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**A physiologically based pharmacokinetic and pharmacodynamic
model for paraoxon in rainbow trout**

Abbas, Richat Rexiati, Ph.D.

The Ohio State University, 1994

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**300 N. Zeeb Rd.
Ann Arbor, MI 48106**

A PHYSIOLOGICALLY BASED PHARMACOKINETIC AND PHARMACODYNAMIC
MODEL FOR PARAOXON IN RAINBOW TROUT

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the
Degree Doctor of Philosophy in the Graduate School of The
Ohio State University

By

Richat R. Abbas, B.S.

* * * * *

The Ohio State University

1994

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DEDICATION

To My Family

Especially,

My Parents, My Wife, My Daughter and Son

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In Vivo Acetylcholinesterase Inhibition in Rainbow Trout
after Water Exposure to Paraoxon. R. Abbas and W. L.

Hayton. Presented at the 25th Annual Pharmaceutics Graduate Student Research Meeting, West Lafayette, Indiana. 1993.

Paraoxon Tissue Distribution and AChE Inhibition in Rainbow Trout. R. Abbas and W. L. Hayton. Pharmaceutical Research. Vol. 10, No. 10, s-365, 1993.

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Chapter I

INTRODUCTION

A. BACKGROUND

Pesticides are a large and diverse group of chemicals that people encounter daily. They are deliberately added to the environment for the purpose of controlling undesirable organisms. In the United States, the use of synthetic pesticides has increased more than 40-fold in the past three decades [1], and it continues to increase rapidly. Organophosphate insecticides (OPs) are among the most heavily used groups of synthetic pesticides. They are extensively used in agriculture and urban pest control with worldwide annual production exceeding 100 million pounds [2]. The popularity of OPs is mainly due to their high pest control capacity and relatively nonpersistent nature in the environment.

However, the OPs are not only highly toxic to the target species but also to nontarget species, including humans and fish [3]. They therefore constitute a

substantial threat of severe or even fatal accidental, intentional, or occupational poisoning. Marco et al., (1987) reported that pesticide poisonings occur at a rate of about 500,000 each year, result in about 20,000 annual fatalities worldwide, and that the OPs are responsible for more than half of the fatalities [4].

There are many sources of human exposure to insecticides other than via direct contact at the application site. Consumption of contaminated seafood is a common means of exposure. OPs enter the aquatic environment through aerial drift, runoff from application sites, or direct introduction for insect control purposes. They then become rapidly distributed through the action of wind and water. Agricultural runoff from fields and grazing lands is considered the major route of OP movement into water [5]. After entering surface water, OP residues may attach to suspended material, deposit on the bottom sediment, or accumulate in fish. In fish, OPs may cause acute toxicity or accumulate at a sublethal level. The degree of accumulation depends primarily on the physicochemical properties of OPs and many other secondary factors. The presence of OP residues in fish is a major commercial and ecological concern.

B. PARATHION AND PARAOXON

Parathion (O,O-diethyl O-p-nitropheny phosphorothioate) and its oxygen analog paraoxon (O,O-diethyl O-p-nitropheny phosphate) were synthesized by Schrader in 1944 [3]. Due to a wide range of insecticidal activities and suitable physical and chemical properties such as low volatility and sufficient stability in water and mild alkali [6], parathion became one of the most widely used organophosphate insecticides worldwide [3]. However, it exhibits high mammalian toxicity by all routes of exposure and parathion is involved in a large number of acute poisoning cases every year [3, 7]. It is responsible for more poisoning of humans than any other OP. Parathion tends to accumulate in fish because of its relatively high lipid solubility (log octanol/water partition coefficient=3.56) [8] and low water solubility (20 µg/ml). Its bioconcentration factor, the ratio between the concentration of the compound in trout and in water at steady-state, is 335 (Table 1.1).

Table 1.1. Physicochemical Properties and Toxicity of Parathion and Paraoxon.

Property	Parathion	Paraoxon
Water solubility (mg/L)	20 ^a	2400 ^a
Log P	3.56 ^b	1.49 ^b
Bioconcentration factor (trout)	335 ^b	
IC50 (trout, Mol)	10 ^{-5c}	10 ^{-8d}

^a Budavari et al., 1989.

^b Mourik and de Jong, 1978.

^c Forsyth and Chambers, 1989.

^d Johnson and Wallace, 1987.

The accumulation of parathion and paraoxon in fish could signal cause for concern about the environmental hazard that these chemicals might pose in aquatic food chains. The fate and toxicity of OPs in aquatic animals can be influenced by many different rate-limiting factors such as uptake kinetics, internal distribution, rates of biotransformation into active and inactive metabolites, and interaction of the active metabolites with the target site.

C. METABOLISM

The acute toxicity of parathion is mainly due to its inhibition of acetylcholinesterase (AChE) activity [9, 10]. However, parathion is an inherently weak cholinesterase inhibitor with an IC_{50} in the range of $10^{-5}M$ [11]. When it is activated via oxidative desulfuration to form its oxygen analog and active metabolite paraoxon ($IC_{50}=10^{-8}M$ [42]), it is a potent inhibitor of AChE (EC 3.1.1.7).

Biotransformation is an important factor that influences the toxicity and persistence of insecticides. Metabolic disposition of parathion can be classified into two general categories: metabolic activation by oxidative desulfuration to the oxygen analog, paraoxon, and detoxification by the hydrolysis of either parathion or

paraoxon to biologically inactive metabolites such as *p*-nitrophenol and diethyl thiophosphate or diethyl phosphate. Figure 1.1 illustrates these major metabolic pathways for parathion [13, 14]. Reaction 1 is oxidative desulfuration catalyzed by the cytochrome P-450-dependent monooxygenases. It results from an attack on the phosphorus atom by oxygen and the formation of an unstable phosphooxathiiran intermediate, which then undergoes release of sulfur and the formation of the oxygen analog paraoxon [15-18]. The released sulfur atom can bind covalently to P-450 and hinder subsequent oxidation reactions [19]. In reaction 2 oxidative hydrolysis (dearylation reaction) of parathion is mediated by the cytochrome P-450-dependent monooxygenases. Reaction 3 is the dearylation of paraoxon, which is catalyzed by the A-esterase paraoxonase (EC 3.1.1.2). Reactions 2 and 3 are all detoxification steps in which the products are not inhibitors of AChE and incapable of being transformed to compounds that capable of inhibiting AChE. *p*-Nitrophenol, the metabolic production of paraoxon and/or parathion, undergoes sulfation (reaction 4) and glucuronidation (reaction 5) and produces *p*-nitrophenol sulfate and *p*-nitrophenol- β -D-glucuronide [20].

The reactions that occur in mammals have also been demonstrated both *in vitro* and *in vivo* in aquatic organisms [14, 21-24]. However, there is a wide range of species

difference in the metabolic disposition rates of parathion. Wallace and Dargan [25] reported that the rates of parathion desulfuration, parathion hydrolysis and paraoxon hydrolysis are significantly greater in both rats and mice than trout. In fact activation of parathion to paraoxon, and detoxification of paraoxon by hydrolysis are insignificant in trout.

In addition to the enzymatic biotransformations, parathion can undergo non-enzymatic oxidative desulfuration to form paraoxon. This reaction utilizes oxygen and is enhanced by UV light [13, 14].

Figure 1.1. Metabolic pathway of parathion. Reaction 1 and 2 are mediated by the cytochrome P-450-dependent monooxygenases requiring NADPH and oxygen. Reaction 3 is catalyzed by paraoxonase; 4 and 5 are conjugation reactions.

D. MECHANISM OF TOXICITY

AChE is one of many enzymes important for the proper functioning of the nervous systems and the mechanism of OP toxicity is mainly due to the inhibition of AChE activity by phosphorylation of the hydroxyl group at the active site of the enzyme. The function of AChE is to terminate the action of neurotransmitter acetylcholine by catalyzing its hydrolysis at the junctions of the various cholinergic nerve endings [26]. Extensive studies by Nachmansohn and Wilson and their colleagues [27] of the reaction of AChE with different substrates and inhibitors have demonstrated the presence of two binding sites at the active center of the enzyme: the anionic subsite and the esteratic subsite (serine hydroxyl). The anionic subsite bonds with the cationic part of acetylcholine. Nucleophilic attack occurs at the esteratic subsite on the acyl carbon of acetylcholine. The catalytic mechanism is shared by other serine esterases, where a serine hydroxyl group at the esteratic site is rendered highly nucleophilic through a charge-relay system that involves neighboring histidine and aspartic acid residues. During the nucleophilic attack, enzyme and ester form a tetrahedral intermediate that collapses to an acetylated enzyme with release of choline. The acetylated enzyme is labile to hydrolysis, which results in the formation of acetate and active enzyme. The OPs act

as true hemisubstrates and interact with the AChE in a way similar to that of acetylcholine. However, the OP inhibitors do not have a quaternary nitrogen and react only with the esteratic site (serine hydroxyl) of AChE. The hydrolysis of the phosphorylated AChE is very slow and the catalytic activity of the enzyme for acetylcholine is lost [26].

There are three important steps in the interaction between AChE and acetylcholine or inhibitor -- (1) complex formation; (2) acetylation, phosphorylation; and (3) deacetylation, dephosphorylation (1.1).

Complex formation is governed by an association constant K_A ($K_A = k_1/k_{-1}$), which determines the affinity of the enzyme for the substrate. The association constant is large for acetylcholine and the phosphate inhibitors, and enzyme-substrate complex formation is thereby favored. Acetylation (k_2) and deacetylation (k_3) steps for acetylcholine are very fast so that hydrolysis of acetylcholine and regeneration of AChE occur rapidly. With the phosphate inhibitors, phosphorylation (k_2) is moderately fast, and dephosphorylation (k_3) is very slow, and accumulation of

phosphorylated enzyme results. Turnover numbers (the number of molecules hydrolyzed per minute by one molecule of enzyme) have been estimated to be 300,000 for acetylcholine and 0.008 for dimethyl phosphates. Therefore, AChE rapidly hydrolyzes acetylcholine and is irreversibly inhibited by OPs [3]. The terms 'reversible' and 'irreversible' are relative and reflect a wide difference in the duration of action [27]. However, the term 'irreversible' might be better suited to describe the recovery of OP because after reaction with AChE, the intact OP molecule is not recovered when the enzyme activity is recovered [2].

The potency of OP insecticides to inhibit AChE depends on the degree of phosphorylation. The degree of phosphorylation is governed by the property of the substituents on the P atom. Because the transphosphorylation reaction relies on interaction of the phosphorus atom with the unshared pair of electrons of the serine-hydroxyl oxygen, the electrophilicity of the phosphorus atom has a critical role in the reaction of insecticide with enzyme. Electron donating atoms or groups on the atom reduce anti-AChE activity of the OP. For example, parathion, with a sulfur atom which is electron donating, is an extremely poor inhibitor of AChE. However, replacing sulfur with oxygen (i.e. paraoxon) enhances anti-AChE activity of the compound because of its electron

withdrawing nature. Paraoxon is a potent inhibitor of AChE [2].

E. TOXIC MANIFESTATIONS

Inhibition of AChE activity results in accumulation of acetylcholine in cholinergic synapses, and excessive stimulation of cholinergic pathways in central and peripheral nervous systems. The over stimulation of cholinergic receptors causes multiple signs and symptoms, because of widespread distribution of the cholinergic neurons throughout central and peripheral nervous systems. Intoxication by OPs is indicated by the following major signs: headache, dilated pupils, blurry vision, nausea, difficulty breathing, tremors and convulsions. Severe poisoning causes death due to respiratory failure that results from bronchiolar constriction, excessive production of bronchiolar mucus, paralysis of the respiratory muscles and inhibition of the respiratory control center in the medulla oblongata of the brain [26].

F. BIOCHEMICAL ASPECTS OF AChE INHIBITION

Organophosphate insecticides (such as paraoxon) inhibit AChE activity by phosphorylating the serine hydroxyl group, and also react irreversibly with two other esterases -- carboxylesterase (CaE; EC 3.1.1.1) and butyrylcholinesterase (BuChE; EC 3.1.1.8). Inactivation of these noncritical esterases produces no toxic effect [28, 29]. CaE (Aliesterase) is present in many tissues, e.g., liver, kidney, intestine, plasma and muscle [30]. This serine esterase catalyzes the hydrolysis of carboxylesters. BuChE (also known as pseudo or nonspecific cholinesterase) is presented predominately in plasma. The phosphorylation of the serine hydroxyl groups of CaE and BuChE by paraoxon are important only as potential detoxification processes because they stoichiometrically reduce the amount of paraoxon available to inhibit AChE [31]. However, as the whole body level of BuChE in rats is 1000 times less than that of the CaE [32], and the *in vivo* inhibition of BuChE does not potentiate OP toxicity in mice [33], the reaction of OPs with BuChE is an insignificant detoxification pathway. However, the phosphorylation of CaE by OPs is a significant route of detoxification. Pretreatment of rats with triorthocresyl phosphate (TOCP), a strong inhibitor of CaE with weak AChE activity, reduces the malathion LD50 from 1100 to 10 mg/kg, a 110-fold potentiation of malathion

toxicity [34]. Similarly, paraoxon toxicity is also potentiated by selective blocking of the CaE binding sites with other compounds [35], and conversely induction of CaE activity by pretreatment with aldrin reduces the toxicity of paraoxon [36].

Another important detoxification pathway for paraoxon is catalytic hydrolysis by A-esterase (paraoxonase). A-esterase is widely distributed among several tissues of rats and other mammals [37] but not in fish; thus in contrast to mammals paraoxonase plays no significant role in the detoxification of paraoxon in fish tissues [25].

In addition to the enzymatic detoxification of OPs, reactivation of the phosphorylated AChE also plays an important role in recovery of the active enzyme. Phosphorylated AChE undergoes spontaneous hydrolysis, but the rate depends on the nature of the alkyl substituents. In the case of paraoxon, the rate of dephosphorylation is very slow, but it can be accelerated by treating with a nucleophilic agent such as 2-pyridine aldoxime methiodine (2-PAM, pralidoxime) and hydroxylamine [38]. 2-PAM has been most widely used as a therapeutic agent to treat OP poisoning. The phosphorylated enzyme can lose an alkyl group, which leaves the phosphorylated AChE charged at physiological pH, and not susceptible to activation by an

oxime. Consequently, the effectiveness of 2-PAM in reactivating the phosphorylated AChE is dependent on its early administration after poisoning. This dealkylation process is known as "aging", and the charged, phosphorylated AChE is called "aged" AChE [39]. "Aged" AChE is permanently deactivated, and recovery of the active AChE is via *de novo* enzyme synthesis.

G. OBJECTIVES

Physiologically based pharmacokinetic and pharmacodynamic (PBPK-PD) models have been extensively used in risk assessment and prediction of pharmacokinetic and pharmacodynamic behavior in non-test mammals [40-49]. In the aquatic environment, physiologically based pharmacokinetic (PBPK) models have been developed for methotrexate in sting rays [50], phenol red in dogfish shark [51], waterborne organic chemicals [52, 53], and pyrene [54] in rainbow trout. However, none of the present PBPK models in aquatic animals have related the toxicological effect to the target organ concentration, which would increase the significance of the model both for examination of the mechanism of toxicity of a chemical and for risk assessment.

There are significant differences among species in their susceptibility to OP toxicity. In particular, fish are less sensitive than mammals. Interspecies difference in toxicity of OP have been associated with: 1) kinetics of AChE inhibition such as affinity of binding and rate of phosphorylation, 2) hydrolysis of OP by CaE, 3) metabolic activation and detoxification, 4) reactivation and aging of phosphorylated AChE, 5) *in vivo* synthesis and degradation rates of AChE, and 6) uptake and distribution of OPs into target organs [55-58]. Although, a significant amount of work has been done to explore mechanisms for interspecies differences in OP toxicity by characterizing these several influences independently or qualitatively [20,24,55-65], few efforts have been made to assemble and study these factors together in a prospective manner to form a quantitative model of OP toxicity in fish. Such a model would aid in the assessment of the risk involved in the exposure of aquatic animal to OPs, and of the consequent risk to humans by consumption of OP-exposed fish. In addition, currently there is no information available on pharmacokinetics of parathion and paraoxon in fish. The pharmacokinetics of parathion and paraoxon would be useful to examine whether species differences in the pharmacokinetics are also involved in interspecies differences in toxicity.

The objectives of the investigation are to characterize the pharmacokinetics of parathion and paraoxon after intravascular administration and water exposure, develop a PBPK-PD model for paraoxon in rainbow trout, and validate the model experimentally by water exposure of trout to paraoxon. This model accounts for the uptake of paraoxon from water, its distribution into tissues, AChE inhibition in the tissues of interest, detoxification and removal of paraoxon by CaE, and depuration of paraoxon from fish. Because the level of free AChE is mainly governed by its synthesis and degradation rates in fish [58], these rate constants are also determined and incorporated into the model. The model should be useful in providing insights into the relationships among OP exposure, tissue concentrations and the consequent toxic effect. It would also provide a basis for risk assessment and would permit prediction of OP pharmacokinetics and pharmacodynamics in other species and under other exposure conditions.

Chapter II

DETERMINATION OF PARATHION AND ITS ACTIVE METABOLITE PARAOXON IN BIOLOGICAL SAMPLES BY GAS CHROMATOGRAPHY

A. ABSTRACT

A sensitive capillary gas chromatographic method was developed for simultaneous determination of organophosphate insecticide parathion and its active metabolite paraoxon in biological samples. This method involved a simple liquid-liquid extraction of parathion and paraoxon from water, plasma and tissues, and capillary gas chromatographic determination using electron capture detection and splitless injection; malathion was used as an internal standard. A gradient oven temperature program was used, and the injection port and detector temperatures were 200°C and 300°C, respectively. These techniques allowed quantitative determination of parathion and paraoxon at 9-210 ng/ml levels with recoveries that ranged from 79.4-110.3% for tissues, and 91.9-100.0% for plasma and water. The within-day and between-day coefficients of variation were less than 8.1%. The method was used to characterize the pharmacokinetics of parathion and paraoxon, and the tissue distribution of paraoxon in rainbow trout.

B. INTRODUCTION

Analytical methods for determination of parathion and its active metabolite paraoxon have been mostly directed towards monitoring the residues in agricultural and food products, and in environmental matrices [66-69]. Although high-performance liquid chromatography and gas chromatography have been employed for pharmacokinetic studies of parathion and paraoxon in plasma [70,71], the sensitivities of the published assays are insufficient to fully characterize the pharmacokinetics of parathion and paraoxon after intravenous administration. Due to the high toxicity of paraoxon, only small doses can be tolerated and a very sensitive analytical method is needed to determine parathion and paraoxon concentrations in biological samples.

This chapter described a simple liquid-liquid extraction and a sensitive capillary gas chromatographic method for determination of parathion and paraoxon concentrations in biological fluids, and validation procedures of this method.

C. MATERIALS AND METHODS

1. Reagents and Chemicals

Analytical grade parathion (99.5%) was a gift from Scheming, Denmark, while paraoxon (97.0%) and malathion (98.0%) were provided by Chem Service (West Chester, PA). Iso-octane, hexane and methanol were HPLC/GC grade. All other chemicals were reagent grade.

2. Instrumentation

All analyses were performed with a Hewlett-Packard (HP) Model 5890, series II, gas chromatograph equipped with a electron capture detector and a model 7673 autosampler. GC parameters and data integration were controlled by HP Chemstation software. The chromatographic column was a fused silica capillary column, 25m x 0.31-mm I.D., coated with a crosslinked 5% phenyl methyl silicone film of 0.52 micron thickness

3. GC operating conditions

Samples were introduced to the capillary through a splitless injector that was set at 200°C. After injection of a 1- μ l sample, the purge valve was closed for 2 min. Initially, the oven temperature was held at 70°C for 1 min, then brought up to 200°C at 50°C/min, held at 200°C for 8 min, and then brought up to 250°C at 70°C/min and held at 250°C for 3 min; The detector temperature was 300°C; nitrogen was used as carrier gas, total flow rate was 50 ml/min and total run time was 15.3 min.

4. Samples and standards

Plasma, brain, heart, white muscle, liver and kidney were obtained from unexposed rainbow trout and spiked with parathion, paraoxon, and internal standard malathion. Water (10 ml) was also spiked with parathion, paraoxon and internal standard. Stock solutions of parathion, paraoxon and internal standard malathion were prepared in hexane.

5. Assay methods

Plasma samples were extracted three times with 3 ml of isooctane, using 200 μ l methanol and concentrated sodium chloride as protein denaturing agents. The extracts were centrifuged for 3 min at 5°C and 3000 x g (IEC PR-7000 Centrifuge, International Equipment Company). The supernate was collected and evaporated under a stream of nitrogen. The residue was reconstituted in 0.1 to 1.0 ml of iso-octane, and 1- μ l aliquots were injected on to the GC.

Brain, heart, white muscle, liver and kidney were homogenized (Omni Mixer Homogenizer) in 100 mM phosphate buffer pH 7.4 containing 3 mM EDTA. The tissue samples were extracted three times with 3 ml of hexane for 5 min by rotation, using 400 μ l of concentrated sodium chloride and 1 ml of methanol as protein denaturing agents. The extracts were centrifuged for 5 min at 5°C and 4500 x g. Water samples were extracted three times with 3 ml of hexane only. The supernatants were collected and evaporated under a stream of nitrogen. The residue was reconstituted in 0.5 to 1.0 ml of iso-octane, and 1- μ l aliquots were injected on to the GC.

D. VALIDATION PROCEDURES

Individual plasma and tissue samples were analyzed for possible chromatographic interference with each analyte or the internal standard (Fig. 2.1).

Extraction recovery of parathion and paraoxon in plasma and water was determined by spiking with standard parathion and paraoxon, and were extracted as described above. The extraction recovery of paraoxon in brain, heart, white muscle, liver and kidney was determined by spiking these tissues with standard paraoxon, and extracted as described above.

Stability of parathion and paraoxon in stored samples of plasma were characterized by spiking rainbow trout plasma with 214 ng/ml of parathion and 190 ng/ml of paraoxon separately, and stored for various times at -20°C. The plasma samples were assayed for parathion and paraoxon (Table 2.1).

The linearity of the assay was examined by constructing standard curves in fish plasma for paraoxon and parathion with concentration range of 9.3 to 200 ng/ml and 15 to 210 ng/ml respectively, and determining the coefficient of correlation. The standard curves were obtained by plotting

the peak area ratio of parathion or paraoxon vs internal standard against the concentration of parathion or paraoxon (Fig. 2.2 and 2.3).

Within-day precision of the method was determined by spiking fish plasma with different concentrations of parathion or paraoxon in five replicates. Between-day precision was assessed by analyzing fish plasma samples spiked at three different concentrations on five different days.

E. RESULTS AND DISCUSSION

Chromatographic separation was completed within 15.3 min and no interference peaks were observed with either parathion and its metabolite paraoxon, or the internal standard malathion. Typical chromatograms of both blank fish plasma and spiked fish plasma sample (Figure. 2.1) indicated that there were no interference peaks from the plasma; the retention times were 9.58, 10.6 and 11.2 min for paraoxon, internal standard malathion and parathion.

The satisfactory separation was obtained by properly programming the oven temperature, such as utilization of two different temperature gradients. The limits of detection

(the detector signal at least twice the mean noise signal level, $S/N \geq 2$) were 5 and 8 ng/ml for paraoxon and parathion, respectively. The recovery data for parathion and paraoxon in plasma and water, and paraoxon in tissues are listed in Tables 2.2, 2.3 and 2.4. The extraction recovery to be 79.4% - 110.3% for tissues, and 91.9% - 100.0% for plasma and water.

Parathion and paraoxon were found to be stable in fish plasma during the assay period (Table 2.1). Addition of EDTA to tissue samples stabilized paraoxon since EDTA binds calcium, which is essential for paraoxonase activity.

Table 2.1. Stability of Parathion and Paraoxon in Plasma at -20°C. Initial concentrations of parathion and paraoxon in the plasma were 214 ng/ml and 190 ng/ml. The plasma concentrations were determined at various time up to 23 day.

Time (day)	Paraoxon (% remaining)	Parathion (% remaining)
0	100.0	100.0
1	92.6	97.9
2	99.5	94.6
3	98.9	99.5
4	99.5	99.5
7	93.6	100.0
23	92.6	97.9

Table 2.2. Recovery Data for Parathion Concentration in Plasma and Water.

	Concentration (ng/ml)	Recovery (%) (mean $\pm$ SD, n=3)
Plasma	15.0	98.7 $\pm$ 7.8
	30.0	96.2 $\pm$ 2.1
	60.0	94.6 $\pm$ 1.4
	120.0	99.5 $\pm$ 6.4
	200.0	95.6 $\pm$ 4.9
Water	10.0	94.8 $\pm$ 2.4
	40.0	92.7 $\pm$ 1.9
	200.0	91.6 $\pm$ 3.5

Table 2.3. Recovery Data for Paraoxon Concentration in Plasma and Water.

	Concentration (ng/ml)	Recovery (%) (mean $\pm$ SD, n=3)
Plasma	10.0	99.9 $\pm$ 8.5
	20.0	100.0 $\pm$ 5.3
	40.0	94.1 $\pm$ 3.6
	100.0	98.2 $\pm$ 2.0
	200.0	99.6 $\pm$ 1.3
Water	10.0	93.9 $\pm$ 1.4
	40.0	91.9 $\pm$ 2.7
	200.0	93.4 $\pm$ 1.8

Table 2.4. Recovery Data for Paraoxon Concentrations in Fish Tissues.

	Concentration (ng/ml)	Recovery (%) (mean $\pm$ SD, n=3)
Brain	50.0	95.3 $\pm$ 4.8
	100.0	98.4 $\pm$ 5.3
	200.0	101.7 $\pm$ 6.5
Heart	50.0	110.3 $\pm$ 4.3
	100.0	105.7 $\pm$ 6.7
	200.0	98.8 $\pm$ 5.5
Muscle	50.0	81.4 $\pm$ 4.7
	100.0	85.6 $\pm$ 5.2
	200.0	79.4 $\pm$ 3.1
Liver	50.0	82.5 $\pm$ 6.8
	100.0	86.1 $\pm$ 5.1
	200.0	85.6 $\pm$ 5.6
Kidney	50.0	84.5 $\pm$ 6.1
	100.0	82.7 $\pm$ 6.6
	200.0	98.9 $\pm$ 5.4

The within-day and between-day coefficients of variation (CV) (Tables 2.5 and 2.6) were less than 8.1%.

This method has been used to analyze pharmacokinetic study samples. An example of the plasma concentration-time profiles for fish is shown in Chapter 3 (Figure. 3.1)

In conclusion, a method based on capillary GC and simple liquid-liquid extraction has been developed and validated for analyzing parathion and its active metabolite paraoxon in fish tissues and water. This method was shown to be sufficiently specific, sensitive, precise, and accurate for use in pharmacokinetic and pharmacodynamic studies.

Table 2.5. Within-day Coefficients of Variation (CV) in Determination of Parathion and Paraoxon Concentrations.

	Concentration (ng/ml)		CV (%)
	<u>Added</u>	<u>Found</u> (mean $\pm$ SD, n=5)	
Paraoxon	10.0	10.2 $\pm$ 0.8	7.8
	40.0	38.4 $\pm$ 1.2	3.1
	200.0	198.3 $\pm$ 1.4	0.7
Parathion	15.0	14.5 $\pm$ 0.4	2.8
	60.0	57.4 $\pm$ 0.3	0.5
	210.0	205.5 $\pm$ 1.2	0.6

Table 2.6. Between-Day Coefficients of Variation (CV) in Determination of Parathion and Paraoxon Concentrations.

	Concentration (ng/ml)		
	<u>Added</u>	<u>Found</u>	CV (%)
	(mean $\pm$ SD, n=5)		
Paraoxon	10.0	10.7 $\pm$ 0.5	5.1
	40.0	38.6 $\pm$ 3.1	8.0
	200.0	196.6 $\pm$ 2.5	1.3
Parathion	15.0	14.8 $\pm$ 0.7	4.7
	60.0	56.3 $\pm$ 0.9	1.7
	210.0	204.2 $\pm$ 1.6	0.8

Figure 2.1. Typical GC chromatograms of blank (A) and spiked fish plasma (B), peaks correspond to: 9.58 min = paraoxon, 10.6 min = malathion, and 11.2 min = parathion.

Figure 2.2. Standard curve for determination of paraoxon concentration. Rainbow trout plasma was spiked with paraoxon, concentrations ranged from 9.30 to 200 ng/ml, and the concentration was determined by GC. The coefficient of determination was 0.998, $n=3$.

Figure 2.3. Standard curve for determination of parathion concentration. Rainbow trout plasma was spiked with parathion, concentrations ranged from 15.0 to 210 ng/ml, and the concentration was determined by GC. The coefficient of determination was 0.999, $n=3$.

Chapter III

PHARMACOKINETICS OF PARATHION AND PARAOXON IN RAINBOW TROUT

A. ABSTRACT

Fish are less sensitive than mammals to organophosphate (OP) insecticide toxicity. The species differences have been mainly investigated by biochemical studies of AChE and organophosphate interaction. To examine whether species differences in the pharmacokinetics of the organophosphate insecticides were also involved in their differential toxicity, rainbow trout were fitted with a dorsal aorta cannula and administered parathion and its active metabolite paraoxon intraarterially (IA) and via water exposure. Serial blood samples were removed and the plasma concentrations of parathion and paraoxon were determined by capillary GC with ec detection. Plasma protein binding was determined by equilibrium dialysis and ultrafiltration. After IA injection the plasma concentration-time profiles of parathion and paraoxon were multiexponential, with terminal $t_{1/2}$ of 56.7 and 0.532 hr. The steady-state volume of distribution and total body clearance (CL_b) for parathion and paraoxon were 1,325 and 1,060 ml kg^{-1} , and 19.9 and 2,900 ml $hr^{-1}kg^{-1}$; the plasma unbound fractions were 1.23%

and 52.5%. The large difference in CL_b between parathion and paraoxon appeared to result primarily from differences in plasma protein binding. Both parathion and paraoxon had greater persistence in trout than rat, suggesting that sensitivity differences were unrelated to pharmacokinetic differences.

B. INTRODUCTION

Pharmacokinetic models have been used increasingly in aquatic systems as an important tool to determine bioaccumulation by accurately estimating uptake, distribution, metabolism and excretion of organic chemicals in fish [73-81]. Kinetic parameters are useful for comparison of chemicals and test species [82], and for providing a basis to predict the fate of chemicals in the natural ecosystem.

Interspecies comparisons of organophosphate toxicity demonstrate that fish species are more resistant to parathion and paraoxon than mammalian species [14, 55]. The species differences have been investigated primarily from biochemical aspects [55-58]. Although the pharmacokinetics of parathion and paraoxon have been reported in several mammalian species such as rat, rabbit, dog and pig [70, 83-

85], there is little information available on the pharmacokinetics of parathion and paraoxon in fish. Pharmacokinetic information would be useful for examination of whether species differences in the pharmacokinetics are also involved in interspecies differences in toxicity. In addition, characterization of the biological half-life, total body clearance and volume of distribution at steady state of parathion and paraoxon would be useful for aquatic risk assessment.

The purpose of this study was to characterize the pharmacokinetics of parathion and paraoxon in rainbow trout, and to examine whether there are species related differences in parathion and paraoxon pharmacokinetics that contribute to the differential toxicity between fish and mammals.

C. MATERIALS AND METHODS

1. Chemicals

Analytical grade parathion (99.5%) was a gift from Scheming (Denmark). Paraoxon (97.0%) and malathion (98.0%) were from Chem Service Inc. (West Chester, PA). Paraoxon was contaminated with its degradation product *p*-nitrophenol which was readily visible in alkaline conditions. Paraoxon

was purified using a mixture of methylene chloride and 5% sodium bicarbonate. p-nitrophenol was removed from paraoxon until its yellow color was no longer visible in the aqueous phase. The purity of paraoxon was measured after drying the organic phase.

2. Fish and treatments

Rainbow trout (*Oncorhynchus mykiss*) (140 g; Fresh Water Farms, Urbana, Ohio) were acclimated to the laboratory and temperature (12°C) of study for a minimum of 4 weeks. The vivarium was windowless and had an automatically timed photoperiod that were set at 07:00 to 19:00. The acclimation tanks were "Living Stream" systems built by Frigid Units, Inc., Toledo, OH. These systems aerate the water, maintain the temperature, and filter water through a charcoal filter, with an average recirculation time of 1.5 min. Dechlorinated Columbus Municipal water was added continuously to the holding aquarium at a rate of 1.5 liters/min. The raceway was cleaned daily and water temperature, pH, and dissolved oxygen were measured daily; the acceptable ranges for these parameters were 11-13°C, 6.5-8.0, and >9.6 mg/liter (>80% saturation), respectively. Our monitored values were within these ranges. Fish were fed a commercially available soft, moist food obtained from

Rangen Inc. (Buhl, ID). Fish were not fed on the day preceding their experimental use. When signs of disease were apparent, e.g., lethargy, erratic swimming, skin lesions, eroded or reddish fins, the fish was removed from the holding aquarium.

3. Cannulation of fish

A trout that had been fasted for 24 hr was transferred to 8 liters of water containing 100 mg/L MS222 for anesthesia. An airstone was placed in the water and oxygen was bubbled into the water. After a few minutes, the anesthetized fish was transferred ventral side up to a brace lined with moistened cheese cloth, and a submersible pump was used to irrigate the gill with oxygenated water containing MS222. A hole was drilled in the snout using a Dremel motor tool. Two sutures were placed on the dorsal surface of the oral cavity: one approximately midway between the snout and leading edge of the first gill arch and the second immediately posterior to the hole drilled into the snout. A cannula made from Teflon tubing (28 gauge thin wall) and attached to a 5/8" x 25 gauge needle and 1 ml syringe filled with heparinized non-nutrient teleost saline [115] was inserted into the dorsal aorta using a 2" 18 gauge I.V. catheter. The catheter was inserted at the midline

between the second and third gill arches at approximately a 30° angle. Prior to the insertion the cannula tubing was filled with saline and then inserted into the fish slowly until blood appeared in the tube. The center poke was removed and the tubing was threaded into the catheter and 7 cm into the artery. The catheter was removed, taking care not to pull out the cannula. The cannula tubing was disconnected from the needle and threaded through the hole drilled in the snout and then re-connected to the needle. The cannula was tied with the two sutures placed in the roof of the mouth and a third suture was placed on the dorsal side of the head, immediately posterior to the hole drilled through the snout and firmly secured to the cannula [116,117]. The gills were irrigated with anesthetic free oxygenated water until operculum movement began and then the fish was immediately transferred to an isolation tank for 24 hr to allow complete recovery before the experiment.

Table 3.1 Analysis of water quality used for the experiment.

Parameter	Analysis
Hardness(as CaCO_3), mg/l	10
Nitrate-Nitrite, N mg/l	0.75
Phosphorus Total, P mg/l	0.16
Lead Total, Pb $\mu\text{g/l}$	<3
Mercury Total, Hg $\mu\text{g/l}$	<0.5
pH	7.1
Dissolved Oxygen (% saturation)	>90%

D. EXPERIMENTAL DESIGN

1. Intraarterial administration of parathion and paraoxon in rainbow trout

Due to the limited solubility of both paraoxon and parathion [6], the dosing solution for paraoxon was made of 50% propylene glycol in water, and for parathion 95% dimethyl sulfoxide (DMSO) in water. The amount of parathion or paraoxon administered was determined accurately by GC analysis. The dose was administered by drawing blood into the cannula, switching to the syringe containing the dose and injecting it into the aorta. Each trout received a 100 μ l (10 mg/kg) bolus dose of parathion or a 50 μ l (5.7 mg/kg) bolus dose of paraoxon. Immediately after injection the cannula was rinsed three times by pulling blood into the cannula and pushing it back into the aorta. The cannula was also flushed by infusing heparinized saline to remove paraoxon or parathion bound to the cannula tubing and to prevent contamination of the initial blood samples. Preliminary experiments established that there was no residual drug in the cannula after dosing.

2. Sampling times

Serial blood samples were removed at various times that were selected based on a pilot study (paraoxon, 5, 10, 15, 30, 45, 60, 90, 120, 180, and 300 min; parathion, 0.167, 0.333, 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, 24 and up to 500 hr.). 100-200 microliters of blood was removed at each sampling time and 5 ml was removed at the last sampling time. The blood was collected in a heparinized test tube, and centrifuged, and the separated plasma stored at -20°C until it was analyzed by gas chromatography. The volume of blood removed as sample did not exceed 15% [88] of the estimated total blood volume of trout (4% of body weight) [89].

3. Sample preparation and analysis

Plasma samples were extracted three times with 3 ml of isooctane, using 200 µl methanol and concentrated sodium chloride as protein denaturing agents. The extracts were centrifuged for 3 min at 5°C and 3000 x g (IEC PR-7000 Centrifuge, International Equipment Company). The supernate was collected and evaporated under a stream of nitrogen. The residue was reconstituted in 0.1 to 1.0 ml of isooctane, and 1-µl aliquots were injected on to the GC. The

more detailed description of the analytical procedures were described in Chapter II.

4. Plasma protein binding studies

The binding of parathion to plasma pooled from several trout was measured using an equilibrium dialysis apparatus (20-Cell Equilibrium Dialyzer, Spectrum Medical Industries, Inc., Los Angeles, CA). Plasma samples spiked with parathion were dialyzed against non-nutrient teleost Ringers solution [90] in Teflon cells that had 0.25 ml chambers; a regenerated cellulose membrane (Spectra/Por 2) with molecular weight cutoff of 12-14,000 was used. The cells were rotated at 15 rpm in a 12°C water bath for 12 hr. Preliminary experiments indicated that equilibrium was reached by 12 hr. Subsequently, the buffer and plasma compartments were sampled to obtain unbound and total concentration.

Protein binding of paraoxon was determined by ultrafiltration. Paraoxon was incubated with pooled trout plasma at 12°C for 2 hr to allow equilibrium. Following equilibration, the paraoxon concentration was determined, and 0.5 ml aliquots were pipetted into ultrafiltration filter tubes (Free Level™, Syva Co., Palo Alto, CA) that

had a molecular weight cut-off of 12-14,000. To separate bound and unbound paraoxon, the filter devices were centrifuged at 12°C for 10 min at 2,000 x g (IEC PR-7000 Centrifuge System). The concentrations of paraoxon in the plasma (total concentration) and filtrate (unbound concentration) were determined by GC.

5. Water exposure to parathion

Parathion stock solution in ethanol was added to an exposure tank that contained 94 liters of dechlorinated water maintained at 12°C; it was dispersed by stirring with a high speed electric-motor-driven propeller for 25 min in total darkness to avoid ultraviolet-light-catalyzed conversion to paraoxon [13]. After 24 hours, the cannulated trout (330 - 400 g) were placed into the solution. To maintain oxygen near saturation, oxygen was bubbled into the exposure tanks using a glass micro diffuser (ASTM, Porosity E). Samples of exposure water (20 ml) were taken before fish were placed in the exposure system and concurrently with blood samples during the exposure at various times up to 124 hr. The volume of blood sampled was controlled and did not exceed 15% of the estimated blood volume (4% body weight) of trout. The concentrations of parathion and its metabolite paraoxon in plasma and water

samples were determined using the GC method. The oxidative conversion of parathion to paraoxon in water was minimized by treating the water with sodium thiosulfate.

6. Water exposure to paraoxon

A stock solution of paraoxon was made in ethanol and the concentration was determined accurately by GC. The solution of paraoxon in ethanol was added to an exposure tank containing 80 liters of dechlorinated water maintained at 12°C and dissolved by stirring with a high speed electric-motor-driven propeller for 20 min. The cannulated trout (140 - 150 g) were placed into the solution. The oxygen saturation in the water was maintained as described above. Samples of blood and water were removed at each of the following exposure times: 0, 0.25, 0.5, 1, 1.5, 2, 4, 6, 12 and 24 hr. The concentration of paraoxon in plasma was determined along with its water concentration.

E. DATA ANALYSIS

The plasma concentration-time profiles of parathion and paraoxon were analyzed using both model dependent [91, 92] and "model independent" (statistical moment theory) [93]

methods. In the model independent analysis, the area under the plasma concentration-time curve ($AUC_{0 \rightarrow t}$) was calculated by the logarithmic trapezoidal rule ($AUC_{0 \rightarrow \infty} = AUC_{0 \rightarrow t} + AUC_{t \rightarrow \infty}$; $AUC_{t \rightarrow \infty} = C_t/k$, where, k is the terminal rate constant) [94]. The total body clearance was calculated as {dose} divided by $\{AUC_{0 \rightarrow \infty}\}$, and the volume of distribution at steady state (Vd_{ss}) was calculated by the method of Benet and Galeazzi (3.1) [95].

$$Vd_{ss} = MRT \cdot CL_b \quad (3.1)$$

For model-dependent analysis, the model that described the data satisfactorily was based on the pattern of residuals, the sum of weighted, squared deviations of the fitted equation, and the visual evaluation of the goodness of fit [96]. The plasma concentration-time profiles of paraoxon were described by a two compartment model for instantaneous bolus input with elimination from central compartment, which assumed first order kinetics [91,92]. The following model-based equation was fitted to the plasma concentration-time profile of paraoxon (weight: y^{-2}) from each fish using PCNONLIN (Version 4.2), a pharmacokinetic data analysis program (SCI software, ClinTrials Inc., Lexington, Kentucky). The program used a least-squares regression algorithm to determine the values for the

pharmacokinetic parameters from the best fit of the following equation (3.2):

$$C_p = A e^{-\alpha t} + B e^{-\beta t} \quad (3.2)$$

A and B are the intercepts, and α and β are hybrid rate constants; β is the slope of the linear portion of a semilog plot of plasma concentration-time data. The values of α and β were related to the model rate constant by (3.3,3.4):

$$\alpha + \beta = k_{10} + k_{12} + k_{21} \quad (3.3)$$

$$\alpha\beta = k_{10} k_{21} \quad (3.4)$$

After intraarterial administration of parathion, the plasma concentration-time profiles were analyzed using a three compartment model (one central compartment and two reversibly linked peripheral compartments; weight: y^{-2}), and the associated triexponential equation (3.5):

$$C_p = A e^{-\alpha t} + B e^{-\beta t} + C e^{-\gamma t} \quad (3.5)$$

A, B and C corresponded to the three zero-time intercepts of feathered lines of parathion concentration-time profile, and α , β and γ were the hybrid rate constants.

F. RESULTS AND DISCUSSION

The plasma concentration-time profile of paraoxon in rainbow trout declined biexponentially, Figure 3.1, which indicated that the plasma pharmacokinetics were adequately described by a two compartment model. The plasma concentration-time profile of parathion was fitted by a three compartment model, and the computer-predicted concentrations were similar to the experimentally determined concentrations, Figure 3.2. Tables 3.2 and 3.3 showed the pharmacokinetic parameters of paraoxon and parathion from model dependent and model independent analyses. Similar values were obtained from the two analyses, which further indicated that the two-compartment and the three-compartment models adequately described the disposition of paraoxon and parathion in rainbow trout.

Table 3.2. Pharmacokinetic Parameters of Paraoxon in Rainbow Trout after Intravascular Administration.

	V_{ss} (ml/kg)	CL_b (ml/hr/kg)	$T_{1/2}^{**}$ (hr)	f_{free} (%)
Compartmental	1080 ± 508	3020 ± 1249	0.528 ± 0.08	
Moment	1040 ± 279	2780 ± 270	0.535 ± 0.09	
Mean	1060	2900	0.532	
				52.5 ± 2.1

* Values are mean ± SD, n=5.

** Harmonic mean half-life [97].

Table 3.3. Pharmacokinetic Parameters of Parathion in Rainbow Trout after Intravascular Administration.

	V_{ss} (ml/kg)	CL_b (ml/hr/kg)	$T_{1/2}^{**}$ (hr)	f_{free} (%)
Compartmental	1370 ± 193	21.4 ± 7.1	56.1 ± 2.8	
Moment	1280 ± 233	18.3 ± 8.3	57.3 ± 14.6	
Mean	1325	19.9	56.7	
				1.23 ± 0.08

* Values are mean ± SD, n=5.

** Harmonic mean half-life [97].

Figure 3.1. Paraoxon plasma concentration-time profile in rainbow trout after intraarterial administration of 5.7 mg/kg paraoxon; the profile is from a single representative trout (351 g body weight). Symbols represent the experimentally determined concentrations and the line represents the least-squares fit of the two compartment model based biexponential equation.

Figure 3.2. Parathion plasma concentration-time profile in rainbow trout after intraarterial administration of 10 mg/kg parathion; the profile is from a single representative trout (559 g body weight). Symbols represent the experimentally determined concentrations and the line represents the least squares fit of the three compartment model based triexponential equation.

Paraoxon and parathion had similar apparent volumes of distribution, even though the binding to plasma protein was remarkably different (Table 3.2 and 3.3). Parathion has a larger log P value than does paraoxon (3.56 vs. 1.49), and this higher fat solubility may indicate that parathion would tend to concentrate more in fatty tissue and perhaps in other tissues as well, leading to a larger apparent volume of distribution. However, this tendency was apparently offset by the much higher binding of parathion in the plasma. The influence of plasma protein binding on the volume of distribution can be shown clearly from the unbound volume of distribution (V_{ss}/f_{free}) of parathion (92,200 ml/kg) and paraoxon (1,860 ml/kg).

The difference between paraoxon and parathion in plasma protein binding also appeared to account in part for the difference in their total body clearance values. Since parathion was 1.23% unbound in plasma as compared with the 52.5% fraction free of paraoxon, the much longer plasma half-life of parathion (56.7 hr) was attributable to the much smaller plasma free fraction and the slow redistribution from the tissues into blood due to its high lipophilicity.

The total body clearance of paraoxon in trout (2,900 ml/hr/kg) was greater than the cardiac output in trout at 12°C (2,070 ml/hr/kg) [79]. This suggested that branchial excretion was a primary pathway of elimination in trout, in addition to the irreversible removal of paraoxon by covalent binding to AChE and CaE. Hepatic blood flow was only 60.0 ml/hr/kg [79], and the liver could not have been a significant contributor to the total body clearance value of paraoxon. On the other hand, the clearance of parathion (20.0 ml/hr/kg) was less than the hepatic blood flow, which allowed for the possibility that parathion was cleared by the liver. This agreed with the observation that chemicals with log P values greater than three are poorly excreted by the gills, and that chemicals with log P values smaller than three (i.e. paraoxon) are mainly excreted via gills [97].

Figure 3.3 shows that parathion was rapidly taken up from water and that arterial blood/water equilibrium occurred within 30 min. The plasma/water concentration ratio was about 100, which reflected the high degree of plasma protein binding of parathion. These data suggested that the permeability of the gill epithelium to parathion was very high and that the rate-limiting factor in the uptake of parathion was the delivery capacity of water to the absorption site; in other words, water-flow-rate limited uptake [80]. It should be noted that the blood sampling

site was immediately distal to the gill, and the carrying capacity of plasma was high due to the plasma protein binding. Figure 3.4 showed that paraoxon was also rapidly taken up from water by trout over 1 hr period, and that the plasma and water concentrations reached equilibrium rapidly. The relatively larger volume of exposure water maintained a constant paraoxon concentration in the water during the experiment. The plasma/water concentration ratio was about 1.5, which reflected the low degree of plasma protein binding of paraoxon ($f_{free} = 0.525$).

The concentrations of paraoxon, which were formed metabolically from parathion after water exposure and intraarterial administration, were not detectable. This further confirmed the observation by Wallace and Dargan that parathion is poorly metabolized by trout [25]. The initial exposure concentration of parathion was approximately 20% of the acute 96 hr LC50 [13]. No obvious adverse effects or mortalities were observed that might be attributed to parathion or paraoxon exposure.

A comparison of the pharmacokinetics of parathion and paraoxon in trout and rat showed that both compounds are poorly metabolized in trout, but have large intrinsic metabolic clearance in rat [25]. Therefore, total body clearance values for parathion in trout ($CL_b = 20.0$ ml/hr/kg)

and rat ($CL_b=3,000$ ml/hr/kg) [98] were expected to differ because of the interspecies differences in metabolic clearance. Even though there are large differences in the hydrolysis of paraoxon between trout and rat [25], the total body clearance values of paraoxon were similar [98], which might be due to different pathways of elimination of paraoxon in trout (branchial) and in rat (liver).

In conclusion, this study demonstrated that the apparent volumes of distribution of parathion and paraoxon were similar in fish due to a combination of different lipophilicity and plasma protein binding. The differences in total body clearance of parathion and paraoxon in fish were due to the differences in plasma protein binding and the significant branchial excretion of paraoxon. Finally, the fish-mammal differences in sensitivity to organophosphate toxicity appear unrelated to pharmacokinetic differences. In addition, the prolonged half-life of parathion suggests that parathion may persist in fish, which leads to a direct concern for human consumption of organophosphate poisoned fish.

Figure 3.3. Parathion concentration-time profile in rainbow trout after water exposure to a 75 ng/ml parathion solution; the profile is from a single representative trout (339 g body weight).

Figure 3.4. Paraoxon concentration-time profile in rainbow trout after water exposure to a 75 ng/ml paraoxon solution; the profile is from a single representative trout (140 g body weight).

Chapter IV

A PHYSIOLOGICALLY BASED PHARMACOKINETIC AND PHARMACODYNAMIC MODEL FOR PARAOXON IN RAINBOW TROUT

A. ABSTRACT

A physiologically based pharmacokinetic and pharmacodynamic model was developed to describe paraoxon uptake, tissue distribution and AChE inhibition in fish after water exposure. The PBPK-PD model structure consisted of brain, heart, liver, kidney and muscle, which were interconnected by blood circulation. The blood flow rates to the organs and tissues were obtained from the literature. The tissue:blood partition coefficients for paraoxon were determined experimentally at steady state. Zero-order synthesis and first-order degradation of AChE were determined by fitting the results from a dose-response study to a steady-state linear model. The uptake and depuration clearances describing the exchange of paraoxon at the gill were determined using a compartmental pharmacokinetic model. The agreement between simulated and experimentally obtained values indicated that this model is an appropriate tool to predict organophosphate exposure, tissue distribution and the resulting toxic effect.

B. INTRODUCTION

Physiologically based pharmacokinetic (PBPK) modeling is an important tool in toxicology and medical fields [40-42]. It utilizes known anatomical and physiological values to characterize the disposition of a chemical. Actual tissue masses and blood flow rates to tissues are used to predict the concentration of a chemical in a tissue or group of kinetically related body regions. A set of differential equations describes the delivery of a compound to a tissue (based on blood flow), distribution into the tissue (using a tissue/blood partition coefficient for each tissue group), and elimination which may be in the form of metabolism or excretion via the kidney, liver (bile) or gill. A PBPK model leads to a comprehensive understanding of the uptake, disposition and toxicity of chemicals, and provides a basis to extrapolate or scale the model to other conditions and species beyond those of the original experiment [40,42].

The concept of using known anatomical and physiological functions as a basis for pharmacokinetic models dates back to Teorell's pioneering work in the 1930s [100,101]. Although the term *physiologically based pharmacokinetics* was introduced about 1973 by Dedrick [51], PBPK models were developed in the 1960s for various drugs to describe their uptake and disposition [102,103]. Subsequently, PBPK

modeling has been used in risk assessment [43-45] and successfully employed to extrapolate kinetic data among mammalian species [46-49].

PBPK models were first adapted to aquatic species by Zaharko, Bungay and coworkers [50, 51]. They modified a model developed in the mouse and simulated the distribution of methotrexate in sting ray (*Dasyatidae sabina* and *D. sayi*) [50] and the distribution and elimination of phenol red in the dogfish shark (*Squalus acanthus*) [51] after intravenous administration. Neither model, including the one recently developed by Law et al. in rainbow trout after intravenous administration of pyrene [54], incorporated the environmentally relevant route of exposure (branchial), which limited the application of these models in aquatic toxicology and risk assessment. The important function of gill uptake, accumulation and disposition of chemicals in fish was incorporated and successfully simulated by Nichols and coworkers in their PBPK models for waterborne organic chemicals in rainbow trout, catfish and fathead minnows [52,53,104,105]. None of the present PBPK models in aquatic animals have, however, incorporated the toxicologic effect related to the target organ concentration, which would increase the significance of the model in examining the mechanism of toxicity of chemicals.

Paraoxon, a directly acting inhibitor of acetylcholinesterase (AChE), is a potent toxicant of the cholinergic nervous system. Its toxicity is representative of the other OP insecticides and may be readily quantified by measurement of the activity of acetylcholinesterase. In addition, paraoxon can be found in aquatic environments due to the non-enzymatic oxidative conversion of the widely used organophosphate insecticide (OP) parathion. Its presence in the aquatic environment directly threatens aquatic animals and consequently it poses a risk to humans who consume exposed fish. Therefore, paraoxon was selected as an appropriate model compound for the development of the physiologically based pharmacokinetic and pharmacodynamic model in fish.

The objectives of this investigation were: (1) to develop a physiologically based pharmacokinetic and pharmacodynamic model for paraoxon in rainbow trout; (2) to validate the model by comparing the experimentally determined time course of paraoxon concentration and AChE inhibition in selected tissues of trout with the model predicted values; (3) to extrapolate the validated model to a high exposure concentration, and to multiple oral exposure.

C. PHYSIOLOGICALLY BASED PHARMACOKINETIC (PBPK) MODEL

The approach for developing a PBPK model is summarized in the following schematic diagram.

Figure 4.1. Schematic of the steps involved in the development of physiologically based pharmacokinetic models.

The PBPK model development for a chemical is based upon an understanding of the biochemical and physicochemical properties of the substance and the physiological characteristics of the species. The mechanisms of the toxicity, necessary biochemical constants, physicochemical properties and physiological constants of the species of interest are then obtained from the literature and if necessary, by experiment. The planned uses for the model and the available information are considered in formulation of a model. The subsequent procedures in the development of the model involve model simulation, comparison of the simulation results with experimental data, model refinement, and completion of validation with a sensitivity analysis.

The formulation of a model involves five general steps: model assumptions, selection of tissue compartments, model parameterization, mathematical formulation of the model, and simulation and experimental validation of the model [41].

1. Model assumptions

A PBPK model is based upon the physiology and anatomy of the species of interest. The body is divided into several physiological compartments. Each compartment represents a particular organ or several organs and/or

tissues lumped together. In each compartment, the drug concentration is assumed to be uniform, and each compartment has anatomical significance; e.g., brain, heart, liver, highly perfused and poorly perfused tissues. Each organ compartment is viewed as consisting of three homogeneous subcompartments: 1) the capillary blood volume (vascular space) through which the organ is perfused with blood, 2) the interstitial water which forms a matrix for the tissue cells, 3) and the intracellular space consisting of the tissue cells that comprise the organ. The transport of the chemical into the organ compartment occurs through influx with the afferent blood in the vascular subcompartment. Each subcompartment is considered to be a well-mixed phase, and the efferent blood therefore has the same concentration as the vascular subcompartment. The chemical crosses the capillary wall and diffuses into the interstitial water; it then moves across the cellular membrane from the interstitial fluid into the cell [41,92]. In each subcompartment, chemical may be metabolized or bound to an enzyme. The specific binding to a particular enzyme may cause toxic action; in the case of OP, it irreversibly binds to the physiological enzyme AChE and thereby produces neurotoxicity by overstimulation of the cholinergic nervous system as a result of a high concentration of acetylcholine

For a low molecular weight, non-ionized, lipid-soluble chemical, blood perfusion-limited transport is usually assumed; i.e. chemical movement within a compartment is much more rapid than the rate of delivery of the chemical to the compartment by the blood. In other words, exchange of chemical between capillary blood and interstitial water is considered to be very rapid and cell membrane is considered to be very permeable to the chemical. In this case the concentration of chemical in the venous blood exiting a compartment is in equilibrium with that in the other subcompartments. For very polar, highly ionized or charged chemicals on the other hand, membrane permeation is assumed to be rate limiting in the overall transport process[92].

The following assumptions were made for developing the PBPK model for paraoxon in trout, based on its physicochemical properties and the physiology of the trout.

- (1) Paraoxon distribution is blood flow rate limited.
- (2) Each tissue compartment is well mixed and homogeneous.
- (3) Tissue compartments are interconnected only through the circulatory system.
- (4) Paraoxon binds reversibly to tissue, and irreversibly to AChE and CaE in each tissue compartment.

(5) Uptake and elimination of paraoxon are linear.

2. Selection of tissue compartments

The composition and number of compartments are based on the determinants of chemical distribution, metabolism and toxicity. The model usually includes the tissues in which the drug exerts a pharmacologic or toxicologic effect, the tissues that are involved in the metabolism and elimination of the drug, the fluids that are easily sampled and the tissue spaces that contain relatively large amounts of drug.

The tissue compartments selected for the PBPK model of paraoxon were based on the physiology of fish [52]. The gill was included because of its importance as an uptake and excretory organ in fish; brain and heart were selected as they are sites where paraoxon may exert its toxicity; liver was included because of its involvement in metabolism. White muscle and kidney were selected because of their role in the distribution of OP. In addition, muscle has a significant role in accumulation of toxic chemicals in fish due to its relatively large mass. The model structure is shown in Figure 4.2.

Figure 4.2. Flow diagram of the physiologically based pharmacokinetic model for paraoxon in rainbow trout. Arrows with dotted lines in and out of the gill compartment indicate exchange of paraoxon between blood and water.

D. PHARMACODYNAMIC MODEL FOR AChE INHIBITION

The pharmacodynamics of AChE inhibition was modeled based on the biochemical interaction between OP and AChE. The concentration of free AChE in each tissue is determined by the OP concentration, the bimolecular inhibition rate constant for reaction of OP with AChE, the reactivation rate constant of AChE bound to OP, the synthesis rate of new AChE, and the basal degradation rate of AChE. The normal physiological level of AChE results from a balance between basal degradation of AChE and the synthesis of new enzyme. In addition to the reaction with AChE, OP also binds to CaE, and this reaction has a significant role in removing OP from the target site, as described previously in Chapter One.

Wallace and Herzberg [58] reported that the reactivation of inhibited AChE in trout is insignificant and that once the enzyme is inhibited, the recovery of AChE activity is only via *in vivo* enzyme synthesis. Therefore, in trout the free AChE level is mainly governed by the OP concentration, bimolecular inhibition rates for interaction between OP and enzymes, zero-order synthesis rate of new enzyme, and first-order degradation rate of AChE. The AChE inhibition model in trout was therefore assembled according to these interactions among OP, AChE and CaE (Figure 4.3 inhibition model).

Figure 4.3. Schematic diagram of the pharmacodynamic model for interaction of paraoxon with AChE and CaE, synthesis and degradation of the enzymes.

E. MODEL PARAMETERIZATION

1. Blood flow

Blood flow rates to the tissues in rainbow trout were taken from the values reported by Barron et al. [79] and Nichols et al. [52]. Barron and coworkers determined the blood flow distribution in rainbow trout using ^{57}Co -labeled microspheres. This method results in underestimation of the total blood flow to liver and kidney receiving portal blood supply because of the series arrangement of the capillary bed [52,79]. Nichols et al. [52] adjusted the blood flow values for liver and kidney based on the blood circulation features of these organs as reported in the literature. Since the brain blood flow was not reported by Barron et al. [79], it was modeled after the value reported by Cameron [106]. Cardiac output of rainbow trout at 12°C was also from the value determined by Barron and coworkers using the indicator dilution method. The arterial blood flow to tissues and cardiac output are listed in Table 4.1.

Table 4.1. Blood flow rates used in the PBPK model of rainbow trout.

Parameter	% of cardiac output	Flow (ml/hr)
Liver	2.90	4.80
Kidney	5.60	9.28
W. Muscle	60.0	99.4
Heart	0.00291	0.00480
Brain*	0.00399	0.00661
Cardiac output (12°C)		2070 ml/hr/kg

Calculated for small trout based on data from Barron et al. [79] and Nichols et al. [52].

* From Cameron, 1974 [106].

2. Tissue volume

The values used for most tissue volumes were those reported by Barron et al., which were comparable to the values reported and used by Nichols et al. and Law et al. in their PBPK models. Brain volume was calculated as a percentage of body weight [106]. The tissue volumes of rainbow trout used in the model are listed in the Table 4.2.

Table 4.2. Tissue volumes of rainbow trout used in the PBPK model.

Parameter	% of body weight	volume (ml)
Liver	1.26	1.0
Kidney	0.841	0.672
W. Muscle	66.0	52.8
Heart	0.172	0.138
Brain	0.154	0.123
Plasma	4.0	3.20

3. Partition coefficient

This partition coefficient is the ratio of the drug concentration in tissue to the drug concentration in plasma (C_p) at equilibrium. The partition coefficient may be determined by several approaches; e.g., *in vitro* by vial equilibration [107], ultracentrifugation [108], and equilibrium dialysis [109,110] methods, or *in vivo* following a steady-state infusion of chemical [105,111]. Calculation of the partition coefficient value depends on the particular organ. For all noneliminating organs, the partition coefficient is simply obtained by dividing the concentration of chemical in the tissue by the steady state plasma concentration. For an elimination organ, the calculation is more complex since a correction factor must be included to account for the influence of elimination on the equilibrium distribution [92]. The correction factor includes organ blood flow (Q_i), intrinsic clearance with respect to free drug concentration (CL_{int}) and fraction free in plasma (f_f), which is in the form of $[(Q_i + f_f * CL_{int}) / Q_i]$. It is one over extraction ratio; with high intrinsic clearance drug, the partition coefficient should be calculated based on organ blood flow and clearance.

The *in vitro* vial equilibration method [107] is frequently used to determine partition coefficient for

volatile organic chemicals. It involves comparison of the change in the concentration of a chemical in the headspace of test vials containing tissues with reference vials. The *in vitro* partition coefficients of non-volatile organic chemicals are determined by equilibrium dialysis and ultrafiltration techniques. These *in vitro* methods have the advantages of simplicity and speed. However, due to lack of blood flow, they may not provide the true *in vivo* equilibrium distribution coefficient.

The constant-rate intravenous infusion technique is frequently used to achieve steady state drug concentration for *in vivo* determination of partition coefficients in mammalian species. However, for a relatively long half life drug such as paraoxon, use of a single infusion rate would require considerable time to achieve steady state. To shorten the time to steady state, an initial bolus dose with an infusion starting immediately after it could be used, but this approach might place the concentration of paraoxon in the toxic range. Infusion to steady state by the method described by Wagner [112] overcomes the problem. In this approach, the clearance and half life values of the compound are used to calculate two sequential infusion rates and the duration of each infusion. A similar approach was used for trout except that the paraoxon was administered via the

water bathing the fish, a sufficiently large volume of exposure water was used to insure that the paraoxon concentration would not decline during the approach to steady state. To use this approach in fish, a preliminary experiment was necessary to determine that the steady state plasma concentration. At steady state, blood was withdrawn from the dorsal aorta, the fish was rapidly sacrificed and tissues excised and processed as described in the Validation section. Paraoxon concentrations in plasma and tissues were determined by a capillary GC assay. Partition coefficients were calculated as the ratio of the tissue and plasma concentrations at steady state (Table 4.3). The blood to plasma paraoxon concentration ratio was also determined (0.913).

Table 4.3. Partition Coefficients used in the PBPK model of paraoxon.

Ratio	Partition Coefficient
Plasma/water	1.46
Liver/plasma	5.89
Brain/plasma	3.90
Heart/plasma	2.91
Muscle/plasma	1.00
Kidney/plasma	0.451
Blood/plasma	0.913

4. Determination of uptake clearance

The uptake and depuration parameters of paraoxon are important components of the model and they were incorporated into the model as uptake and depuration clearance constants. The uptake and depuration clearance values were determined by fitting the time course of water and plasma concentrations after water exposure to a clearance-based compartmental model.

Pharmacokinetic analysis involved two types of model, a clearance-based model and a rate constant-based model. Clearance can be defined as the volume of a fluid which has been completely cleared of a chemical per unit of time. A clearance constant is related to a rate constant in that clearance (Cl) is the product of a rate constant (K) and the volume of the corresponding reference fluid (V). Clearance and apparent volume of distribution are independent parameters which characterize discrete, identifiable processes (uptake, distribution and elimination). They are determined by the physicochemical properties of the chemical and the physiology of the animal, while a rate constant is a dependent parameter whose value is determined by the corresponding clearance and volume. Thus, a change in a rate constant may be due to a change in clearance, a change in volume, or a change in both [99].

The experimental design for uptake studies in aquatic animals generally includes either a flow-through exposure where the water concentration of chemical is kept constant using a dilution apparatus [113], or a static exposure where the fish is placed in a fixed volume of water containing some initial concentration of chemical [114]. The advantages of continuous flow studies are the maintenance of optimum water quality conditions and the simplicity of the calculations involved in estimating the uptake and elimination constants. However, constant exposure is rare in the real world, and the difficulty and cost of setting up and maintaining a dilution apparatus for extended periods of time can be a constraint on experimental design in the laboratory.

4.1. Fish and treatment

Rainbow trout (*Oncorhynchus mykiss*) (140 g; Fresh Water Farms, Urbana, Ohio) were acclimated to the laboratory and temperature (12°C) of study for a minimum of 4 weeks. The vivarium was windowless and had an automatically timed photoperiod that were set at 07:00 to 19:00. The acclimation tanks were "Living Stream" systems built by Frigid Units, Inc., Toledo, OH. These systems aerate the water, maintain the temperature, and filter water through a

charcoal filter, with an average recirculation time of 1.5 min. Dechlorinated Columbus Municipal water was added continuously to the holding aquarium at a rate of 1.5 liters/minute. The raceway was cleaned daily and water temperature, pH, and dissolved oxygen were measured; the acceptable ranges for these parameters were 11-13°C, 6.5-8.0, and >9.6 mg/liter (>80% saturation), respectively. Our monitored values were within these ranges. Fish were fed a commercially available soft, moist food obtained from Rangen Inc. (Buhl, ID). Fish was not fed on the day preceding their experimental use. When signs of disease were apparent, e.g., lethargy, erratic swimming, skin lesions, eroded or reddish fins, the fish was removed from the holding aquarium.

4.2. Cannulation

A trout that had been fasted for 24 hr was transferred to 8 liters of water containing 100 mg/L MS222 for anesthesia. An airstone was placed in the water and oxygen was bubbled into the water. After a few minutes, the anesthetized fish was transferred ventral side up to a brace lined with moistened cheese cloth, and a submersible pump was used to irrigate the gill with oxygenated water containing MS222. A hole was drilled in the snout using a

Dremel motor tool. Two sutures were placed on the dorsal surface of the oral cavity: one approximately midway between the snout and leading edge of the first gill arch and the second immediately posterior to the hole drilled into the snout. A cannula made from Teflon tubing (28 gauge thin wall) and attached to a 5/8" x 25 gauge needle and 1 ml syringe filled with heparinized non-nutrient teleost saline [115] was inserted into the dorsal aorta using a 2" 18 gauge I.V. catheter. The catheter was inserted at the midline between the second and third gill arches at approximately a 30° angle. Prior to the insertion the cannula tubing was filled with saline and then inserted into the fish slowly until blood appeared in the tube. The center poke was removed and the tubing was threaded into the catheter and 7 cm into the artery. The catheter was removed, taking care not to pull out the cannula. The cannula tubing was disconnected from the needle and threaded through the hole drilled in the snout and then re-connected to the needle. The cannula was tied with the two sutures placed in the roof of the mouth and a third suture was placed on the dorsal side of the head, immediately posterior to the hole drilled through the snout and firmly secured to the cannula [116,117]. The gills were irrigated with anesthetic-free oxygenated water until operculum movement began and then the

fish was immediately transferred to an isolation tank for 24 hr to allow complete recovery before the experiment.

4.3. Experimental design

Stock solution of paraoxon was made in ethanol and the concentration of paraoxon was determined accurately by GC. Paraoxon was added to a tank containing 80 liters of dechlorinated water maintained at 12°C, and dissolved by stirring with a high speed electric-motor-driven propeller for 20 min. The cannulated trout was placed into the solution. The oxygen saturation in the water was maintained as described above. Samples of blood and water were removed at each of the following exposure times: 0, 0.25, 0.5, 1, 1.5, 2, 4, 6, 12 and 24 hr. The concentrations of paraoxon in plasma were determined along with their water concentrations using a GC method as described in chapter II.

4.4 Results

The time course of paraoxon concentration in plasma and water were analyzed by fitting to a compartment model (Figure 4.4). Uptake and depuration clearances were estimated from the following equations (4.1,4.2) ($Cl_u=0.651$

ml/min/g; $Cl_d=0.468$ ml/min/g), and these values were used in the PBPK model assuming they were proportional at per gram basis.

$$dC_w/dt = (Cl_d [C_p] - Cl_u [C_w])/V_w \quad (4.1)$$

$$dC_p/dt = (Cl_u [C_w] - Cl_d [C_p])/V_p \quad (4.2)$$

where C_w and C_p were paraoxon concentrations in water and plasma, respectively; Cl_u and Cl_d were uptake and depuration clearances; V_w was exposure water volume, and V_p was volume of distribution of paraoxon in plasma.

Figure 4.4. Concentration time profiles of paraoxon in water and plasma after water exposure of trout to a 75 ng/ml paraoxon solution. The line represents predicted values, and points represent experimentally determined values.

5. Bimolecular inhibition rate constants

The reaction of paraoxon with AChE and CaE is governed by bimolecular inhibition rate constants (k_i). The value of the rate constant is a function of the phosphorylation rate constant (K_p) and the association rate constant (k_a). The bimolecular AChE inhibition rate constant (K_{AChE}) was modeled after the value reported by Johnson and Wallace for rainbow trout [57]. The inhibition rate constant for CaE was taken from the value determined by Gearhart et al. [118]. Briefly, an appropriate concentration of inhibitor was preincubated with the S-9 fraction of tissue homogenate. The preincubation was stopped by adding 3.0 ml of 1 mM acetylthiocholine and dithiobisnitrobenzoate at five time points between 0 and 5 min for trout. To measure the remaining AChE activity ($[E]_t$), the absorbance was recorded at 412 nm. The rate constant values were then calculated [119,120]. The following reaction scheme describes the inhibition process and defines the rate constants.

$$K_m = \frac{k_{-1} + k_p}{k_1} \quad (4.3)$$

Assuming $k_p \ll k_{-1}$

$$K_m = \frac{k_{-1}}{k_1} = k_D = 1/k_a \quad (4.4)$$

$$K_{eq} = \frac{k_1 k_p}{k_{-1}} = k_p/k_D = k_a k_p = k_i \quad (4.5)$$

[E] and [I] represent the concentrations of free enzyme and inhibitor, respectively; [EI]_R is the concentration of the reversible enzyme-inhibitor complex, k_a is the association constant, k_D is the dissociation constant, and k_p is the phosphorylation constant. The fraction of activity remaining at time t is calculated by dividing the remaining activity by original uninhibited enzyme activity ($[E]_t/[E]_0$). Least-squares linear regression of the natural log of the fraction of E remaining ($[E]_t/[E]_0$) versus time resulted in a line at each OP concentration with slope = $-k_{app}$ (the apparent rate constant for phosphorylation). A double reciprocal plot of the inhibitor concentration versus k_{app} resulted in a line with slope = $1/k_1$, Y-intercept = $1/k_p$ and X-intercept = $-1/k_D = -k_a$ [119].

6. Determination of synthesis and degradation rates of AChE ----- a dose-response approach

6.1. Background

Time course studies of paraoxon in tissues and AChE inhibition after water exposure of trout to a low concentration of paraoxon revealed that the continued presence of paraoxon led to a new steady state level of AChE activity in the tissues (Chapter IV, Section H). Since the reactivation of organophosphate-inhibited AChE is insignificant in trout [57], the establishment of the new steady state activity of AChE may have resulted from continued rapid synthesis of new enzyme during the time course of inhibition by a low level of paraoxon. If the turnover of AChE is sufficiently rapid, then its rate of formation and degradation should be incorporated in the physiologically based pharmacokinetic and pharmacodynamic model because the turnover of AChE would contribute to its activity.

One approach to characterizing the synthesis and degradation rates of AChE is the method described by Wenthold et al. [121]. In this method, AChE is labeled by administration of ^{14}C -L-leucine. Labeled AChE is isolated by a solubilization and fractionation work up that involves differential and density gradient centrifugation, gel

filtration and electrophoresis. AChE is quantified by the amount of associated radioactivity. This technique would characterize the rapidly turning over intracellular pool of AChE. However, this approach seems inappropriate for the model, because it appears that a majority of the newly synthesized AChE is not exported to the cell surface. Rather it is degraded intracellularly [122]. In the context of the PBPK-PD model, the turnover of the total extracellular enzyme is important rather than the intracellular pool. Therefore, determination of the effective synthesis and degradation rates of AChE was undertaken by measurement of the total enzyme activity.

6.2. Fish and Treatment

Rainbow trout (*Oncorhynchus mykiss*) (70 - 100 g; Fresh Water Farms, Urbana, Ohio) were acclimated to the laboratory and temperature (12°C) of study for a minimum of 4 weeks. The wet lab was windowless with an automatically timed photoperiod that was set at 07:00 to 19:00. The acclimation tanks were "Living Stream" systems built by Frigid Units, Inc., Toledo, OH. These systems aerate the water, maintain the temperature and filter the water through a charcoal filter with an average recirculation time of 1.5 min. Dechlorinated Columbus Municipal water was added

continuously to the holding aquarium at a rate of 1.5 liters/minute. The raceway was cleaned daily and water temperature, pH, and dissolved oxygen were measured; the acceptable ranges for these parameters were 11-13°C, 6.5-8.0, and >9.6 mg/liter (>80% saturation), respectively. Our monitored values were within these ranges. Fish were fed a commercially available soft, moist food obtained from Rangen Inc., (Buhl, ID). Fish were not fed on the day preceding their experimental use. When signs of disease were apparent; e.g., lethargy, erratic swimming, skin lesions, eroded or reddish fins, fish were removed from the holding aquarium.

6.3. Experimental Design

Trout were exposed to paraoxon by placing them into a 100, 200, 400 ng/ml solution of paraoxon maintained at 12°C in 80 liters of water. The exposure time was 24 hr. Three trout were exposed for each exposure time. The water concentration of paraoxon was determined before and after each exposure. At the end of each exposure time, trout were removed from the exposure solution, rinsed, and were sacrificed by a blow to the head. Tissues were excised, rinsed free of blood, patted dry, divided into two parts,

weighed and frozen in liquid nitrogen and stored at -20°C until analysis.

6.4. Determination of Paraoxon Concentration

Paraoxon concentration in the tissues was determined by a capillary gas chromatographic method (Chapter II).

6.4.1. Materials

Analytical grade parathion (99.5%) was provided by Scheming, Denmark. Paraoxon (97.0%) and malathion (98.0%) were provided by Chem Service (West Chester, PA). Iso-octane, hexane and methanol were HPLC/GC grade. All other chemicals were reagent grade.

6.4.2. Method

The tissues were homogenized (Omni Mixer Homogenizer) in 100 mM phosphate buffer pH 7.4 containing 3 mM EDTA. The tissue samples were extracted three times with 3 ml of hexane for 5 min by rotation, using 400 μl sodium chloride and 1 ml of methanol as protein denaturing agents. The extracts were centrifuged for 5 min at 5°C and $4500 \times g$ (IEC

PR-7000 Centrifuge, International Equipment Company). The supernatants were collected and evaporated under a stream of nitrogen. The residue was reconstituted in 0.5 to 1.0 ml of iso-octane, and 1- μ l aliquots were injected onto the GC as described in Chapter II.

6.5. AChE activity determination

AChE activity was determined using the method described by Ellman et al. [123]. It is an extremely sensitive and specific method, and applicable to either small amounts of tissue or to low concentrations of enzyme. The assay is the standard method used to measure AChE activity at the physiological temperature of the species studied (12°C for trout). The principle of the method is based upon an AChE-catalyzed color-producing reaction where the concentration of AChE is proportional to the rate of color formation:

where AChE hydrolyzes the substrate acetylthiocholine to thiocholine and acetate, the thiocholine then reacts with dithiobisnitrobenzoate to produce the yellow anion of 5-thio-2-nitrobenzoic acid. The amount of AChE is

proportional to the change in absorbance at 412 nm, the $A_{\max}$ for 5-thio-2-nitrobenzoate.

6.5.1. Chemicals

Acetylthiocholine chloride, acetylcholine chloride, 5,5 dithiobis-2-nitrobenzoic acid, NaH_2PO_4 , Na_2HPO_4 , disodium ethylenediaminetetraacetic acid, sodium azide, bovine serum albumin, sodium potassium tartrate, sodium carbonate, copper sulfate, sodium hydroxide, and trichloroacetic acid were purchased from Sigma Chemical Co., St. Louis, MO.

6.5.2. Instrumentation

KONTRON UVIKON 860 Spectrophotometer, Kontron Instruments. Proportional Temperature Control, Neslab Instrument, Inc. Newington, NH.

6.5.3. Assay Procedure

The tissues were cut into small pieces and diluted to 20% with homogenization buffer (1 mM acetylcholine chloride in pH 7.4, 100 mM phosphate buffer). The mixture was homogenized in an Omni Mixer homogenizer (OMNI

International, Waterbury, CT) with the homogenization vessel held in an ice and acetone mixture. The tissue was homogenized for 15-45 seconds with the homogenizer operated at 60% of its maximum power. After ensuring that no large chunks of tissue remained, the homogenate was centrifuged at 9000g for 20 min in the cold room (about 12°C) using a temperature equilibrated Eppendorf microcentrifuge. A portion of the supernatant for each tissue was diluted to an appropriate concentration with homogenization buffer for AChE activity determination and the remainder of the homogenate was kept for later determination of protein content. A UV spectrophotometer (KONTRON UVIKON 860 Spectrophotometer) was used to measure the absorbance at the wavelength of 412 nm. The temperature was maintained at 12°C by the proportional temperature control unit.

One hundred microliters of dithiobisnitrobenzoate solution and two hundred microliter of acetylthiocholine solution were placed in each of four microcuvettes. In each of the three cuvettes, 200 µl of diluted tissue homogenate was added; to the fourth cuvette, 200 µl homogenization buffer was added for use as a control. Each solution was mixed by covering the cuvette with parafilm and inverting it. Shaking was avoided because it resulted in a foam. The absorbance was monitored for 5 min and the change in

absorbance was calculated after subtracting the change in absorbance of the blank. The enzyme activity was calculated as: nmol substrate hydrolyzed per min per gram of tissue = change in absorbance/min divided by 13,600, where 13,600 was the extinction coefficient at 412 nm of 5-thio-2-nitrobenzoate.

The AChE activity in tissue from paraoxon exposed trout was compared with the activity in tissues from trout that were held under same conditions without paraoxon present (Table 4.6). To block paraoxon interaction with AChE after tissue samples were removed, tissues were excised and placed into cold buffer that contained acetylcholine.

6.6. Protein Concentration Determination

Protein content in the tissues was determined by a modification of the Lowry method [124]. The portion of the homogenate supernatant which was saved for protein measurement was diluted and made up to 1.0 ml volume with double distilled water. One-tenth milliliter of 0.15% sodium deoxycholate solution was then added, and the mixture was vortexed and allowed to stand for 10 min to solubilize the proteins. One-tenth ml of 72% trichloroacetic acid was then added, the mixture was vortexed to precipitate the

proteins, and centrifuged at 5,000g for 25 min using the Eppendorf microcentrifuge.

The supernatant was discarded, and the pellet which contained the protein was dissolved by addition of 0.1 ml 1N sodium hydroxide. One milliliter of a 2% sodium carbonate, 1% copper sulfate, and 2% sodium potassium tartrate mixed in the proportion of 100:1:1 was added, and allowed to stand for 30 min. The absorbance was then read at 660 nm against a reference blank treated in a similar manner except that the starting point was distilled water. A standard curve was made by including standards with each determination of protein content in tissues. Bovine serum albumin (fatty acid free) dissolved in 1% sodium azide as a preservative was diluted to make the standard curve (Figure 4.5). The AChE activity was then expressed on a per milligram protein basis.

Figure 4.5. Representative standard curve for determination of protein concentration in the tissue samples from rainbow trout ($r^2 = 0.999$).

6.7. Data analysis

A model was developed that was based on the reaction of paraoxon with AChE, and the zero-order synthesis and first-order degradation of free AChE, Figure 4.6. The following model based equation was derived for the steady state case by assuming that the reactivation of the phosphorylated enzyme was insignificant, which is the case for trout [57]:

$$1/\text{AChE} = (K_{\text{AChE}}/R_o) [\text{PO}] + K_D/R_o \quad (4.6)$$

where K_{AChE} is bimolecular inhibition rate constant of AChE, R_o is the zero-order synthesis rate of AChE, K_D is the first-order degradation rate of AChE and $[\text{PO}]$ is the paraoxon concentration in the tissue.

The results of the dose-response study were plotted as $1/\text{AChE}$ vs paraoxon, and analyzed by linear regression analysis. The coefficient of determination (r^2) was greater than 0.898. The results of the plot are presented in Figures 4.7-4.9, and the synthesis and degradation rates of AChE is listed in Table 4.4.

Figure 4.6. Schematic diagram of the model of paraoxon (PO) reaction with AChE, zero-order synthesis and first-order degradation of AChE for rainbow trout.

6.8. Results

Table 4.4 *In vivo* synthesis and degradation rates of AChE in the tissues of rainbow trout.

Tissue	R_o (nmole/min)	K_D (1/hr)
Brain	7.67×10^{-3}	7.31×10^{-5}
Liver	5.52×10^{-3}	4.65×10^{-3}
Muscle	1.31×10^{-2}	5.83×10^{-4}
Heart	1.18×10^{-2}	3.23×10^{-3}

Figure 4.7 Concentration-response relationships in brain of rainbow trout, analyzed by linear regression analysis. Coefficient of determination (r^2) was 0.899.

Figure 4.8. Concentration-response relationships in muscle of rainbow trout, analyzed by linear regression analysis. Coefficient of determination (r^2) was 0.912.

Figure 4.9 Concentration-response relationships in heart rainbow trout, analyzed by linear regression analysis. Coefficient of determination (r^2) was 0.914.

Figure 4.9 (continued)

Concentration-response relationships in liver rainbow trout, analyzed by linear regression analysis. Coefficient of determination (r^2) was 0.738.

The dose response study demonstrated (Figures. 4.3, 4.4 and 4.5) that the inhibition of AChE activity was dose dependent. The observed complete inhibition of AChE activity indicated that increasing paraoxon concentration in the tissues overcomes the synthesis of new AChE. It further indicated the importance of incorporating the zero-order synthesis (R_0) and first-order degradation (K_d) rates of AChE into the model. Numerical values for R_0 and K_d were determined from the data shown in Figures 4.7-4.9 by using linear regression analysis. The synthesis and degradation rates of AChE in liver was determined by fitting three experimental point, and the last was not included since it was considered as an outlier. The synthesis and degradation rates of AChE were also used for CaE [118].

F. MATHEMATICAL FORMULATION OF THE MODEL

For the simulation of the PBPK model, mass balanced differential equations were written to describe uptake of paraoxon from water, delivery of the compound by blood to the tissue, distribution into the tissue, metabolism in the tissue, and depuration from fish into the water. The paraoxon concentration in each tissue compartment depended upon the plasma perfusion rate of the tissue (Q_i), the

tissue volume (V_i), the paraoxon concentration in the plasma (C_p), and in the exposure water (C_w), the tissue-to-plasma partition coefficient at steady state (R_i), and the depuration of paraoxon. For each tissue, the equation also described the interaction of paraoxon with AChE and CaE, AChE activity, CaE activity, zero-order synthesis rate (R_{oi}) and first-order degradation rate (K_{di}) of AChE and CaE.

The mass balanced differential equations for the PBPK-PD model were as follows:

$$\text{Water (w): } d[PO]_w/dt = Cl_d \cdot [PO]_p/V_w - Cl_u \cdot [PO]_w/V_w \quad (4.7)$$

Plasma (p):

$$d[PO]_p/dt = (Cl_u[PO]_w + Q_b[PO]_b/R_b + Q_h[PO]_h/R_h + Q_l[PO]_l/R_l + 0.4Q_m[PO]_m/R_m + Q_k[PO]_k/R_k - Cl_d[PO]_p - (Q_b + Q_h + Q_l + Q_m + Q_k)[PO]_p)/V_p - K_{CaE}[CaE]_p[PO] \quad (4.8)$$

Kidney (k):

$$d[PO]_k/dt = (Q_k[PO]_p + 0.6Q_m[PO]_m/R_m - Q_k[PO]_k/R_k)/V_k - K_{AChE}[AChE]_k[PO]_k - K_{CaE}[CaE]_k[PO] \quad (4.9)$$

$$d[AChE]_k/dt = R_{Ok} - K_{dk}[AChE]_k - K_{AChE}[AChE]_k[PO]_k \quad (4.10)$$

$$d[CaE]_p/dt = R_{Ok} - K_{dk}[CaE]_k - K_{CaE}[CaE]_k[PO]_k \quad (4.11)$$

Liver (l):

$$d[PO]_l/dt = (Q_l[PO]_p - Q_l[PO]_l/R_l - CL_h[PO]_l/R_l)/V_l - K_{AChE}[AChE]_l[PO]_l - K_{CaE}[CaE]_l[PO]_l \quad (4.12)$$

$$d[AChE]_l/dt = R_{Ol} - K_{dl}[AChE]_l - K_{AChE}[AChE]_l[PO]_l \quad (4.13)$$

$$d[CaE]_p/dt = R_{Ol} - K_{dl}[CaE]_l - K_{CaE}[CaE]_l[PO]_l \quad (4.14)$$

Other tissues (i):

$$d[PO]_i/dt = (Q_i[PO]_p - Q_i[PO]_i/R_i)/V_i - K_{AChE}[AChE]_i[PO]_i - K_{CaE}[CaE]_i[PO]_i \quad (4.15)$$

$$d[AChE]_i/dt = R_{Oi} - K_{di}[AChE]_i - K_{AChE}[AChE]_i[PO]_i \quad (4.16)$$

$$d[CaE]_i/dt = R_{Oi} - K_{di}[CaE]_i - K_{CaE}[CaE]_i[PO]_i \quad (4.17)$$

Table 4.5. Symbols used in the PBPK-PD model for paraoxon in rainbow trout.

Parameter/ Variable	Symbol
Paraoxon concentration	$[PO]_i$
AChE concentration	$[AChE]_i$
CaE concentration	$[CaE]_i$
partition coefficient (Tissue/plasma)	R_i
Zero order synthesis rate	R_o
First order degradation rate	K_D
Bimolecular rate constant of AChE	K_{AChE}
Bimolecular rate constant of CaE	K_{CaE}
Blood flow to tissue	Q_i
Tissue volume	V_i
Hepatic clearance	CL_h
Uptake clearance	Cl_u
Depuration clearance	Cl_d
Subscripts: b = brain; l=liver; k = kidney; m, = white muscle; h = heart, p = plasma, w = water; and i = any tissue except specified.	

G. SIMULATION OF THE MODEL

Simulation is the system behavior predicted by solving the differential equations representing the quantitative interrelationships among the various model parameters under specified conditions. Due to the complexity of this model, it turned out to be impractical to use NONLIN84 and PCNONLIN software to perform the simulation. Initial model simulation was performed by numerical integration of the differential equations with the "Continuous System Modeling Program III (CSMP III), an IBM program available on the IBM 3090/600J mainframe computer at The Ohio State University. CSMP III uses FORTRAN IV for the various differential equation statements that describe the model. The model and its inputs were described by a combination of differential equation statements and functional block statements. Initial conditions, parameters, computer increments, and ranges were input as numerical data. The differential equations were then numerically integrated using one of the several available numerical integration methods to give the output in both tabular and graphical forms. The advantages of CSMP III are that it is extremely fast, handling up to 300 differential equations, it is relatively simple to write the program statements, and a choice of five integration methods is available, depending on the requirements of the model.

The variable step Runge-Kutta method was used to automatically adjust the time increment of integration to meet changing integration conditions.

CSMP was developed by IBM in early 1970s. While it is available on the campus mainframe computer, there has been lack of maintenance and updating of this software. Problems with stability were frequently encountered during the simulation. Also the program was not readily controlled from a terminal, and graphics features and sensitivity analysis of the model were not supported. To overcome these deficiencies, the final part of the simulation and sensitivity analysis were performed using SIMUSOLV software, which is a integration software package developed by the Dow Chemical Company (Midland). Its primary functions are to simulate the behavior of physical systems, analyze steady state conditions, perform sensitivity analysis, and produce documentation in the form of graphs and tables of values. SIMUSOLV is also a FORTRAN-based simulation software, and its programming structure and integration methods are similar to those of the CSMP software.

H. MODEL VALIDATION

Model validation involves the comparison of the model predicted values with experimentally determined data in various defined tissues, model refinement and sensitivity analysis. Since PBPK models are only simplified representations of the actual complex system, only those system variables that are determined to be critical determinants are described in detail. The purpose of the validation process then is to determine whether all major determinants/processes that are essential for describing the system behavior have been adequately identified and characterized. When model predictions and experimental data agree, there is a higher level of confidence for using the model to make predictions that can be used for decision-making, i.e., risk assessment.

Sensitivity analysis of a PBPK model refers to the evaluation of the effect of a change in the value of a particular parameter on the tissue concentration or toxic effect predicted by the model. Sensitivity is expressed as the magnitude of change in the endpoint of interest (e.g., AChE inhibition) as a function of change in the value of a particular model parameter. It involves determination of

the response of the system to the model input parameter. Model refinement is a procedure in which the model assumptions and the model structure are re-evaluated and modified if the model fails to adequately predict the experimental data.

1. Experimental

1.1. Fish and treatment

Rainbow trout (*Oncorhynchus mykiss*) (70 - 100 g; Fresh Water Farms, Urbana, Ohio) were acclimated to the laboratory and temperature (12°C) of study for a minimum of 4 weeks. The wet lab was windowless with an automatically timed photoperiod that was set at 07:00 to 19:00. The acclimation tanks were "Living Stream" systems built by Frigid Units, Inc., Toledo, OH. These systems aerate the water, maintain the temperature and filter water through a charcoal filter, with an average recirculation time of 1.5 min. Dechlorinated Columbus Municipal water was added continuously to the holding aquarium at a rate of 1.5 liters/minute. The raceway was cleaned daily and water temperature, pH, and dissolved oxygen were measured; the acceptable ranges for these parameters were 11-13°C, 6.5-8.0, and >9.6 mg/liter (>80% saturation), respectively. The

monitored values were within these ranges. Fish were fed a commercially available soft, moist food obtained from Rangen Inc. (Buhl, ID). Fish were not fed on the day preceding their experimental use. When signs of disease were apparent e.g., lethargy, erratic swimming, skin lesions, eroded or reddish fins, fish were removed from the holding aquarium.

1.2. Water exposure

Trout were exposed to paraoxon by placing them into a 75 ng/ml solution of paraoxon maintained at 12°C in an 80 liter volume of water. The exposure times were 4, 8, 12, 24, 48, 72, 96, and 120 hr. Three trout were exposed for each exposure time. The water concentrations of paraoxon were determined before and after each exposure. At the end of each exposure time, trout were removed from the exposure solution, rinsed and anesthetized with MS-222 (100 mg/L) in an ice-water-mixture. Blood samples were removed via the dorsal aorta using a 1-ml syringe and a 23-G needle. After blood was withdrawn, the trout were sacrificed by a blow to the head. Brain, heart, liver, white muscle and kidney were excised, rinsed free of blood, patted dry, divided into two parts, weighed, frozen in liquid nitrogen, and stored at -20°C until analysis.

1.3. Determination of paraoxon concentrations

Plasma samples were extracted three times with 3 ml of isooctane, using 200 μ l methanol and concentrated sodium chloride as protein denaturing agents. The extracts were centrifuged for 3 min at 5°C and 3000 x g (IEC PR-7000 Centrifuge, International Equipment Company). The supernatant was collected and evaporated under a stream of nitrogen. The residue was reconstituted in 0.1 to 1.0 ml of iso-octane, and 1- μ l aliquots were injected onto the GC.

Brain, heart, white muscle, liver and kidney were homogenized (Omni Mixer Homogenizer) in 100 mM pH 7.4 phosphate buffer containing 3 mM EDTA. The tissue samples were extracted three times with 3 ml of hexane for 5 min by rotation, using 400 μ l sodium chloride and 1 ml of methanol as protein denaturing agents. The extracts were centrifuged for 5 min at 5°C and 4500 x g. Water samples were extracted three times with 3 ml of hexane only. The supernatant was collected and evaporated under a stream of nitrogen. The residue was reconstituted in 0.5 to 1.0 ml of iso-octane, and 1- μ l aliquots were injected onto the GC.

1.4. AChE Activity Determination

AChE activity was determined using the method described by Ellman et al. [123]. Detailed description of the assay was presented in this Chapter, Section E-6.5.

1.5. Protein concentrations determination

Protein content in the tissues was determined by a modification of the Lowry method [124] as described in this Chapter, Section E - 6.6.

Table 4.6. Model Parameters for AChE Inhibition in Rainbow Trout.

Tissue	Initial AChE (nmole/mg protein)	K_{AChE}^* Bimolecular rate constant (nmole ⁻¹ Min ⁻¹)
Brain	32.9 ± 3.3	1.06 × 10 ⁻³
Heart	3.62 ± 1.4	
Liver	2.89 ± 0.19	
Kidney	0.40 ± 0.034	
Muscle	8.70 ± 0.53	
plasma	0.133 ± 0.010	

* Taken from Johnson and Wallace, 1987 [57].

Table 4.7. Model Parameters for CaE Inhibition in Rainbow Trout.

Tissue	Initial* CaE (nmole/mg protein)	K_{CaE}^{**} Bimolecular rate constant (nmole ⁻¹ Min ⁻¹)
Brain	1.60	5.10
Heart	1.40	
Liver	459.8	
Kidney	34.6	
Muscle	65.0	
plasma	41.1	

* Taken from Mawell et. al., 1987b and 1988 [32,126].

** Taken from Johnson and Wallace, 1987 [57].

2. Validation

The agreement between the physiologically based pharmacokinetic and pharmacodynamic model prediction and experimental data was tested by comparing the model predicted concentration-time profiles of paraoxon and the AChE level in various tissues with the values from paraoxon water exposed trout. The theoretical predictions of the concentration-time profiles of paraoxon and AChE activity versus time were considered to agree with experimental values if they were within one standard deviation of the mean of the experimental values. In addition, the comparison of the model output with experimental observations was also made by measuring the coefficient of determination (r^2) [125].

3. Data analysis

The coefficient of determination, r^2 , was calculated by the following equation [125]:

$$r^2 = 1 - \sum \text{dev}^2 / \text{Sy}^2 \quad (4.7)$$

where $\text{Sy}^2 = \sum y_{\text{obs}}^2 - (\sum y_{\text{obs}})^2 / N$, $\text{dev}^2 = (y_{\text{obs}} - y_{\text{pred}})^2$; N represents the number of determinations; y_{obs} and y_{pred} represent experimental and model predicted values, respectively.

Goodness of fit

The goodness of fit between experimentally determined values and PBPK-PD model predicted values was evaluated by using the coefficients of determination (r^2); which exceeded 0.9 in all cases

4. Sensitivity analysis

The sensitivity of the model predicted response to variation of each model parameter was measured by incremental sensitivity analysis [126]. Parameters were varied one at a time by a chosen increment, and computer simulation was used to generate the model response to these incremented values.

Table 4.8. Sensitivity of brain AChE inhibition to changes in the PBPK-PD model parameters

Parameter	Percent Change in AChE inhibition when model parameter is increased 10% ^a		
	Brain	muscle	Liver
[AChE] _o	+1.0	<0.1	<0.1
[CaE] _o	+1.8	<0.1	<0.1
Q	-0.8	+0.8	<0.1
R	<0.1	<0.1	<0.1
R _{ob}	+17.9		
K _{Db}	-1.5		
CL _h ^b		<0.1	
K _{AChE} ^b		-3.0	
K _{CaE} ^b		+2.8	

^a Model predictions of percent change in brain AChE inhibition at 48 hr after water exposure trout to 75 ng/ml paraoxon.

^b General model parameters that do not vary with tissue.

I. MODEL EXTRAPOLATION

Once the PBPK model was validated, it was used to predict the target tissue concentration of paraoxon and consequent AChE levels for various exposure conditions. The usefulness of the model lies with its ability to project the original validated model to other routes of administration, species and dosage regimes (different doses or exposure concentrations). To demonstrate the ability of this PBPK-PD model for paraoxon in fish to project dosage regime and route of administration effects, the validated model for water exposure was modified to predict high water concentration exposure (high dose) and multiple oral exposure. With high water concentration exposure, the brain AChE activity inhibition was simulated because of the important central role of brain AChE in the toxicological response to OP exposure.

Since there is a possibility of contamination of fish food with toxic pesticides at the processing and application site in fresh water fish farms, there is a chance of repeated oral exposure of fish to pesticides. Therefore, this model was also used to predict paraoxon plasma concentration during repeated oral exposure to paraoxon.

J. RESULTS

This study with rainbow trout exposed to paraoxon provided four types of kinetic information: (1) the time course of chemical concentration in arterial blood, (2) the time course of chemical in exposure water, (3) the time course of chemical concentration in trout tissues, and (4) the time course of AChE activity inhibition in corresponding tissues. These data were subsequently used to evaluate the accuracy of the PBPK-PD model simulations. The characterizations of the concentration-time profiles of paraoxon in static exposure water, rainbow trout plasma and tissues are shown in Figures 4.10-4.12. Paraoxon was rapidly taken up by fish from water with plasma concentrations reaching equilibrium with water concentration within 1 hr (Figure 4.10). The plasma/water distribution coefficient was 1.50, which reflected the moderate degree of plasma protein binding of paraoxon (f_{free}). The plasma/water distribution coefficient of paraoxon determined in this study was comparable with the *in vitro* partition coefficient value reported by Jepson et al. [127].

Paraoxon was rapidly distributed into muscle, kidney, heart, brain and liver (4.10-4.12). In kidney and muscle, paraoxon concentration reached steady state by 4 hr. However, in liver, brain and heart, the steady state

concentrations of paraoxon were reached by 24 hr. The *in vivo* partition coefficients of paraoxon determined at steady state (Table 4.3) indicated that the steady state tissue concentrations were in the order: liver > brain > heart > muscle > kidney.

The time courses of *in vivo* AChE inhibition in tissues of trout after water exposure to paraoxon were shown in Figures 4.13-4.16. Paraoxon inhibited AChE activity in all tissues studied. The maximum inhibition of AChE activity occurred after 24 hr in brain (58.9%), heart (51.2%) and liver (49.6%); in muscle and kidney the maximum inhibition were 15.9% and 36.6% after 12 hr.

In addition to the irreversible reaction of paraoxon with AChE, paraoxon also reacts with CaE irreversibly and stoichiometrically. CaE is abundant in liver, muscle, kidney and plasma, and protects against OP toxicity. The irreversible interaction of CaE with OP was incorporated in the model. The model simulated time courses of CaE activity inhibition were shown in Figures 4.17-4.20. The model predicted maximum CaE activity inhibition by paraoxon after water exposure was greater than 95% in liver, kidney and plasma (Figures 4.17, 4.19 and 4.20), and 87% in muscle (Figure 4.18). Figure 4.21 shows time course of AChE

inhibition in brain in the presence and absence of CaE in the model.

The experimentally determined concentration-time profiles of paraoxon and the time course of AChE inhibition in various tissues of trout after water exposure agreed with the PBPK-PD model simulated values. The predicted values were within the error limits of the experimental values for most time points, and the coefficients of determination (r^2) were greater than 0.9. They all indicated that the model performance was successful in predicting the experimentally determined time courses of the exposure water, plasma and tissue concentrations of paraoxon and AChE activity.

The sensitivity of the pharmacodynamic response of the model to changes in the model parameters was analyzed by incremental sensitivity analysis. AChE activity inhibition in brain was chosen as the model response because of the important central role of brain AChE in the response to paraoxon. The effects of parameter variation on AChE activity was evaluated based on the change in brain AChE activity. Table 4.8 shows the results of the analysis of the sensitivity of brain AChE to 10% increases in several of the model parameters and inputs. The magnitude of the change in inhibition of brain AChE defined the relative

importance of each parameter in generating the model response. The zero order synthesis rate of brain AChE (R_{ob}) produced the greatest change in brain AChE inhibition (+17.9%; a positive change indicates a decreased inhibition), followed by initial level of CaE in brain ($[CaE]_0$, +1.8). The bimolecular rate constant of AChE was also influential (K_{AChE} , -3.0%) as was the bimolecular rate constant of CaE (K_{CaE} , +2.8%). In addition, there were only small effects from a 10% change in the initial values of AChE, blood flow, the degradation rate constant of brain AChE (K_{Db}) and the muscle/plasma partition coefficient (R). All other model parameters produced very little effect (<0.1%) on the model predicted inhibition of brain AChE. The effect of changes in the bimolecular inhibition rate constants for reaction of paraoxon with AChE and CaE were roughly equal but in opposite directions. Since bimolecular inhibition rate constants do not normally vary, this analysis suggests that zero order synthesis rate of brain AChE and initial CaE are likely the most important parameters to produce variation in inhibition of brain AChE by paraoxon.

The validated model was extrapolated to high water concentration exposure, and the model predicted brain AChE inhibition was greater than 90% (Figure 4.21), which agreed

with the experimentally determined AChE inhibition value from the dose response study (Figure 4.7). The simulated result from multiple oral exposure is shown in Figure 4.22. The model predicted a steady state level of paraoxon plasma concentration after repeated oral exposure every 6 hr for 5 days.

Figure 4.10. Concentration-time profiles of paraoxon in water, plasma and liver after exposure of trout to a 75 ng/ml solution of paraoxon. Points represent experimentally determined values, the lines depict computer simulation of the PBPK-PD model. Each point represents the mean $\pm$ SD, $n=3$ fish.

Figure 4.11. Concentration-time profiles of paraoxon in brain and white muscle after exposure of trout to a 75 ng/ml solution of paraoxon. Points represent experimentally determined values, the lines depict computer simulation of the PBPK-PD model. Each point represents the mean $\pm$ SD, $n=3$ fish.

Figure 4.12. Concentration-time profiles of paraoxon in heart and kidney after exposure of trout to a 75 ng/ml solution of paraoxon. Points represent experimentally determined values, the lines depict computer simulation of the PBPK-PD model. Each point represents the mean $\pm$ SD, $n=3$ fish.

Figure 4.13. Time course of AChE inhibition in liver and white muscle after exposure of trout to a 75 ng/ml solution of paraoxon. Points represent experimentally determined values, the lines depict computer simulation of the PBPK-PD model. Each point represents the mean $\pm$ SD, n=3 fish.

Figure 4.14. Time course of AChE inhibition in heart after exposure of trout to a 75 ng/ml solution of paraoxon. Points represent experimentally determined values, the lines depict computer simulation of the PBPK-PD model. Each point represents the mean $\pm$ SD, $n=3$ fish.

Figure 4.15. Time course of AChE inhibition in brain after exposure of trout to a 75 ng/ml solution of paraoxon. Points represent experimentally determined values, the lines depict computer simulation of the PBPK-PD model. Each point represents the mean $\pm$ SD, n=3 fish.

Figure 4.16. Time course of AChE inhibition in kidney after exposure of trout to a 75 ng/ml solution of paraoxon. Points represent experimentally determined values, the lines depict computer simulation of the PBPK-PD model. Each point represents the mean $\pm$ SD, $n=3$ fish.

Figure 4.17. Time course of liver CaE activity inhibition in rainbow trout after exposure of trout to a 75 ng/ml solution of paraoxon. The line depict computer simulation of the PBPK-PD model.

Figure 4.18. Time course of muscle CaE activity inhibition in rainbow trout after exposure of trout to a 75 ng/ml solution of paraoxon. The line depict computer simulation of the PBPK-PD model.

Figure 4.19. Time course of kidney CaE activity inhibition in rainbow trout after exposure of trout to a 75 ng/ml solution of paraoxon. The line depict computer simulation of the PBPK-PD model.

Figure 4.20. Time course of plasma CaE activity inhibition in rainbow trout after exposure of trout to a 75 ng/ml solution of paraoxon. The line depict computer simulation of the PBPK-PD model.

Figure 4.21. The model predicted time course of brain AChE activity inhibition in rainbow trout in the presence and the absence of CaE in the model. The solid line depicts computer simulation of the PBPK-PD model with CaE activity in the model, and the dotted line depicts computer simulation without incorporating CaE activity into the model

Figure 4.22. The PBPK-PD model extrapolated time course of AChE inhibition in brain after exposure of trout to high concentration of paraoxon (concentration = 400 ng/ml,). The dotted line represents the extrapolated brain AChE activity inhibition.

Figure 4.23. PBPK-PD model predicted concentration-time profiles of paraoxon in plasma after repeated oral administration of paraoxon to trout (every 6 hr for 5 days).

K. DISCUSSION

The validity of the PBPK-PD model that has been developed to describe the paraoxon uptake, tissue distribution and AChE inhibition was supported by the good agreement between the model predictions and experimental data for the concentration-time profiles of paraoxon and time course of AChE activity inhibition by paraoxon in a variety of tissues (Figure 4.10-4.16). Sensitivity analysis suggested that brain AChE level, predicted by the model, is most sensitive to variability in the zero order synthesis rate of AChE and initial CaE activity.

The importance of the zero order synthesis of AChE was also demonstrated by the time course of the paraoxon tissue distribution and AChE activity inhibition after water exposure of trout to paraoxon (75 ng/ml). In the continuing presence of paraoxon in the tissue, AChE activity declined, to more than 41% of its initial value, and a new steady state of AChE activity was established in all the tissue studied (Figure 4.13-4.16) corresponding to the steady state concentration of paraoxon. Since the reactivation of the paraoxon inhibited AChE is negligible in trout and the inhibition of trout AChE is essentially irreversible [57], the sole means for restoring AChE activity was via *de novo* enzyme synthesis. The establishment of the new steady state

level of AChE activity appeared to result from the continued rapid synthesis of new enzyme during the time course of inhibition by a constant concentration of paraoxon in the tissues. In the absence of AChE synthesis, the model predicts that the AChE activity would soon decline to zero. The model predicts an inverse relationship between the steady-state levels of paraoxon and AChE activity. This relationship was evaluated by a dose response study. The dose response study demonstrated that the inhibition of AChE activity was dose dependent (Figures 4.7-4.9). The observed complete inhibition of AChE activity in dose response study indicated that increasing paraoxon concentration in the tissues overcomes the synthesis of new AChE. The dose response study also suggested that the fish brain was the most sensitive tissue in response to the paraoxon concentration (Figure 4.7), which agreed with the observation by Chambers and Levi [2].

The sensitivity analysis also suggested that the variation of CaE initial level in tissue had direct impact on the AChE inhibition of paraoxon. The simulation of the PBPK-PD model without incorporating the interaction of paraoxon with CaE demonstrated that the brain AChE activity inhibition was increased when compared with the result of the simulation of the model in the presence of CaE (Figure

4.21). It indicates the important role of CaE in determining the availability of paraoxon to inhibit brain AChE, and consequent detoxification action of CaE against OP toxicity, which is also suggested by pharmacological investigations. When CaE activity is selectively inhibited by cresylbenzodioxaphosphorin oxide at a dose that does not inhibit AChE, the dose of OP to inhibit brain AChE as well as to produce lethality is reduced [31,33]

The model predicted that the maximum CaE activity inhibition was greater than 95% in liver, kidney and plasma (Figures 4.17, 4.19 and 4.20), and 87% in muscle (Figure 4.18), while AChE activity inhibition in these tissues was still less than 53%. It further demonstrated the protective effects of CaE in these tissues against OP toxicity by irreversibly and stoichiometrically removing OP from the system [36]. Based on the Initial level of CaE in the tissues (Table 4.7), the protective action of CaE in liver, muscle and plasma would be more significant than the other tissues.

The observed intertissue differences in paraoxon concentration (Figure 4.10-4.12) were corresponded to the partition coefficients of the tissues. Since the partition coefficient reflects the affinity of the tissue to a

chemical, the intertissue differences of paraoxon concentration were due to the different affinity of various tissues and differences in the blood flow to the tissues.

The successful prediction of the experimental data has confirmed the accuracy of the model assumptions. Examination of the concentration-time profiles of paraoxon and the time course of AChE inhibition indicated that there were no apparent relationships between paraoxon concentration and AChE inhibition in the tissues of trout, since the AChE inhibition depends on the free concentration of paraoxon in the tissues.

The objective of this research was to develop a comprehensive quantitative physiologically based pharmacokinetic and pharmacodynamic model for paraoxon in fish that accurately predicts the uptake, disposition and corresponding AChE inhibition of organophosphate insecticides, while remaining simple enough to be applied to problems in aquatic toxicology and environmental risk assessment. The PBPK model that incorporated chemical uptake from water by gills developed by Nichols et al. [52,53], provided a means of modeling an environmentally relevant route of exposure (water exposure). The present work extended this model approach by incorporation the

pharmacodynamics of the toxic chemical. This comprehensive model parameterized with physiological information from the literature has accurately predicted the uptake and disposition of paraoxon and the corresponding AChE activity inhibition of paraoxon in trout.

The influence of blood flow, to distribute organophosphate cholinesterase inhibitor, on the subsequent inhibition of AChE activity was suggested by Maxwell et al. in a model developed in rat [126], which agreed with the result of the present study. Intertissue variation in blood flow was found to be the important factor in explaining the intertissue difference in the rate of *in vivo* inhibition of cholinesterase by paraoxon.

Chapter V

SUMMARY OF OBSERVATIONS AND CONCLUSIONS

The objectives of the investigation were (1) to develop and validate a simple liquid-liquid extraction and a sensitive capillary gas chromatographic method for determination of parathion and paraoxon in biological fluids, (2) to characterize the pharmacokinetics of parathion and paraoxon in rainbow trout, and examine whether there are species related differences in parathion and paraoxon pharmacokinetics that contribute to the differential toxicity that has been observed between fish and mammals, and (3) to develop a PBPK-PD model for paraoxon in rainbow trout, and validate the model experimentally by water exposure of trout to paraoxon.

Chapter two described the development of a sensitive capillary gas chromatographic method for simultaneous determination of organophosphate insecticide parathion and its active metabolite paraoxon in biological samples. This method involved a simple liquid-liquid extraction of parathion and paraoxon from water, plasma and tissues, and

capillary gas chromatographic determination using electron capture detection and splitless injection; malathion was used as an internal standard. A gradient oven temperature program was used, and the injection port and detector temperatures were 200°C and 300°C, respectively. The method allowed quantitative determination of parathion and paraoxon at 9-210 ng/ml levels with recoveries that ranged from 79.4-110.3% for tissues, and 91.9-100.0% for plasma and water. The within-day and between-day coefficients of variation were less than 8.1%. The method was used to characterize the pharmacokinetics of parathion and paraoxon, and the tissue distribution of paraoxon in rainbow trout.

Chapter three described the determination and analysis of the pharmacokinetics of parathion and paraoxon in rainbow trout. Fish are less sensitive to organophosphate insecticides than are mammals. The species differences had mainly been investigated by biochemical studies of AChE and organophosphate interaction. To examine whether species differences in the pharmacokinetics of the organophosphate insecticides were also involved in their differential toxicity, rainbow trout were fitted with a dorsal aorta cannula and administered parathion and its active metabolite paraoxon intraarterially (IA) and via water exposure. Serial blood samples were removed and the plasma

concentrations of parathion and paraoxon were determined by capillary GC with ec detection. Plasma protein binding was determined by equilibrium dialysis and ultrafiltration. After IA injection the plasma concentration-time profiles of parathion and paraoxon were multiexponential, with terminal $t_{1/2}$ of 56.7 and 0.532 hr. The steady-state volume of distribution and total body clearance (CL_b) for parathion and paraoxon were 1,325 and 1,060 ml kg^{-1} , and 19.9 and 2,900 ml $hr^{-1} kg^{-1}$; the plasma unbound fractions were 1.23% and 52.5%. The large difference in CL_b between parathion and paraoxon appeared to result primarily from differences in plasma protein binding. Both parathion and paraoxon had greater persistence in trout than rat, suggesting that sensitivity differences were unrelated to pharmacokinetic differences.

In chapter four a physiologically based pharmacokinetic and pharmacodynamic model was developed to describe paraoxon uptake, tissue distribution and AChE inhibition in fish after water exposure. The PBPK/PD model structure consisted of brain, heart, liver, kidney, and muscle that were interconnected by blood circulation. The blood flow rates were obtained from the literature. The tissue:blood partition coefficients for paraoxon were determined experimentally at steady state. Zero-order synthesis and

first-order degradation of AChE were determined by fitting the results from a dose-response study to a steady-state linear model. The uptake and depuration clearances describing the exchange of paraoxon at the gill were determined using a compartmental pharmacokinetic model. The agreement between simulated and experimentally obtained values indicated that this model was an appropriate tool to predict organophosphate exposure, tissue distribution and resulting toxic effect. The usefulness of this model was demonstrated by extrapolating it to a high concentration water exposure and multiple repeated oral exposure conditions. The model was also used to quantitatively estimate the degree of protection that CaE provided the fish. The major contribution of the present work was to incorporate the pharmacodynamics of toxic chemical in the fish model, to provide a relatively simple and physiologically realistic approach for determining uptake, disposition, and consequent toxic effects by using free swimming fish. This comprehensive model parameterized with physiological information from literature has accurately predicted the uptake, disposition and corresponding AChE activity inhibition of paraoxon in trout.

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APPENDIX A.

Representative results of compartmental analysis of paraoxon and parathion concentration-time profiles.

1. Paraoxon

Time (hr)	Paraoxon observed concentration ($\mu\text{g/ml}$)	Paraoxon predicted concentration ($\mu\text{g/ml}$)
0.083	5.354	5.181
0.167	1.401	1.522
0.25	1.059	0.8662
0.41	0.6970	0.6182
0.667	0.3781	0.4433
1	0.2430	0.2903
1.5	0.1802	0.1537
2	0.0974	0.0814
3	0.0242	0.0228
5	0.00169	0.00179

$A = 5.661 \mu\text{g/ml}$, $B = 0.510 \mu\text{g/ml}$, $\alpha = 7.821 \text{ hr}^{-1}$,

$\beta = 1.354 \text{ hr}^{-1}$.

2. Parathion

Time (hr)	Parathion observed concentration ($\mu\text{g/ml}$)	Parathion predicted concentration ($\mu\text{g/ml}$)
0.17	34.99	35.01
0.333	5.678	5.665
0.5	4.257	4.228
0.75	3.999	3.997
1	3.878	3.836
1.5	3.634	3.545
2	4.387	3.286
4	2.095	2.514
8	1.991	1.732
12	1.382	1.407
24	1.079	1.096
48	0.9501	0.8193
72	0.6914	0.6166
97	0.5903	0.4587
120	0.5151	0.3494
144	0.2701	0.2631
168	0.1724	0.1979
234	0.0759	0.0906
312	0.0446	0.0359
384	0.0205	0.0154

$A = 705.3 \mu\text{g/ml}$, $B = 3.149 \mu\text{g/ml}$, $C = 1.664 \mu\text{g/ml}$,

$\alpha = 18.93 \text{ hr}^{-1}$, $\beta = 0.358 \text{ hr}^{-1}$, $\gamma = 0.0124 \text{ hr}^{-1}$

APPENDIX B.

Representative results of PBPK/PD model simulation of paraoxon concentration-time profile and time-course of AChE inhibition in rainbow trout.

1. Paraoxon Plasma concentration

Time (hr)	simulated concentration ($\mu\text{g/ml}$)
0	0.000
0.24	10.884
0.48	25.536
0.72	37.195
0.96	58.677
1.2	88.298
1.44	110.375
1.68	111.572
1.74	112.061
1.8	112.281
1.86	112.479
1.92	112.645
1.98	112.871
2.04	113.058
2.1	113.262
2.16	113.592
2.4	113.863
4	113.650
4.8	113.574
7.2	113.352
8	113.279

9.6	113.134
12	112.912
14.4	112.682
16.8	112.445
19.2	112.204
21.6	111.960
24	111.716
26.4	111.472
28.8	111.228
31.2	110.984
33.6	110.741
36	110.499
38.4	110.257
40.8	110.015
43.2	109.774
45.6	109.533
48	109.293
50.4	109.054
52.8	108.815
55.2	108.576
57.6	108.338
60	108.101
62.4	107.864
64.8	107.627
67.2	107.391
69.6	107.156
72	106.921
74.4	106.687
76.8	106.453
79.2	106.220
81.6	105.987
84	105.754
86.4	105.522
88.8	105.291
91.2	105.060
93.6	104.830
96	104.600
98.4	104.371
100.8	104.142
103.2	103.914
105.6	103.686
108	103.458
110.4	103.232

112.8	103.005
115.2	102.779
117.6	102.554
120	102.329

2. Paraoxon water concentration

Time (hr)	simulated concentration ($\mu\text{g/ml}$)
0	75.132
2.4	74.945
4	74.827
4.8	74.769
7.2	74.595
8	74.538
9.6	74.425
12	74.258
14.4	74.093
16.8	73.929
19.2	73.767
21.6	73.605
24	73.443
26.4	73.282
28.8	73.122
31.2	72.961
33.6	72.801
36	72.642
38.4	72.482
40.8	72.324
43.2	72.165
45.6	72.007
48	71.849
50.4	71.692
52.8	71.535
55.2	71.378
57.6	71.221
60	71.065
62.4	70.910
64.8	70.754
67.2	70.599
69.6	70.444
72	70.290
74.4	70.136
76.8	69.982
79.2	69.828
81.6	69.675

84	69.523
86.4	69.370
88.8	69.218
91.2	69.067
93.6	68.915
96	68.764
98.4	68.613
100.8	68.463
103.2	68.313
105.6	68.163
108	68.014
110.4	67.865
112.8	67.716
115.2	67.567
117.6	67.419
120	67.271

3. Paraoxon liver concentration

Time (hr)	simulated concentration ($\mu\text{g/ml}$)
0	0.000
2.4	113.936
4	172.153
4.8	207.486
7.2	331.708
8	375.739
9.6	460.300
12	563.817
14.4	630.564
16.8	666.545
19.2	683.333
21.6	690.067
24	692.062
26.4	691.944
28.8	690.995
31.2	689.674
33.6	688.215
36	686.693
38.4	685.146
40.8	683.583
43.2	682.017
45.6	680.446
48	678.883
50.4	677.339
52.8	675.825
55.2	674.248
57.6	672.699
60	671.188
62.4	669.649
64.8	668.119
67.2	666.594
69.6	665.072
72	663.553
74.4	662.037
76.8	660.526
79.2	659.018
81.6	657.512

84	656.010
86.4	654.513
88.8	653.018
91.2	651.527
93.6	650.038
96	648.554
98.4	647.074
100.8	645.596
103.2	644.121
105.6	642.651
108	641.184
110.4	639.720
112.8	638.259
115.2	636.803
117.6	635.347
120	633.897

4. Paraoxon heart concentration

Time (hr)	simulated concentration ($\mu\text{g/ml}$)
0	0
2.4	48.8594
4	75.6492
4.8	87.7818
7.2	120.333
8	130.132
9.6	148.451
12	173.204
14.4	195.217
16.8	214.891
19.2	232.511
21.6	248.304
24	262.459
26.4	275.145
28.8	286.512
31.2	296.687
33.6	305.797
36	313.946
38.4	321.231
40.8	327.734
43.2	333.535
45.6	338.701
48	343.291
50.4	347.362
52.8	350.964
55.2	354.137
57.6	356.923
60	359.358
62.4	361.475
64.8	363.302
67.2	364.865
69.6	366.186
72	367.293
74.4	368.195
76.8	368.919
79.2	369.478

81.6	369.885
84	370.157
86.4	370.306
88.8	370.342
91.2	370.273
93.6	370.111
96	369.863
98.4	369.538
100.8	369.142
103.2	368.682
105.6	368.165
108	367.592
110.4	366.973
112.8	366.313
115.2	365.611
117.6	364.873
120	364.106

5. AChE inhibition in liver

Time (hr)	simulated AChE inhibition (%)
0	0
2.4	21.6024
4	29.7765
4.8	32.8779
7.2	40.0779
8	42.0377
9.6	45.4855
12	49.5235
14.4	52.2298
16.8	53.8301
19.2	54.6775
21.6	55.0848
24	55.2592
26.4	55.3201
28.8	55.3284
31.2	55.3128
33.6	55.2869
36	55.2567
38.4	55.2249
40.8	55.1921
43.2	55.1588
45.6	55.1256
48	55.0927
50.4	55.0595
52.8	55.0263
55.2	54.9927
57.6	54.9599
60	54.9266
62.4	54.8938
64.8	54.8606
67.2	54.8277
69.6	54.7948
72	54.7616
74.4	54.7287
76.8	54.6958
79.2	54.6631
81.6	54.6301

84	54.5972
86.4	54.5644
88.8	54.5318
91.2	54.4992
93.6	54.4664
96	54.4336
98.4	54.4014
100.8	54.3685
103.2	54.3356
105.6	54.3031
108	54.2706
110.4	54.2381
112.8	54.2055
115.2	54.1734
117.6	54.1408
120	54.1083

6. AChE inhibition in heart

Time (hr)	simulated AChE inhibition (%)
0	0.000
2.4	11.973
4	18.611
4.8	21.537
7.2	28.897
8	30.932
9.6	34.469
12	38.671
14.4	41.857
16.8	44.302
19.2	46.207
21.6	47.718
24	48.939
26.4	49.941
28.8	50.776
31.2	51.480
33.6	52.080
36	52.596
38.4	53.042
40.8	53.430
43.2	53.768
45.6	54.064
48	54.323
50.4	54.550
52.8	54.749
55.2	54.923
57.6	55.075
60	55.209
62.4	55.325
64.8	55.425
67.2	55.512
69.6	55.586
72	55.649
74.4	55.702
76.8	55.746
79.2	55.782
81.6	55.810

84	55.832
86.4	55.848
88.8	55.858
91.2	55.862
93.6	55.863
96	55.859
98.4	55.851
100.8	55.840
103.2	55.826
105.6	55.809
108	55.790
110.4	55.768
112.8	55.744
115.2	55.718
117.6	55.690
120	55.660

7. CaE inhibition in liver

Time (hr)	simulated CaE inhibition (%)
--------------	---------------------------------

0	0
2.4	56.1978
4	74.8177
4.8	80.9221
7.2	91.7068
8	93.7059
9.6	96.3399
12	98.2882
14.4	99.0903
16.8	99.4153
19.2	99.5457
21.6	99.5978
24	99.6185
26.4	99.6268
28.8	99.6302
31.2	99.6312
33.6	99.6316
36	99.6318
38.4	99.6317
40.8	99.6316
43.2	99.6315
45.6	99.6314
48	99.6313
50.4	99.6312
52.8	99.6311
55.2	99.6312
57.6	99.6309
60	99.6308
62.4	99.6308
64.8	99.6306
67.2	99.6306
69.6	99.6304
72	99.6304
74.4	99.6303
76.8	99.6302
79.2	99.6301

81.6	99.6302
84	99.6299
86.4	99.6298
88.8	99.6297
91.2	99.6296
93.6	99.6295
96	99.6294
98.4	99.6293
100.8	99.6292
103.2	99.6291
105.6	99.6292
108	99.6289
110.4	99.6288
112.8	99.6287
115.2	99.6286
117.6	99.6285
120	99.6284

8. Paraoxon plasma concentration after repeated
oral exposure of paraoxon in trout

Time (hr)	simulated plasma concentration ($\mu\text{g/ml}$)
0	0.000
1	5.581
2	0.147
3	0.010
4	0.002
5	0.001
7	331.516
8	176.930
9	95.206
10	50.833
11	26.803
12	13.921
12	13.921
12	13.921
13	408.662
14	223.461
15	121.319
16	65.225
17	34.630
18	18.116
18	18.116
18	18.116
19	412.144
20	225.543
21	122.564
22	65.968
23	35.067
24	18.366
24	18.366
24	18.366
25	412.729
26	226.110

27	123.068
28	66.380
29	35.380
30	18.587
30	18.587
30	18.587
31	413.135
32	226.636
33	123.622
34	66.899
35	35.828
36	18.947
36	18.947
36	18.947
37	413.564
38	227.184
39	124.228
40	67.506
41	36.394
42	19.446
42	19.446
42	19.446
43	414.101
44	227.811
45	124.919
46	68.220
47	37.095
48	20.104
48	20.104
48	20.104
49	414.769
50	228.573
51	125.756
52	69.099
53	37.983
54	20.972
54	20.972
54	20.972
55	415.661
56	229.547
57	126.802
58	70.187
59	39.087

60	22.070
60	22.070
60	22.070
61	416.804
62	230.743
63	128.052
64	71.472
65	40.385
66	23.362
66	23.362
66	23.362
67	418.101
68	232.088
69	129.434
70	72.875
71	41.798
72	24.772
72	24.772
72	24.772
73	419.557
74	233.553
75	130.909
76	74.355
77	43.277
78	26.245
78	26.245
78	26.245
79	420.974
80	235.020
81	132.403
82	75.861
83	44.785
84	27.749
84	27.749
84	27.749
85	422.526
86	236.562
87	133.945
88	77.399
89	46.315
90	29.268
90	29.268
90	29.268

91	424.072
92	238.106
93	135.484
94	78.934
95	47.843
96	30.788
96	30.788
96	30.788
97	425.618
98	239.644
99	137.016
100	80.464
101	49.367
102	32.303
102	32.303
102	32.303
103	427.142
104	241.166
105	138.538
106	81.977
107	50.875
108	33.804
108	33.804
108	33.804
109	428.677
110	242.684
111	140.048
112	83.486
113	52.377
114	35.298
114	35.298
114	35.298
115	430.176
116	244.185
117	141.544
118	84.977
119	53.863
120	36.777