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Pharmacokinetics and pharmacodynamics of chlorpyrifos in adult male Long-Evans rats following repeated subcutaneous exposure to chlorpyrifos

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Abstract

Chlorpyrifos (CPF) is a commonly used organophosphorus pesticide. Several pharmacokinetic and pharmacodynamic studies have been conducted in rats in which CPF was administered as a single bolus dose. However, there is limited data regarding the pharmacokinetics and pharmacodynamics following daily exposure. Since occupational exposures often consist of repeated, daily exposures, there is a need to evaluate the pharmacokinetics and pharmacodynamics of CPF under exposure conditions which more accurately reflect real world human exposures. In this study, the pharmacokinetics and pharmacodynamics of CPF were assessed in male Long Evans rats exposed daily to CPF (0, 3 or 10mg/kg/day, s.c. in peanut oil) over a ten day study period. Throughout the study, multiple pharmacokinetic (urinary TCPy levels and tissue CPF and metabolite levels) and pharmacodynamic (blood and brain AChE activity) determinants were measured. Average blood AChE activity on day ten was 54 and 33 percent of baseline among animals in the 3 and 10mg/kg/day CPF treatment groups, respectively, while average brain AChE activity was 67 and 28 percent of baseline. Comparable dose-response relationships between brain AChE inhibition and blood AChE inhibition, suggests that blood AChE activity is a valid biomarker of brain AChE activity. The pharmacokinetic and pharmacodynamic measures collected in this study were also used to optimize a rat physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) model for multiple s.c. exposures to CPF based on a previously published rat PBPK/PD model for CPF following a single bolus injection. This optimized model will be useful for determining pharmacokinetic and pharmacodynamic responses over a wide range of doses and durations of exposure, which will improve extrapolation of results between rats and humans.

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Keywords

Chlorpyrifos; Pesticide, Pharmacokinetics; Pharmacodynamics; Subcutaneous; Cholinesterase

1. Introduction

The effects of repeated occupational and environmental exposures to organophosphorus pesticides (OPs) are poorly understood, although human and animal studies consistently identify neurotoxicity as the primary endpoint of concern (Bushnell and Moser 2006; Costa 2006; Rohlman et al. 2010). The OP chlorpyrifos (CPF) continues to be a human health concern due to its continued use worldwide and documented occupational and environmental human exposures (Alexander et al. 2006; Farahat et al. 2011; Farahat et al. 2010; Garabrant et al. 2009). The mechanism(s) of neurotoxicity following repeated exposures to CPF at levels that do not cause acute toxicity remains an area of active investigation as does the identification of biomarkers that reliably predict individuals at risk for neurotoxicity following occupational and/or environmental exposures to this OP. The development of well-defined pharmacokinetic and pharmacodynamic parameters in animal models that simulate relevant human exposures are needed for optimal mechanistic studies, which in turn will better inform human studies of occupational exposures to CPF and related OPs.

CPF requires metabolic activation to significantly inhibit acetylcholinesterase (AChE), which is thought to mediate the acute toxicity of this compound (Sultatos 1994). Upon entry into the body, CPF undergoes cytochrome P-450 (CYP) mediated metabolism to its active metabolite, chlorpyrifos-oxon (CPF-oxon) (Ma and Chambers 1994) which is the metabolite primarily responsible for the inhibition of not only AChE, but also other B-esterases such as butyrylcholinesterase (BuChE) and carboxylesterase (CE). CPF-oxon is enzymatically hydrolyzed by A-esterases such as paraoxonase 1 (PON-1) (Pond et al. 1998; Sultatos and Murphy 1983) to form the detoxified metabolites diethylphosphate (DEP) and 3,5,6 trichloropyridinol (TCPy). The inhibition of B-esterase by CPF-oxon can also result in the formation of DEP and TCPy (Chanda et al. 1997). The parent compound CPF can also be metabolized by CYP enzymes to form diethylthiophosphate (DETP) and TCPy (a CPF-specific biomarker of exposure) (Ma and Chambers 1994), which are readily excreted in urine (Nolan et al. 1984).

Pharmacokinetic studies of CPF in rat models have primarily been performed in animals administered a single bolus dose (Busby-Hjerpe et al. 2010; Marty et al. 2007; Smith et al. 2009; Timchalk et al. 2007; Timchalk et al. 2002), with the exception of one study that investigated the pharmacokinetics of CPF in neonatal rats (Marty et al. 2007) and another that studied combined exposure to nicotine and CPF (Lee et al. 2010). Since occupational exposures often consist of repeated daily exposures (Farahat et al. 2011; Farahat et al. 2010; Farahat et al. 2003), there is a need to evaluate the pharmacokinetics and pharmacodynamics of CPF and other OPs under exposure conditions which more accurately reflect real world human exposures.

The objective of the current study, therefore, was to assess the pharmacokinetics and pharmacodynamics of CPF in rats following repeated daily exposures to CPF. An important consideration in the design of these studies was the route of exposure since this is known to influence the pharmacokinetics and pharmacodynamics of CPF (Carr and Nail 2008; Marty et al. 2007; Smith et al. 2009). Neurobehavioral studies of chronic or subchronic CPF exposures in rats have utilized several different exposure routes (Bushnell et al. 2001; Icenogle et al. 2004; Moser 2000; Moser et al. 2005), including oral gavage and

subcutaneous injection. While oral gavage is commonly used in pharmacokinetic and pharmacodynamic studies (Lee et al. 2010; Lowe et al. 2009; Smith et al. 2009), it activates stress responses (Brown et al. 2000), which may interfere with neurobehavioral endpoints. Subcutaneous (s.c.) dosing has also been used in a number of CPF-related studies (Bushnell et al. 2001; Icenogle et al. 2004; Slotkin et al. 2006) and is proposed to result in a slow sustained release of the pesticide into the systemic circulation (Marty et al. 2007; Pope et al. 1991; Smith et al. 2009), which approximates most human dermal exposures (Gallo and Lawryk 1991). In light of these considerations, and the fact that most of the internal dose following occupational CPF exposures is thought to result from dermal exposure (Durham et al. 1972; Farahat et al. 2010; Methner and Fenske 1994), the current study employed s.c. injections to administer CPF to adult male Long-Evans rats at 0, 3 or 10mg/kg/day over a ten day study period. Throughout the study, multiple pharmacokinetic (urinary TCPy levels and tissue CPF and metabolite levels) and pharmacodynamic (blood and brain AChE activity) determinants were measured. Herein, we report the use of these pharmacokinetic and pharmacodynamic determinants to adapt a previously published rat physiologically-based pharmacokinetic and pharmacodynamic (PBPK/PD) model for CPF based on a single bolus injection (Smith et al. 2009; Timchalk et al. 2002) for better fit with multiple s.c. exposures to CPF. The optimization of this model for exposure scenarios more relevant to occupational exposure will improve extrapolation of mechanistic studies in rat models to humans.

2. Materials and Methods

2.1. Chemicals

Chlorpyrifos (CAS # 2921-88-2) and chlorpyrifos-methyl (CAS # 5598-13-0) were purchased from ChemService Inc (West Chester, PA, USA). Acetylthiocholine iodide (CAS # 1866-15-5), 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB, CAS # 69-78-3), ethopropazine (CAS # 1094-08-2), tetraisopropyl pyrophosphoramidate (iso-OMPA, CAS # 513-00-8), N-(t-butyltrimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA, CAS # 77377-52-7) and Triton X-100 (CAS # 9002-93-1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydromatrix was purchased from Varian (Walnut Creek, CA, USA). ^{13}C - ^{15}N -3,5,6-TCPy was a gift from Steve Hutton at Dow Agrosiences (Indianapolis, IN, USA).

2.2. Animals

All procedures using animals were carried out with the approval of the Institutional Animal Care and Use Committees of Oregon Health & Science University (Portland, OR) and the University of California (Davis, CA). Male Long-Evans rats (251 – 289g) were obtained from Charles River Laboratories (Hollister, CA), housed one animal per cage in metabolic cages (Techniplast USA, Exton, PA or Hoeltge, Inc., Cincinnati, OH) and maintained on a 12hr photocycle at a controlled temperature ($22 \pm 2^\circ\text{C}$). Rats were fed Lab Diet 5001 rodent chow (Lab Diet, Brentwood, MO) *ad libitum*. Rats (12 per treatment group) were administered CPF at 3 or 10mg/kg/day or an equal volume (300 μL) of vehicle (peanut oil) injected s.c. into the subscapular region. Animals were monitored daily for signs of cholinergic toxicity, including excessive secretions, tremors, ataxia, failure to groom and weight loss. Total daily urine was collected and the volume determined from animals one day prior to the first CPF injection (day 0, pre-exposure) and on each day throughout the remainder of the study. Whole blood (approximately 500 μL) was collected from the saphenous vein into Microtainer tubes containing potassium EDTA (BD, Franklin Lakes, NJ) on days 0 (pre-exposure), 2, 4, 7 and 10. On study days 4 and 10, brains and livers were harvested from a subset of animals immediately upon euthanasia. Brains were rinsed in ice-cold phosphate-buffered saline, microdissected on ice and isolated brain regions were flash

frozen; livers were rinsed in ice-cold phosphate-buffered saline and flash frozen. Brain and liver samples were stored at -80°C until further analysis.

2.3. Urinary analysis

An aliquot of each urine sample was analyzed for the CPF-specific metabolite TCPy by negative-ion chemical ionization gas chromatography-mass spectrometry, with ^{13}C - ^{15}N -3,5,6-TCPy as an internal standard, as described previously (Farahat et al. 2010). The creatinine concentration of each urine sample was determined using the Jaffe reaction (Fabiny and Ertingshausen 1971). Daily TCPy excretion was determined using the following equation:

$$\text{TCPy}_d = \frac{\text{Urine}_d \times \text{CR}_d \times \text{TCPy}_c}{\text{TCPy}_{\text{MW}} \times 1,000} \quad (1)$$

where TCPy_d = daily TCPy excretion (μmol); Urine_d = daily urine excretion volume (ml); CR_d = daily urinary creatinine excretion (mg creatinine/ml urine); TCPy_c = creatinine-adjusted urinary TCPy concentration (ng TCPy/mg creatinine); and TCPy_{MW} = molecular weight of TCPy (198.5).

2.4. AChE activity in blood and brain

An aliquot of whole blood from each animal was used to measure hemoglobin (Hgb) levels using an Hgb Pro Hemoglobin meter (ITC, Edison NJ). After Hgb was measured, the remainder of the blood was diluted 1:25 with ice-cold 0.03% Triton phosphate buffer (pH 7.4), vortexed to lyse cells and stored at -80°C until AChE analysis. Whole blood AChE activity analysis was determined on a Cobas Fara II (Roche, Indianapolis, IN, USA) clinical chemistry analyzer at room temperature using 50 μl diluted whole blood, 5 μl acetylthiocholine (56.6mM) as a substrate for AChE (Ellman et al. 1961), 1.1 μl ethopropazine (6mM) as an inhibitor of BuChE (Naik et al. 2008), and 11 μl of the color reagent, DTNB (10mM). Final assay volume including phosphate buffer (pH 7.4) was 300 μl . Whole blood AChE activity was normalized to hemoglobin content. AChE activity in the cerebellum was determined on a SpectraFluor Plus (Tecan San Jose, CA) at room temperature using 10 μl tissue homogenate, 10 μl acetylthiocholine (2mM) as a substrate for AChE (Ellman et al. 1961), 3 μl iso-OMPA (0.1mM) as an inhibitor of BuChE (Reiner et al. 1993) and 6 μl DTNB (10mM). Final assay volume including phosphate buffer (pH 8.0) was 200 μl . Brain AChE activity was normalized to total protein as determined using the BCA assay according to the manufacturer's directions (Pierce, Rockford, IL).

2.5. Tissue analysis for CPF and metabolites

Levels of CPF, CPF-oxon and TCPy in brain and liver samples were determined by gas chromatography/electron capture detector (GC-ECD). Approximately 0.2g of each tissue sample was weighed and mixed with 2g hydromatrix before the addition of 50 μl of the internal standard (chlorpyrifos-methyl). Samples were then transferred to an extraction cell (Dionex, Sunnyvale, CA, USA) loaded with 3g of hydromatrix and topped off with hydromatrix. The extraction was carried out by accelerated solvent extraction using an accelerated solvent extractor ASE300 (Dionex, Sunnyvale, CA, USA) using ethyl acetate as the solvent extractor under the following experimental conditions: pressure, 1500 psi; temperature, 60°C ; static time, 5 min; heat time, 5 min; flush volume, 150%; purge time, 90 sec; and 2 extraction cycles. Extracts were collected and concentrated to approximately 1ml under a gentle nitrogen stream in a TurboVapII Concentration System (Zymark, Hopkinton, MA, USA). Sample concentrates were loaded onto pre-conditioned 130mg Bond Elut Certify SPE columns (Varian, Palo Alto, CA, USA) and elution was carried out by $2 \times 2\text{ml}$

ethyl acetate: acetone (50:50). The eluates were collected and dried under nitrogen and were then reconstituted in 0.98ml toluene. After the addition of MTBSTFA (20 μ l) each sample was derivatized at 70°C for 1 hour. GC/ECD analysis was carried out with an Agilent 6890 GC with a capillary column (HT-8, 50m $\times$ 0.22mm i.d., 0.25 μ m film thickness; SGE). A sample blank and two quality control spike samples (spike level: CPF and TCPy: 5 and 50 ng/ml; CPFO: 25 and 250 ng/ml) prepared in the same sample matrix were included in each batch run. Concentrations were determined against a corresponding calibration curve of each standard established during each batch run. The limits of quantification for CPF, TCPy and CPF-oxon were 3, 5 and 15nmol/L, respectively.

2.6. Pharmacokinetic/pharmacodynamic analysis

For the purposes of this study, pharmacokinetic parameters included urinary TCPy excretion and tissue concentration of CPF and metabolites in brain and liver, while the pharmacodynamic parameters were considered to be AChE activity in the blood and brain. For all model simulations, a previously reported rat PBPK/PD model for CPF exposure was used, which has been described in detail elsewhere (Smith et al. 2009; Timchalk et al. 2002). In brief, the rat PBPK/PD model describes the time course of absorption, distribution, metabolism and excretion of CPF, CPF-oxon and TCPy, and the inhibition of B-esterases by CPF-oxon in rats following a single s.c. exposure (Smith et al. 2009; Timchalk et al. 2002). The PBPK/PD model utilizes a two-compartment system to simulate CPF movement from the s.c. dosing site to the mixed blood compartment (Smith et al. 2009). The model utilizes several physiologically based pharmacokinetic parameters including organ volumes, blood flow rates, partition coefficients for CPF and CPF-oxon, metabolic constants for CPF metabolism by CYPs and PON1, and the fraction of CPF and CPF-oxon bound in blood (Smith et al. 2009; Timchalk et al. 2002). The CYP mediated metabolism of CPF was limited to the liver and brain compartments. Metabolism by CYPs and PON1 were all described as Michaelis-Menten processes (Timchalk et al. 2002). Interactions of the oxon with B-esterase enzymes were modeled as second-order processes occurring in multiple compartments including the blood and brain (Timchalk et al. 2002). To describe the pharmacodynamic interaction between oxon and B-esterase enzymes, the model utilizes a number of pharmacodynamic parameters such as enzyme turnover, enzyme activity, enzyme degradation, bimolecular inhibition rate and reactivation rate (Smith et al. 2009; Timchalk et al. 2002). The blood kinetics and urinary TCPy elimination were described with a one-compartment model utilizing a first-order rate of urinary elimination (Timchalk et al. 2002).

The PBPK/PD model parameters for a s.c. CPF exposure were originally obtained from a single exposure study (Smith et al. 2009) and as such several model parameters had to be refitted in order for the model to fit the current animal data derived from repeated s.c. exposures. First-order transfer rate constants for the movement of CPF between the different s.c. compartments and mixed blood were optimized (Table 1) using urinary TCPy concentrations from *in vivo* exposures. Using tissue specific values for CPF concentration and AChE inhibition, additional modeling parameters were refitted, including K_m for hepatic metabolism of CPF to CPF-oxon and TCPy and the blood/brain partition coefficient for CPF (Table 1). Brain metabolism of CPF to CPF-oxon was described as a Michaelis-Menten process (Chambers and Chambers 1989). The V_{max} (0.00313 μ mol/l/hr) for this metabolic rate was scaled to fit the *in vivo* system and the K_m was fit using brain data from the current study. The degradation rate (k_d : 0.01 hr⁻¹) for blood AChE was that of earlier estimates (Timchalk et al. 2002). All PBPK/PD model simulations were conducted using acslX (Aegis Technologies Group; Huntsville, AL).

3. Results

Male Long-Evans rats received daily s.c. administration of 0, 3 or 10mg CPF/kg/day (N=12/treatment). None of the CPF-treated rats displayed symptoms of overt toxicity or exhibited significant weight loss relative to vehicle controls over the 10-day exposure (data not shown). Based on the daily urinary TCPy excretion for animals administered CPF at 3 or 10mg/kg/day, the cumulative μ moles of TCPy excreted over the ten day study and the percentage of the molar CPF dose excreted was determined (Table 2). The percentage of molar dose excreted each day was similar between the 3 and 10mg/kg/day CPF dose groups and ranged from 8 to 23% with higher percentages of the dose being excreted at latter time points. PBPK/PD model simulations for urinary TCPy excretion were fitted to the cumulative urinary TCPy data from animals exposed daily to 3 or 10mg CPF/kg/day (s.c.) over the ten day study period. Good model fits were obtained by optimizing the first-order transfer rate of CPF into the blood (K_{sc} : 0.052 hr⁻¹), peripheral s.c. compartment (K_{sp} : 0.86 hr⁻¹) and from the peripheral s.c. compartment to the central s.c. compartment (K_{ps} : 0.016 hr⁻¹) (Table 1).

Average blood AChE activity, as a percent of baseline, decreased with increasing dose and time, ranging from 83 to 54% and 67 to 33% among animals in the 3 and 10mg/kg/day CPF treatment groups, respectively (Figure 1). PBPK/PD model simulations for whole blood AChE activity were fit to the blood AChE inhibition data from the animals (Figure 1). Model simulations for the blood concentration of CPF in animals exposed subcutaneously to 3 or 10mg/kg/day of CPF are shown in Figure 1.

The average hepatic concentrations of CPF on study days 4 and 10 were 0.002 μ mol/l and 0.024 μ mol/l for 3mg/kg/day exposed rats and 0.048 μ mol/l and 0.153 μ mol/l for 10mg/kg/day exposed rats (Figure 2). Hepatic concentrations of TCPy on days 4 and 10 were 0.207 μ mol/l and 0.446 μ mol/l for 3mg/kg/day exposed rats and 0.717 μ mol/l and 1.305 μ mol/l for 10mg/kg/day exposed rats. The hepatic concentration of CPF-oxon was below the level of detection (15 nmole/L) in all animals. PBPK/PD model simulations for hepatic CPF concentration were fitted to the hepatic CPF tissue concentrations of the animals (Figure 2). Adequate model fits were obtained by adjusting hepatic metabolism parameters for the conversion of CPF to TCPy (K_m : 38.5 μ mol/l) and CPF to CPF-oxon (K_m : 3.54 μ mol/l) (Table 1).

The average brain AChE activities on days 4 and 10 were 84 and 67% of control for 3mg/kg/day treated animals and 67 and 28% of control for 10mg/kg/day treated animals (Figure 3). Average brain concentrations of CPF on study days 4 and 10 were 0.02 μ mol/l and 0.06 μ mol/l for 3mg/kg/day exposed rats and 0.102 μ mol/l and 0.246 μ mol/l for 10mg/kg/day exposed rats (Figure 3). The average brain CPF concentration was consistently higher than hepatic CPF concentration at similar time points and CPF doses. Levels of CPF-oxon and TCPy in the brain were below the limits of detection in all animals with the exception of one animal from the 10 mg/kg/day treatment group, in which brain TCPy concentration was detected at 0.013 μ mol/l on day 10. PBPK/PD model simulations for brain CPF concentration, and brain AChE activity were fit to the animal data (Figure 3). Good fits were achieved for the concentration of CPF in the brain by optimizing the blood/brain partition coefficient for CPF (4.0). The V_{max} (0.00313 μ mol/l/hr) for brain metabolism of CPF to CPF-oxon was determined by scaling hepatic metabolism by the ratio of brain/hepatic metabolism (2.3E-4) (Chambers and Chambers 1989), while the K_m (57 μ mol/l) was determined by fitting the model to AChE activity (Table 1).

4. Discussion

Previous pharmacokinetic studies of CPF (Marty et al. 2007; Smith et al. 2009) following a single injection suggest that s.c. administration results in a slow sustained absorption of CPF from the injection site into the systemic system, which in turn results in slower metabolism and excretion of CPF as TCPy. Specifically, Marty *et al.* (2007) reported that s.c. injection of ^{14}C -labeled CPF (DMSO) resulted in a depot of ^{14}C -activity at the injection site with a slow peak time for blood TCPy concentrations and hypothesized that CPF is slowly released from the injection site over time. A similar hypothesis was made by Smith *et al.* (2009) after comparing the pharmacokinetics of CPF via multiple routes of exposure and vehicles of administration in rats. Consistent with these previous reports, the TCPy concentration excreted in the urine of animals receiving repeated daily s.c. injections of CPF revealed that there is a relatively slow excretion of the molar daily s.c. dose of CPF with about 9 and 21% of the dose being excreted on days 2 and 10, respectively (Table 2). This level of urinary TCPy is a smaller percentage of the administered CPF dose than previously reported values of 39% and 44% at 48 hours following a single s.c. injection of $5\mu\text{g/kg}$ CPF in corn oil or DMSO, respectively (Smith et al. 2009). The observation by Smith *et al.* (2009) that the percent of CPF dose excreted following s.c. administration is dependent on the injection vehicle, likely explains the discrepancies in excreted dose between the current study, in which peanut oil was used as a vehicle, and these previous reports.

Comparing PBPK/PD parameters from the current study with previously reported data (Smith et al. 2009) indicates that subcutaneous administration of CPF using peanut oil as a vehicle requires a smaller first order transfer rate into blood (k_{sc} : 0.052 and 0.34 hr^{-1} , respectively), the peripheral s.c. compartment (k_{sp} : 0.86 and 1.46 hr^{-1} , respectively) and from the peripheral s.c. compartment to the central s.c. compartment (k_{ps} : 0.016 and 0.04 hr^{-1} , respectively) than administration in corn oil. Additionally, a larger first order transfer rate for CPF into blood (k_{sc} : 0.052 and 0.03 hr^{-1} , respectively) and the peripheral s.c. compartment (k_{sp} : 0.86 and 0.07 hr^{-1} respectively) and a similar rate of transfer from the peripheral s.c. compartment to the central s.c. compartment (k_{ps} : 0.016 and 0.03 hr^{-1} , respectively) are predicted for peanut oil relative to DMSO (Smith et al. 2009).

PBPK/PD model simulations were fit to the animal data for hepatic and brain CPF concentrations as determined after 4 or 10 days of repeated daily injections but were simulated for CPF concentrations in blood since samples were not available for determining blood CPF concentrations. Model simulations predict that CPF concentration would be highest in the blood followed by the brain and then the liver. Experimental animal data were consistent with these predictions, e.g., for any given time and dose, CPF concentrations were significantly higher in the brain than in the liver. This can be explained by the differences between liver and brain metabolism of CPF as well as chemical partitioning into the tissues. The liver has high metabolic activity towards CPF allowing it to quickly convert CPF to its metabolites (Ma and Chambers 1994), and as a result, hepatic metabolism of CPF has a large influence on the hepatic concentration of CPF. The brain has much lower metabolic activity towards CPF (Chambers and Chambers 1989) and as such localized brain metabolism of CPF will not have a large impact on the brain concentration of CPF. However, localized brain metabolism of CPF does have a dramatic effect on AChE inhibition in the brain (Smith et al. 2009). The difference between liver and brain metabolism of CPF is further supported by analytical data of TCPy levels in these tissues. TCPy was detected in the livers of animals from both dosing groups (3 and 10mg/kg/day) at both time points (days 4 and 10); in contrast, TCPy in the brain was below the level of detection in all but one animal in the 10mg/kg/day treatment group on day 10. Based on tissue specific concentrations of CPF and kinetic parameters for CPF metabolism, neither liver nor brain metabolism is thought to be saturated even though animals were exposed to

relatively high concentrations of CPF. This can be attributed to the s.c. exposure route which results in a slow sustained release of CPF into the body. Similar doses of CPF administered by other exposure routes, such as iv or oral, may saturate metabolism because a large concentration of CPF reaches the tissue in a relatively short time period.

The blood/brain partition coefficient for CPF of 4.0 in the current study (Table 1) is notably lower than previous estimates (12.5 to 33) (Lowe et al. 2009; Timchalk et al. 2002). The difference in the blood/brain partitioning of CPF could be attributed to the repeated administration of an oil vehicle (corn oil) which may change the blood chemistry of the animals (Lowe et al. 2009). Higher oil/lipid levels in the plasma could result in higher partitioning of CPF to the blood and result in a lower partition coefficient for the brain. A similar phenomenon was reported when late pregnancy rats with altered lipid levels were administered CPF (Lowe et al. 2009).

On study day ten, average whole blood AChE activity was suppressed from baseline by 46 and 67% in 3 and 10mg/kg/day CPF-treated animals, respectively. The magnitude of whole blood AChE inhibition seen in animals from the current study is similar to previous reports of red blood cell AChE inhibition in Egyptian agricultural workers that have significant dermal exposures to CPF (Farahat et al. 2011; Farahat et al. 2010) and exhibit significant neurobehavioral deficits compared to a control population (Abdel Rasoul et al. 2008; Farahat et al. 2003). Average AChE activity in the cerebellum was inhibited by 33 and 72% of control following 10 days of exposure to 3 and 10mg/kg/day CPF, respectively. Carr and Nail (2008) observed a 63% inhibition in cerebellum AChE activity from preweanling rats exposed to seven daily s.c. injections of CPF (5mg/kg/day, DMSO), which is consistent with the present findings. In the current study, no attempts were made to describe the recovery of brain AChE. Chiappa et al. (1995) reported that adult rats exposed to five weekly s.c. injections of CPF (15, 30 or 60mg/kg) had global brain AChE activity that was 20 – 50 percent of control, which then recovered to 70 – 90 percent of control during a five week recovery period.

The magnitude of brain AChE inhibition is known to vary within different regions of the brain. Chiappa et al. (1995) examined the regional variation of AChE inhibition in rats exposed to weekly s.c. injections of CPF (60mg/kg) and reported that the greatest amount of inhibition occurred in the forebrain followed by the hippocampus and then the cerebellum. Bushnell et al. (1994) reported that adult rat cortex is slightly more sensitive than the hippocampus to weekly s.c. injections of CPF. Regional differences in rat brain AChE inhibition also exist following a single s.c. exposure to CPF (Bushnell et al. 1993; Dam et al. 2000). Gender-selective differences in brain AChE inhibition have also been reported with male neonatal rats (post natal day 1) exposed to a single s.c. CPF injection being more sensitive than females; however this gender effect was much less notable by post natal day 11 (Dam et al. 2000). The route of exposure has also been shown to result in differences in brain AChE inhibition. Carr and Nail (2008) reported a larger magnitude of brain AChE inhibition in preweanling rats following repeated s.c. administration of CPF compared to an oral exposure. PBPK/PD modeling of a single s.c. exposure to CPF also predicts a greater magnitude and duration of brain AChE inhibition than that following an oral exposure (Smith et al. 2009). Smith *et al.* (2009) suggested that the increased and prolonged inhibition of brain AChE inhibition following a s.c. exposure to CPF is due to slow release of CPF from the injection site, sequestration of CPF in the brain and localized brain metabolism of CPF to CPF-oxon. Experimental data obtained in animals following repeated daily s.c. injections supports this suggestion, demonstrating higher CPF concentrations in the brain than the liver (Figures 2 and 3). Limited work has been conducted on CPF metabolism in the brain; however, there is evidence that there is the potential for localized brain metabolism of CPF to CPF-oxon (Chambers and Chambers 1989). CPF metabolism in the brain could have

important implications since a small amount of CPF-oxon formed locally at the target site may have a greater impact on toxicity than a large amount of CPF-oxon formed in the liver, most of which likely does not reach the brain. The current PBPK/PD model simulations suggest that low level brain metabolism ($V_{\max} = 0.00313 \mu\text{mol/l/hr}$) of CPF to CPF-oxon can accurately simulate the magnitude of AChE inhibition observed in the experimental animal data. Further metabolism studies should be conducted to better assess the capabilities of the brain to metabolize CPF.

Careful consideration should be taken when choosing a PBPK/PD model for estimating the pharmacokinetics and pharmacodynamics of CPF following exposure. The current study used a s.c. route of exposure as a surrogate for dermal CPF exposure, which mostly occurs in occupational exposures. These data will differ from other exposure routes such as oral exposure, which is common for infants and children due to CPF exposure through food, water, and hand-to-mouth behaviors (Freeman et al. 2005). For these situations, a PBPK/PD model for oral CPF exposure (Smith et al. 2009; Timchalk et al. 2002) would be more suitable.

In conclusion, multiple pharmacokinetic and pharmacodynamic measurements taken from rats exposed daily for 4 or 10 days to two different doses of CPF (3 and 10mg/kg/day, s.c.) were used to modify a previously reported single exposure rat PBPK/PD model (Smith et al. 2009) to fit data collected following multiple s.c. injections. Urinary TCPy excretion data showed that the majority of the CPF dose was not excreted from the animals, suggesting that CPF is absorbed and excreted slowly following a repeated s.c. dosing regimen. There was also a large depression of brain AChE activity in CPF-treated animals, which is likely due to the relatively high brain levels of CPF and localized metabolism of CPF to CPF-oxon. The pharmacokinetics of brain AChE inhibition was similar to that of blood AChE inhibition, suggesting that blood AChE is a valid biomarker of brain AChE activity. Importantly, the relative degree of blood AChE inhibition in rats following this repeated s.c. injection paradigm was similar to the magnitude of blood AChE inhibition reported in a population of agricultural workers exposed to high dermal concentrations of CPF over a period of 9 to 17 days (Farahat et al. 2011; Farahat et al. 2010), suggesting that this rat exposure model is appropriate for mechanistic studies of CPF-induced neurotoxicity following occupational exposures. A PBPK/PD rat model for multiple s.c. exposures to CPF will also allow for simulations of pharmacokinetic and pharmacodynamic responses over a wide range of doses and durations of exposure to improve efforts to extrapolate results from rats to humans.

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Research Highlights

- The pharmacokinetics/pharmacodynamics of chlorpyrifos were assessed in rats.
- Results show that blood AChE activity is a valid biomarker of brain AChE activity.
- Results were used to update a PBPK/PD model for chlorpyrifos exposure.
- Updated PBPK/PD model will improve extrapolation between rats and humans.

Figure 1. Experimental data (symbols) and physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model simulations (lines) for whole blood AChE activity (top panels) and chlorpyrifos concentration in the blood (bottom panels) from male Long-Evans rats following daily subcutaneous administration of 3 or 10mg/kg/day of chlorpyrifos. The data represent the mean $\pm$ SD of 12 animals for days 0-4 and 6 animals for days 5-10.

Figure 2. Experimental data (symbols) and physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model simulations (lines) for chlorpyrifos concentration in the liver of male Long-Evans rats following daily subcutaneous administration of 3 or 10mg/kg/day of chlorpyrifos. The data represent the mean $\pm$ SD of 3 animals.

Figure 3. Experimental data (symbols) and physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model simulations (lines) for brain AChE activity (top panels) and chlorpyrifos concentration in the brain (bottom panels) from male Long-Evans rats following daily subcutaneous administration of 3 or 10mg/kg/day of chlorpyrifos. The data represent the mean $\pm$ SD of 3 animals.

Table 1

Optimized pharmacokinetic and pharmacodynamic model parameters for repeated subcutaneous administration of chlorpyrifos in peanut oil to adult male Long-Evans rats

Parameter	Value
Ksc: first order transfer rate of CPF ^a from s.c. compartment to mixed blood	0.052 hr ⁻¹
Ksp: first order transfer rate of CPF from the s.c. compartment to the peripheral s.c. compartment	0.86 hr ⁻¹
Kps: first order transfer rate of CPF from the peripheral s.c. compartment to the s.c. compartment	0.016 hr ⁻¹
Km liver CPF to TCPy ^b	38.5 µmol/l
Km liver CPF to CPF-oxon ^c	3.54 µmol/l
Vmax brain CPF to CPF-oxon	0.00313 µmol/l/hr
Km brain CPF to CPF-oxon	57 µmol/l
Partition coefficient brain/blood for CPF	4.0

^a Chlorpyrifos;

^b 3,5,6 trichloropyridinol;

^c Chlorpyrifos-oxon

Table 2

Cumulative urinary 3,5,6-trichloro-2-pyridinol (TCPy) excretion and percent of chlorpyrifos (CPF) dose excreted following daily subcutaneous administration of CPF to male Long-Evans rats

Treatment	Cumulative urinary TCPy excretion (μmole)	Percent of cumulative administered molar CPF dose excreted
3mg/kg/day		
Day 1	0.19 ± 0.3	8.0 ± 8.8
Day 2	0.42 ± 0.3	9.7 ± 6.8
Day 3	0.68 ± 0.3	11.9 ± 5.6
Day 4	0.96 ± 0.3	13.2 ± 4.5
Day 5	1.4 ± 0.4	15.0 ± 3.9
Day 6	1.8 ± 0.5	16.4 ± 4.2
Day 7	2.2 ± 0.5	18.2 ± 4.0
Day 8	2.6 ± 0.5	19.0 ± 3.3
Day 9	3.0 ± 0.5	19.8 ± 2.9
Day 10	3.5 ± 0.5	20.7 ± 2.6
10mg/kg/day		
Day 1	0.50 ± 0.5	5.1 ± 5.9
Day 2	1.2 ± 0.7	8.0 ± 5.3
Day 3	2.0 ± 0.8	10.3 ± 4.5
Day 4	3.0 ± 0.9	12.4 ± 3.9
Day 5	4.2 ± 0.7	13.6 ± 2.6
Day 6	5.5 ± 1.2	15.7 ± 3.6
Day 7	7.5 ± 1.6	18.8 ± 4.2
Day 8	8.9 ± 1.7	19.9 ± 4.0
Day 9	10 ± 2.1	20.9 ± 4.5
Day 10	12 ± 2.3	22.7 ± 4.5

Vales are the average ± S.D. of N = 12 animals for days 1 - 4 and 6 animals for days 5 - 10.