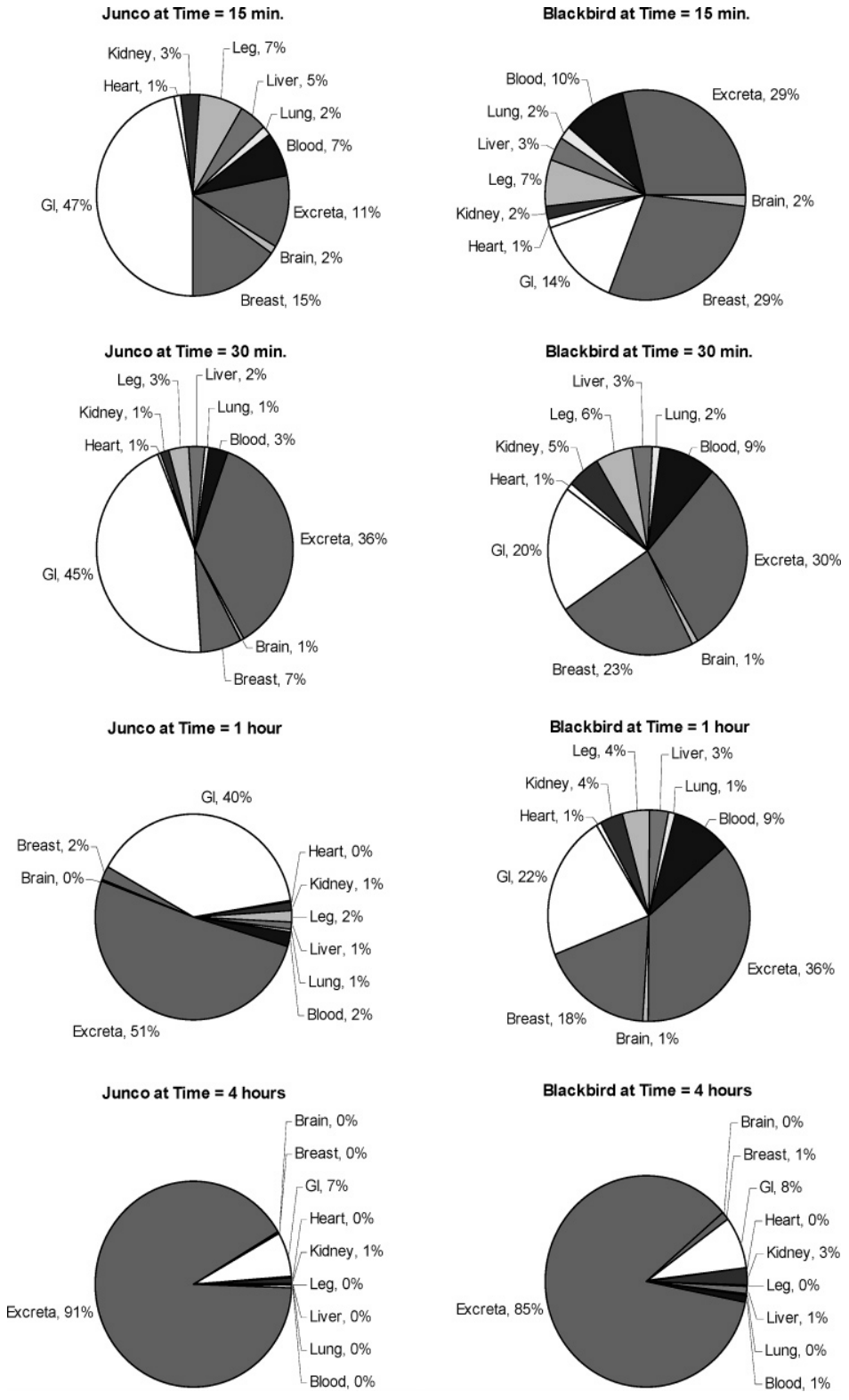


Figure 2.1: Percentage of radioactive CPTH and metabolites in each tissue at four sampling times.

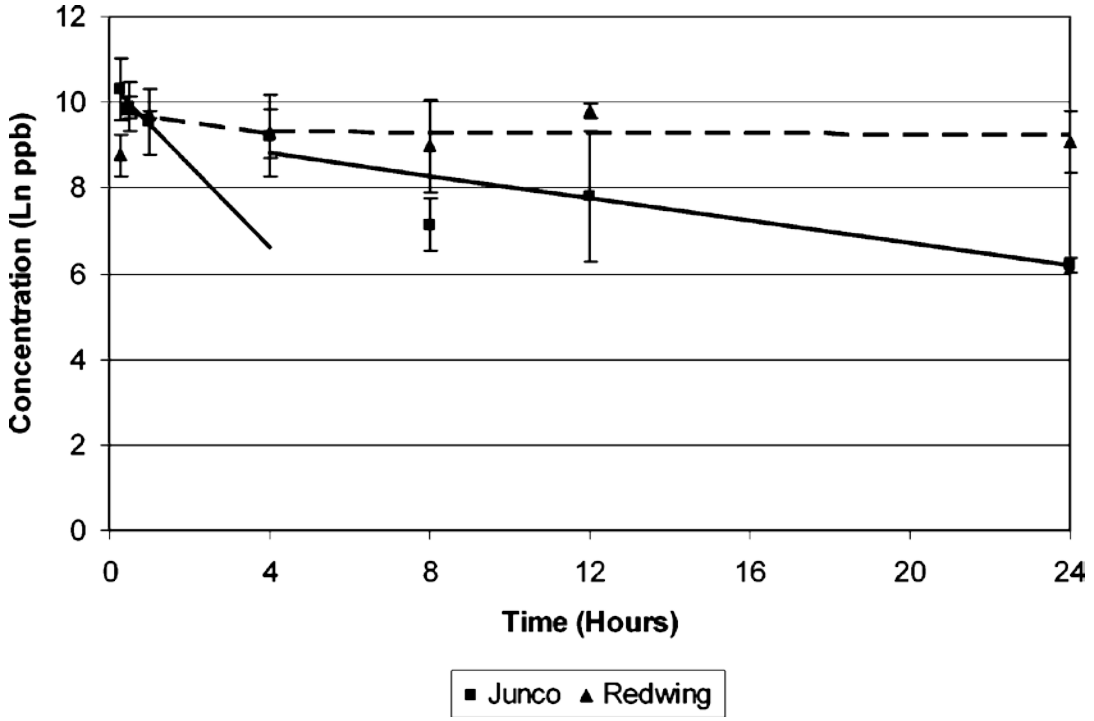


Figure 2.2: Logarithmic plot of elimination of radiolabeled CPTH and metabolites from kidney of red-winged blackbirds and dark-eyed juncos following a single oral dose (n = 3 per time point; error bars indicate standard deviation).

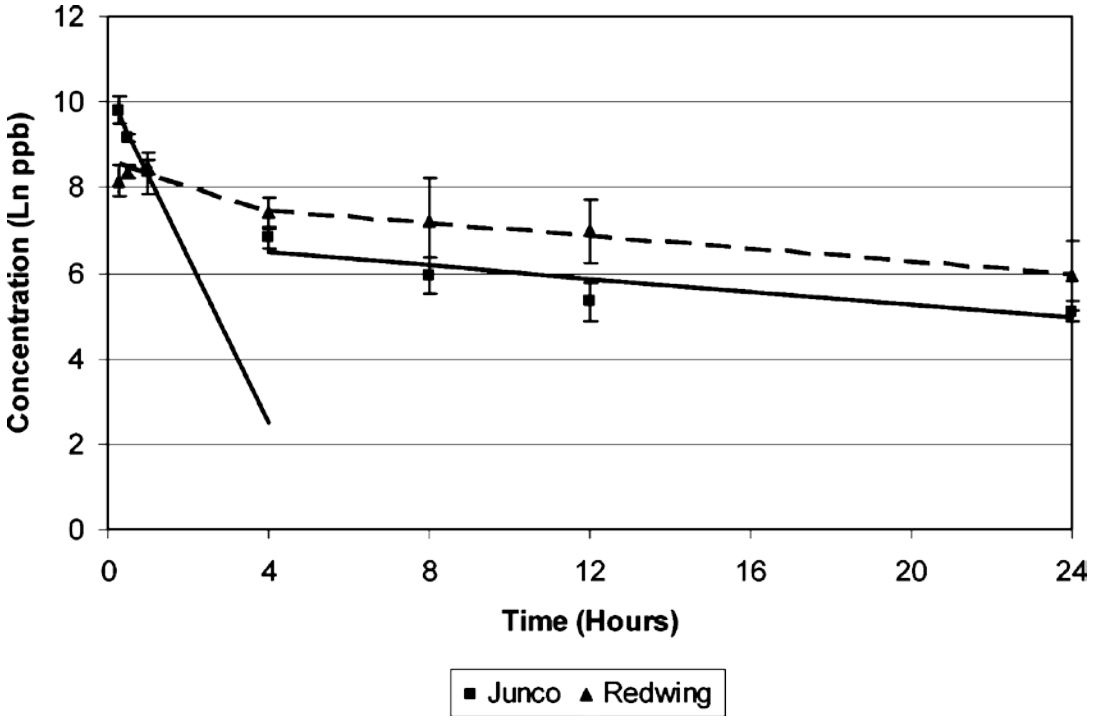


Figure 2.3: Logarithmic plot of elimination of radiolabeled CPTH and metabolites from liver of red-winged blackbirds and dark-eyed juncos following a single oral dose (n = 3 per time point; error bars indicate standard deviation).

The plots of RR in the blood versus time also demonstrate one very distinct feature. There is a virtual lack of any type of uptake curve for CPTH (Figure 2.4). This suggests that CPTH delivered in this manner is readily absorbed and rapidly perfused through the body. This is further supported by a comparison of oral LD_{50} to intraperitoneal (*ip*) LD_{50} values for CPTH in starlings (DeCino et al., 1966). The value for an oral exposure has been reported at 3.8 mg/kg, whereas that for an *ip* injection was found to be 3.5 mg/kg. Examination of the half-life of elimination values (Tables 2.2 and 2.3) reveals that the radioactivity took longer to clear from blackbirds than from juncos as evidenced by the longer half- lives. This is further bolstered by the differences in whole body elimination rates between the two species (Figure 2.5). This could be partially due to the larger body mass of the blackbird as opposed to the junco. However, because the data were evaluated as concentrations and

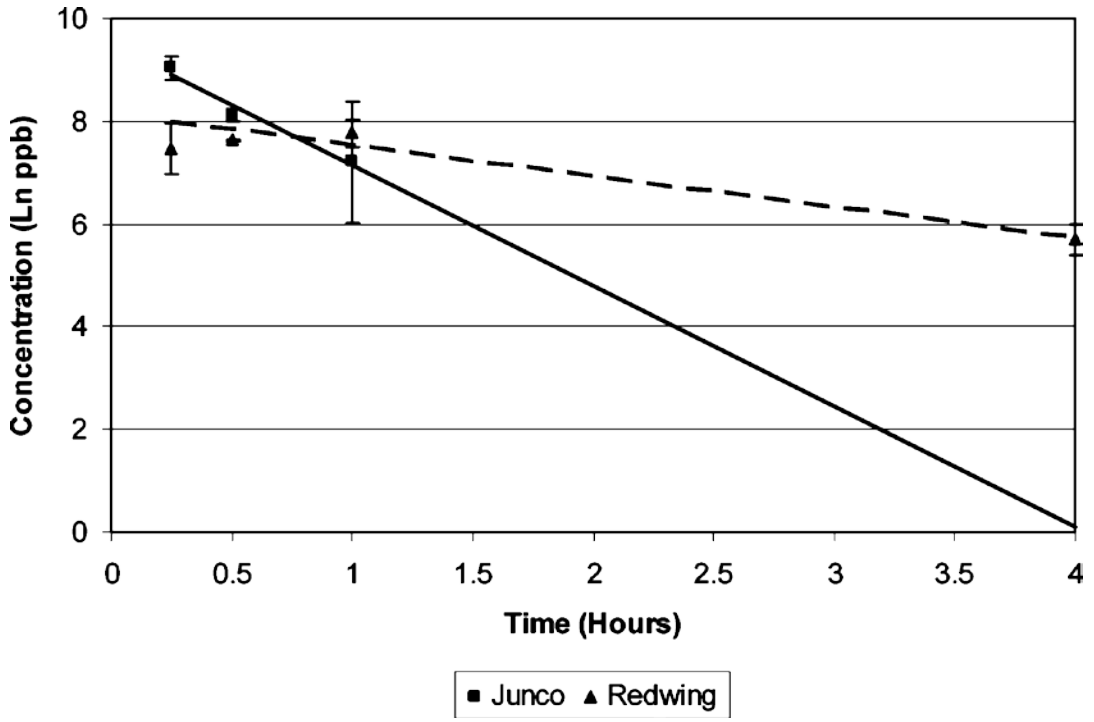


Figure 2.4: Logarithmic plot of elimination of radiolabeled CPTH and metabolites from whole blood of red-winged blackbirds and dark-eyed juncos following a single oral dose (n = 3 per time point; error bars indicate standard deviation).

adjusted for body mass, it is equally likely that any differences observed are due to differential metabolism between the two species. The R^2 values for each tissue type in the first elimination phase demonstrated a reasonable fit to a linear model in most cases (Table 2.2). The R^2 values for the second elimination phase demonstrated a far less desirable fit for a linear regression (Table 2.3). As the residue levels approached background levels, the variability of the results increased, resulting in lower R^2 values overall. In the case of several tissue types, the slope of the regression line was found to not be significantly different from zero ($p = 0.10$). In the first elimination phase, junco GI tract and kidney and blackbird kidney did not meet the criteria for a linear regression. Therefore, no

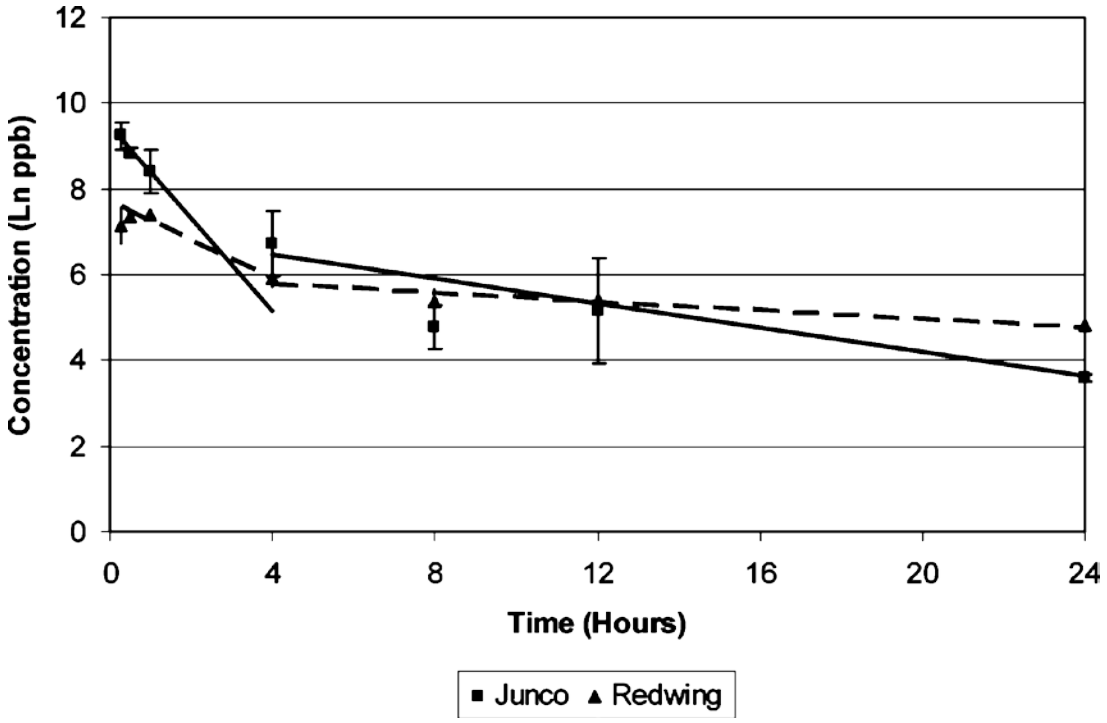


Figure 2.5: Logarithmic plot of elimination of radiolabeled CPTH and metabolites from whole body of red-winged blackbirds and dark-eyed juncos following a single oral dose (n = 3 per time point; error bars indicate standard deviation).

elimination rate constants or half-lives were calculated for these tissues. In the second elimination phase, rate constants could not be generated for junco brain and blood or for blackbird kidney.

Elimination of CPTH from the kidney of both bird species was slower than for most other tissue types. The difference in elimination was even more pronounced for blackbird than for junco (Figure 2.2). This further supports the hypothesis that CPTH is metabolized differently in junco than it is in blackbird. The slope of the line for junco kidney very closely mirrors that for liver tissue (Figures 2.2 and 2.3). Results of a two-tailed t test reveal that the slopes of the elimination curves, which are directly related to the half-life, for junco kidney and junco liver are not statistically different ($p = 0.3239$). Conversely, the half-life of elimination for blackbird kidney could not be calculated because it has no significant slope.

Previous research has hypothesized that the mode of action for CPTH toxicity involves damage to the kidneys (Giri et al., 1976), more specifically, damage to proximal tubular cells of the kidney (Apostolou, 1969; DeCino et al., 1966; Mull et al., 1972). Additionally, observations of increased blood uric acid levels (Apostolou, 1969) have been made. The appearance of uric acid deposits in the abdominal cavities of exposed birds is also used as a method of determining exposure in certain cases (Cummings et al., 2003; DeCino et al., 1966; Johnston et al., 1999).

Renal damage of this type can be indicative of a highly reactive chemical that may be able to covalently bind to tissues. Such a highly reactive chemical species could cause the extensive tissue damage observed in the previously mentioned studies, resulting in a failure of normal kidney functions and the subsequent uric acid increases. The fact that the RR in the kidney of the more sensitive blackbird species did not change significantly over time in comparison to the that of the less sensitive junco points to the kidney as a possible site of action. This observation is consistent with the hypothesis that covalent binding is occurring in the kidney of blackbirds to a greater degree than

in the kidney of exposed juncos, especially when compared to the rapidly declining RR in the other compartments of the blackbird.

To further investigate potential tissue binding, samples collected at the 15 minute exposure time were subjected to serial extraction with various solvents as well as an acid reflux. The purpose of these extractions was to remove as much radioactivity from the tissues as possible prior to combustion of the remaining pellet. A very robust measure of extractable versus non-extractable

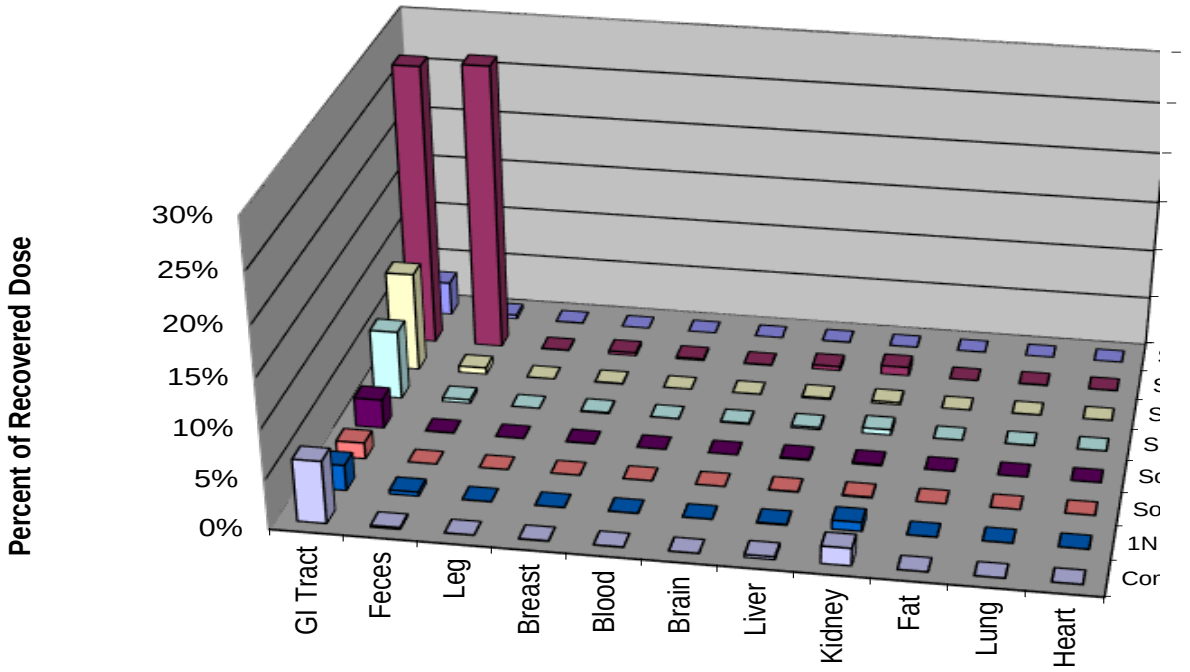


Figure 2.6: Bound and extractable recovery of radioactivity from junco tissues and fluids 15 minutes after receiving a single, oral dose of ^{14}C CPTH.

radioactive residue was determined as a result of this experiment. In the less sensitive junco species, tightly bound residues were found in both the kidney and the GI tract (Figure 2.6). A similar pattern was observed in the more sensitive blackbird species, with bound residue also being observed in the

liver (Figure 2.7). In both the junco and blackbird GI tract, the amount of bound radioactivity, expressed as a percentage of the administered dose, was around 6-7%. In the junco, the bound radioactivity in the kidney was only around 2% while it was 10% in the blackbird.

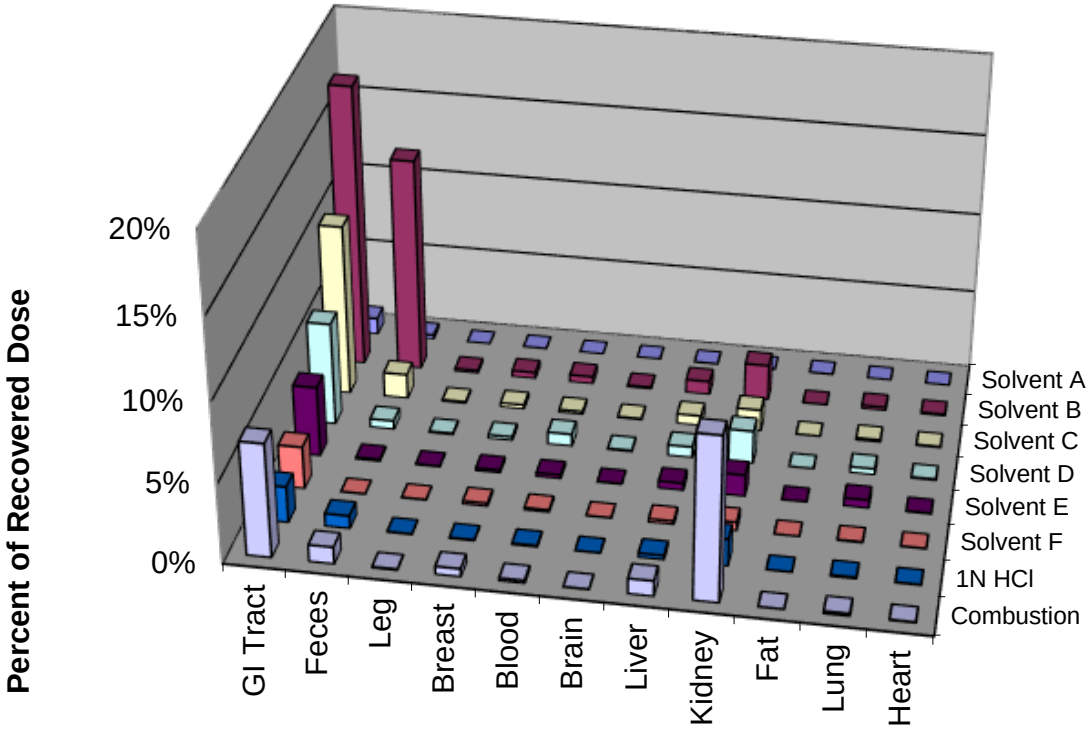


Figure 2.7: Bound and extractable recovery of radioactivity from blackbird tissues and fluids 15 minutes after receiving a single, oral dose of ^{14}C CPTH.

Although it is apparent that CPTH or more likely one of its metabolites is strongly retained by the kidney of red-winged blackbirds, the bulk of the parent and metabolites are rapidly excreted from the bodies of both sensitive and non-sensitive species. Factoring in observations from previous studies

that time to death is typically >24 hours (DeCino et al., 1966), it seems fairly clear that carcasses of exposed birds found in the field are unlikely to contain significant residues of CPTH or its metabolites. Furthermore, it is likely that any metabolites present might be tightly bound to tissues and thus biologically unavailable to any predators that might consume them. These findings are significant with respect to estimating potential secondary exposure of wildlife that may consume CPTH-containing pest bird carcasses which could cause mortality in non-target birds. The results presented further suggest that future research aimed at elucidating the bio-activation pathway by which CPTH is retained by renal tissues of susceptible bird species would greatly increase our understanding of the mode of action of CPTH and possibly lead to the development of more effective and ecologically friendly avicides.

2.4.1 Summary

Our central hypothesis for Phase 1 was that CPTH would be rapidly excreted from most organs. This has been proven with the results listed above. We have addressed the specific questions asked by the hypothesis as well:

- The excretion profile for CPTH exhibited a 2 phase behavior with a rapid phase lasting approximately 4 hours and a slow phase lasting 24 hours or more in most tissues.
- The excretion profile was the same for most tissues tested in both a sensitive and less sensitive species of bird.
- The relative residue levels were higher in the blackbird (a more sensitive species) than in the junco (a less sensitive species).
- The rate of elimination was established for all tissues tested (Tables 2.3 and 2.4).
- There was significant tissue binding, particularly in the kidney, of both bird species tested.

CHAPTER 3 – PHASE 2 – *IN-VITRO* EXPERIMENTS

3.1 Introduction

3-Chloro-*p*-toluidine hydrochloride (CPTH) has been used successfully to control pest bird species, but a deeper understanding of its mode of action is required. This chemical appears to pose minimal hazard to mammalian species (LD_{50}s greater than 1000 mg/kg) while being extremely toxic to the target species (LD_{50} for starlings <10 mg/kg). In addition, CPTH appears to be less toxic to non-target bird species such as hawks and songbirds (LD_{50}s between 100 and 500 mg/kg). However, concerns surround use of this chemical on a wide-spread basis. Very little is known about the metabolism and fate of this chemical after it is consumed by a bird. A more in-depth understanding of this facet of CPTH is required to assuage concerns with its continued use.

Toxic substances can cause damage to exposed tissues in many ways. The chemical can cause damage directly or it can require biotransformation to convert it to its toxic form. In some cases multiple biotransformation steps might be required to cause toxicity. The site of toxic action can be general and spread throughout the body or can be quite specific and localized to a target organ. As a primary mechanism for filtering and removal of toxicants or waste products, the kidney is a potential target of damage for many toxic substances.

What information is known of CPTH comes from pathological examination of exposed birds. CPTH was found to cause damage to the proximal convoluted tubules in sensitive avian species. Experiments with [^{14}C]-CPTH demonstrated that significantly more radiolabeled material bound tightly to the kidneys of sensitive species than a less sensitive species. These observations, along with an observed elevation in the level of uric acid in the birds, lead us to classify this compound as a nephrotoxin. The hypothetical mode of action involves bio-activation resulting in damage to the

kidney and interruption of normal excretion processes leading to death by uric acid poisoning. Experiments to investigate the mechanistic metabolism and provide insight into the mode of action of CPTH were designed and conducted.

3.1.1 Hypothesis

Our hypothesis for this aim is that a reactive metabolite can be formed *in-vitro* and that it can attach to a target compound. Specifically, the questions we will attempt to answer are:

- Can we use mass spectrometry to identify the metabolites of CPTH?
- Can a reactive metabolite be formed *in-vitro*?
- Can glutathione or another suitable molecule be used as a target compound for this metabolite?

3.2 Materials and Methods

In order to investigate the mechanistic toxicology of CPTH, comparisons of metabolism between various species were performed. Several species of birds (mallard duck (*Anas platyrhynchos*), boat-tailed grackle (*Quiscalus major*), and red-winged blackbird (*Agelaius phoeniceus*)) were chosen for inclusion in this research. Blackbirds and grackles are classified as highly sensitive species with respect to CPTH intoxication (LD_{50} = 1 to 8.0 mg/kg), while mallards are more resistant (LD_{50} = 100 mg/kg) (Eisemann et al., 2003). Birds selected for inclusion in this research were free of any exposures to veterinary drugs or toxicants and were donated from other research projects conducted at the USDA's National Wildlife Research Center (NWRC). All birds were euthanized humanely in accordance with American Veterinary Medical Association standards and practices under the supervision of the Attending Veterinarian at the NWRC. All procedures involving animals were carried out with the approval of the Animal Care and Use Committee.

All chemicals and solvents were obtained from either Fisher Scientific (Pittsburgh, PA) or Sigma Aldrich (St. Louis, MO). All chemicals were reagent grade.

3.2.1 Sub-cellular Fraction Preparation

The liver and kidneys from multiple individuals were immediately removed from euthanized birds, pooled, weighed, homogenized in an ice-cold homogenization buffer, and subjected to differential centrifugation. The buffer solution consisted of 250 mM sucrose, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 25 mM potassium chloride, 5 mM magnesium chloride, and 0.1 mM ethylenediaminetetraacetic acid (EDTA). The homogenization buffer was prepared in ultra-pure water and was adjusted to pH 7.4 with 1 M potassium hydroxide. Tissues were minced with scissors in 2 volumes (v/w) of homogenization buffer. The tissues were then homogenized with three passes of a Potter-Elvehjem homogenizer that was kept on ice. The homogenized tissues were placed in centrifuge tubes and subjected to differential centrifugation. The S-9 fraction was defined as the supernatant fraction obtained from an organ homogenate by centrifuging at 9000 × g for 20 minutes in a suitable medium; this fraction contained both cytosol and microsomes. The S-9 fraction was collected as a sub-sample of the supernatant following centrifugation at 9,000 × g for 10 minutes at 4 °C. This fraction contains both cytosol and microsomes and is somewhat more representative of the metabolic activity of the intact organ. The remaining supernatant was transferred to a clean centrifuge tube and placed in the centrifuge at 15,000 × g for 20 minutes at 4 °C. The supernatant was removed and again transferred to a clean centrifuge tube that was centrifuged at 105,000 × g for 60 minutes at 4 °C. This produced the cytosol fraction which was collected as the supernatant of this centrifugation step. The pellet from this centrifugation step is the microsomes. These were gently rinsed three times with

homogenization buffer before being resuspended in homogenization buffer with 3 passes of a Potter-Elvehjem homogenizer that was kept on ice. Following transfer to a clean centrifuge tube, the sample was centrifuged at 105,000 × g for a second time. The supernatant was removed and discarded. The microsomes were resuspended in 1 volume (v/w) of homogenization buffer with 3 passes of a Potter-Elvehjem homogenizer that was kept on ice.

After collection, 1 mL fractions of S-9, cytosol, and microsomes were flash frozen in liquid nitrogen. The tubes were stored in a freezer at -80 ºC until used. In later experiments, the results were normalized for protein content of the fraction being tested. Protein content was determined using a standard Bradford assay kit (Sigma Aldrich, St. Louis, MO).

3.2.2 In-vitro Experiments

In-vitro enzyme experiments were conducted in plastic 1.5 mL snap-cap tubes. A 100 µL aliquot of enzyme source (either microsomes, cytosol, or S-9) and 100 µL of a cofactor solution consisting of 16.5 mM nicotinamide adenine dinucleotide phosphate (NADP), 17 mM glucose-6-phosphate, and 5 mM glucose-6-phosphate dehydrogenase were added to a tube containing enough phosphate buffer (100 mM; pH 7.2) to produce 1 mL of solution. In some experiments, between 20 and 100 µL of acetyl coenzyme A solution was added to the reaction tubes. This solution was prepared by dissolving 2 mg of acetyl coenzyme A in 10 mL of phosphate buffer. All solutions were stored in a refrigerator at 4 °C until used. Depending on the experiment being conducted, a quantity of CPTH or one of its metabolites, that were prepared in phosphate buffer, were added and the tubes were placed in a shaking water bath at 37 ºC for a fixed period of time. The reaction times varied between 15 minutes and 2 hours depending on the study design of the particular experiment. At the end of the reaction, a 500 µL aliquot of 1% acetic acid in methanol was added to each tube. The tubes were

stoppered, vortexed, and placed on ice for 10 minutes. The tubes were then centrifuged at 9,000 × g for 5 minutes and the supernatant removed for analysis via high performance liquid chromatography (HPLC).

3.2.3 Liquid Chromatographic Analysis

All samples were injected into an Agilent 1200 series liquid chromatograph equipped with an auto injector, quaternary pump, temperature-controlled column compartment and diode array detector (Table 3.1). Separation was achieved using a 2.1 x 50 mm Phenomenex Kinetex XB-C18 column (100 Å; 2.6 µm).

Table 3.1: Liquid Chromatograph/Ultra Violet Conditions

Mobile Phase A:	20 mM acetic acid		
Mobile Phase B:	20 mM acetic acid in 9:1 methanol:water		
Gradient:			
Time (min)	% A	% B	Flow Rate (mL/min)
0.0	80	20	0.400
1.0	80	20	0.400
1.1	60	40	0.400
6.0	20	80	0.400
6.1	0	100	0.800
Injection Volume:	5 µL		
Column Temperature:	60 °C		
Run Time:	8 minutes		
Post Time:	4.5 minutes		
Detector:	UV @ 241 nm		

Metabolites were collected and purified prior to identification. A fraction collector was attached to the HPLC system and multiple injections were made of *in-vitro* experiments conducted using red-winged blackbird hepatic S-9 with a reaction time of 60 minutes. The fractions collected were pooled and purified using a Phenomenex Strata-X solid phase extraction column (SPE). Briefly, the fractions were placed in a heated water bath at approximately 40 °C under a stream of nitrogen to

remove methanol from the fraction. The aqueous portion of the fraction was loaded onto a 200 mg SPE cartridge which had been conditioned with methanol and water. After loading the fraction onto the SPE, it was washed with a solution of 30% methanol in water. The metabolites were eluted in methanol, evaporated to dryness under a stream of nitrogen, and reconstituted via the addition of 500 µL of a diluent consisting of 0.1% formic acid in 80:20 water:methanol. Identification of metabolites was performed using an Agilent 1200 series liquid chromatograph coupled to an Agilent 6410 mass spectrometer (LC/MS/MS). The HPLC was equipped with an auto injector, binary pump, and temperature-controlled column compartment. Separation was achieved using the same column as previously described.

Table 3.2: Liquid Chromatograph/Mass Spectrometer Conditions

Mobile Phase A:	0.1% formic acid		
Mobile Phase B:	0.1% formic acid in methanol		
Gradient:			
Time (min)	% A	% B	Flow Rate (mL/min)
0.0	80	20	0.350
1.0	80	20	0.350
6.0	20	80	0.350
6.5	0	100	0.350
Injection Volume:	10 µL		
Column Temperature:	40 °C		
Run Time:	8 minutes		
Post Time:	4 minutes		
Scan Range:	50 to 1000 m/z		
Fragmentor Voltage:	65 V		
Polarity:	Positive		
Gas Temperature:	350 °C		
Gas Flow:	Nitrogen @ 11 L/minute		
Nebulizer Pressure:	35 psi		
Capillary Voltage:	4000 V		

3.3 Results and Discussion

Combining CPTH with hepatic S-9, microsomes, or cytosol did not produce any detectable metabolism in any species tested until a source of an acetyl donor group such as acetyl coenzyme A was added. Upon addition of acetyl coenzyme A, CPTH was rapidly metabolized to two metabolites that were initially designated as Metabolite A and Metabolite B (Figure 3.1). Metabolite A was formed very rapidly upon addition of acetyl coenzyme A and had a longer retention time in a reversed-phase liquid chromatography system. We can conclude from its relative retention that was less polar than the parent molecule (CPTH). A second metabolite (Metabolite B) was observed following formation of Metabolite A. The retention time of Metabolite B was much shorter than that of either CPTH or Metabolite A, suggesting it was more polar and more water soluble than CPTH. No other significant metabolites were observed in chromatograms from *in-vitro* experiments. This includes experiments with Phase II metabolic systems (sulfates and glucuronides) or with bovine serum albumin or glutathione as targets for reactive metabolites (data not shown).

Pooled fractions of Metabolite A produced from hepatic microsomes were injected into the LC/MS/MS using the parameters outlined in Table 3.2 and produced the fragments shown in Figure 3.2. A product ion scan was conducted on the largest ion (184 m/z) producing the fragments shown in Figure 3.3. Based on the interpretation of the mass spectra, Metabolite A was assigned a structure and identified as 3-chloro-4-methylacetanilide (CAT). The formation of this metabolite appeared to be a non-enzymatic process as it did not require the presence of cellular material to occur. The lack of metabolism without the presence of an acetyl source indicated that the acetylation of CPTH was a necessary first step in the metabolic pathway.

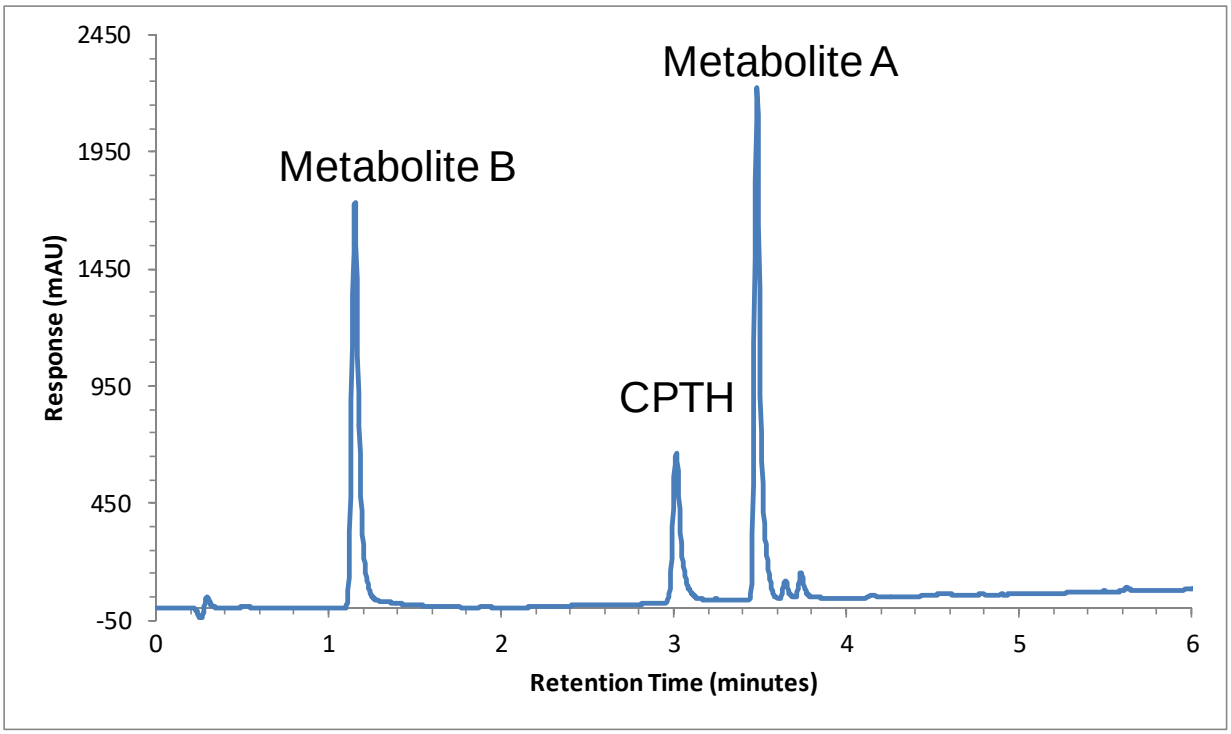


Figure 3.1: Chromatogram of red-winged blackbird hepatic S-9 sample injected using the conditions found in Table 3.1.

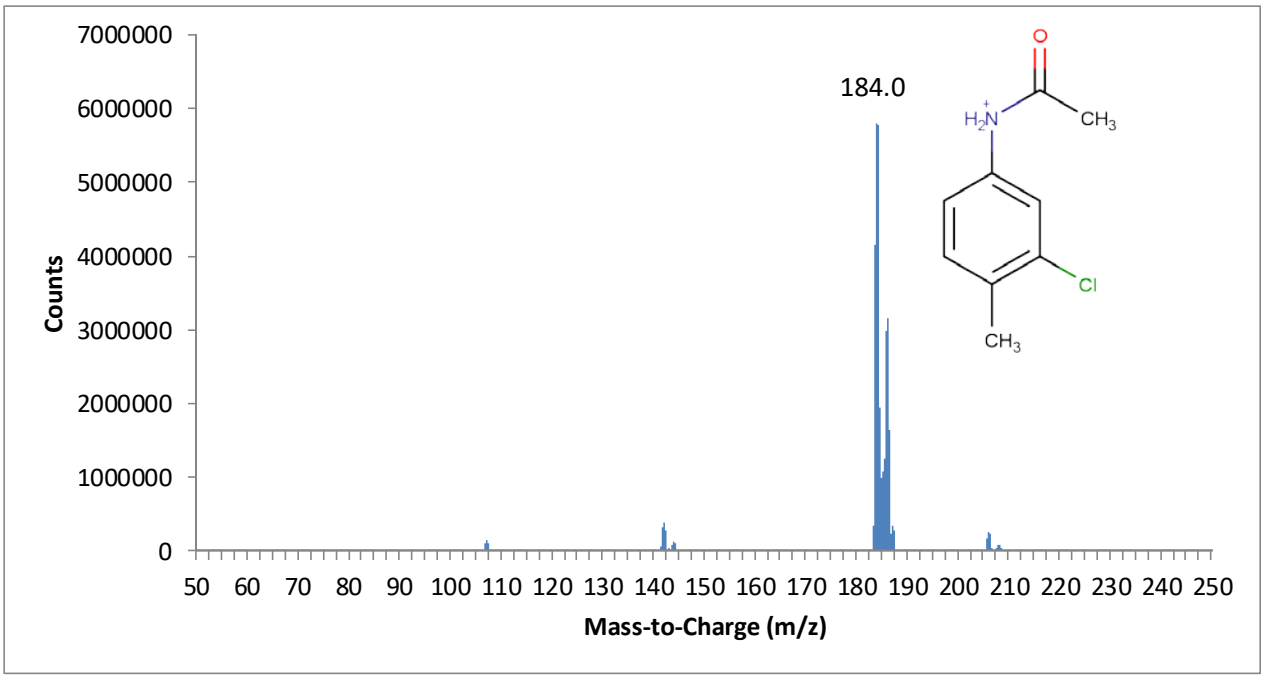


Figure 3.2: LC/MS Spectra of Metabolite A injected using the conditions found in Table 3.2.

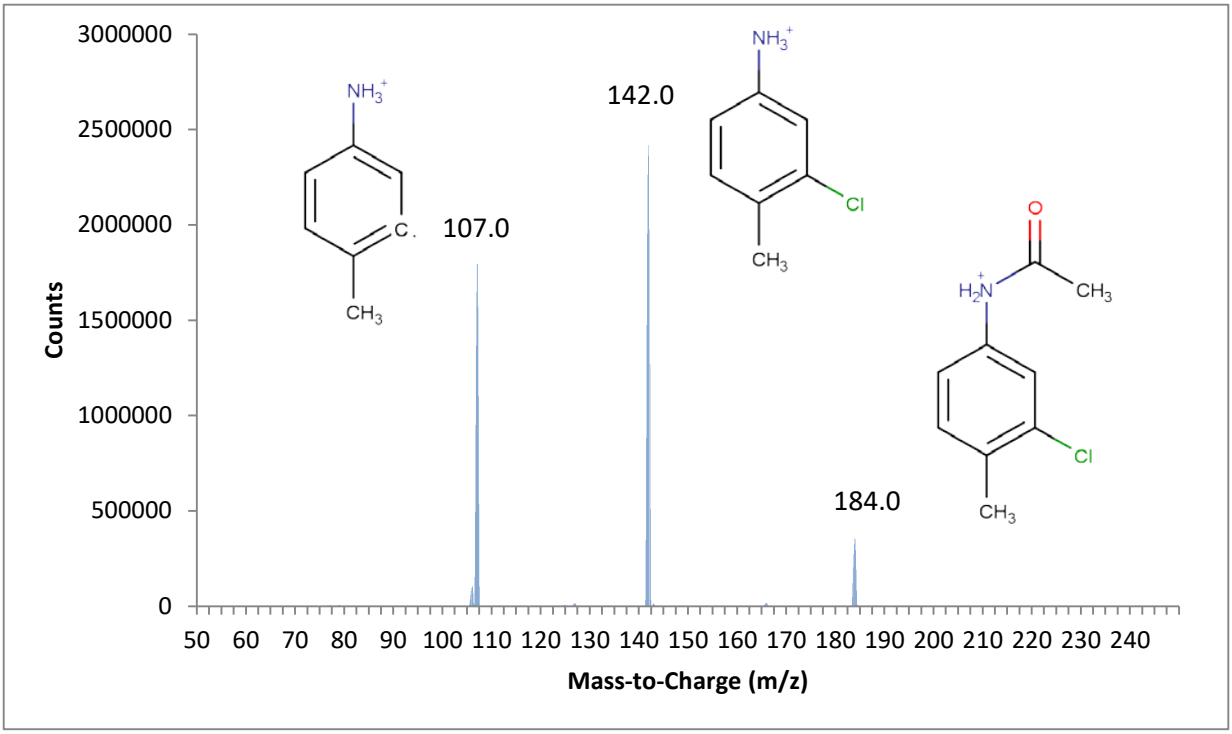


Figure 3.3: LC/MS/MS Spectra of the 184.0 m/z peak of Metabolite A injected using the conditions found in Table 3.2.

Pooled fractions of Metabolite B produced from hepatic microsomes were injected into the LC/MS/MS and produced the fragments shown in Figure 3.4. A product ion scan was conducted on the largest ion (200 m/z) producing the fragments shown in Figure 3.5. Based on the interpretation of the mass spectra, Metabolite B was assigned a structure and identified as N-[3-chloro-4-(hydroxymethyl)phenyl]acetamide (OH- CAT). The hydroxylation reaction was likely a cytochrome P-450 mediated action since the presence of microsomes was necessary for it to occur. Mass spectral analysis of this product indicated that the site of hydroxylation was likely on the benzyl methyl. Hydroxylation of this site did not occur without prior acetylation of the amine group. The acetyl group was necessary to initiate and/or stabilize this enzymatic process.

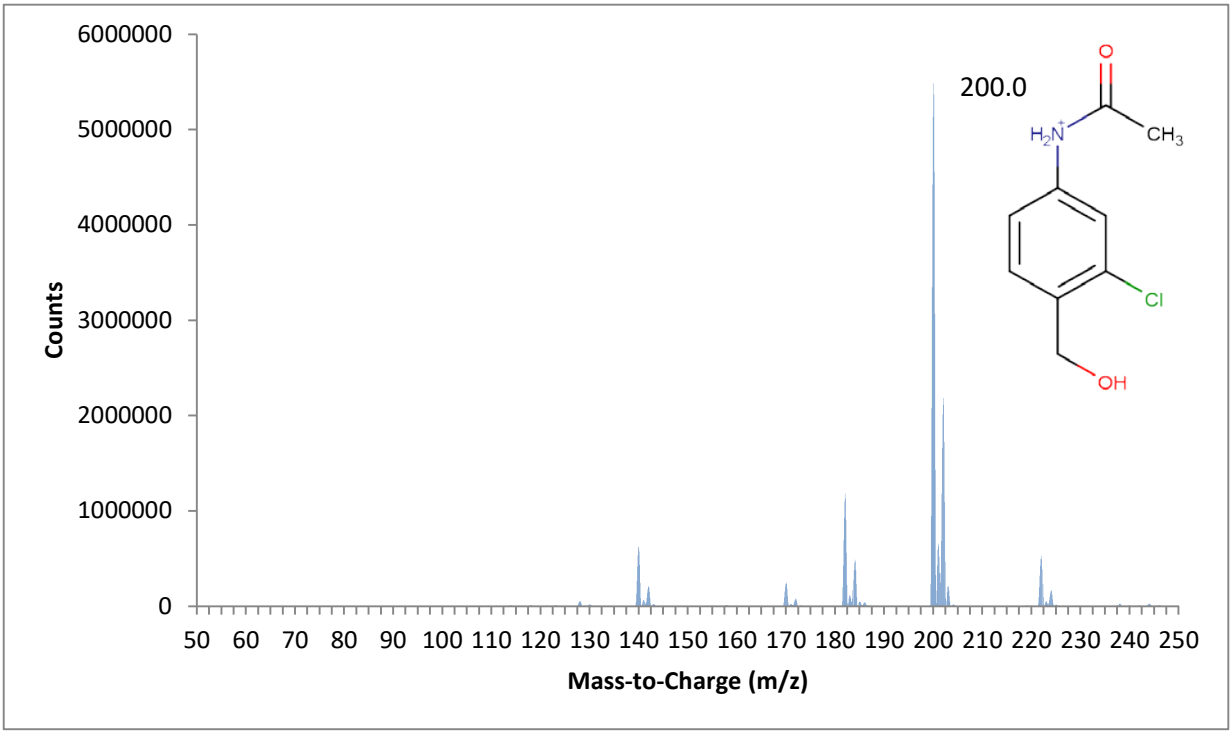


Figure 3.4: LC/MS Spectra of Metabolite B injected using the conditions found in Table 3.2.

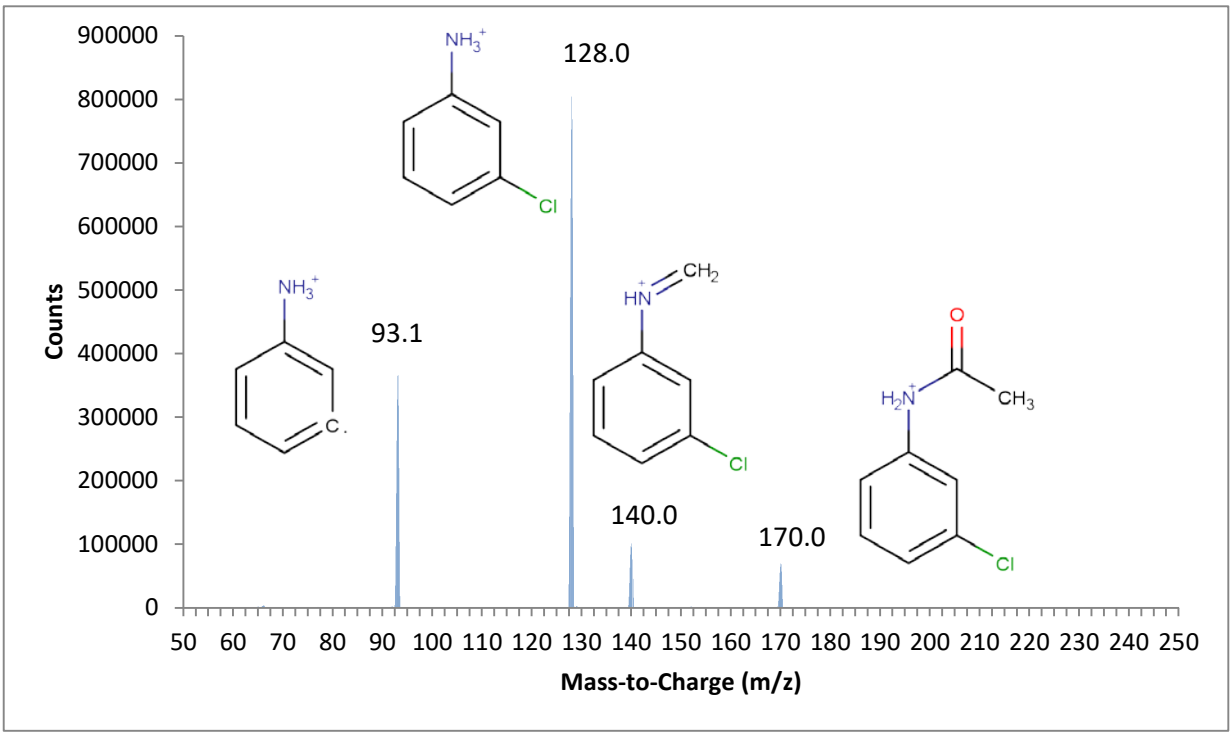


Figure 3.5: LC/MS/MS Spectra of the 200.0 m/z peak of Metabolite B injected using the conditions found in Table 3.2.

To verify the identification of the two metabolites, sources of each were found. CAT was available for purchase from Sigma Aldrich (St. Louis, MO). OH-CAT was custom synthesized by Richman Chemical (Lower Gwynedd, PA). Standards prepared from these sources were injected in the LC/MS/MS. The retention time and spectral fragmentation patters were an exact match for those of the metabolites generated during the *in-vitro* experiments, confirming the identifications.

Initial *in-vitro* experiments were conducted using [^{14}C]-CPTH material left over from experiments conducted in Phase 1. These experiments were conducted by combining hepatic S-9 and renal microsomes in the same reaction vessel. This was an attempt to examine the interplay between the two organs. All three species of birds rapidly acetylated the CPTH substrate. All three also demonstrated a capacity to deacetylate the CAT, reverting it back to CPTH, although the rate of deacetylation was much higher for mallard than for either grackle or blackbird. Low levels of OH-CAT were observed in all species studied (Figures 3.6 to 3.8).

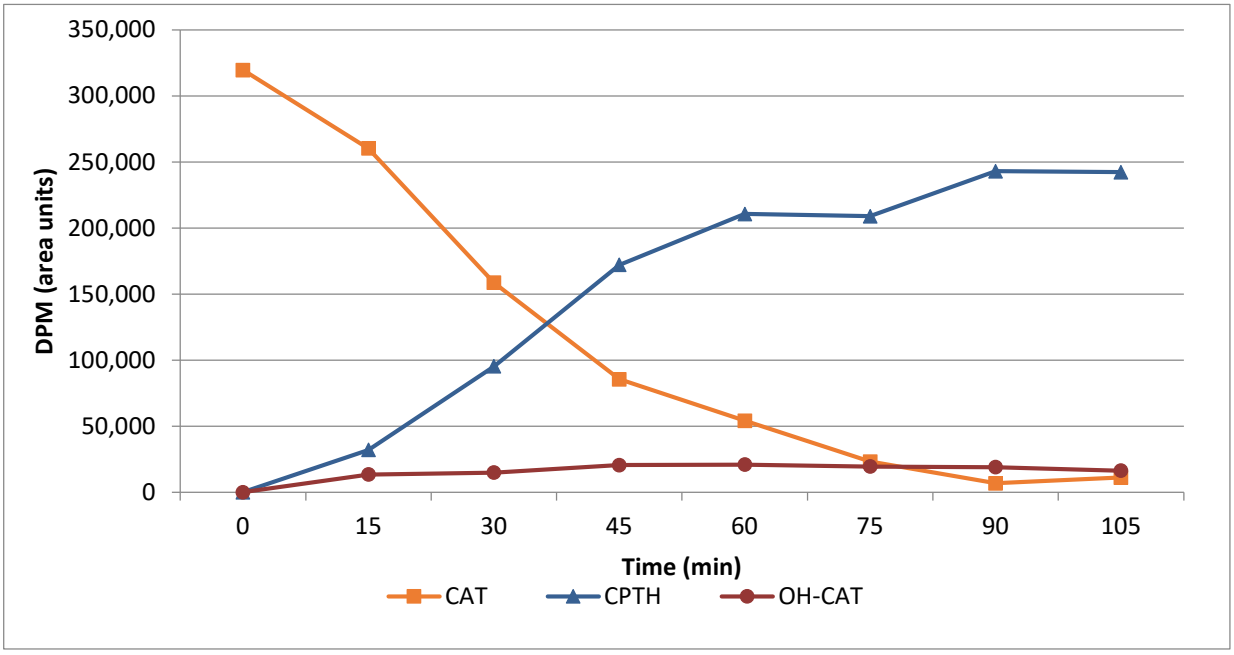


Figure 3.6: *In-vitro* results from mallard duck hepatic S-9 and renal microsome experiment.

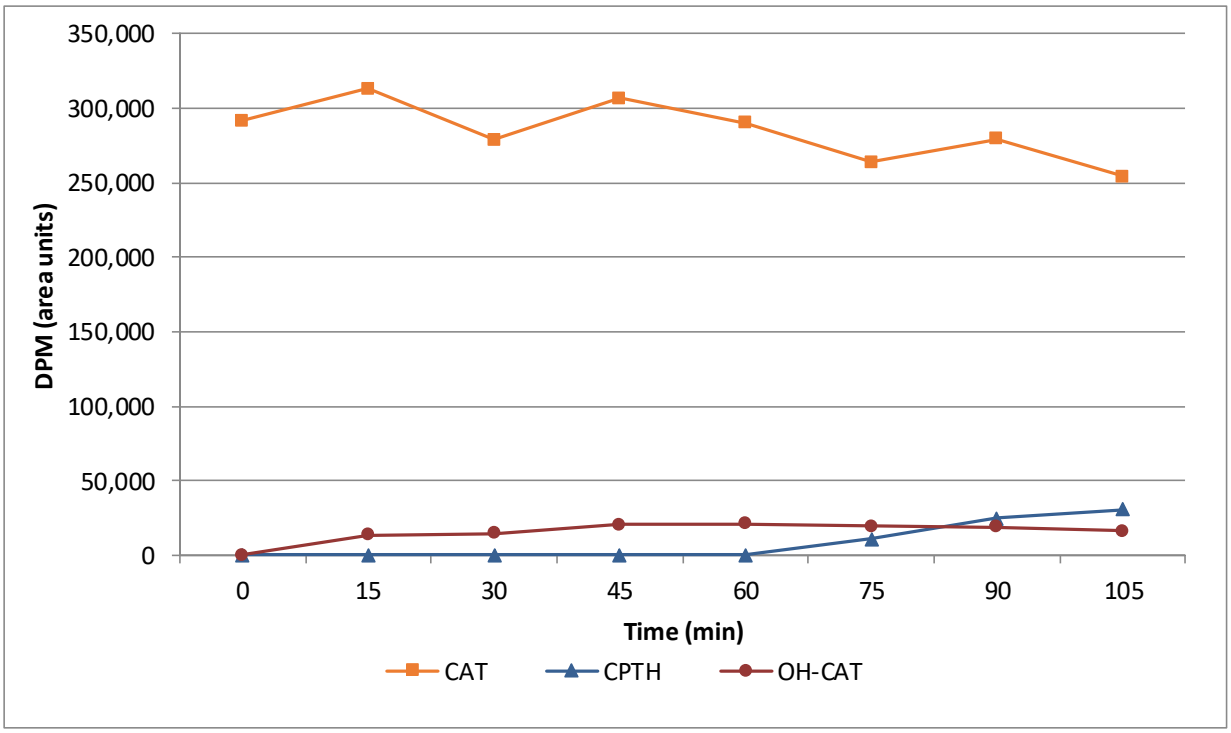


Figure 3.7: *In-vitro* results from boat-tailed grackle hepatic S-9 and renal microsome experiment.

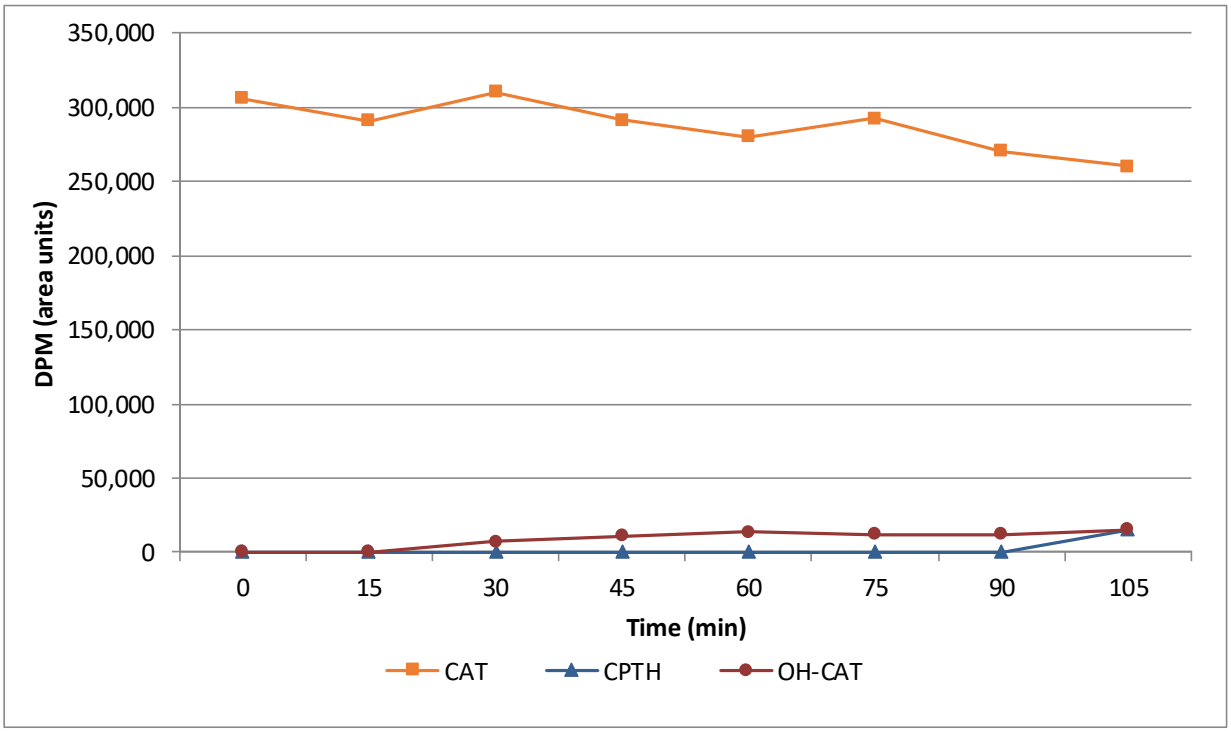


Figure 3.8: *In-vitro* results from red-winged blackbird hepatic S-9 and renal microsome experiment.

To examine the rate of deacetylation in the kidney, additional experiments were conducted in which CAT was used as the substrate (Figure 3.9). These experiments were conducted on the same three species as previously discussed. The rate of deacetylation was calculated by finding the slope of a linear regression created from each set of data. The rates of deacetylation were 4.0 ng CPTH/min/mg protein for mallard duck, 0.20 ng CPTH/min/mg protein for boat-tailed grackle, and 0.16 ng CPTH/min/mg protein for red-winged blackbird. Red-winged blackbirds and boat-tailed grackles are classified as highly sensitive species with respect to CPTH intoxication (LD_{50} = 1 to 8 mg/kg), while mallard ducks are more resistant (LD_{50} = 100 mg/kg). This suggests that deacetylation may be a protective mechanism in the metabolism of CPTH.

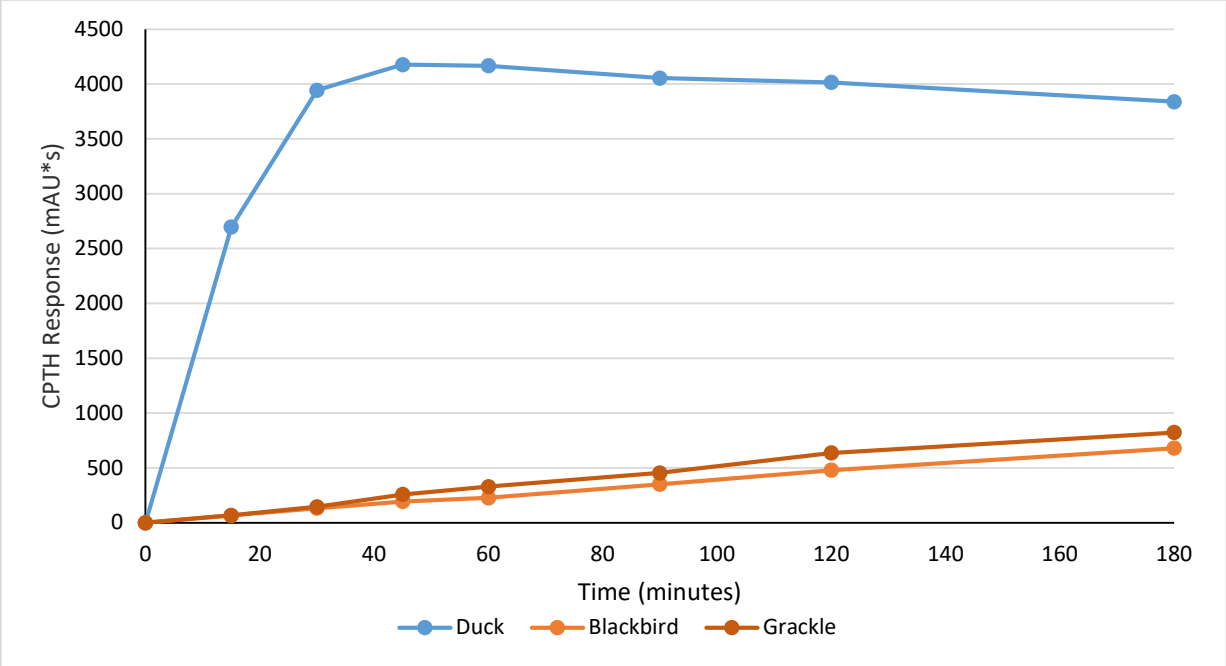


Figure 3.9: *In-vitro* results from renal microsome experiments with mallard duck, boat-tailed grackle, and red-winged blackbird.

Following these preliminary experiments, attempts were made to refine and optimize the parameters for the *in-vitro* experiments with hepatic S-9. Experiments in which the amount of acetyl coenzyme A was varied while the concentration of CPTH added was held constant demonstrated a nearly linear relationship between the two parameters (Figure 3.10).

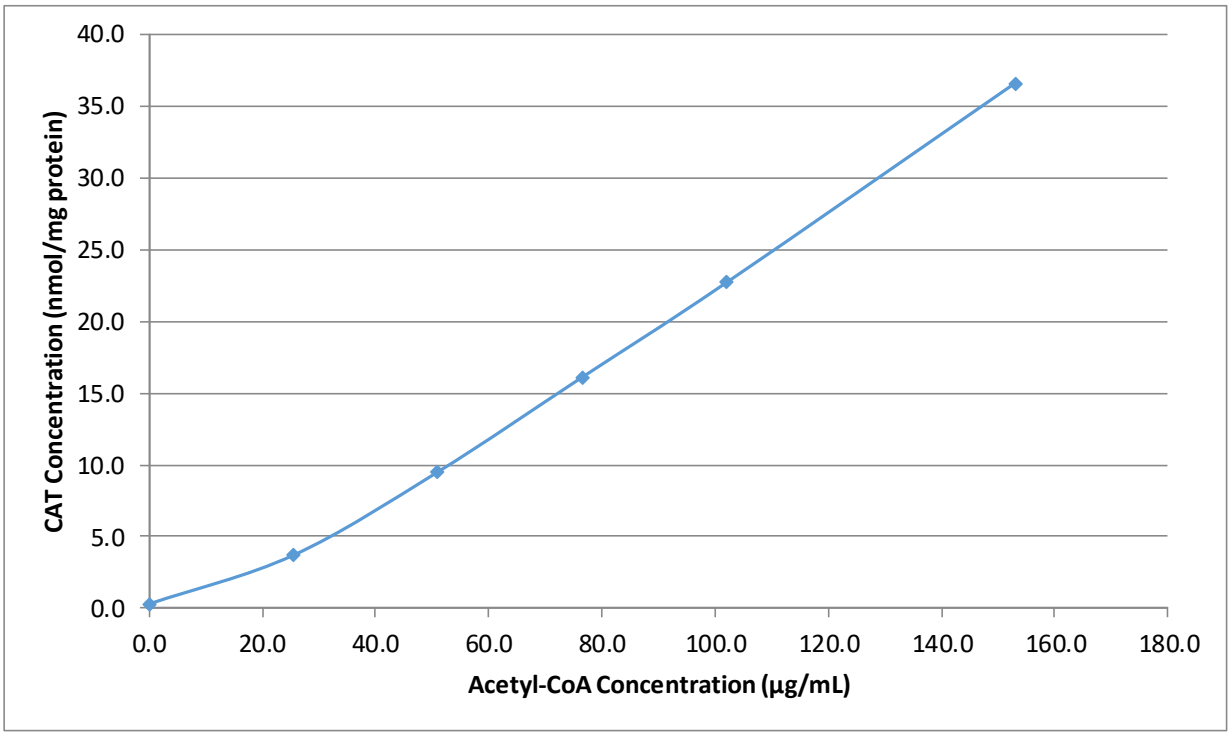


Figure 3.10: Production of CAT in red-winged blackbird hepatic S-9 as a function of varied concentration of acetyl coenzyme A.

When CPTH concentration was increased, the amount of CAT produced was increased proportionally (data not shown). The amount of OH-CAT produced did not follow this same trend. The amount of OH-CAT produced actually decreased at higher concentrations of CPTH substrate (Figure 3.11).

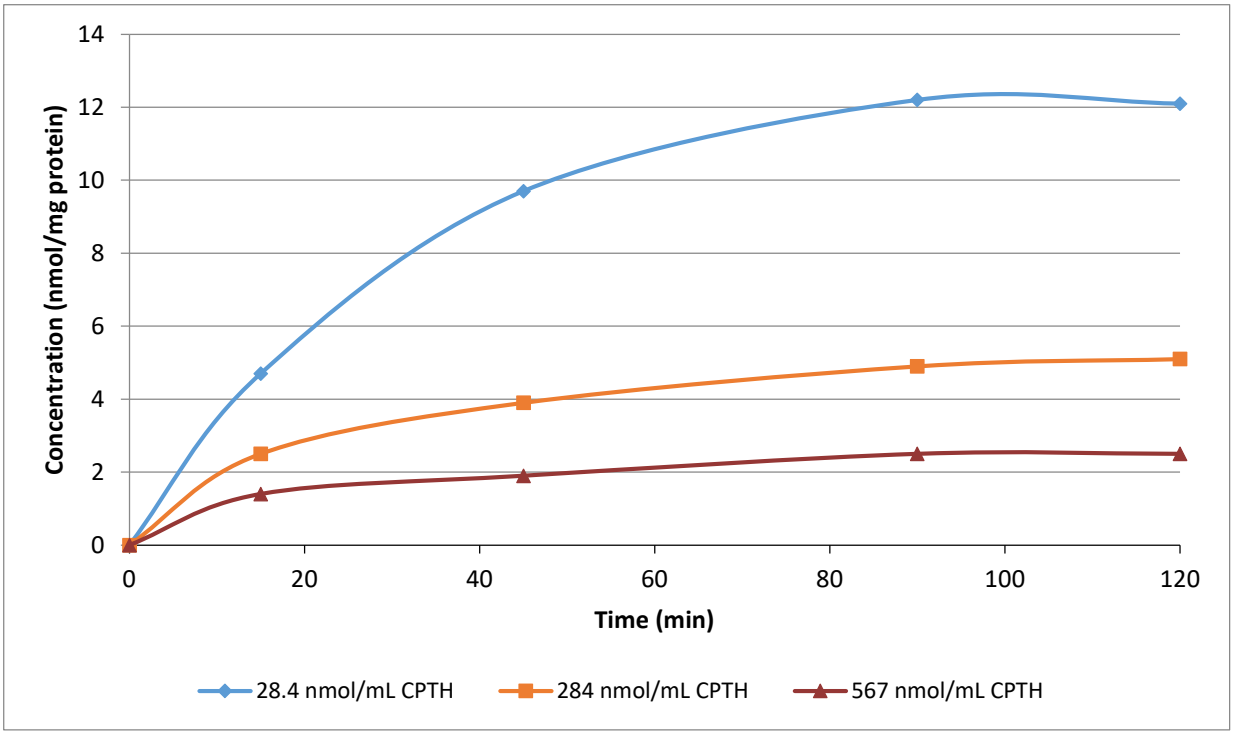


Figure 3.11: Production of OH-CAT in red-winged blackbird hepatic S-9 as a function of varied concentration of CPTH.

In-vitro experiments were conducted to investigate the metabolic pathway. Combining CPTH with hepatic S-9 did not produce any detectable metabolism until a source of acetyl-co-A was added. Upon addition of acetyl-co-A, CPTH was rapidly metabolized to CAT (Figure 3.12). Acetylation is a Phase II biotransformation mechanism by which an acetyl group is transferred to the target chemical. Addition of an acetyl group lowers water solubility and can provide a means to stabilize the formation of ionic species. In the case of CPTH this biotransformation occurred readily in the liver. Following acetylation a slower hydroxylation step was observed. This metabolite would likely be carried throughout the body via circulation and enter the kidney. The formation of this metabolite appears to occur non-enzymatically as it did not require the presence of cellular material. The lack of metabolism without the presence of an acetyl source indicated that the acetylation of CPTH was a necessary first step in the metabolic pathway.

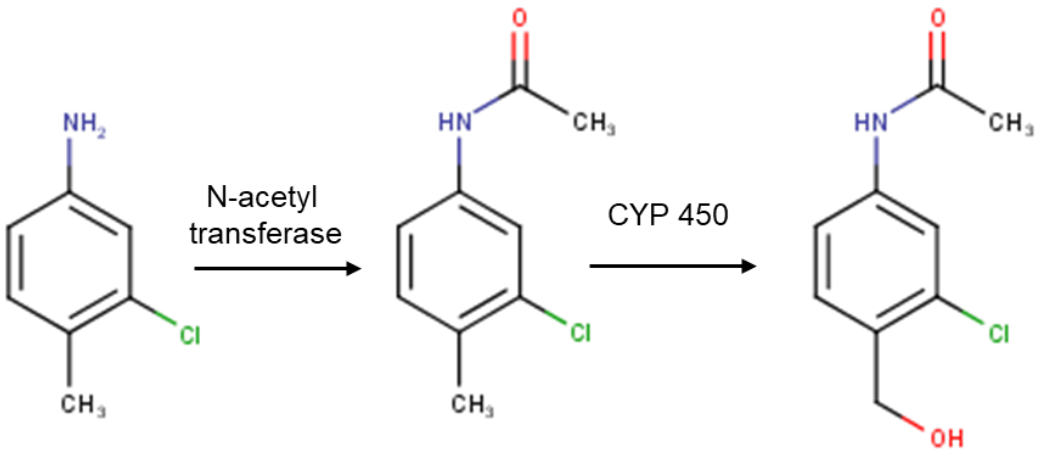


Figure 3.12: Proposed metabolic pathway for biotransformation of CPTH to CAT to OH-CAT.

The second step in the pathway is the formation of a hydroxyl metabolite of CAT (OH-CAT). The hydroxylation reaction was likely a cytochrome P-450 mediated action. The presence of microsomes was necessary for it to occur. This metabolite appeared to be quite stable as it was successfully isolated from S-9 preparations and analyzed by LC/MS/MS. The concentration of OH-CAT reached a steady state and did not appear to react with proteins (Figure 3.11). Therefore, an additional metabolic step would seem to be required to form a reactive metabolite. Mass spectral analysis of this product indicated that the site of hydroxylation was likely on the benzyl methyl group Figures 3.4 and 3.5). Hydroxylation of this site does not occur without prior acetylation of the amine group. This suggested that the acetyl group was necessary to initiate and/or stabilize this enzymatic process.

Considering the presence of strongly bound radioactivity found in Phase 1, it was surprising that no metabolic binding to target molecules (bovine serum albumin or glutathione) was observed in any of the experiments conducted with those substrates. Additional biotransformation of the parent or one of its metabolites appears to be necessary to cause the tissue damage observed in exposed birds.

Renal deacetylation is a ubiquitous biotransformation process that removes an acetyl group from a compound. It is possible that this step could create a reactive metabolite that would be responsible for the damage observed in the kidneys of exposed birds. Additional experiments detailed in Chapter 5 could shed some light on this issue.

3.3.1 Summary

Our central hypothesis for Phase 2 was that a reactive metabolite could be formed *in-vitro* and that it could attach to a target compound. While two metabolites of CPTH (CAT and OH-CAT) were identified, neither was found to react with any target molecule evaluated. We have also answered the following specific questions related to the hypothesis:

- Mass spectrometry was used to identify the two metabolites. Reference standard materials were purchased for each metabolite to confirm the identities.
- Neither of the metabolites were reactive with any target molecules tested.
- No reaction with target molecules (glutathione, sulfate, glucuronide, bovine serum albumin, and cysteine) was observed from any *in-vitro* experiments.

CHAPTER 4 – PHASE 3 – *IN-VIVO* EXPERIMENTS

4.1 Introduction

3-Chloro-*p*-toluidine hydrochloride (CPTH) is a slow-acting toxicant in avians. Because the time to death (1-3 days) is well after exposure, very few analyses of collected carcasses have found significant CPTH residues (Johnston, et al., 1999; Stahl and Johnston, 2006). Public concerns surround the possibility for secondary hazard or non-target exposures from the use of CPTH. CPTH intoxication is often suspected when mysterious bird deaths are noted by members of the general public or conservation groups. Demonstrating that CPTH was not responsible for these die offs would bolster the continued responsible use of this compound.

In one study with grackles, the concentration present in the tissues was found to increase with increasing exposure levels (Johnston et al., 1999). Given that the anticipated exposure dose for a red-winged blackbird would be around 25 mg/kg (Eisemann et al., 2001), the odds of detecting exposure in tissues from these birds are very low. Once a bird is exposed to a lethal dose of CPTH, time to death is typically 1 to 2 days. The observed half-life of elimination for CPTH has been estimated to be 5.4 hours in sensitive species. Therefore, detection of a lethal exposure to CPTH is almost impossible to achieve as the toxicant has left the body of the bird prior to death. An indirect measurement of exposure (through the use of a metabolite or biomarker) would be very valuable to support the continued use of this important damage control agent.

In Chapter 3 *in-vitro* experiments led to the identification of two CPTH metabolites: 3-chloro-4-methylacetanilide (CAT) and N-[3-chloro-4-(hydroxymethyl)phenyl]acetamide (OH-CAT). Although *in-vitro* experiments can be quite valuable in the identification of previously unknown metabolites, they cannot completely replicate all the biological processes present in an intact

organism. In order the fully investigate the metabolism and fate of CPTH and search for more metabolites and target proteins, *in-vivo* experiments are required. An *in-vivo* exposure experiment with CPTH was conducted, and GI tract, liver, kidney, and breast muscle were collected. The tissues were analyzed by an existing gas chromatography/tandem mass spectrometry (GC/MS/MS) method and a newly developed liquid chromatography/tandem mass spectrometry (LC/MS/MS) method (Chapter 3).

4.1.1 Hypothesis

In Phase 3 we will use the information gathered from Phase 2 to look for metabolites of CPTH in the tissues of birds exposed to CPTH *in-vivo*. We will also attempt to answer several specific questions related to this study goal.

- What are the residue levels of CPTH and its metabolites in birds that were given a field-relevant dose of CPTH?
- Can the metabolites identified in Phase 2 be used as a diagnostic test for field exposures to CPTH?
- How does a newly developed liquid chromatography technique compare to a previously used gas chromatography technique?

4.2 Materials and Methods

4.2.1 Trapping of birds

Thirty-seven red-winged blackbirds (mixed gender) were trapped from wild populations in Colorado and transported to the National Wildlife Research Center's Outdoor Animal Research Facility. The birds were held in quarantine for not less than 7 days. They were maintained in group

housing during quarantine and given free access to water and a maintenance diet through the study. All procedures involving animals were carried out with the approval of the NWRC Animal Care and Use Committee (NWRC protocol QA-2708).

4.2.2 Purification of CPTH

CPTH is sold as Compound DRC-1339 Concentrate in several formulated products for use by certified pesticide applicators. The purity limits for these products are 94.1 to 99.9 % CPTH. Significant impurities within the material could confound the results of these dosing tests as impurities within the material could be detected instead of the active ingredient (CPTH). Therefore, the material was purified before use in the *in-vivo* portion of the study.

Compound DRC-1339 Concentrate (0.5 kg) was weighed into a 2-L beaker. Methanol (1.5 L) was added and heated to approximately 40 °C on a stirring hot plate. To this was stirred in 20 mL of a 1 N HCl solution in water and 30 g of activated charcoal. The solution was allowed to stir for approximately 1 hour until it was nearly colorless. It was then filtered through a Whatman #1 filter paper in a Buchner funnel, reserving the liquid. The liquid was evaporated in a rotary evaporator under vacuum until a wet powder consistency was achieved. The powder slurry was filtered through a Whatman #1 filter on a Buchner funnel, with the liquid being reserved. The powder was covered with approximately 200 mL of acetonitrile and, after a 2 minute wait, vacuum was applied. The filtrate was discarded to waste. The process of rotary evaporation and filtering was repeated with the methanol solution until no more powder was obtained. The white, crystalline product was allowed to dry in a fume hood overnight. The purified CPTH powder was stored in a refrigerator at 4 °C until used. Purity was confirmed by HPLC-UV prior to the initiation of the *in-vivo* portion of the study and found to be 99.9%.

4.2.3 Synthesis of Surrogate Compound

A surrogate compound was used for all chromatographic analyses. As a deuterated material was not commercially available, it was synthesized. Deuterated d_6-CPTH was synthesized following the procedures in Hurlbut et al. (1998) with modification of one step. The initial nitration reaction was accomplished using sodium mordenite following the procedures established by Smith and Fry (1989). The mordenite increased the yield of the *p*-nitrotoluene significantly over the original procedure. The reactions yielded a very pale yellow powder with a purity of >99% by GC/MS.

4.2.4 Administration of Dose

Red-winged blackbirds were randomly assigned to test or control groups and exposed to one of three doses of the purified CPTH (8 birds per treatment level) via oral gavage and held for three days. Oral gavage was accomplished by using a #9 gelatin capsule containing the appropriate mass of CPTH material and packed with D-mannitol (an inert material) to fill the unused space in the capsule. The birds were held in a supine position and the capsule was delivered using a stainless steel dosing syringe. Dosage levels were approximately 12.5, 25, and 37.5 mg/kg based on an average weight of 60 g for a red-winged blackbird. A control group of 8 birds received a capsule containing only D-mannitol. Four additional red-winged blackbirds were exposed to a 37.5 mg/kg dose of CPTH which has been labelled with deuterium (d_6-CPTH). Birds in both the test and control groups were held in individual cages for the remainder of the study. Twice daily, the condition of the birds was observed and noted. Deceased birds were removed; their weight and the time were recorded. No individuals from the control group expired during the exposure period. All birds that survived for full 3-day exposure period were euthanized with CO_2. All birds were euthanized humanely in accordance with American Veterinary Medical Association standards and practices.

4.2.5 Tissue Collection

All birds, including the control group, were necropsied to remove tissue samples for analysis. An incision was made in the skin covering the abdomen, and the birds were skinned completely. A lateral incision was then made and a pair of scissors used to cut the breast bone on each side. The kidneys, GI tract (from esophagus to cloaca with contents included), liver, and a portion of the breast muscle were removed from each bird and individually flash frozen with liquid nitrogen. The samples were ground to a fine powder with a chilled mortar and pestle in the case of kidney and liver samples or through the use of a freezer mill (SPEX CertiPrep, Metuchen, NJ) and stored in individual containers. Kidney samples were stored at -80°C. All other samples were stored at -30 °C.

4.2.6 Gas Chromatography-Mass Spectrometry Analysis

The GI tract, liver, and breast muscle were analyzed for CPTH content using National Wildlife Research Center Method 174A "Determination of 3-Chlor-*p*-toluidine Hydrochloride (CPTH) in Quail GI Tract, Liver, and Breast Muscle by GC/MS/MS" (2014). Briefly, the homogenized samples were fortified with a surrogate compound (d_6-CPTH) and extracted with 20% acetonitrile (Fisher Scientific, Pittsburgh, PA)/80% 1 M hydrochloric acid (Fisher Scientific, Pittsburgh, PA). The sample extract was made basic (pH approximately 10) with the addition of 2 M sodium hydroxide (Fisher Scientific, Pittsburgh, PA) and extracted with hexane (Fisher Scientific, Pittsburgh, PA). The hexane extract was cleaned-up using a silica solid phase extraction (SPE) cartridge (Biotage, Charlotte, NC). The CPTH was eluted from the SPE with a solution of *p*-toluidine (Sigma Aldrich, St. Louis, MO) in *n*-butyl acetate (Fisher Scientific, Pittsburgh, PA). Analysis was performed using an Agilent 7890A Gas Chromatograph attached to an Agilent 7000B QQQ Mass Spectrometer

(Santa Clara, CA). Separation was achieved using a 15 m x 0.25 mm ID, 0.250 µm, DB5-MS UI column (Agilent Technologies, Santa Clara, CA) connected through a purged ultimate union to a 0.65 m length of 0.15 mm ID fused silica. This arrangement allowed the use of a post-run backflush to minimize contamination of the detector with high boiling temperature matrix components. Other instrument parameters are in Table 4.1.

Table 4.1: Gas Chromatograph/Mass Spectrometer Conditions

Carrier Gas:	Helium			
Inlet Liner:	ultra-inert liner, splitless, single taper with wool			
Injection Port:	250 °C; pulsed splitless; 15.9 psi; constant-flow			
Injector Pressure:	pulse at 70 psi for 1 min; split vent at 60 mL/min at 2.5 min			
Injection Volume:	1 µL using gastight 10-µL syringe with PTFE plunger			
	Temperature Program:			
Time (min)	Temperature (°C)	Rate (°C/minute)		
0.00	70	—		
2.00	70	—		
7.25	175	20		
8.50	300	100		
12.00	300	—		
Collision Cell:	2.25 mL/min He quench gas; 1.5 mL/min nitrogen collision gas			
Ion Source:	electron impact			
Source Temperature:	230 °C			
Solvent Delay:	4.5 min			
Compound	Precursor Ion (m/z)	Product Ion (m/z)	Dwell (ms)	Collision Energy (V)
d_6-CPTH	148.9	112.2	20	19
d_6-CPTH	146.9	112.2	40	15
d_6-CPTH	112.2	81.2	20	19
CPTH	140.9	106.2	50	15.5
CPTH	139.9	105.2	20	16
CPTH	139.9	77.2	20	19

4.2.7 Liquid Chromatography-Mass Spectrometry Analysis

Samples of kidney, liver, GI tract, and breast muscle (200 g) were individually fortified with a surrogate compound (d_6-CPTH) and extracted with 2 mL of ultra-pure water followed by the

addition of 2 mL of acetonitrile that had been saturated with potassium hydroxide (Fisher Scientific, Pittsburgh, PA). The tissue sample was homogenized with four passes of a Potter-Elvehjem homogenization tube at room temperature. The homogenized sample was transferred to a 15-mL plastic centrifuge tube and 1 g of a QuEChERS salt packet was added (Agilent Technologies, Santa Clara, CA). QuEChERS stands for Quick, Easy, Cheap, Effective, Rugged, and Safe. It is a dispersive solid phase extraction (dSPE) technique which uses the addition of salt to create a partition in a previously miscible solution of acetonitrile and water (Anastassiades et al., 2003) with the aqueous layer comprising the bottom of the two layers. In this case, the salt packet contained magnesium sulfate and sodium chloride in a 4:1 (w/w) ratio. Immediately after addition of the salt mixture, the sample was vortexed and then placed in a centrifuge at 6,000 rpm for 5 minutes.

A proprietary dSPE material, which was specifically designed to remove lipid materials, was used to clean-up the samples (EMR-Lipid, Agilent Technologies, Santa Clara, CA). The dSPE material was activated by the addition of 2 mL of ultra-pure water. The upper, organic layer of the sample from the partition produced previously was removed and added to the dSPE tube. The tube was vortexed and placed in a centrifuge at 6,000 rpm for 5 minutes. The entire liquid layer from the dSPE tube was added to a 15-mL plastic centrifuge tube that contained a QuEChERS salt mixture containing magnesium sulfate and sodium chloride in a 4:1 (w/w) ratio. The tube was vortexed and then centrifuged at 6,000 rpm for 5 minutes.

A portion (1 mL) of the upper, organic layer was removed and transferred to a 1.5-mL micro-centrifuge tube. Hydrochloric acid (100 µL of a 1 N solution) was added and the sample vortexed. The sample was placed in a Speed Vac (Thermo Fisher, Maltham, MA) for approximately one hour. The sample was monitored until approximately 100 µL remained in the tube. The sample was not allowed to dry completely as this was found to have a negative impact on the recovery of CPTH.

The sample was reconstituted by the addition of 100 µL of methanol and 800 µL of 0.1% formic acid. The reconstituted sample was filtered through a 0.45 µm Teflon filter (Samplicity filter system, EMD Millipore, Billerica, MA) directly into an LC vial. The sample was then injected into an Agilent 1200 series liquid chromatograph coupled to an Agilent 6410 mass spectrometer (LC/MS/MS). A Waters X-Bridge C18, 3 µm, 2.1 x 50 mm LC column (Milford, MA) was used to achieve separation under the parameters in Table 4.2. At the start of each analytical run on the

Table 4.2: Liquid Chromatograph/Mass Spectrometer Conditions

Mobile Phase A:	0.1% formic acid
Mobile Phase B:	0.1% formic acid in acetonitrile
Gradient:	

Time (min)	% A	% B	Flow Rate (mL/min)
0.0	90	10	0.350
3.0	90	10	0.350
7.0	60	40	0.350
11.0	0	100	0.350

Injection Volume:	20 µL
Column Temperature:	50 °C
Run Time:	13.0 minutes
Post Time:	3.0 minutes
Polarity:	Positive
Gas Temperature:	350 °C
Gas Flow:	Nitrogen @ 9 L/minute
Nebulizer Pressure:	25 psi
Capillary Voltage:	4000 V

Compound	Precursor (m/z)	Product (m/z)	Dwell (ms)	Frag. (V)	Collision Energy (V)
OH-CAT	200	170	100	80	10
OH-CAT	200	128	100	80	18
OH-CAT	200	93	100	80	35
d_6-CPTH	148	113	100	100	20
d_6-CPTH	148	94	100	100	40
CPTH	142	107	100	90	15
CPTH	142	106	100	90	30
CPTH	142	88.9	100	90	35
CAT	184	142	100	90	15
CAT	184	107	100	90	25
CAT	184	106	100	90	45

instrument, a sequence of five injections was made to assess the suitability of the chromatographic system for analysis of samples. The RSD of the ratio of the analyte's area response to the area response of the surrogate compound (d6-CPTH) was determined.

At the start of each day's analysis, a spike check (SC) was prepared at each fortification level. The SC sample was prepared by adding the appropriate aliquots of a solution containing CPTH, CAT, and OH-CAT and a surrogate solution containing d6-CPTH to a vial containing a diluent. These SC samples were injected at the beginning of the analysis for each batch.

4.3 Method Validation Samples

The repeatability and performance of the methods were assessed using a surrogate avian species. Coturnix quail (*Coturnix japonica*) were used as control matrix for the preparation of fortified quality control samples. Quail were chosen due to the ability to readily obtain large quantities of tissue from each individual. The red-winged blackbirds are much smaller and it would have taken a large number of individuals to provide enough matrix for both the LC and GC analyses. Both birds are granivores, and quail were an adequate representative of red-winged blackbirds.

Quail were purchased from a hatchery in Colorado and their tissues were processed according to the procedures outlined in Chapter 4.2.5. A minimum of 7 replicates at each fortification level were prepared by adding an appropriate aliquot of a fortification solution to a sample of control quail tissue. The samples were then extracted in the same manner as the study samples. For the gas chromatographic assay, control quail liver, GI tract, and breast muscle were fortified at 20, 50, 100, 300, and 1000 ng/g. For the liquid chromatographic assay, control quail kidney, liver, GI tract, and breast muscle were fortified at 5, 10, 20, 500, and 5000 ng/g.

Additional quality control efforts were undertaken with the LC assays. Study samples and quality control samples were randomly assigned in batches of 10 study samples and 13 quality control samples. Within each batch, one sample was randomly selected for replicate analysis and a different sample was selected as a matrix fortification. The matrix was fortified with 500 ng/g of CPTH, CAT, and OH-CAT. This randomized design, combined with the additional replicates and matrix spikes were used to demonstrate the ruggedness and repeatability of the newly developed LC method.

4.4 Results and Discussion

4.4.1 Chromatographic Improvements

As discussed in Chapter 3, the polarity of CPTH and its two major metabolites (CAT and OH-CAT) are very different. During initial method development, it was observed that while CPTH had very high water solubility, both of its metabolites were not as soluble. This created a difficulty in analyzing samples by LC techniques. This difficulty was overcome by incorporating methanol as a co-solvent for the preparation of high level stock solutions of CAT and OH-CAT. After adjusting the solvent system, both CAT and OH-CAT were very easily chromatographed on the LC system, and all three compounds produced adequate peaks. Symmetry factor, as calculated by the instrument's software, was 1.56, 1.59, and 1.77 for OH-CAT, CAT, and CPTH, respectively (Figures 4.2 to 4.4). An ideal symmetry factor would be 1.0, with larger values indicating less ideal peak symmetry. Numbers above 2.0 are generally considered to be unusable as they interfere with the algorithms used by the instrument's software to integrate the area under the peak.

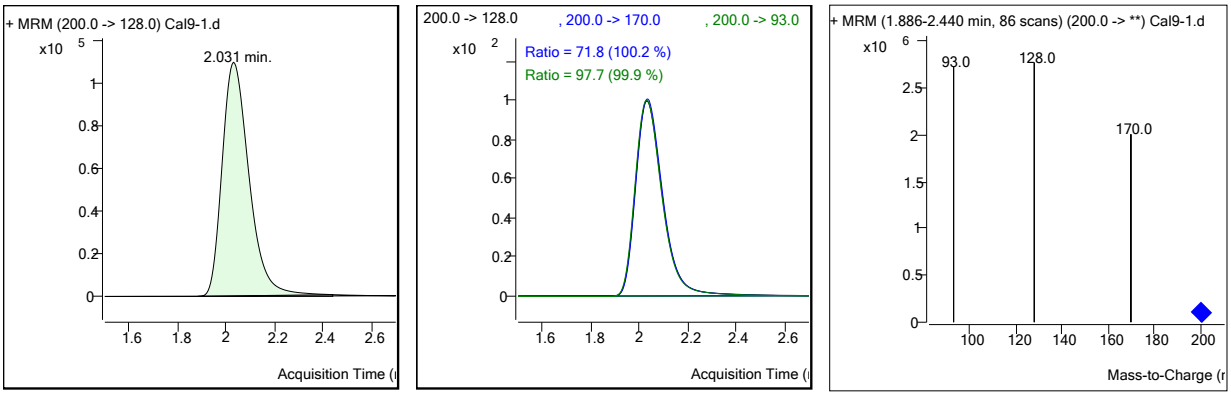


Figure 4.2: LC/MS/MS chromatogram and MRM spectrum of a 1,000 ng/mL OH-CAT standard.

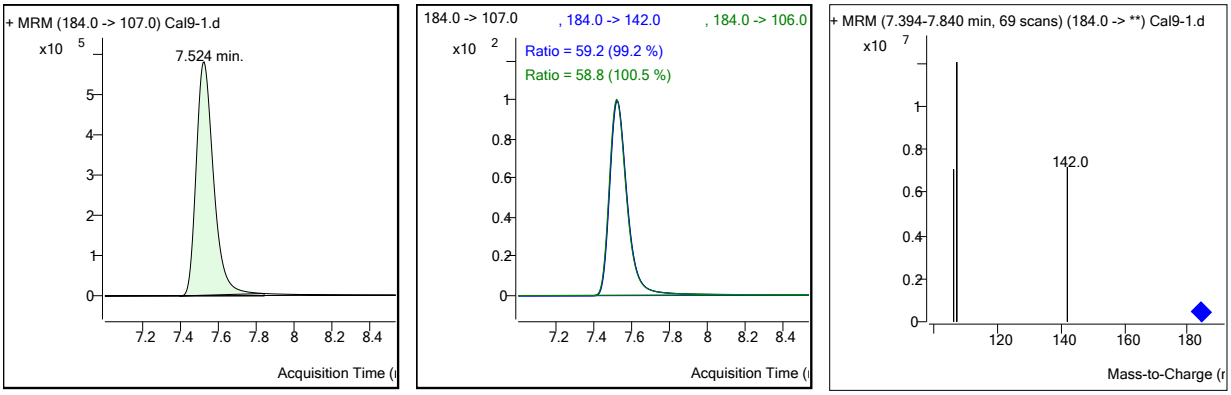


Figure 4.3: LC/MS/MS chromatogram and MRM spectrum of a 1,000 ng/mL CAT standard.

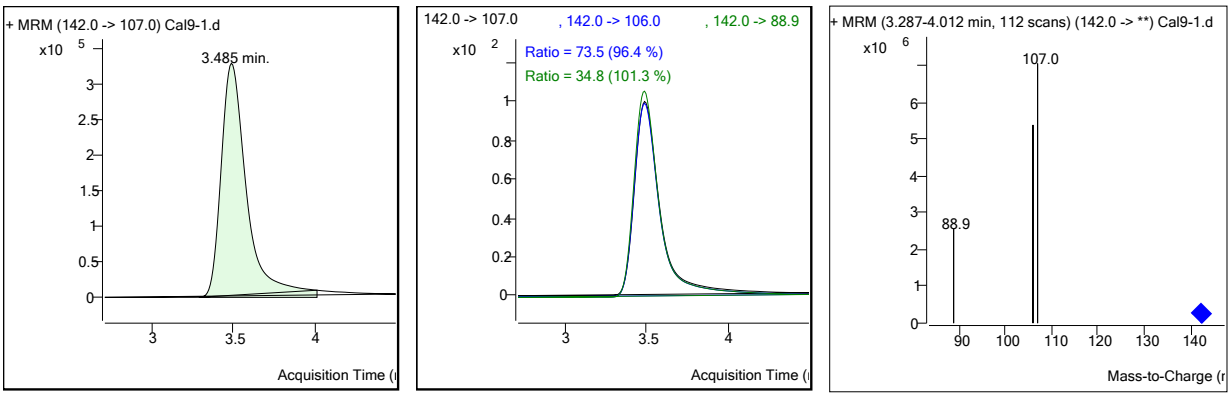


Figure 4.4: LC/MS/MS chromatogram and MRM spectrum of a 1,000 ng/mL CPTH standard.

Attempts were made to improve the performance of the LC system for CPTH. Various LC columns were evaluated, but none resulted in any improvement in performance. The pKa of CPTH

has been reported to be 3.7 (Kimball and Mishalanie, 1994). At the pH of the 0.1% formic acid mobile phase (approximately 2), the CPTH should have been fully protonated. The interaction of this positively charged species with residual silanols on the stationary phase surface of the C18 LC column lead to broadening of the peak. Adjusting the pH of the mobile phase to 6 to deprotonate the CPTH would remove this secondary interaction and improve peak shape. However, the solubility of CPTH in aqueous solutions was quite low and led to the CPTH not being eluted from the LC column at all. It is possible that another type of stationary phase (such as biphenyl or perfluorophenyl) might have produced a sharper peak for CPTH, but all would likely still have residual silanol groups and suffer from the same issues as the C18 stationary phase. The peaks of all three compounds, while not perfectly symmetrical, were sharp enough to reliably integrate and proved suitable for the current study.

On the GC system OH-CAT performed very poorly, while both CAT and CPTH produced very symmetrical and sharp peaks. Symmetry factors, as calculated by the instrument's software, were 4.39, 1.12, and 1.02 for OH-CAT, CAT, and CPTH, respectively (Figure 4.5 to 4.7). OH-CAT was subject to significant signal loss as well, being detectable only at the highest concentration levels.

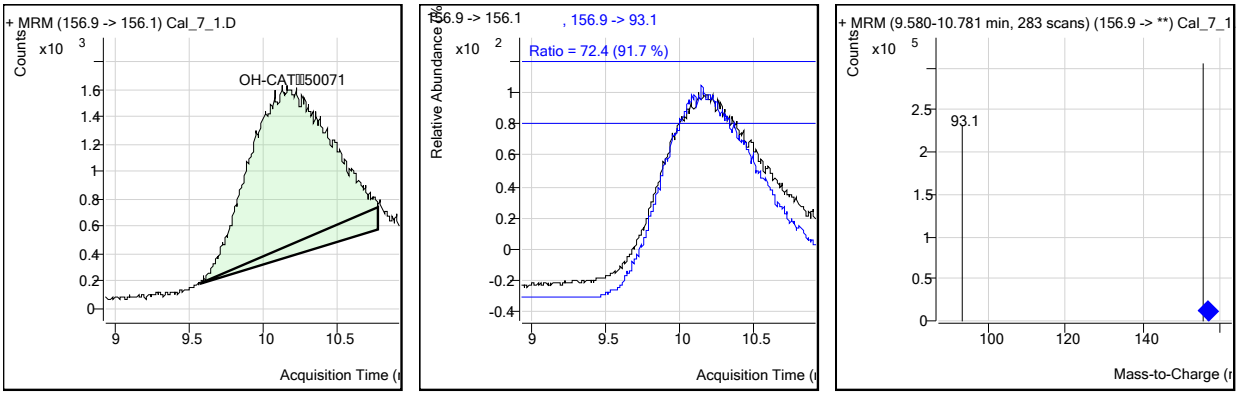


Figure 4.5: GC/MS/MS chromatogram and MRM spectrum of a 5,000 ng/mL OH-CAT standard.

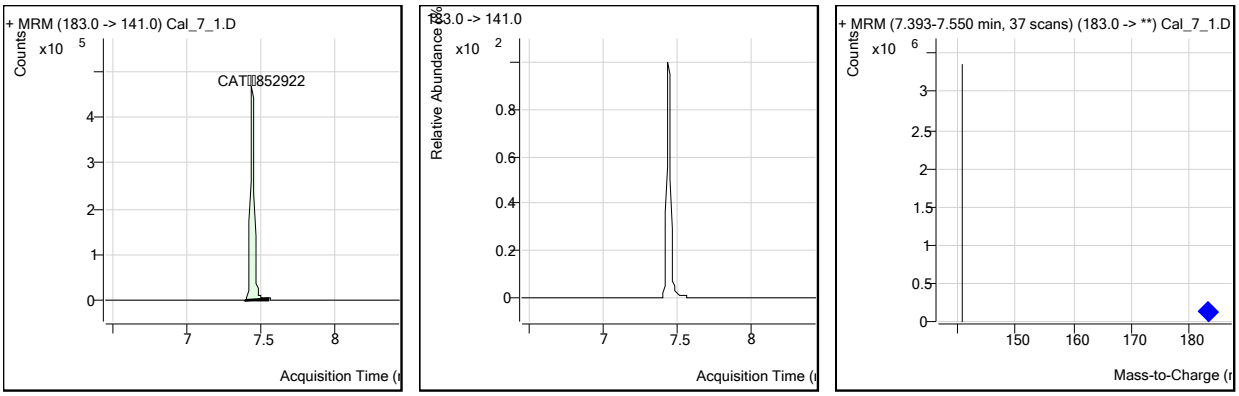


Figure 4.6: GC/MS/MS chromatogram and MRM spectrum of a 5,000 ng/mL CAT standard.

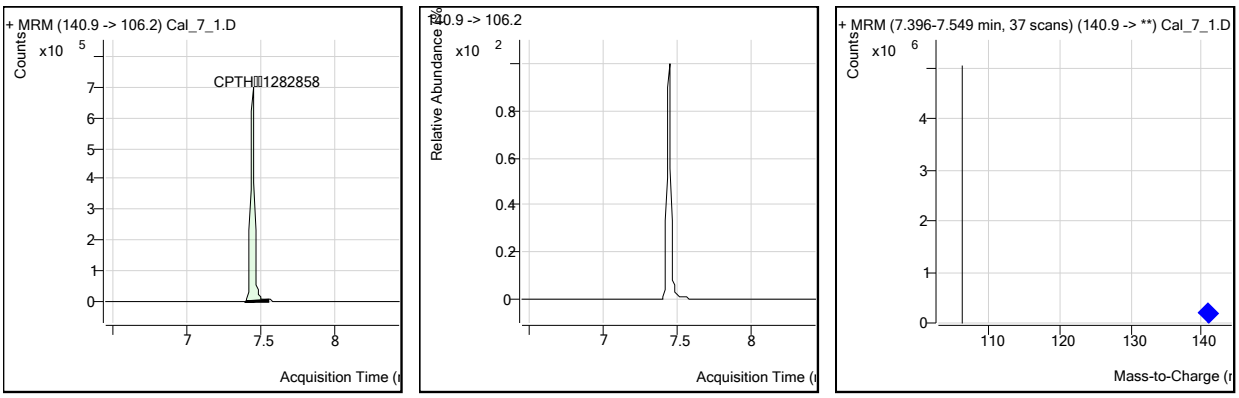


Figure 4.7: GC/MS/MS chromatogram and MRM spectrum of a 5,000 ng/mL CPTH standard.

Therefore, the results of the method validation indicated that results for CPTH and its metabolites were method dependent. Conclusions derived from the *in-vivo* testing would use the CPTH results produced on the GC system and the metabolite results produced on the LC system. Insufficient tissue existed to repeat the analysis of the kidney samples on the GC system, therefore the results for CPTH produced on the LC were used for this tissue only.

4.4.2 Gas Chromatographic Results

The detection limit (DL) for CPTH in red-winged blackbird tissues was estimated from the mean chromatographic response of control samples compared to control samples that had been fortified to

20 ng/g with CPTH. The DL was defined as the concentration of CPTH required to generate a signal equal to 3X the baseline noise (measured peak-to-peak) observed in the baseline at the retention time of CPTH in the control samples. The quantitation limit (QL) for CPTH in red-winged blackbird tissues was estimated in a similar fashion to as the DL with the multiplier of 10X baseline noise used instead of 3X. The DL and QL for GI tract, liver and breast muscle of red-winged blackbirds are shown in Table 4.3. Samples from the control exposure group had observable CPTH responses in several cases. In particular, the baseline noise combined with the presence of detectable CPTH peaks in the breast muscle samples lead to a much elevated DL and QL for this tissue.

Table 4.3: Detection Limit and Quantitation Limits for red-winged blackbird tissue analyzed using GC/MS/MS.

Tissue Type	Detection Limit	Quantitation Limit
GI Tract	2.8 ng/g	9.2 ng/g
Liver	1.1 ng/g	3.8 ng/g
Breast Muscle	4.3 ng/g	14 ng/g

Values below the DL were reported as not detected. For statistical analysis of these samples, a value of one-half of the DL was used. Results between the DL and QL were considered to be qualitative only. Therefore, results between these two values were only reported to two significant digits where other results used three significant digits.

Analyte recoveries from the quality control samples were very good (Table 4.4; Figure 4.8). Breast muscle sample recoveries were significantly lower (α=0.05) than those for either GI tract or liver (SAS General Linear Model (GLM); SAS Institute, Inc., Cary, NC). Breast muscle samples exhibited a much noisier baseline as demonstrated by the elevated DL in breast muscle (Table 4.3). ANOVA results from the same GLM demonstrated a significant effect from tissue type ($F=5.93$; $p=0.0038$) and fortification level ($F=4.10$; $p=0.0042$). There was no significant effect from the

interaction of tissue and level (F=1.39; p=0.2114). The recovery values for breast muscle (particularly at the lowest fortification level) were quite variable, but the GLM results indicate that the recovery values for breast muscle were significantly lower than those in both liver and GI tract.

Table 4.4: Mean CPTH quality control recovery (%) in red-winged blackbirds.

Fortification Level		Tissue Type		
		GI Tract	Liver	Breast Muscle
20 ng/g	Mean ± sd =	103 ± 3.8	101 ± 6.3	106 ± 33
	Range =	99.1 – 110	94.5 – 113	80.9 – 173
	RSD =	3.7%	6.2%	31%
	n =	7	6	7
50 ng/g	Mean ± sd =	97.3 ± 7.7	97.0 ± 6.8	86.2 ±6.2
	Range =	90.0 – 113	88.0 – 109	74.5 – 95.1
	RSD =	7.9%	7.0%	7.2%
	n =	7	7	9
100 ng/g	Mean ± sd =	95.2 ± 2.0	98.6 ± 1.3	87.3 ± 2.5
	Range =	91.9 – 97.1	96.6 – 100	83.5 – 89.6
	RSD =	2.1%	1.3%	2.9%
	n =	7	7	7
300 ng/g	Mean ± sd =	99.3 ± 1.4	103 ± 3.6	97.9 ± 4.9
	Range =	96.6 – 101	99.1 – 109	93.4 – 108
	RSD =	1.4%	3.5%	5.0%
	n =	7	7	7
1000 ng/g	Mean ± sd =	98.8 ± 3.7	106 ± 6.6	88.9 ± 4.2
	Range =	94.0 – 105	93.5 – 113	81.7 – 93.6
	RSD =	3.7%	6.2%	4.7%
	n =	7	7	7

CPTH residues from a field exposure study also demonstrated a high degree of variability (Stahl et al., 2002). In this study the birds were allowed to feed *ad libitum* on a treated bait site. Therefore, the dose level was not known. In a separate study designed to mimic a field exposure, the results for breast muscle were all below the 70 ng/g QL for the method used (Stahl and Johnston, 2006). The current method has a QL in breast muscle which was significantly lower (14 ng/g), demonstrating a clear improvement on previous methods. This was likely due to the use of tandem mass

spectrometer rather than the mass selective detector (single quadrupole) used in the literature method. The results for GI tract samples in the 2006 research were above the QL for the method, but both tissues demonstrated RSD values near 50%. This was consistent with the results from the current study. Also similar between the current study and those in the literature was the difference in concentration of CPTH observed between GI tract and breast muscle. In all cases, the concentration in GI tract was much larger than that for breast muscle in an individual bird.

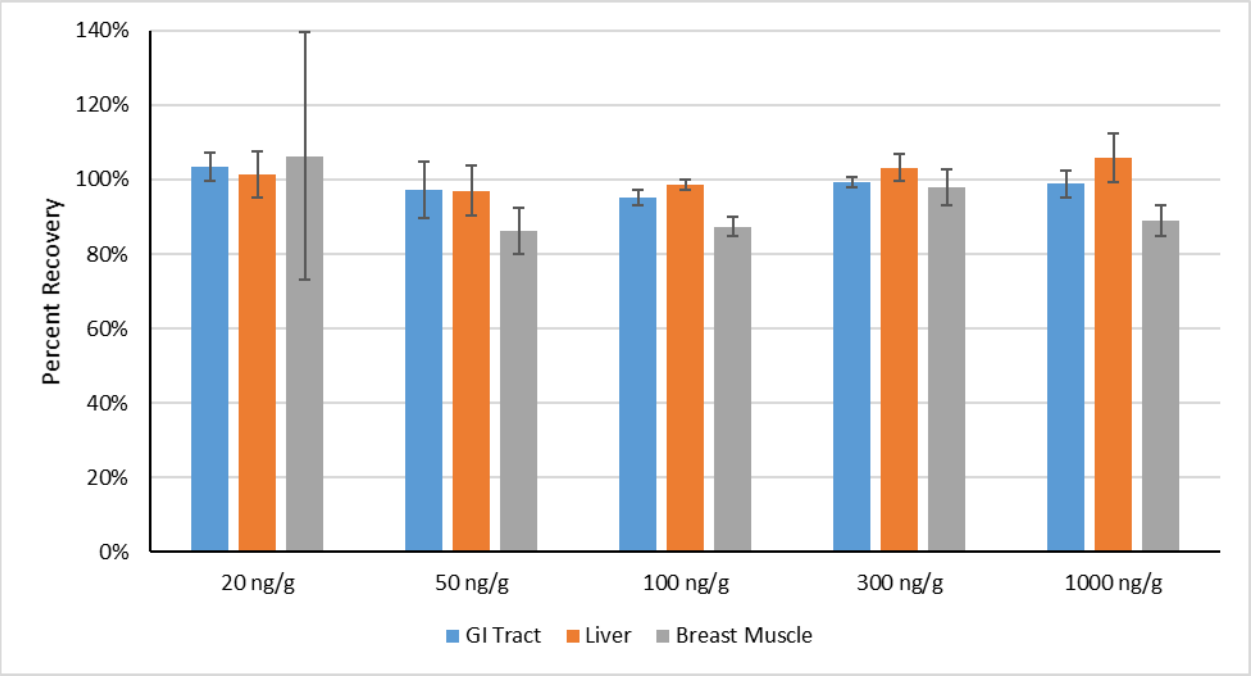


Figure 4.8: Mean quality control recovery for red-winged blackbird tissues fortified with CPTH analyzed by GC/MS/MS (error bars indicate standard deviation).

The residue levels observed in the *in-vivo* study (Table 4.5; Figure 4.9) were higher than anticipated, particularly in the GI tract. Given the rapid excretion observed in the results from Phase 1 of this research, there was an expectation that the GI tract residues would be quite low. There are two possible explanations for this discrepancy. Firstly, the dose administered in Phase 1 was 3.6 mg/kg while the low dose in Phase 3 was 12.5 mg/kg. Time to death is dose dependent for CPTH.

The higher dose used in Phase 3 led to one of the birds in the middle dose level group being found dead approximately 6 hours after receiving the dose of CPTH. All the remaining birds that received a dose were found dead on the morning after receiving the dose with the exception of one bird in the low dose group, which survived for all three days of the study. The lone bird in the low dose group which survived the three days of the study had lower residues in all three tissues compared to other birds in the low group. Secondly, the day the study was conducted was unseasonably cold with nighttime temperatures reaching -10 °C. Combined with the higher dose, the two factors likely contributed to slower digestion and less of the CPTH being excreted prior to death.

Table 4.5: Mean CPTH residue levels (ng/g) in red-winged blackbirds following a single, oral dose analyzed by GC/MS/MS.

Dose Level		GI Tract	Liver	Breast Muscle
12.5 ng/g	Mean ± sd =	66.6 ± 30.1	85.1 ± 36.1	23.8 ± 14.9
	Range =	6.83 – 110	19.8 – 148	4.9 – 50
	RSD =	45%	42%	63%
	n =	8	8	8
25 ng/g	Mean ± sd =	585 ± 820	215 ± 125	42.2 ± 27.2
	Range =	102 – 2400	75.8 – 441	10.3 – 79.5
	RSD =	141%	58%	66%
	n =	7	8	8
37.5 ng/g	Mean ± sd =	559 ± 684	317 ± 145	49.5 ± 21.0
	Range =	107 – 2170	167 – 571	26.4 – 89.5
	RSD =	122%	46%	42%
	n =	8	8	8

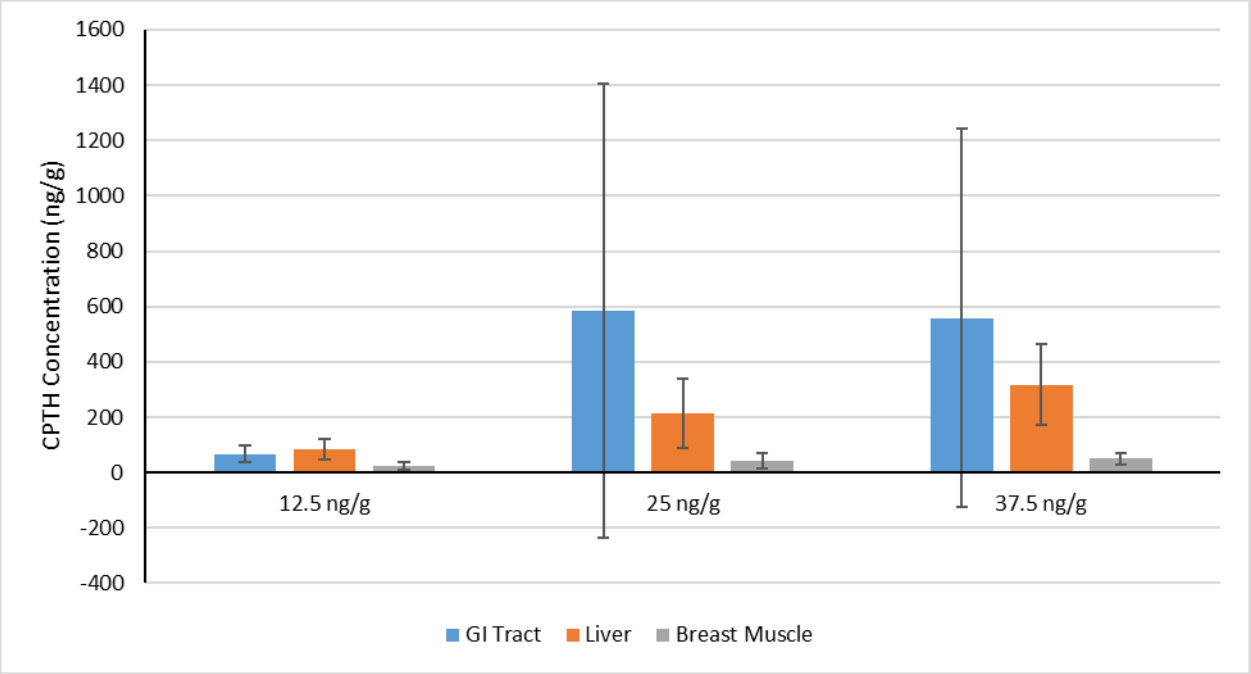


Figure 4.9: Average observed CPTH (ng/g) in red-winged blackbird GI tract, liver, and breast muscle following a single, oral dose analyzed by GC/MS/MS (error bars indicate standard deviation).

4.4.3 Liquid Chromatographic Results

The detection limit (DL) was estimated from the mean chromatographic response of control samples compared to control samples that had been fortified with 5 ng/g of CPTH, CAT, and OH-CAT. The DL was defined as the concentration of analyte required to generate a signal equal to 3X the baseline noise (measured peak-to-peak) observed in the baseline at the retention time of the analyte in the control samples. The method quantitation limit (QL) was estimated in a similar fashion to as the DL with the multiplier of 10X baseline noise used instead of 3X. The DL and QL for GI tract, liver, kidney, and breast muscle of red-winged blackbirds are shown in Table 4.6.

Table 4.6: Detection Limit and Quantitation Limits for red-winged blackbird tissue analyzed using LC/MS/MS.

Tissue Type	Detection Limit	Quantitation Limit
	CPTH	
GI Tract	4.1 ng/g	14 ng/g
Liver	3.8 ng/g	13 ng/g
Kidney	3.8 ng/g	13 ng/g
Breast Muscle	2.5 ng/g	8.3 ng/g
	CAT	
GI Tract	1.6 ng/g	5.4 ng/g
Liver	2.3 ng/g	7.6 ng/g
Kidney	2.1 ng/g	6.9 ng/g
Breast Muscle	2.0 ng/g	6.7 ng/g
	OH-CAT	
GI Tract	1.8 ng/g	5.9 ng/g
Liver	1.6 ng/g	5.4 ng/g
Kidney	3.6 ng/g	12 ng/g
Breast Muscle	2.3 ng/g	7.7 ng/g

Values below the DL were reported as not detected. For statistical analysis of these samples, the value of one-half of the DL was used. Results between the DL and QL were considered to be qualitative only. Therefore, results between these two values were only reported to two significant digits where other results used three significant digits.

Analyte recoveries from the quality control samples hovered near 100% (Tables 4.7 to 4.9; Figures 4.10 to 4.12). RSD values were quite large for all samples, demonstrating significant variability in the method. Several potential sources exist for this variability. Chief among them is the variability observed for samples run on different days. The QC data were subjected to the same GLM procedure as used previously. Results indicated a significant effect for the batch ($F=5.49$; $p=<0.0001$). A significant interaction was also found between batch and compound of interest ($F=3.31$; $p=<0.0001$). The date of analysis (as represented by batch number) had a significant impact on the results.

Table 4.7: Mean CPTH quality control recovery (%) in red-winged blackbirds analyzed by LC/MS/MS.

Fortification Level		Tissue Type			
		GI Tract	Liver	Kidney	Breast Muscle
5 ng/g	Mean ± sd =	131 ± 54	114 ± 26	125 ± 15	126 ± 8.0
	Range =	90-246	81-147	105-146	117-140
	RSD =	41%	23%	12%	6.3%
	n =	7	7	7	7
10 ng/g	Mean ± sd =	107 ± 20	115 ± 16	116 ± 10	124 ± 16
	Range =	64-118	94-142	106-135	104-144
	RSD =	19%	14%	8.6%	13%
	n =	7	7	7	7
20 ng/g	Mean ± sd =	122 ± 14	118 ± 15	114 ± 10	122 ± 15
	Range =	104-144	98-146	96-129	105-149
	RSD =	11%	13%	8.8%	12%
	n =	7	7	7	7
500 ng/g	Mean ± sd =	115 ± 7.8	118 ± 13	113 ± 6.2	110 ± 6.4
	Range =	108-127	99-142	103-121	104-120
	RSD =	6.8%	11%	5.5%	5.8%
	n =	7	9	7	6
5000 ng/g	Mean ± sd =	112 ± 16	125 ± 20	125 ± 11	123 ± 16
	Range =	87-132	109-165	108-142	103-143
	RSD =	14%	16%	8.8%	13%
	n =	7	6	7	7

The type of tissue analyzed had a significant impact on recovery in the QC samples just as it did with the GC technique ($F=6.75$; $p=0.0004$). The recovery values for liver were different than those for GI tract, kidney, and breast muscle ($\alpha=0.05$). The recovery of each compound (CPTH, CAT, and OH-CAT) was also a significant factor ($F=29.54$; $p=<0.0001$). The recovery values for CPTH and OH-CAT were not significantly different from each other, but the recovery levels for CAT were different from both CAT and OH-CAT ($\alpha=0.05$).

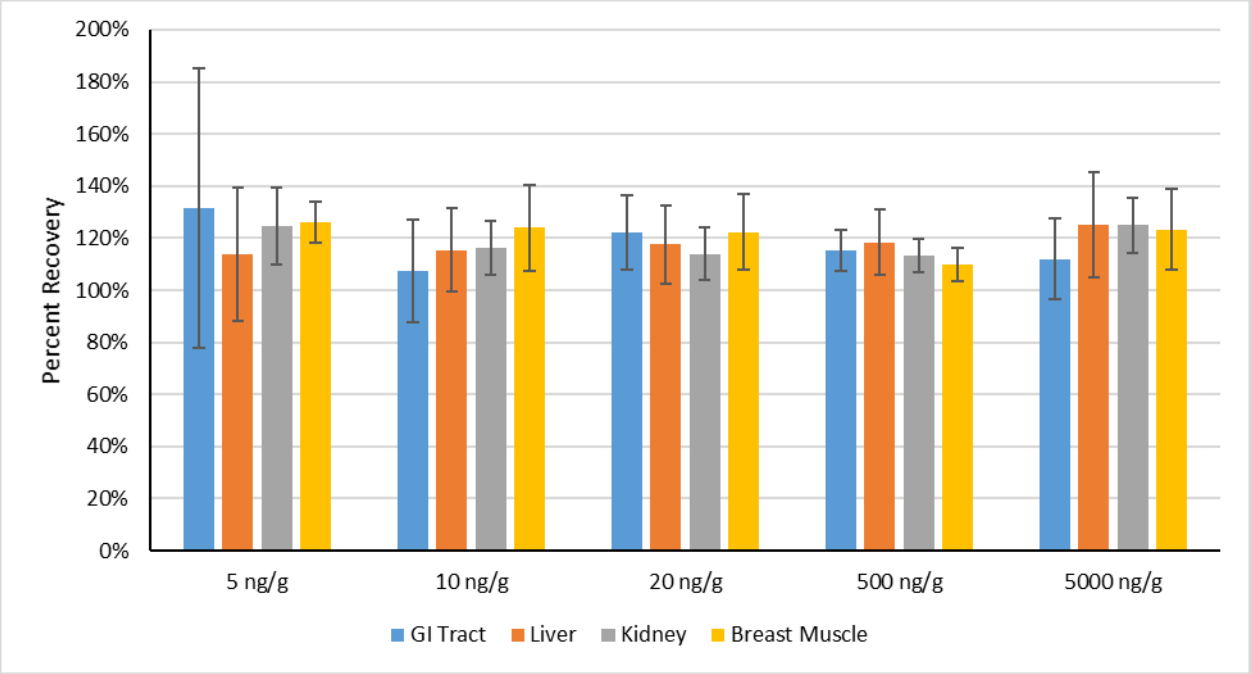


Figure 4.10: Mean quality control recovery for red-winged blackbird tissues fortified with CPTH analyzed by LC/MS/MS (error bars indicate standard deviation).

An additional factor not reflected in the recovery data was instrumental performance. The criteria for instrument suitability, as assessed by repeated injections of a working standard (discussed in Chapter 4.2.7), is typically around 2%. For the data generated on the LC during this study, the RSD values were typically around 3% with values as high as 6% observed in some cases. This variability impacted the injections made on the LC system and contributed to the variability observed in the QC recoveries.

Table 4.8: Mean CAT quality control recovery (%) in red-winged blackbirds analyzed by LC/MS/MS.

Fortification Level		Tissue Type			
		GI Tract	Liver	Kidney	Breast Muscle
5 ng/g	Mean ± sd =	93 ± 27	97 ± 26	87 ± 14	78 ± 22
	Range =	55-135	67-142	71-107	46-100
	RSD =	29%	27%	16%	28%
	n =	7	7	7	7
10 ng/g	Mean ± sd =	85 ± 23	116 ± 21	89 ± 15	97 ± 13
	Range =	35-102	87-158	66-108	79-114
	RSD =	27%	18%	17%	13%
	n =	7	7	7	7
20 ng/g	Mean ± sd =	101 ± 11	116 ± 23	103 ± 18	102 ± 28
	Range =	89-114	94-160	77-124	69-143
	RSD =	11%	20%	17%	27%
	n =	7	7	7	7
500 ng/g	Mean ± sd =	102 ± 11	117 ± 16	103 ± 18	105 ± 14
	Range =	87-120	89-137	62-114	92-128
	RSD =	11%	14%	17%	13%
	n =	7	9	7	6
5000 ng/g	Mean ± sd =	104 ± 13	107 ± 11	109 ± 24	98 ± 23
	Range =	80-116	94-122	66-130	68-130
	RSD =	13%	10%	22%	23%
	n =	7	6	7	7

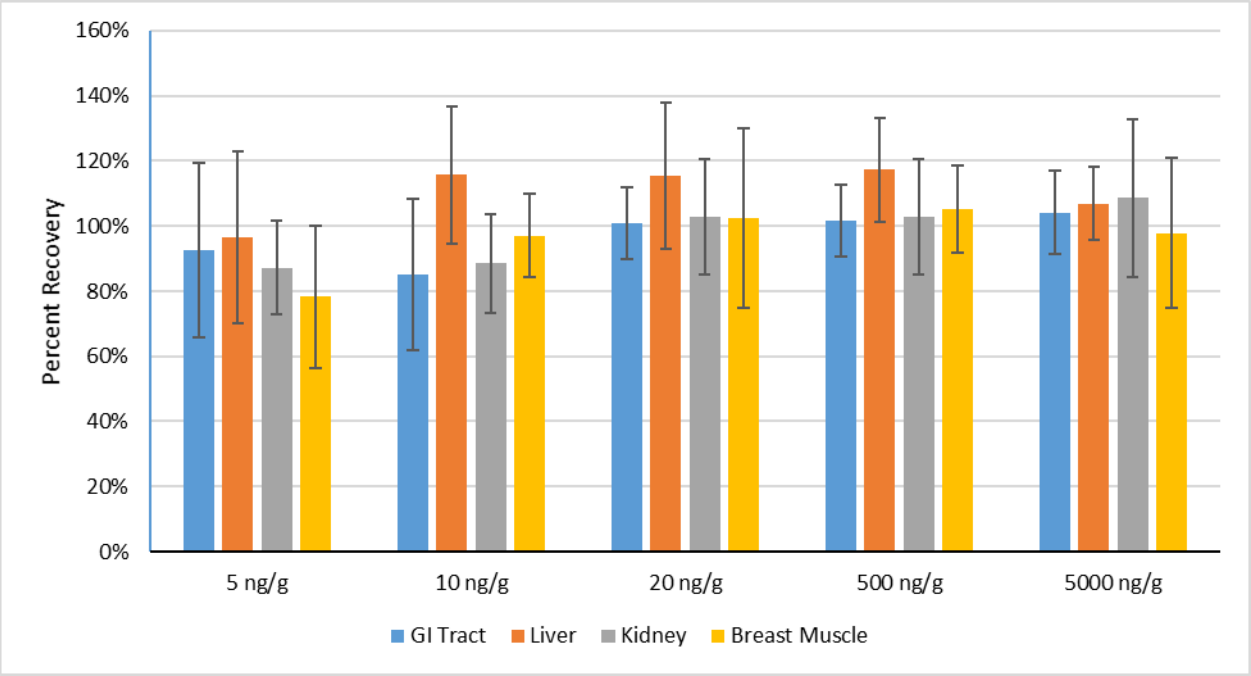


Figure 4.11: Mean quality control recovery for red-winged blackbird tissues fortified with CAT analyzed by LC/MS/MS (error bars indicate standard deviation).

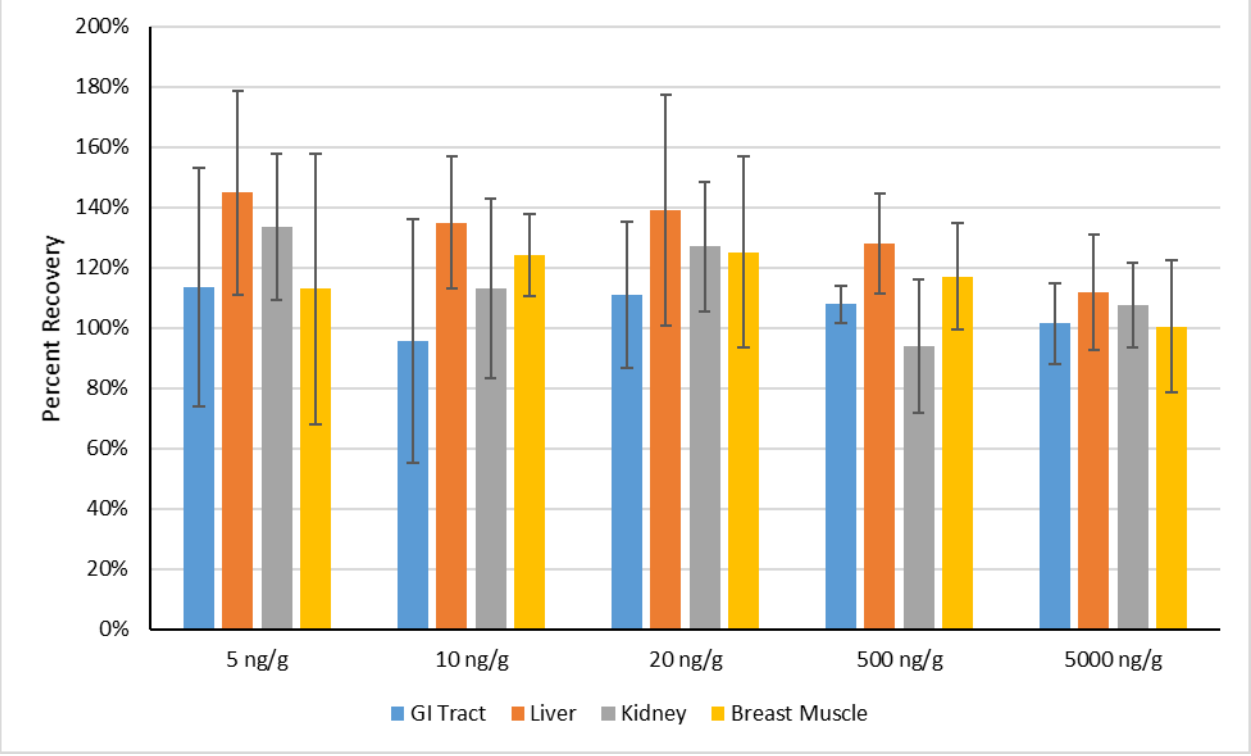


Figure 4.12: Mean quality control recovery for red-winged blackbird tissues fortified with OH-CAT analyzed by LC/MS/MS (error bars indicate standard deviation).

Table 4.9: Mean OH-CAT quality control recovery (%) in red-winged blackbirds analyzed by LC/MS/MS.

Fortification Level		Tissue Type			
		GI Tract	Liver	Kidney	Breast Muscle
5 ng/g	Mean ± sd =	114 ± 40	145 ± 34	134 ± 24	113 ± 45
	Range =	50-183	81-184	90-161	40-153
	RSD =	35%	23%	18%	40%
	n =	7	7	7	7
10 ng/g	Mean ± sd =	96 ± 41	135 ± 22	113 ± 30	124 ± 14
	Range =	35-142	103-161	75-158	109-147
	RSD =	43%	16%	27%	11%
	n =	7	7	7	7
20 ng/g	Mean ± sd =	111 ± 24	139 ± 38	127 ± 21	125 ± 32
	Range =	60-131	93-206	98-160	99-177
	RSD =	22%	27%	17%	26%
	n =	7	7	7	7
500 ng/g	Mean ± sd =	108 ± 6.2	128 ± 17	94 ± 22	117 ± 18
	Range =	100-117	108-152	66-133	97-147
	RSD =	5.7%	13%	23%	15%
	n =	7	9	7	6
5000 ng/g	Mean ± sd =	101 ± 13	112 ± 19	108 ± 14	100 ± 22
	Range =	83-119	89-140	90-125	72-128
	RSD =	13%	17%	13%	22%
	n =	7	6	7	7

Assessment of the daily spike check (SC) samples also demonstrated a high level of variability. The observed recovery of these samples was compared to their theoretical concentration and accuracy values generated. Graphs of each SC as a function of batch number demonstrated significant variation across the batches. As these SC samples were essential analytical standards, observed variability in their accuracy demonstrated significant challenges with the LC system. As discussed previously, this variability could have been due to issues with the variability of the LC system. It was unlikely that the fortification solutions were decomposing or changing over time as no clear trend was found in the graphs (Figures 4.13 to 4.15). The anticipated accuracy of these SC samples was ± 10%. The values on the trend graphs deviated from this expectation in many cases.

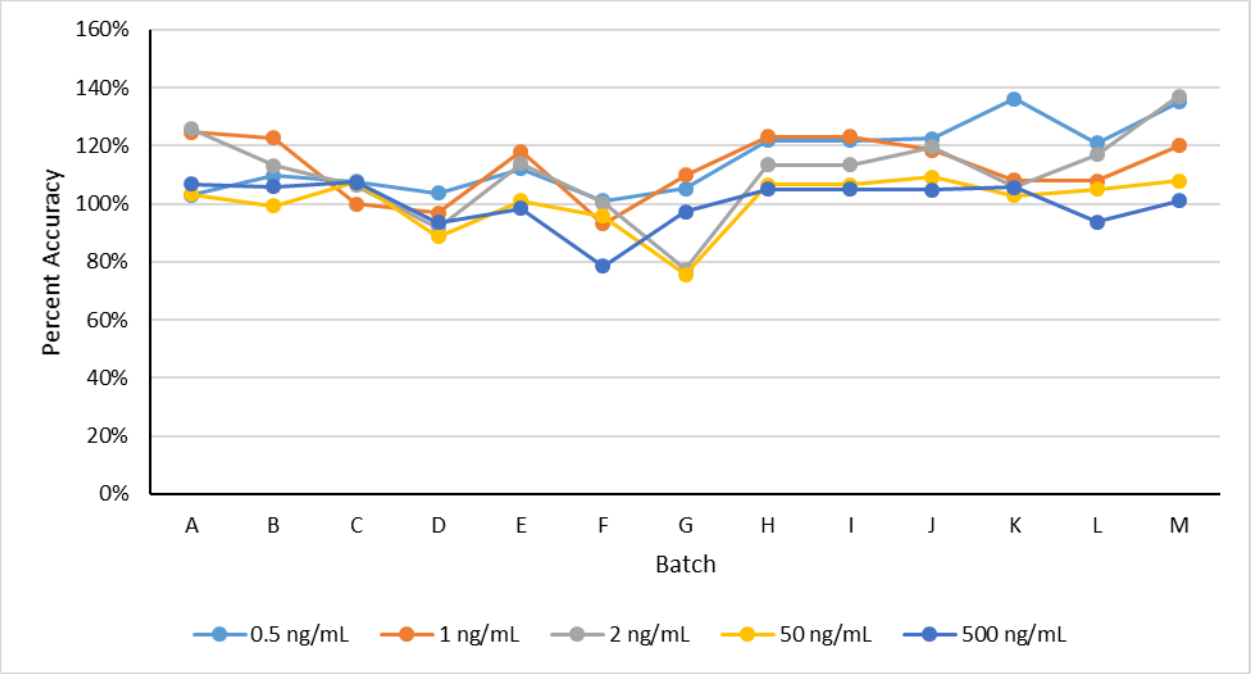


Figure 4.13: Trend in accuracy of Spike Checks prepared for CPTH at the start of each batch.

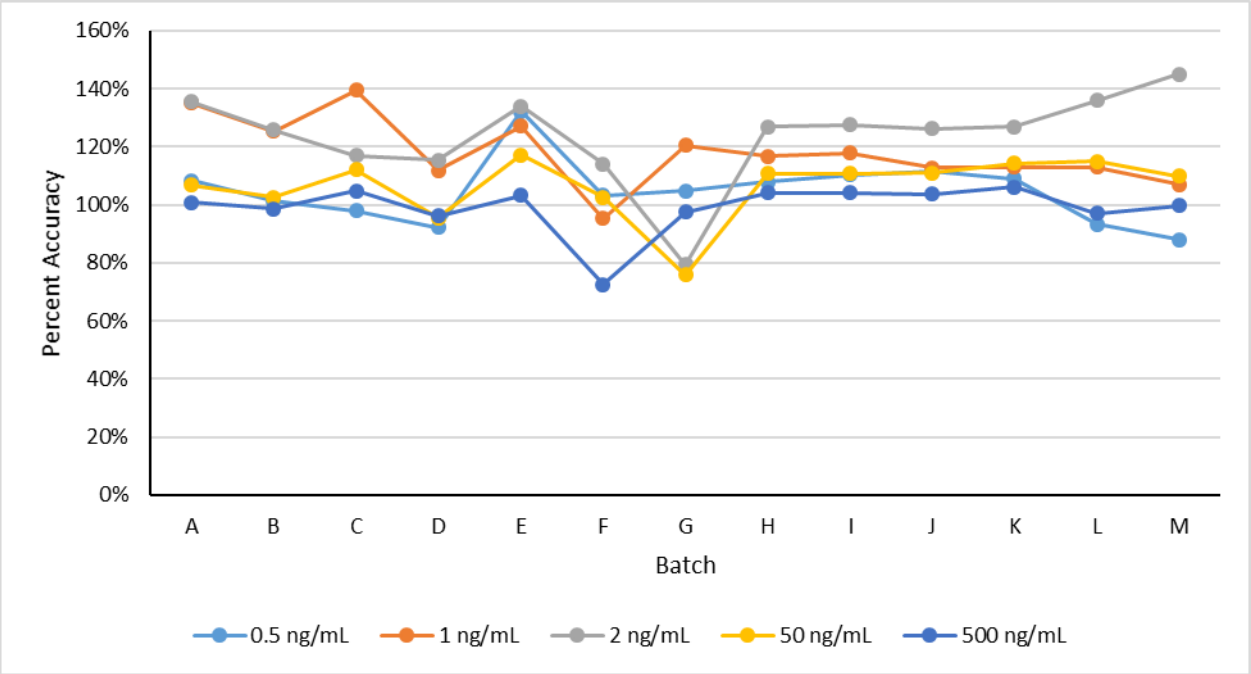


Figure 4.14: Trend in accuracy of Spike Checks prepared for CAT at the start of each batch.

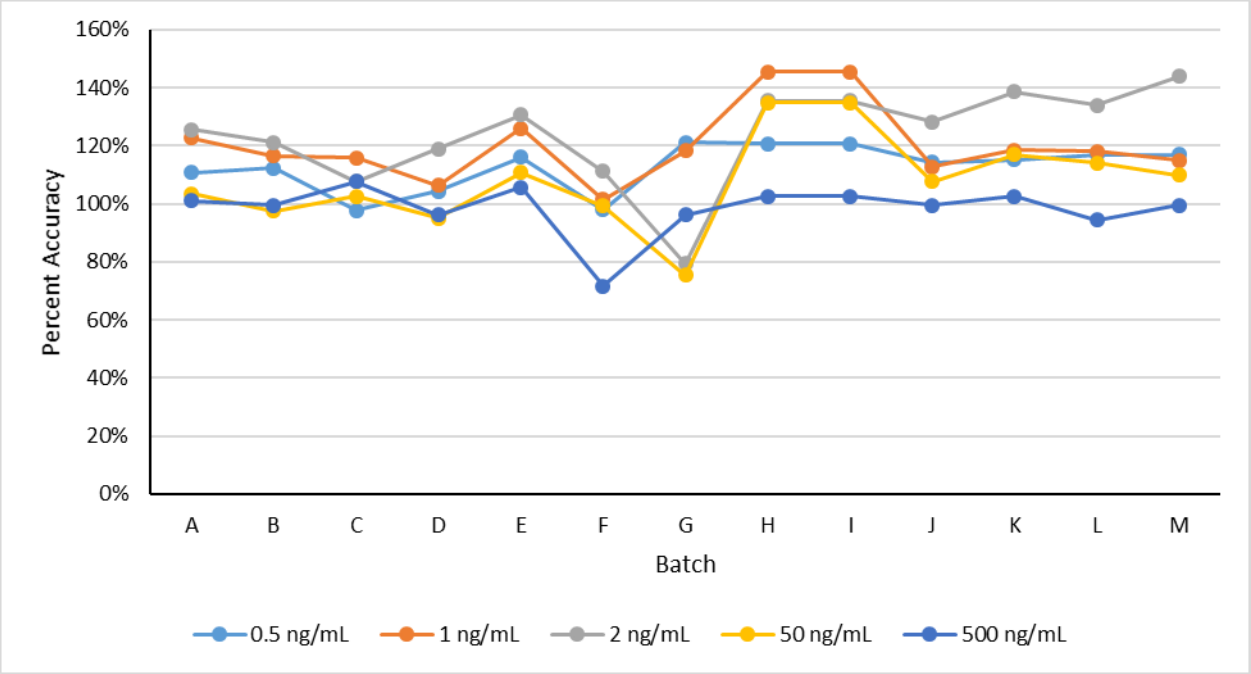


Figure 4.15: Trend in accuracy of Spike Checks prepared for OH-CAT at the start of each batch.

The mass of sample used for the LC technique were quite small (less than 200 mg). Even though homogenization of the samples in liquid nitrogen produced a very fine powder that should have yielded a very homogeneous sample, some variability was observed in the repeated analysis of the same sample. Variation between repeated measures of the same sample were as high as 150% in some cases. Use of a larger sample would likely have improved the variability somewhat, but limitations on the size of a red-wing blackbird's kidney and the use of each sample for two assays (residue analysis and proteomics) dictated that the sample sizes needed to be kept as small as possible. Due to the small tissue mass, the QuEChERS technique was downsized to suit the smaller available tissue mass. The dispersive solid phase extraction technique was initially intended for fruit and vegetable samples in which 10 to 15 g of material is used. The technique relies on competition between the sorbent used and matrix compounds in the sample extract; therefore use of too little matrix can have a negative impact on the performance of QuEChERS techniques. If no sample

matrix exists, it is likely a portion of the target analyte (CPTH, CAT or OH-CAT) would be bound by the sorbent and lost from the sample extract.

A standard approach for evaluating a method's accuracy, selectivity and sensitivity is the use of a surrogate compound. Ideally, surrogates would have been used for each of the three compounds being studied (CPTH, CAT, and OH-CAT). Only the d6-CPTH was available for the present study. However, this did not seem to significantly impact the performance of the method as the CPTH results suffered from the same issues as both CAT and OH-CAT and were not fully corrected for by use of the d6-CPTH. A possibility did exist for the replacement of a deuterium on the d6-CPTH which would have changed its mass and rendered it unavailable to the mass spectrometer, reducing its recovery. The use of a 13-C labeled compound would overcome this limitation and should be pursued if this work is carried on in the future.

All of the above issues with the recovery and performance of the QC samples translated into highly variable results from the analysis of the study samples. The observed residue values for CPTH (Figure 4.16; Table 4.10), CAT (Figure 4.17; Table 4.11), and OH-CAT (Figure 4.18; Table 4.12) demonstrate the same degree of variability seen previously in data generated by the GC technique.

Despite the issues with variability of the LC technique, significant effects were observed in the data. The data generated were subjected to a GLM procedure as previously discussed. The observed residue in each tissue type was significantly different ($F=29.91$; $p=<0.0001$). The residue results for GI tract were significantly different than those for liver, kidney, and breast muscle ($\alpha=0.05$). Dose level of CPTH given to the birds also had a significant effect on the observed residues ($F=40.00$; $p=<0.0001$). Within each tissue type, as the administered dose increased, the observed residue levels increased ($F=7.43$; $p=<0.0001$).

Table 4.10: Mean CPTH residue levels (ng/g) in red-winged blackbirds following a single, oral dose analyzed by LC/MS/MS.

Dose Level		GI Tract	Liver	Kidney	Breast Muscle
12.5 ng/g	Mean ± sd =	27 ± 14	32 ± 25	81 ± 44	13 ± 9.7
	Range =	4.5-46.6	1.7-76.6	3.8-134	1.3-33.0
	RSD =	52%	78%	54%	75%
	n =	8	8	8	8
25 ng/g	Mean ± sd =	260 ± 380	103 ± 62	116 ± 64	34 ± 26
	Range =	41.8-1110	1.9-181	21.9-179	4.5-84.7
	RSD =	150%	60%	55%	76%
	n =	8	8	8	8
37.5 ng/g	Mean ± sd =	240 ± 160	140 ± 120	190 ± 200	38 ± 26
	Range =	26.0-450	29.5-389	41.1-628	16.0-92.2
	RSD =	67%	86%	105%	68%
	n =	8	8	8	8

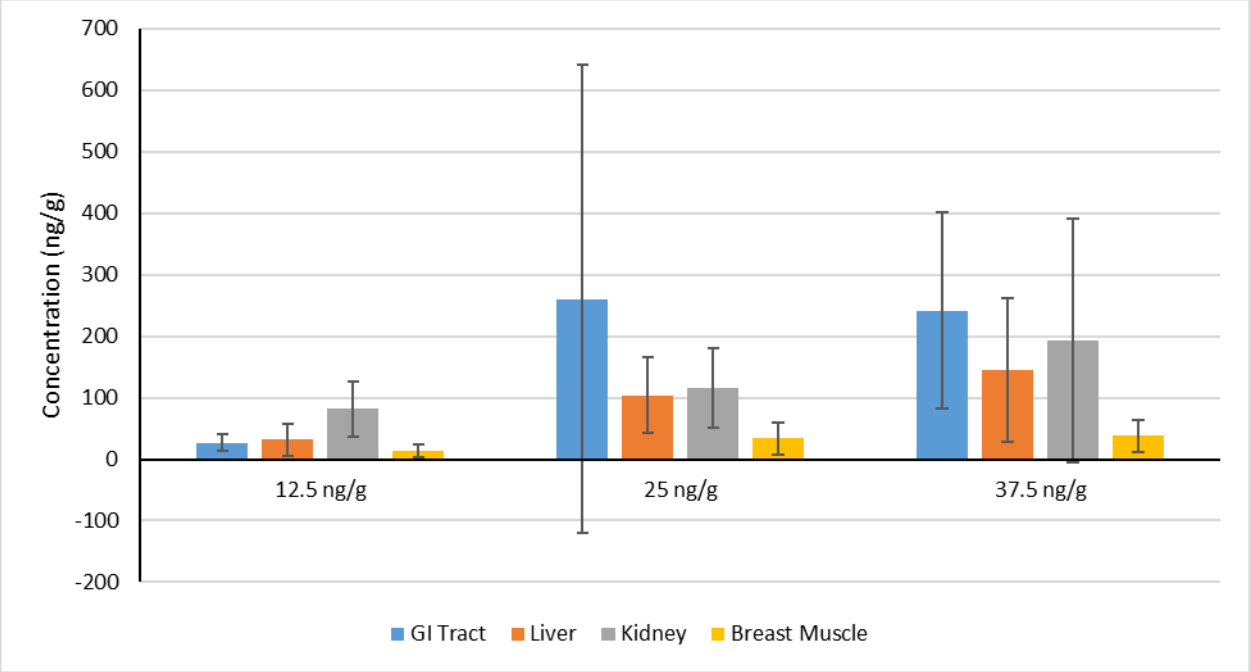


Figure 4.16: Average observed CPTH (ng/g) in red-winged blackbird GI tract, liver, kidney, and breast muscle following a single, oral dose analyzed by LC/MS/MS (error bars indicate standard deviation).

Table 4.11: Mean CAT residue levels (ng/g) in red-winged blackbirds following a single, oral dose analyzed by LC/MS/MS.

Dose Level		GI Tract	Liver	Kidney	Breast Muscle
12.5 ng/g	Mean ± sd =	10.7 ± 4.6	3.1 ± 3.1	2.6 ± 2.7	2.0 ± 1.4
	Range =	0.8-16.2	1.2-9.60	1.1-8.35	1.0-46.2
	RSD =	43%	100%	100%	70%
	n =	8	8	8	8
25 ng/g	Mean ± sd =	120 ± 170	14 ± 10	5.1 ± 5.0	11 ± 10
	Range =	10.8-504	1.2-30.4	1.1-14.6	1.0-27.9
	RSD =	140%	71%	98%	91%
	n =	8	8	8	8
37.5 ng/g	Mean ± sd =	270 ± 310	21 ± 13	39 ± 46	14 ± 10
	Range =	23.2-924	3.6-40.1	1.1-125	4.5-30.3
	RSD =	110%	62%	120%	71%
	n =	8	8	8	8

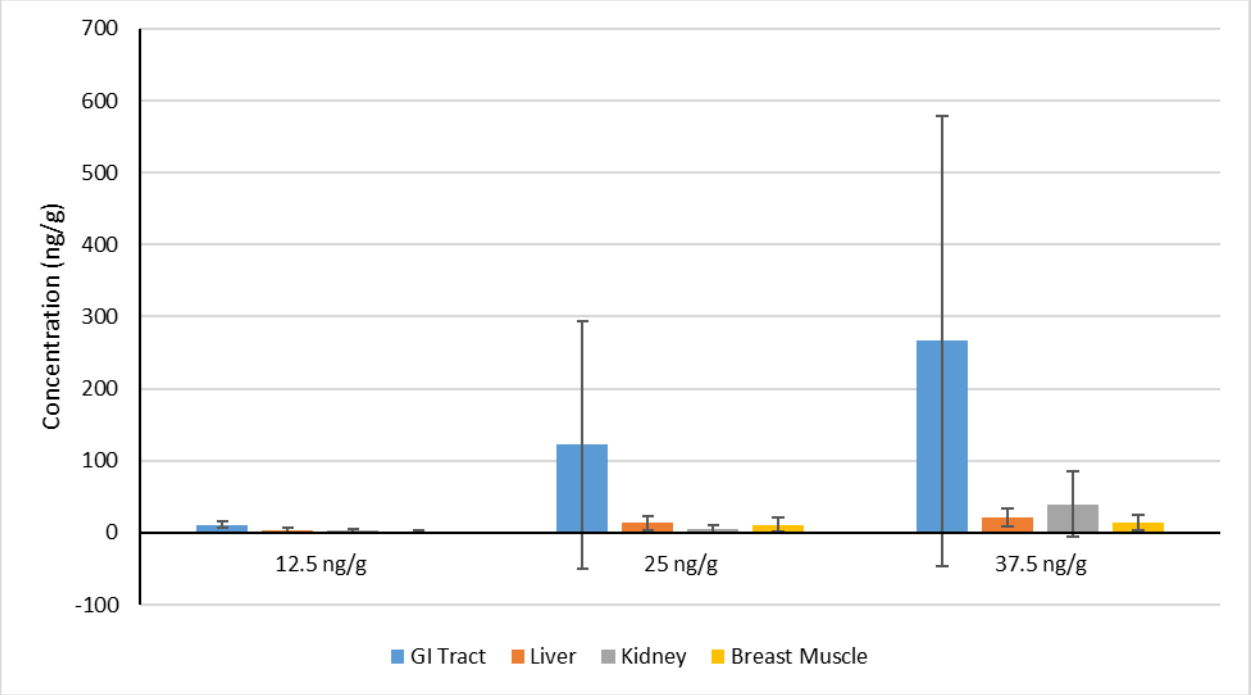


Figure 4.17: Average observed CAT (ng/g) in red-winged blackbird GI tract, liver, kidney, and breast muscle following a single, oral dose analyzed by LC/MS/MS (error bars indicate standard deviation).

Table 4.12: Mean OH-CAT residue levels (ng/g) in red-winged blackbirds following a single, oral dose analyzed by LC/MS/MS.

Dose Level		GI Tract	Liver	Kidney	Breast Muscle
12.5 ng/g	Mean ± sd =	530 ± 310	100 ± 96	24 ± 27	19.1 ± 8.6
	Range =	0.9-926	1.6-177	1.8-77.9	1.2-27.6
	RSD =	58%	96%	89%	110%
	n =	8	8	8	8
25 ng/g	Mean ± sd =	2200 ± 1400	590 ± 660	77 ± 30	100 ± 50
	Range =	517-5020	174-2130	40.7-116	26.6-148
	RSD =	63%	110%	35%	50%
	n =	8	8	8	8
37.5 ng/g	Mean ± sd =	5000 ± 1700	1350 ±740	1300 ± 2600	460 ± 470
	Range =	2140-8200	64.8-2360	69.2-7610	142-1540
	RSD =	34%	55%	200%	100%
	n =	8	8	8	8

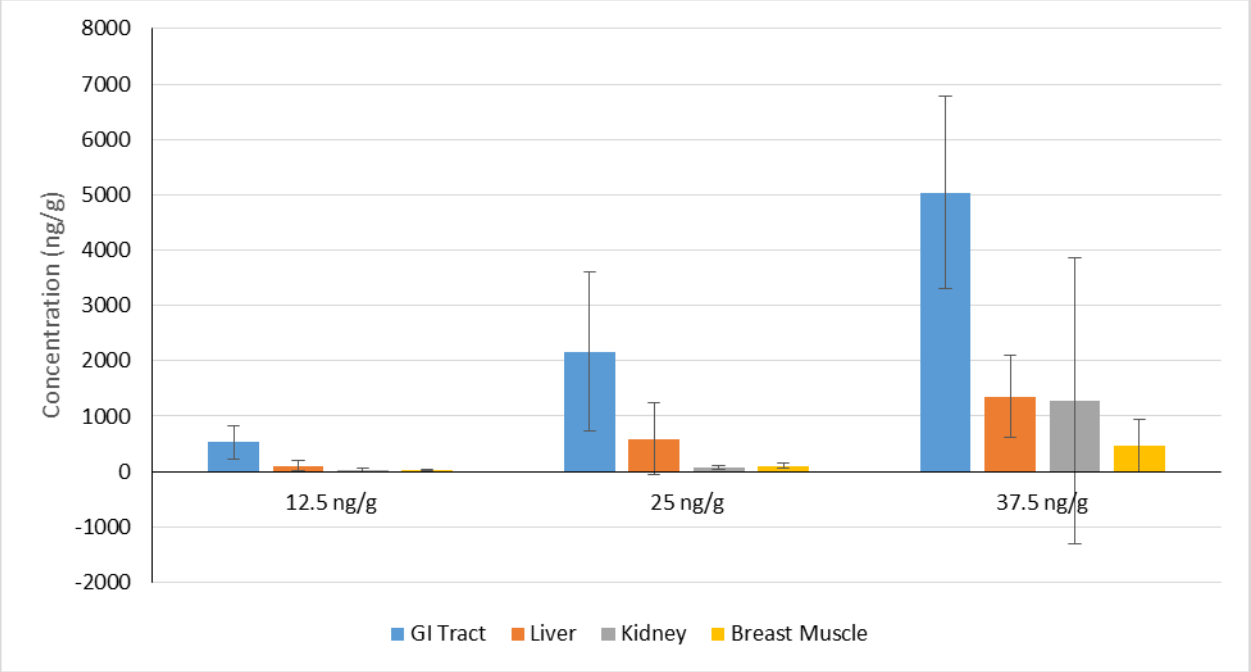


Figure 4.18: Average observed OH-CAT (ng/g) in red-winged blackbird GI tract, liver, kidney, and breast muscle following a single, oral dose analyzed by LC/MS/MS (error bars indicate standard deviation).

The observed values for OH-CAT were quite surprising. As discussed in Chapter 3.3, the amount of OH-CAT generated *in-vitro* was quite low. The opposite was true for the *in-vivo* portion of the study. To further investigate this observation, values were generated for the ratio of CAT to

CPTH and OH-CAT to CPTH within each sample. Results indicated that while the ratio of CAT to CPTH remained relatively steady despite the dose level (Figure 4.19) the ratio of OH-CAT to CPTH increased with increasing dose (Figure 4.20). The ratio of CAT to CPTH was less than 1.0 in most cases. This demonstrated that there was less CAT than CPTH present in most samples, despite being the major metabolite formed in *in-vitro* experiments. The only tissue where this was not true was the GI tract. This indicated that CAT was being excreted at a greater rate than both CPTH and OH-CAT. The ratio of OH-CAT to CPTH was greater than 1 in most cases. There was more OH-CAT present than CPTH in most samples. Whether OH-CAT was being retained at a greater rate than CPTH and CAT or it was simply formed at a high rate is unclear. This finding is in stark contrast with the *in-vitro* experiments where OH-CAT remained low throughout, OH-CAT was the dominant species present in the *in-vivo* study.

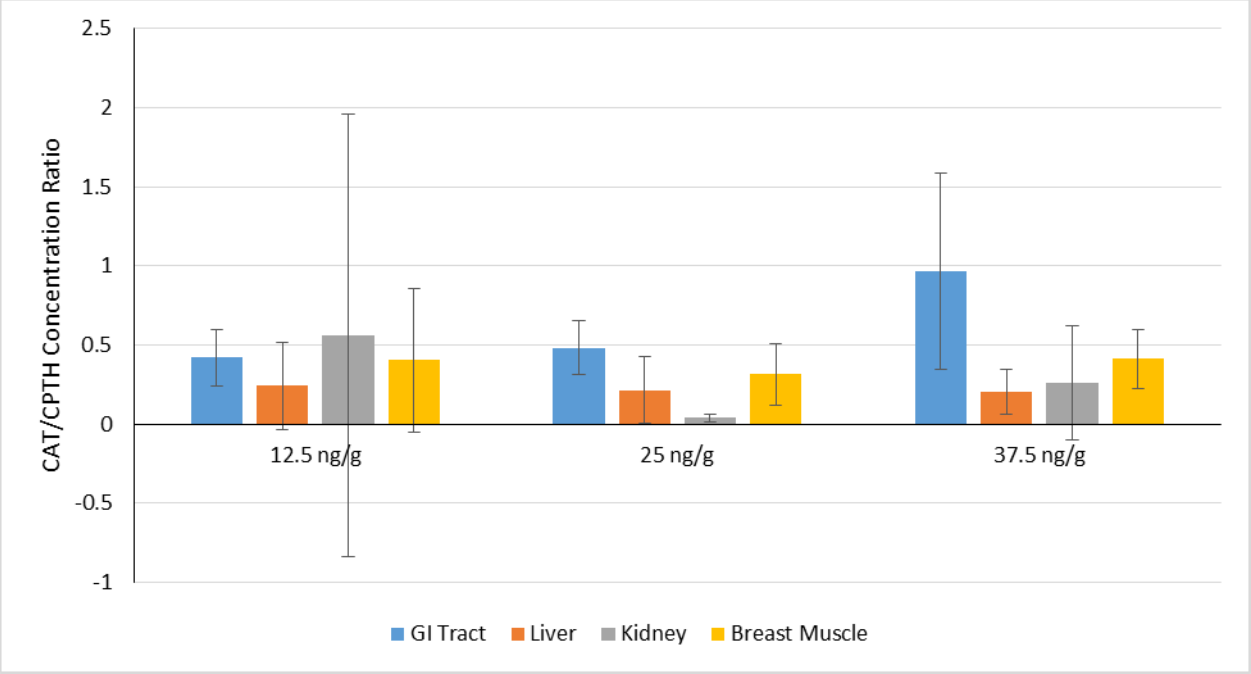


Figure 4.19: Ratio of concentration of CAT to CPTH in red-winged blackbird GI tract, liver, kidney, and breast muscle following a single, oral dose analyzed by LC/MS/MS (error bars indicate standard deviation).

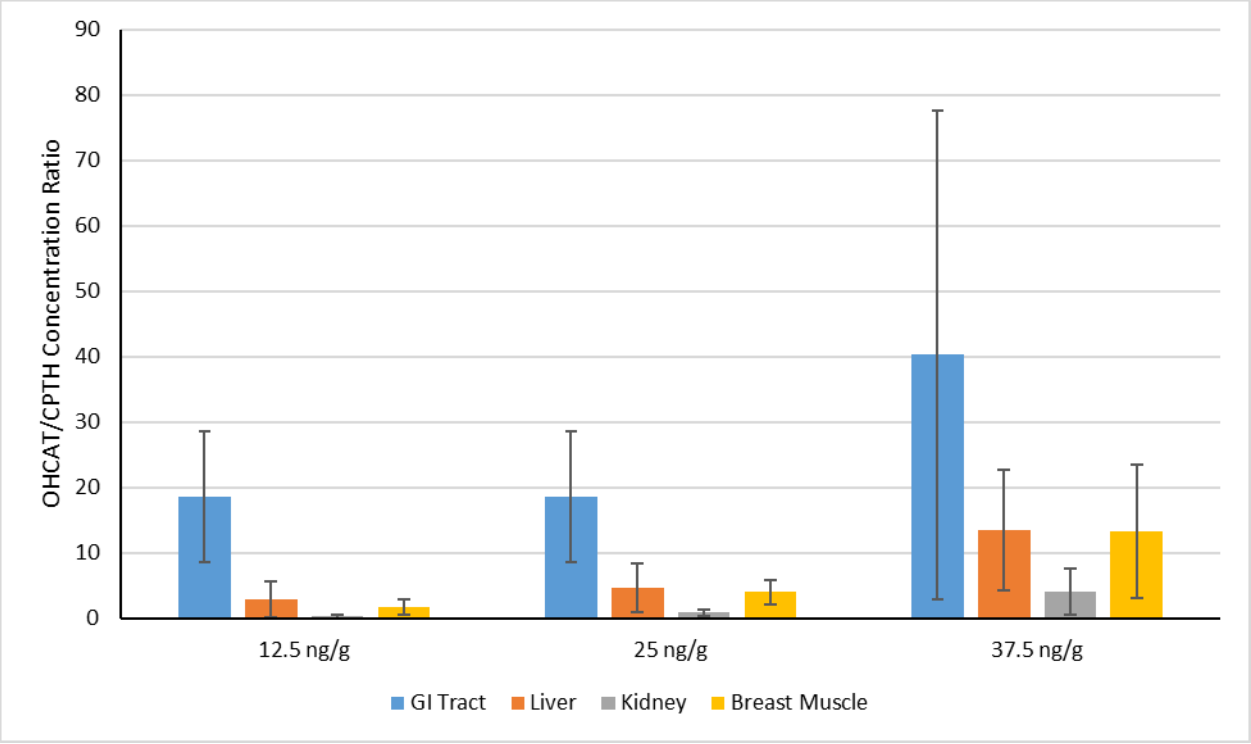


Figure 4.20: Ratio of concentration of OH-CAT to CPTH in red-winged blackbird GI tract, liver, kidney, and breast muscle following a single, oral dose analyzed by LC/MS/MS (error bars indicate standard deviation).

4.4.4 Comparison of LC and GC Results

Residue results were generated for CPTH in GI tract, liver, and breast muscle using both the GC and LC techniques. The data from each technique were combined and subjected to a two-way ANOVA procedure. The observed residue levels were significantly impacted by both tissue type ($F=9.42$; $p=0.0001$) and analytical technique ($F=5.56$; $p=0.0168$). No significant interaction was found between tissue type and analytical technique however ($F=1.70$; $p=0.1862$). The results generated using the GC technique were higher than those generated using the LC technique. However, both techniques demonstrated the same difference in observed residue level between the three tissue types.

The values for the DL for each technique were comparable, demonstrating that either technique would be suitable for the detection of CPTH or its metabolites at trace levels. However, the GC

technique is limited due to the aforementioned solubility and chromatographic performance issues of the OH-CAT metabolite. The GC technique was well suited for the detection of CAT and CPTH.

4.4.5 Extrapolation to Field Data

CPTH residues from a field exposure study demonstrated a high degree of variability (Stahl et al., 2002). In this study the birds were allowed to feed on a treated bait site therefore, therefore the dose level was not known. In a separate study designed to mimic a field exposure, the results for breast muscle were all below the 70 ng/g QL for the method used (Stahl and Johnston, 2006). The current method has a QL in breast muscle which was significantly lower (14 ng/g), demonstrating a clear improvement on previous methods. The results for GI tract samples in the 2006 research were above the QL for the method, but both tissues demonstrated RSD values near 50%. This was consistent with the results from the current study. Also similar between the current study and those in the literature, there was a difference observed between the concentration of CPTH found in GI tract and breast muscle. In all cases, the concentration in GI tract was much larger than that for breast muscle in an individual bird.

Although CPTH and metabolites were detected under laboratory conditions, translation of these techniques to bird carcasses found in the field could be more difficult. Stahl and Johnston (2006) examined the breast muscle and GI tract of mourning doves exposed to CPTH. The exposure was via oral ingestion of rice grains treated with CPTH, therefore the actual dose for each bird was not precisely controlled and likely led to increased variability of the results. Regardless, they were able to observe residues in the GI tract of exposed doves up to 14 days post mortem. Given the results presented in Chapter 4 for OH-CAT and CAT and the relative instability of CPTH, it is likely that

detection of metabolites of CPTH could be accomplished as long as two or three weeks post exposure.

4.4.6 Summary

In Phase 3 we used the information generated from Phase 2 to look for metabolites of CPTH in the tissues of birds exposed to CPTH *in-vivo*. We also answered several specific questions related to the study goal.

- Residue levels of CPTH, CAT, and OH-CAT were measured for three different doses of CPTH given orally to red-winged blackbirds.
- Two different methods were used (GC/MS/MS) and (LC/MS/MS) to quantify these residues.
- Of the two metabolites of CPTH, OH-CAT was the best candidate as a biomarker of CPTH exposure due to the relatively high levels found in the tissues of exposed birds.
- The newly developed LC/MS/MS method was just as sensitive as the GC/MS/MS method, but the results generated via LC/MS/MS were not as repeatable as those from the GC/MS/MS.
- The LC/MS/MS method was able to detect OH-CAT at extremely low levels where the GC/MS/MS was not.

CHAPTER 5 – PHASE 4 – PROTEOMICS AND METABOLOMICS EXPERIMENTS

5.1 Introduction

Proteomics and metabolomics are similar techniques in which the proteins (proteomics) or metabolites (metabolomics) from an experiment are subjected to high resolution mass spectrometry to look for previously unidentified peptides or chemical compounds. Proteomics is a rapidly developing field that combines biochemical techniques for extraction and purification of protein with high resolution mass spectrometry. The pairing of the two techniques allows for the evaluation of very complex mixtures. Specialized data handling software makes it possible to evaluate the data for likely molecules of interest. Evaluation of proteins can be accomplished in two primary ways. The first, called top-down proteomics, uses the introduction of intact proteins to a mass spectrometer. The identified proteins are then subjected to tandem mass spectrometry to obtain fragment ions which can yield the peptide sequence for the targeted protein. The second approach is called bottom-up proteomics. In this approach, proteins are reduced with dithiothritol, alkylated with iodoacetamide, and digested with a proteolytic enzyme to produce peptides that are then introduced into the mass spectrometer. Targeted peptides are then subjected to tandem mass spectrometry just as in top-down proteomics (Tailor et al., 2016). The peptide sequences and information on the specific digestion protocol are fed into a database which can identify the protein from the resultant peptides.

In both top-down and bottom-up approaches the procedure can benefit from enrichment of the target proteins. This can be accomplished through affinity capture or some other anti-body technique. Gel electrophoresis techniques, such as a western blot, could also be used to isolate the specific target proteins before analysis. Radiometric techniques (such as those employed in Phase 1)

could also be used to find the targeted protein in fractions of the protein digest separated by chromatographic procedures. A bottom-up approach was selected for the current research efforts as the specific reaction and target were not known.

Metabolomics functions in a very similar manner to proteomics in that samples are injected and screened for unknown metabolites using high resolution mass spectrometry and powerful data processing software. The technique is quite useful for identifying previously unknown metabolic pathways.

5.1.1 Hypothesis

In Phase 4 we will use proteomics and metabolomics to look for two things: any protein adduct formed by CPTH or one of its metabolites, and previously unknown metabolites that were not detected in Phase 2. Our working hypothesis for this aim is that CPTH forms a reactive metabolite in the kidney of exposed birds which forms a covalent bond with the structural components of the kidney. We also believe that mass spectrometry can be used to detect this adduct or metabolite. The questions related to this hypothesis that we will attempt to answer were:

- Can a protein adduct be identified from a trypsin digest of whole kidney?
- Can additional metabolites be identified in the kidney of birds following an *in-vivo* exposure?
- Can tandem mass spectrometry be used to elucidate the structure of any metabolites?

5.2 Materials and Methods

5.2.1 Protein Extraction

Blackbird kidney tissue (approximately 25 mg) from the blackbirds dosed in Chapter 4.2.4 was placed in a 15-mL plastic centrifuge tube and 14 mL of a lysis buffer was added. The lysis buffer consisted of 5M urea, 2 M thiourea, 2 mM tributylphosphine, 1 mM sodium vanadate, 0.25% CHAPS, 0.25% Tween-20, 0.25% sulfobetaine, 10% isopropanol, 5% glycerol, 12.5% water-saturated isobutanol, and a protease inhibitor tablet (Sigma Aldrich, St. Louis, MO) dissolved in ultra-pure water. The sample was incubated in a water bath at room temperature for 30 minutes then centrifuged at 4,000 rpm for 5 minutes. The protein concentration of each sample was estimated using a protein assay kit (Cytoskeleton Inc., Denver, CO). Based on the determined protein concentration, sufficient sample extract was added to a 1.5-mL micro centrifuge tube to equal 500 µg of protein. The volume was adjusted to 200 µL with ultra-pure water and vortexed. Proteins were precipitated by the addition of 600 µL of methanol and 150 µL of chloroform. Ultra-pure water (450 µL) was added to solubilize and remove salts and buffers. The sample was vortexed and centrifuged at 8,000 rpm for 5 minutes. The aqueous layer was removed and 450 µL of methanol was added. The sample was vortexed and centrifuged again and the supernatant removed.

5.2.2 Trypsin Digestion

The protein pellet was resolubilized in 100 µL of 6 M urea. The sample was vortexed and 5 µL of 200 mM dithiothreitol added. The sample was vortexed and incubated at room temperature for an hour. The process above was repeated with 20 µL of 200 mM iodoacetamide and 200 mM dithiothreitol. The trypsin digestion was accomplished by addition of 775 µL of ultra-pure water and 10 µL of 200 µg/mL sequencing-grade trypsin (Sigma Aldrich, St. Louis, MO). The sample was

incubated in a 37 °C water bath overnight.

The trypsin-digested peptide sample was purified using a C18 Sep-Pak cartridge (Waters, Milford, MA). Briefly, the cartridge was conditioned with a solution of 65% acetonitrile and 35% water containing 0.1% formic acid (Solution B) followed by a solution of 98% water and 2% acetonitrile containing 0.1% formic acid (Solution A). The sample was added to the cartridge and it was washed with Solution A. The peptides were eluted from the cartridge with Solution B. The purified peptide sample was placed in a Speed Vac (Thermo Fisher, Maltham, MA) and the solvent removed. The sample was resuspended in 100 µL of Solution A and placed in LC vials for analysis.

5.2.3 Liquid Chromatography/Mass Spectrometry

Samples from the blackbird control and low dose groups were injected into an Agilent 1200 series liquid chromatograph coupled to an Agilent 6530A quadrapole-time of flight (Q-TOF) mass spectrometer (Table 5.1). Separation was achieved using a 2.1 x 100mm Agilent Poroshell 120 EC-C18 column (100 Å; 2.7 µm).

5.2.4 Metabolomics Experiments

Samples prepared for LC/MS/MS analysis in Chapter 4.2.7 were also injected into the LC-QTOF using the method outlined in Chapter 4.2.7 which was employed for the LC/MS/MS analysis of CPTH, CAT, and OH-CAT. No special handling or changes to the extraction technique were needed. The MS parameters from Chapter 5.2.3 were used in place of the parameters from Chapter 4.2.7.

Table 5.1: Liquid Chromatograph/Mass Spectrometer Conditions

Mobile Phase A:	0.1% formic acid		
Mobile Phase B:	0.1% formic acid in acetonitrile		
Gradient:			
Time (min)	% A	% B	Flow Rate (mL/min)
0.0	97	3	0.400
1.0	85	15	0.400
12.0	70	30	0.400
13.0	0	100	0.400
14.0	0	100	0.400
15.0	97	3	0.400
Injection Volume:	1 µL		
Column Temperature:	40 °C		
Run Time:	15 minutes		
Post Time:	3 minutes		
Scan Range:	100 to 1600 m/z		
Fragmentor Voltage:	175 V		
Polarity:	Positive		
Gas Temperature:	350 °C		
Gas Flow:	Nitrogen @ 10 L/minute		
Sheath Temperature:	350 °C		
Sheath Flow:	Nitrogen @ 12 L/minute		
Nebulizer Pressure:	30 psi		
Capillary Voltage:	3500 V		

5.2.5 Data Handling

Data files obtained from the LC-Q-TOF MS were subjected to molecular feature extraction using Agilent's Profinder software (Version B.06.00, Agilent Technologies, Santa Clara, CA). Molecular Feature Extractor is an untargeted feature finding algorithm that involves finding and quantifying all the known and unknown compounds or metabolites down to the lowest abundance, and extracting all relevant spectral and chromatographic information. The software's parameters were adjusted to look for peptides with charge states up to z=5. Only molecular features with more than two ions, peak height above 4,000 counts, and within 0.25 minutes of peaks in other chromatograms were selected for analysis.

The extracted molecular features were then analyzed by Agilent's Mass Profiler Professional (Version B.12.05, Agilent Technologies, Santa Clara, CA). Mass Profiler Professional (MPP) is a powerful chemometrics platform. MPP provides the ability to evaluate complex mass spectral data and apply statistical processes to identify metabolites or differences in peptides between treatment groups. Only molecular features with a significance above α=0.05 and having greater than a two-fold change between treated and control groups were selected for evaluation (Figure 5.1). A Benjamini-Hochberg test (Benjamini and Hochberg, 1995) was used to test for false discovery of molecular features.

5.3 Results and Discussion

5.3.1 Proteomics Results

The molecular features identified by Profinder were subjected to significance and fold-change testing in MPP. The data were evaluated with critical values of α=0.05 and fold-change of 2. A total of seven compounds met those criteria (Figure 5.1). The green bars in Figure 5.1 represent the critical values for significance and fold change for the plot. The red boxes are molecular features which exceeded both criteria.

Formulas for these compounds were generated by the software based on the spectra for the compounds. Examination of the constructed chromatograms demonstrated that one compound (m/z=459.24332, C24 H34 N4 O3 S) was present in all the control samples, but was completely absent from the treated samples. The other six compounds (Table 5.2) were present in all of the treated samples, but not present in any of the control samples. The compounds identified were not large peptides and none of them were multiply charged, indicating that no protein adduct was found.

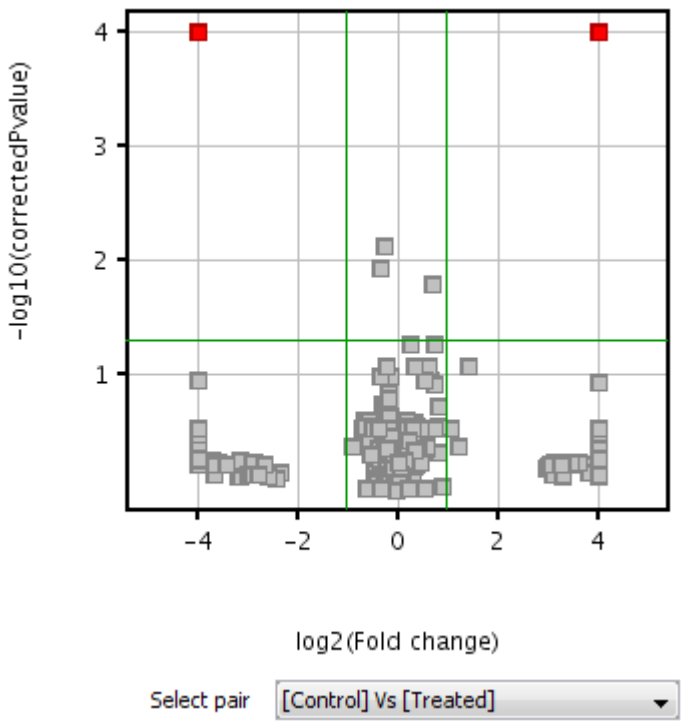


Figure 5.1: Volcano plot of peptide sample generated by MPP software. Red squares indicate molecular features which met both the fold change and significance critical values.

Table 5.2: Compounds identified by MPP software as meeting the significance and fold-change criteria.

m/z	Relative Concentration in Control	Relative Concentration in Treated	Proposed Formula
504.2648	-7.637	7.748	C18 H29 N15 O3
548.2909	-7.536	7.709	C20 H33 N15 O4
592.3160	-7.369	7.436	C20 H35 N18 O4
416.2130	-7.088	7.196	C14 H21 N15 O
553.2505	-6.303	7.282	C14 H30 N11 O5
509.2204	-6.944	7.053	C18 H22 N17 O2
459.2433	7.466	-7.199	C24 H34 N4 O3 S

It is likely that the compound that was present in the control samples but was absent in the treated samples was a target molecule for a reactive metabolite generated by CPTH or one of its metabolites. The ratio of oxygen and nitrogen atoms in this compound do not match any combination of amino acids, indicating that it was not a protein.

Using the values for dose and the physiological characteristics of a typical red-winged blackbird, a theoretical concentration of the adduct was calculated. Assuming approximately 10% of the dose administered was bound tightly to the kidney of blackbirds and that the average kidney weighs approximately 410 mg, a predicted concentration in the kidney of approximately 60 µg/g for a 250 µg dose of CPTH was calculated. This level of response may not have been sufficient for detection by the mass spectrometer.

No sample enrichment techniques were employed in this study. Use of these techniques would necessitate the generation of an antibody or the use of a radioactively tagged molecule. If these enrichment techniques had been used, the likelihood of finding the adducted species would have been greatly improved.

5.3.2 Metabolomics Results

Samples of kidney and liver from both treated and control blackbird dose groups were subjected to the same data handling techniques as those used for the peptide samples. Twenty-five compounds were identified as meeting both the significance and fold change cut-off values. Many of them were very close to the significance and fold change cut-off (Figure 5.2). All of the features were evaluated using a recursive workflow in which the spectra were examined for the presence of a chlorine atom. Eight of the compounds were found to have chlorine atoms present. One of the compounds was identified as CPTH and another as OH-CAT. The remaining six compounds were subjected to a product ion scan using molecular masses found in Table 5.3 and the instrumental parameters found in Chapter 4.2.7.

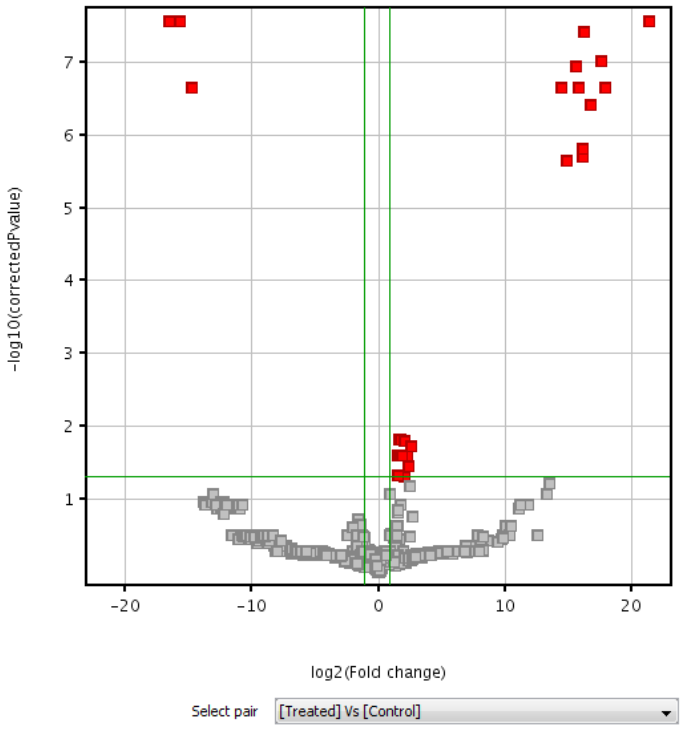


Figure 5.2: Volcano plot of metabolite samples generated by the MPP software. Red squares indicate molecular features which met both the fold change and significance critical values.

Table 5.3: Chlorinated compounds identified by MPP software as meeting the significance and fold-change limits.

m/z	Relative Concentration in Control	Relative Concentration in Treated	Proposed Formula
282.06342	-8.476183	8.230797	C12 H12 Cl N3 O3
284.20575	-1.1423086	0.6082338	C8 H26 Cl N9
298.05804	-8.943059	8.637918	C12 H12 Cl N3 O4
333.06863	-7.3469987	7.4584336	C16 H15 Cl N3 O S
354.0694	-8.050535	7.70402	C18 H18 Cl N O S
411.12408	-9.053984	8.853768	C16 H21 Cl N7 O2 S

None of the compounds had the correct ratio of carbon, hydrogen, and nitrogen to be peptides, indicating that they are likely not a protein adduct. Using a list of likely metabolites, predicted masses for sulfate, glucuronide, glutathione, and cysteine conjugates for CPTH, CAT, and OH-CAT were generated. None of the masses corresponded to anything seen in Table 5.3.

5.3.3 Metabolite Identification

The sample extracts were injected into the LC/MS/MS system and product ion scans taken of six possible metabolites. As all six metabolites were chlorinated, each had an A+2 peak corresponding to the ^{37}Cl isotope. Therefore, each sample was injected twice to produce product scans for the molecular ion corresponding to both the ^{35}Cl and the ^{37}Cl peaks. A mass spectra was produced for each of the pairs of peaks and the data was normalized to the base peak in each spectra. The spectra from the ^{35}Cl containing molecule was plotted in the positive axis and the spectra from the ^{37}Cl containing molecule was plotted in the negative axis. Those ions which align in the plot are not chlorinated, while those which are offset by 2 mass units are chlorinated.

The paired mass spectra for the 315/317 m/z peaks (Figure 5.3) yielded a non-chlorinated product ion at 132 m/z and chlorinated product ions at 140/142, 167/169, 182/184, 195/197, and 209/211 m/z. The chlorinated peaks were all separated by 2 amu, confirming the presence of chlorine in this metabolite. In a similar fashion, the 333/335 m/z peaks yielded chlorinated product ions at 140/142 and 182/184 m/z (Figure 5.4). The 282/284 m/z peaks showed non-chlorinated peaks at 133, 157, and 175 m/z and chlorinated peaks at 140/142, 165/167, 195/197, and 211/213 m/z (Figure 5.5). The 411/413 m/z peaks yielded non-chlorinated peaks at 60 and 185 m/z and chlorinated peaks at 140/142 and 182/184 m/z (Figure 5.6). The 298/300 m/z peaks gave non-chlorinated product ions at 132, 160, and 174 m/z and chlorinated ions at 140/142, 152/154, 167/169, 182/184, 195/197, and 209/211 m/z.

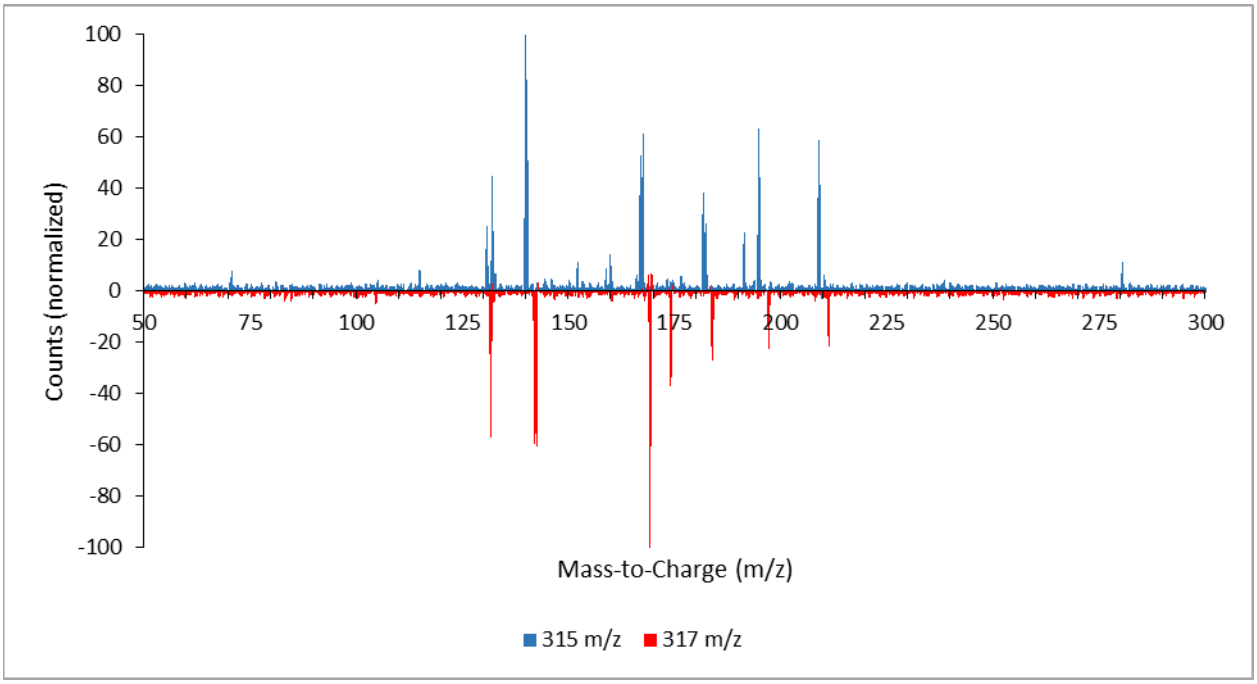


Figure 5.3: Mass spectrum of 315 m/z and 317 m/z peak at retention time 1.694 minutes in the kidney of a blackbird orally dosed with 32.5 mg/kg CPTH.

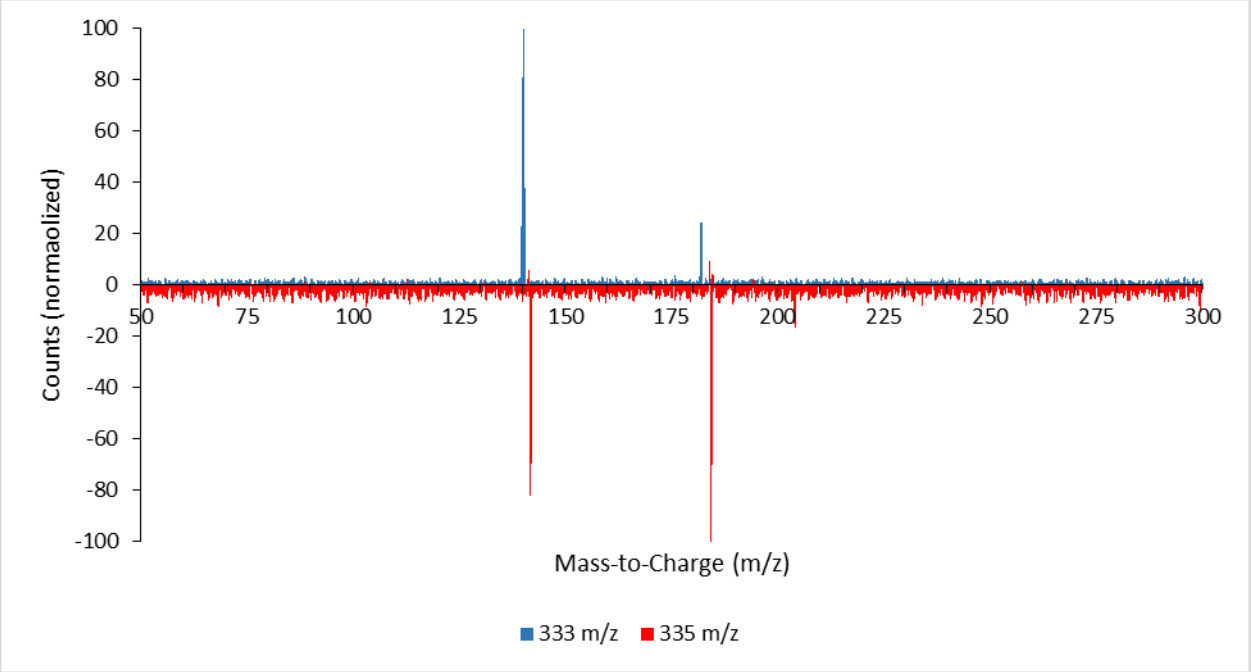


Figure 5.4: Mass spectrum of 333 m/z and 335 m/z peak at retention time 2.297 minutes in the kidney of a blackbird orally dosed with 32.5 mg/kg CPTH.

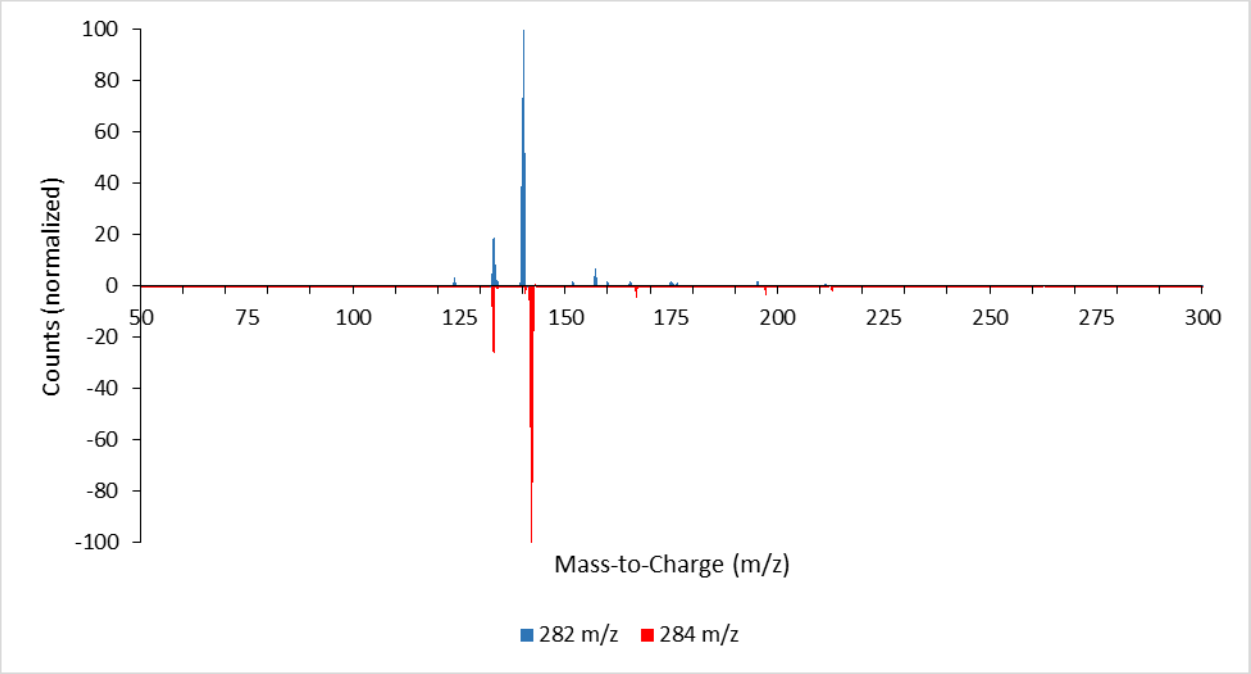


Figure 5.5: Mass spectrum of 282 m/z and 284 m/z peak at retention time 2.620 minutes in the kidney of a blackbird orally dosed with 32.5 mg/kg CPTH.

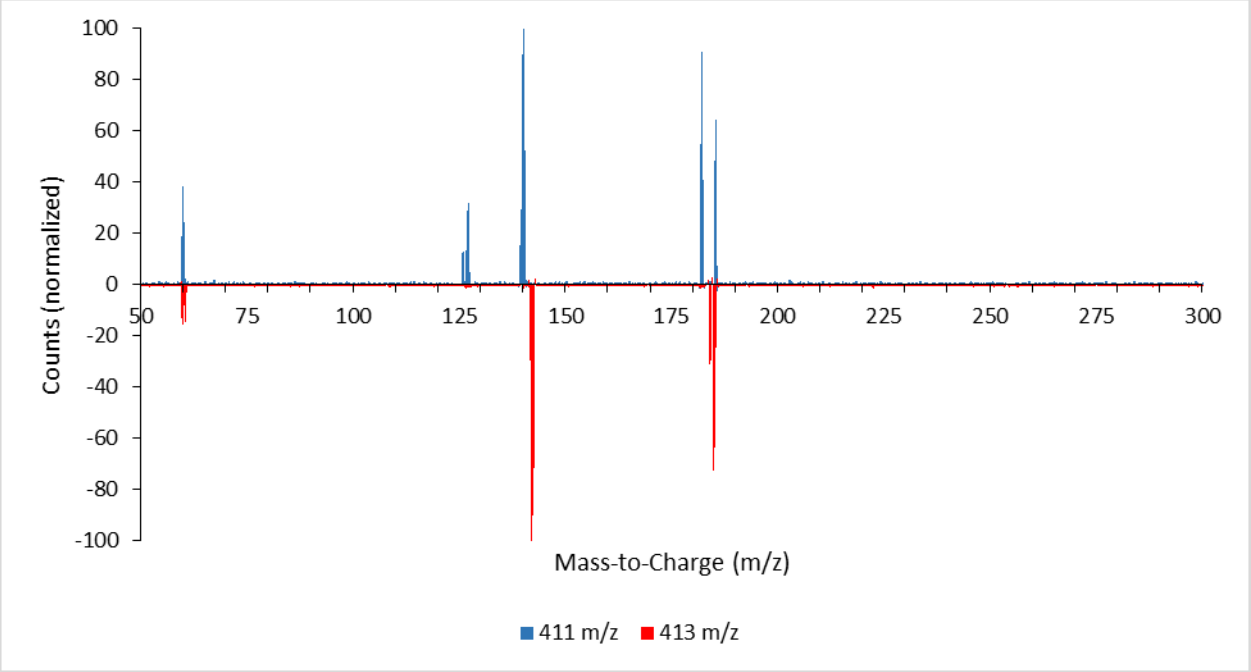


Figure 5.6: Mass spectrum of 411 m/z and 413 m/z peak at retention time 0.941 minutes in the kidney of a blackbird orally dosed with 32.5 mg/kg CPTH.

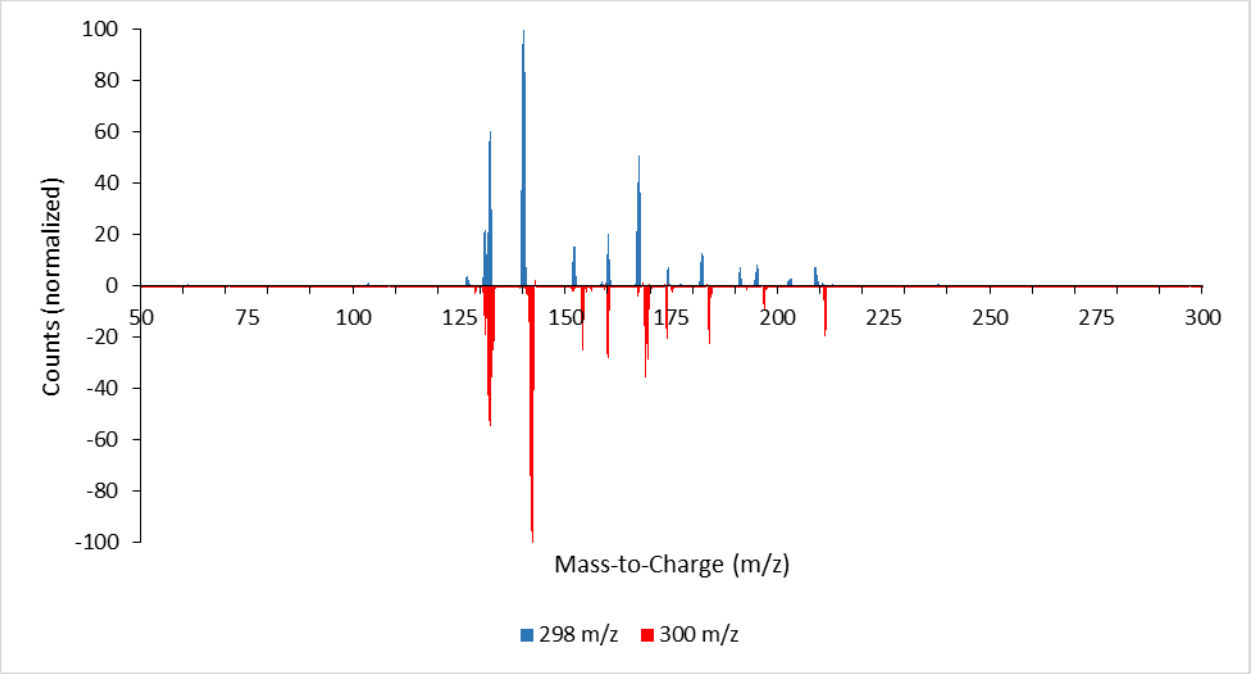


Figure 5.7: Mass spectrum of 298 m/z and 300 m/z peak at retention time 2.635 minutes in the kidney of a blackbird orally dosed with 32.5 mg/kg CPTH.

The mass spectra for these five compounds demonstrated very distinct similarities. All of them shared the 140/142 m/z product ion. Four of the five compounds yielded a 182/184 m/z product ion. Two of the compounds shared a non-chlorinated product ion at 132 m/z while a third produced a product ion at 133 m/z. Proposed structures for these product ions have been generated (Figure 5.8).

The proposed structure for the 182 m/z product ion called into question the metabolic pathway described in Chapter 3.3. If the hydroxylation of CAT were occurring on the benzyl methyl group, as previously proposed, the 182 m/z fragment would not have been possible. The hydroxylation for the reactive compound which produced the metabolites observed above could not have occurred at this position on CAT. While the formation of OH-CAT was proven *in-vivo*, it seems unlikely that it was responsible for the metabolites observed. Another chemical species must have been present which was reactive. It is possible that hydroxylation at the amine could have occurred (N-hydroxylation) via the flavin monooxygenase system (FMO). The FMO system is membrane bound on the

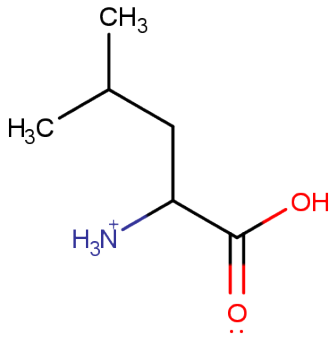
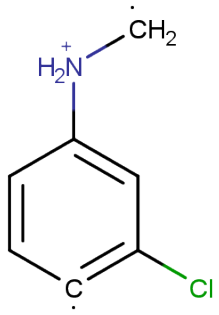
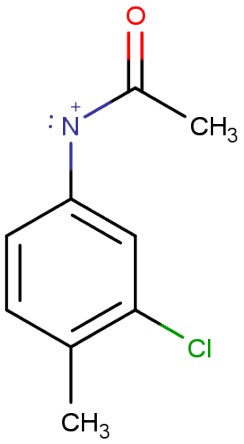
Ion Mass (m/z)	132	140	182
Proposed Structure			

Figure 5.8: Proposed structures for product ions generated from metabolites of CPTH in the kidney of blackbirds orally dosed with 32.5 mg/kg CPTH.

cytoplasmic side of the endoplasmin reticulum that is known to add a hydroxyl to aryl amines such as CPTH (Gan, et al., 2016). Once hydroxylated, the amine could have been transferred to the newly oxygenated amine through N,O-acetyltransferase. This particular NAT is known to transfer an acetyl group from the amine to the hydroxyl amine in aryl-hydroxyl amine species (Gan, et al., 2016). Other compounds which undergo this biotransformation pathway have been shown to form a highly reactive nitrenium ion which binds covalently to DNA and proteins (Gan, et al., 2016; Hein, 2006; Streeter and Hoener, 1988). Given the product ions produced from the detected metabolites and the information on nitrenium ion formation, a new metabolic pathway has been proposed (Figure 5.9).

The structure proposed for the 132 m/z product ion corresponded to leucine. Leucine is an essential amino acid. At physiological pH, the carboxylic acid portion of the molecule would have unpaired electrons which could have formed a strong bond with the nitrenium ion. It is therefore likely that some of the nitrenium ions formed bound covalently to leucine present in the kidney of the exposed blackbirds.

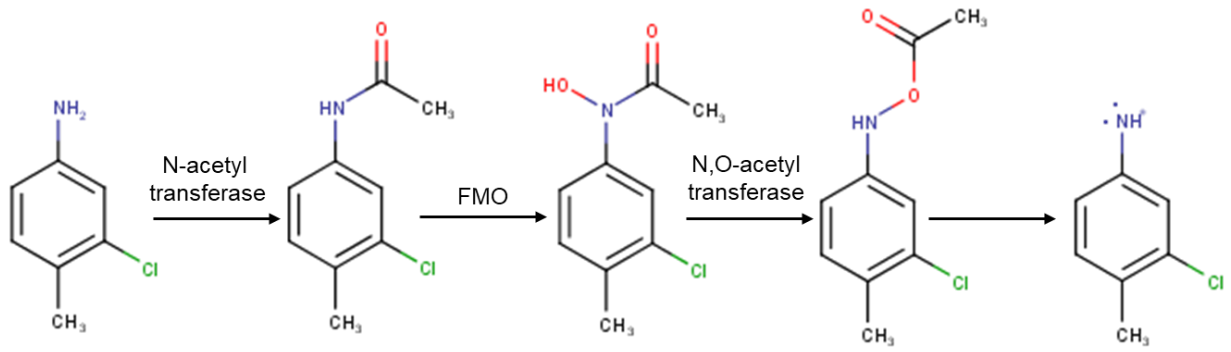


Figure 5.9: Proposed metabolic pathway for the formation of a nitrenium ion.

5.3.4 Summary

In Phase 4 we used proteomics and metabolomics to look for two things: any protein adduct formed by CPTH or one of its metabolites, and previously unknown metabolites that were not detected in Phase 2. Our working hypothesis for this aim was that CPTH formed a reactive metabolite in the kidney of exposed birds which formed a covalent bond with the structural components of the kidney. We also believed that mass spectrometry could be used to detect this adduct or metabolite. While we did not find a protein adduct, we did find additional metabolites that could be attributed to formation of a reactive metabolite in the kidney of exposed birds. We also answered the following questions:

- Compounds were identified in the trypsin digest of kidney tissue from exposed birds which were either up or down regulated following the exposure.
- None of the identified peptides produced a strong enough signal to permit identification of their structure.
- Additional metabolites were found in extracts of kidney tissue of birds following an *in-vivo* exposure.

- Six of these metabolites had chlorine as part of their structures and demonstrated that they were the direct result of exposure to CPTH *in-vivo*.
- Tandem mass spectrometry was used to propose potential structures for these important metabolites.
- A metabolic pathway leading to the formation of a highly reactive nitrenium ion was proposed.

CHAPTER 6 – CONCLUSIONS

6.1 Summary of Findings

The primary hypothesis of this research was that CPTH toxicity arose from a metabolite that bound to tissues in exposed birds, particularly those of the kidney. The results of each phase of the research completed reinforced this hypothesis. In Phase 1 we found that there was significant tissue binding, particularly in the kidney, of both bird species tested. In the kidney of the more sensitive blackbird species, the amount of radioactively labeled CPTH remained relatively constant for the entire 24 hour exposure period. In phase 2 two metabolites were identified from *in-vitro* experiments. The two metabolites (CAT and OH-CAT) were not found to react with any target molecule evaluated (glutathione, sulfate, glucuronide, bovine serum albumin, and cysteine). In phase 3 we demonstrated that CPTH and its two metabolites could be detected for three different doses of CPTH given orally to red-winged blackbirds. In phase 4 we used proteomics and metabolomics to look for two things: any protein adduct formed by CPTH or one of its metabolites, and previously unknown metabolites that were not detected in Phase 2. While we did not find a protein adduct, we did find additional metabolites that could be attributed to formation of a reactive metabolite in the kidney of exposed birds. We were also able to propose a metabolic pathway leading to the formation of a highly reactive nitrenium ion which could be responsible for the tissue damage observed following exposure to CPTH.

6.2 Tissue Binding

Binding of amine containing compounds, such as azo dyes, has been known for many decades. The work of Miller and Miller (1946) found residues tightly bound in the livers of exposed rats.

Intravenous administration of ^{14}C-labeled CAT to starlings demonstrated the ability for CAT to bind covalently to kidney tissues (Siegel and Giri, 1983). The mechanism by which this might occur remains unknown. It is likely that the formation of OH-CAT is necessary for this mechanism to occur since it only forms once CPTH is transformed to CAT. The results from Phase 2 indicate that deacetylation occurs more rapidly in non-sensitive avian species than it does in sensitive avian species, pointing to deacetylation as a protective process. Lowered deacetylase activity in sensitive birds would lead to an increase in the circulating concentration of CAT, which would lead to formation of increased amounts of OH-CAT. Proximal tubule cells have the highest level of biotransformation activity in the kidney, making them a likely target site for the formation of a reactive metabolite.

It is unlikely that hydrogen ion concentration is to blame for the observed damage to the proximal convoluted tubules, as CPTH is a free base at physiological pH. The solubility would be greatly reduced in the blood stream and it would likely readily filter through the kidney and be excreted. The pKa of CAT and OH-CAT have not been measured, but was estimated using Marvin Sketch software (Chem Axon; Cambridge, MA). The estimated pKa values for CAT, OH-CAT, and N-OH-CAT are 14.4, 13.9, and 8.9, respectively. At the physiological pH present in the proximal convoluted tubules, all should be present in an uncharged state. Biotransformation is required to cause the damage observed in the kidney. The proximal tubule cells have the highest capacity for biotransformation of any other cells in the nephron. The damage observed is likely the result of the formation of a reactive metabolite in the proximal tubule cells themselves.

The kidneys make up less than 1% of the mass of the body, but receive up to 25% of cardiac output (Schnellmann, 2013). Any metabolites circulating throughout the body such as CAT and OH-CAT would be carried to the kidney in large quantities where they would come into contact with the

proximal tubule cells. The proximal tubule reabsorbs as much as 80% of solute and filtrate which are initially filtered across the glomerulus. Damage to this section could be catastrophic with respect to removal of impurities in the blood (Schnellmann, 2013).

6.3 Cast Formation

While direct damage to the proximal tubule cells is the most likely mode of action for CPTH's damage to kidneys, it is possible that cast formation in the nephron could cause some of the damage. In mammals excretion of urea is one of the functions of the kidney. This process is slightly different in that birds excrete uric acid rather than urea. Excretion of uric acid occurs through the formation of urine spheres with proteins in the nephron (Casotti and Braun, 2004). It is possible that CPTH or one of its metabolites may interfere with uric acid's binding with these proteins and create a physical blockage in the nephron. This could be the cause of the increased uric acid levels observed in the research of Apostolou (1969). However, this would not explain the extensive kidney damage observed. The increase in uric acid is more likely the result of gross damage to the proximal tubules which interrupts normal excretion of uric acid.

6.4 Future Work

While the ultimate goal of this research was to develop a method to identify and quantify a metabolite which was covalently bound to protein, that goal remains unrealized. It will remain a research priority as the identification of this adduct is critical to understanding the ultimate chemical species responsible for causing the extensive renal damage observed in birds exposed to CPTH. As previously discussed, the use of an anti-body enrichment technique or a radioactively labeled molecule would be the logical next step to achieving this result.

An outcome of this research is that a sensitive LC/MS/MS method has been developed that is able to detect CPTH and its two major metabolites in tissues of exposed birds. This technique was able to confirm the formation of two primary metabolites of CPTH *in-vivo*, which is an important step in confirming the metabolic pathway leading to kidney damage. The results from *in-vivo* exposure to CPTH indicate that the metabolites are present at higher concentrations than the parent CPTH in almost all cases. The levels of OH-CAT in particular were quite high, indicating that it was an excellent candidate as a biomarker for CPTH exposure. While the performance of the method needs to be improved before it can be used for highly quantitative work, its usefulness as a diagnostic tool for exposure to CPTH cannot be overlooked. Additional work should be undertaken to improve the reproducibility and precision of the method.

Species differences in the toxicity of CPTH are still of interest to evaluate the potential secondary hazard of CPTH to raptors and scavengers. Identifying the reason for these species-specific differences may lead to a screening technique that would permit the determination of risk from exposure. In order to investigate the role of OH-CAT in the mode of action, a study in which a non-sensitive species receives a dose of CPTH and the kidneys are removed and analyzed for CPTH and metabolites would be of interest. The lack of OH-CAT present in samples from non-sensitive species would point to its formation as a necessary step for the toxicity of CPTH.

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Title: Absorption, Distribution, and Excretion of [14C]-3-Chloro-4-methylaniline Hydrochloride in Two Species of Birds Following a Single Oral Dose

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Publication: Journal of Agricultural and Food Chemistry

Publisher: American Chemical Society

Date: Dec 1, 2004

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