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Comparative pharmacokinetics of chlorpyrifos versus its major metabolites following oral administration in the rat

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ABSTRACT

Chlorpyrifos (CPF) is a commonly used diethylphosphorothionate organophosphorus (OP) insecticide. Diethylphosphate (DEP), diethylthiophosphate (DETP) and 3,5,6-trichloro-2-pyridinol (TCPy) are products of both *in vivo* metabolism and environmental degradation of CPF and are routinely measured in urine as biomarkers of exposure. Hence, urinary biomonitoring of TCPy, DEP and DETP may be reflective of an individual's contact with both the parent pesticide and exposure to these metabolites in the environment. In the current study, simultaneous dosing of ^{13}C- or ^{2}H-isotopically labeled CPF (^{13}C-labeled CPF, 5 ^{13}C on the TCPy ring; or ^{2}H-labeled CPF, diethyl-D10 (deuterium labeled) on the side chain) were exploited to directly compare the pharmacokinetics and metabolism of CPF with TCPy, and DETP. The key objective in the current study was to quantitatively evaluate the pharmacokinetics of the individual metabolites relative to their formation following a dose of CPF. Individual metabolites were co-administered (oral gavage) with the parent compound at equal molar doses (14 μmol/kg; ~5 mg/kg CPF). Major differences in the pharmacokinetics between CPF and metabolite doses were observed within the first 3 h of exposure, due to the required metabolism of CPF to initially form TCPy and DETP. Nonetheless, once a substantial amount of CPF has been metabolized (≥3 h post-dosing) pharmacokinetics for both treatment groups and metabolites were very comparable. Urinary excretion rates for orally administered TCPy and DETP relative to ^{13}C-CPF or ^{2}H-CPF derived ^{13}C-TCPy and ^{2}H-DETP were consistent with blood pharmacokinetics, and the urinary clearance of metabolite dosed groups were comparable with the results for the ^{13}C- and ^{2}H-CPF groups. Since the pharmacokinetics of the individual metabolites were not modified by co-exposure to CPF; it suggests that environmental exposure to low dose mixtures of pesticides and metabolites will not impact their pharmacokinetics.

1. Introduction

Organophosphorus (OP) insecticides constitute a large family of structurally related pesticides that share a common mode of toxicological action (i.e. cholinesterase inhibiting), and do not appreciably bioaccumulate in humans since they are readily metabolized and excreted. Biomonitoring of OP insecticide exposures has been conducted for approximately two decades and has focused on a broad segment of the population including: pesticide workers and their families (Aprea et al., 1999, 1994, 1997; Curl et al., 2002; Garcia et al., 2000; Loewenherz et al., 1997), pregnant women (Needham, 2005; Perera et al., 2003, 2005; Whyatt et al., 2004), children (Aprea et al., 2000; Koch et al., 2002; Lu et al., 2005, 2001), and the general population (Barr et al., 2004; Heudorf and Angerer, 2001). These biomonitoring studies have served as an important framework for understanding the relationship between pesticide usage, exposure, and the extent of internalized dosimetry across these populations.

Chlorpyrifos (CPF) is one of the most widely used broad spectrum phosphorothionate OP insecticides in the U.S. despite being banned for residential use within the last decade. Fig. 1 displays a generalized scheme for CPF metabolism; wherein, CYP450-mediated oxidative desulfuration converts CPF into its active neurotoxic metabolite, chlorpyrifos-oxon (CPF-oxon), which acts as an acetylcholinesterase (AChE) inhibitor resulting in a buildup of acetylcholine in nerve tissue (Sultatos, 1994; Timchalk and Krieger, 2001). Hepatic and extra-hepatic A-esterases (PON-1), and tissue B-esterases (cholinesterase; ChE) also metabolize CPF-oxon forming 3,5,6-trichloro-2-pyridinol (TCPy) and diethylphosphate (DEP). Alternatively, CYP450-mediated dearylation converts CPF directly into TCPy and diethylthiophosphate (DETP). The metabolite TCPy, either by itself or in a conjugated form (glucuronide or sulfate)

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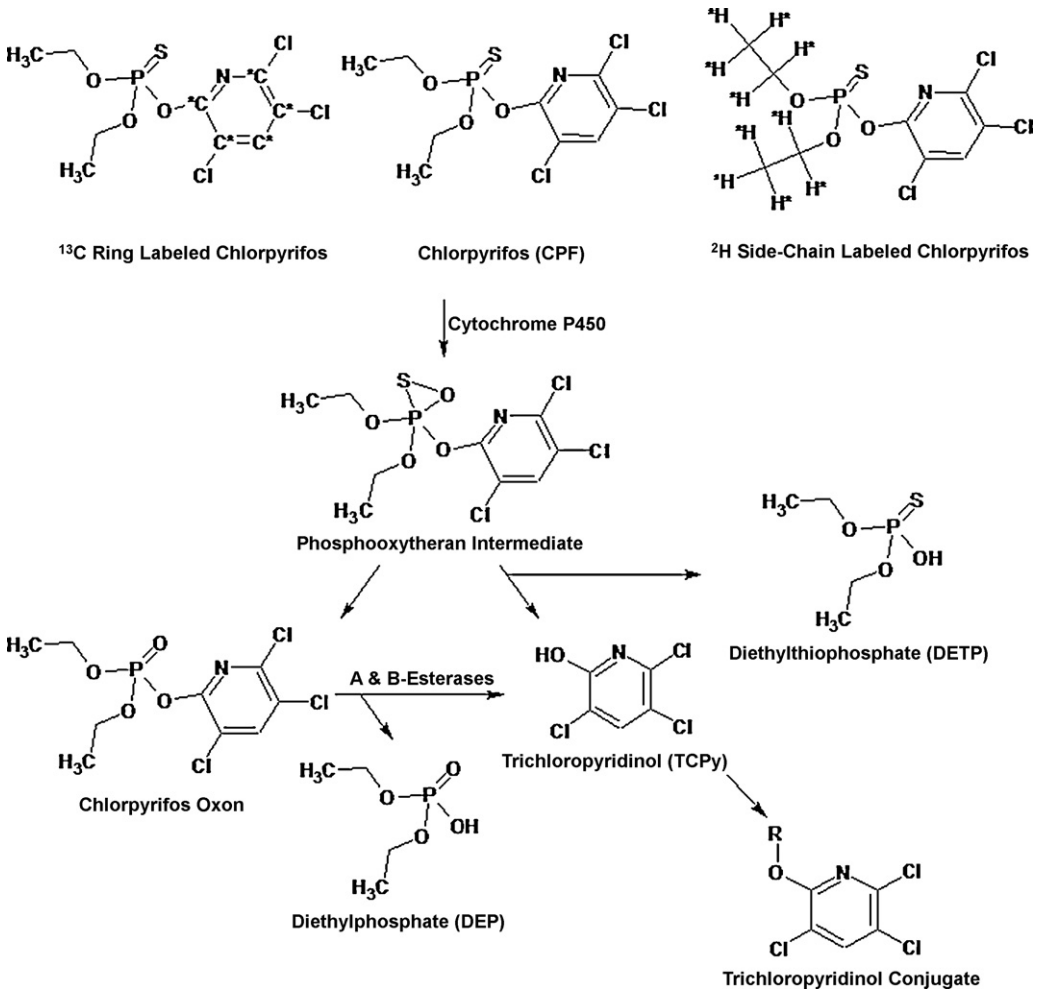

Fig. 1. Metabolic scheme for chlorpyrifos (CPF), ^{13}C-ring labeled CPF, and ^{2}H-side chain labeled CPF. ^{13}C-labeled carbons and ^{2}H-labeled hydrogens are noted by asterisks. The initial metabolism of CPF is mediated by CYP450 to form either chlorpyrifos-oxon (CPF-oxon) or trichloropyridinol (TCPy) and diethylthiophosphate (DETP). CPF-oxon undergoes further metabolism by both A- and B-esterases to form diethylphosphate (DEP) and TCPy, which can undergo phase II sulfation and glucuronidation.

is readily eliminated via the urine (Bakke et al., 1976; Nolan et al., 1984), as are the dialkylphosphate metabolites, DEP and DETP (Griffin et al., 1999).

The CPF metabolite, TCPy, and the non-specific OP metabolites, DEP and DETP, are routinely analyzed in blood and urine to biomonitor for CPF and other dialkylphosphate insecticide exposures, respectively. Detection of the parent insecticide in blood would represent the most accurate method for assessing a specific pesticide exposure (Needham, 2005); however, CPF is rapidly metabolized to TCPy making it difficult to quantify in blood (Griffin et al., 1999; Nolan et al., 1984). Biomonitoring of CPF in blood is further complicated due to the need for sophisticated analytical methods to obtain very low detection limits (pg/g) required for environmentally relevant exposure levels (Barr and Needham, 2002; Whyatt et al., 2003, 2005). Also, the collection of multiple blood samples for large biomonitoring studies is challenging due to the invasive nature of sample collection; an especially important consideration when biomonitoring young children. Hence, urinary biomonitoring of the CPF specific metabolite TCPy and the dialkylphosphate OP metabolites (DEP and DETP) remains the simplest and most common method of estimating CPF and dialkylphosphate OP pesticide exposures.

Further complicating the interpretation of dialkyphosphate and TCPy biomonitoring data is the pervasive presence of these metabolites in the environment (Lu et al., 2005). Several key studies have been conducted that characterized the environmental persistence of OP metabolites (Krieger et al., 2003; Lu et al., 2005; Needham et al., 2005). These studies suggest a potential for overpredicting human exposures due to the pervasive presence of dialkylphosphate metabolites and more specific metabolites, like TCPy, in the environment. Whether these metabolites are readily absorbed, excreted unchanged, or altered before elimination has been an important question. Recent comparative pharmacokinetic studies in the rat suggests that DEP, DETP and TCPy can be readily absorbed and eliminated in the urine of rats and presumably humans (Timchalk et al., 2007a). There is a real potential for these metabolite co-exposures to confound CPF pharmacokinetics, obfuscating the interpretation of biomonitoring data. Hence, monitoring total urinary metabolite levels may be reflective of not only an individual's contact with the parent pesticide, but also exposure with the ubiquitously present individual metabolites. Thus, the presence of TCPy, DEP or DETP in urine or blood may not be indicative of actual CPF exposure.

As previously noted, comparative pharmacokinetic studies of CPF (50 mg/kg) and its key metabolites have been conducted following *in vivo* oral exposure in the rat to equimolar doses of CPF, DEP, DETP, or TCPy (Timchalk et al., 2007a). However, concurrent dosing studies of CPF and metabolites, to mimic real-world mixture exposures, have not been performed due to an inability to analytically distinguish between metabolites dosed individually or metabolized from the parent compound. In the current study, simultaneous dosing of ^{13}C- or ^{2}H-isotopically labeled CPF (^{13}C-

labeled CPF, 5 ^{13}C on the TCPy ring; or ^{2}H-labeled CPF, diethyl-D10 (deuterium labeled) on the side chain: see Fig. 1) were exploited to directly compare the pharmacokinetics and metabolism of CPF with TCPy and DETP when the metabolites were co-administered (oral gavage) with the parent compound at equal molar doses.

The current study was designed to assess the implications of metabolite and parent insecticide co-exposure on the uptake, metabolism and excretion of the key metabolites and parent insecticide in the rat. Although previous studies have evaluated the individual metabolite pharmacokinetics (Timchalk et al., 2007a) there are currently no studies evaluating the potential pharmacokinetic interactions for co-exposures. In this regard, the null hypothesis was that co-exposure to equal molar doses of metabolites (TCPy, DETP or DEP) would impact the pharmacokinetics of CPF derived metabolites. In addition, it was anticipated that these studies would provide needed pharmacokinetic parameters to describe the kinetics for individual metabolite and combined exposures. These data will be particularly useful for understanding the impact of combined exposures on CPF dosimetry and can be further exploited to understand the contribution of metabolite residue exposures on biomonitoring results.

2. Materials and methods

2.1. Chemicals

Diethylphosphate (DEP, CAS 598-02-7, 98% pure) was purchased from Chem Service Inc. (West Chester, PA), TCPy (CAS 6515-38-4, 99% pure) was provided by Dow AgroSciences (Indianapolis, IN), and DETP (CAS 5871-17-0) was purchased from Sigma Aldrich as the potassium salt. The ^{13}C-labeled CPF and ^{2}H-labeled CPF were provided by the CDC (Center for Disease Control, National Center for Environmental Health, Atlanta, GA). All other solvents and reagents, including the derivatizing agent (N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide, CAS 77377-52-7) (MTBSTFA) were of reagent grade or better and purchased from Sigma Chemical Company (St. Louis, MO).

2.2. Animals

Adult male Sprague–Dawley rats with and without indwelling jugular vein cannula were purchased from Charles River Laboratories (Raleigh, NC). All procedures using animals were in accordance with protocols established in the NIH/NRC *Guide and Use of Laboratory Animals* and were reviewed by the Institutional Animal and Care Use Committee of Battelle, Pacific Northwest Division.

2.3. In vitro metabolism

Two naïve adult male Sprague–Dawley rats (0.5 and 0.6 kg) were used to prepare liver microsomes to compare *in vitro* metabolism of labeled and unlabeled CPF. Livers were perfused with phosphate buffered saline (PBS) and excised from rats sacrificed by CO_2 asphyxiation. All microsomal preparation procedures were carried out on ice. Livers were immediately rinsed with PBS and homogenized. Microsomes were prepared following ultracentrifugation protocols as previously described (van der Hoeven and Coon, 1974). Liver tissue homogenate was centrifuged at 9000 × *g* for 20 min at 4 °C. The pellet was discarded and the remaining supernatant was ultracentrifuged at 100,000 × *g* for 1 h at 4 °C. The resulting pellet was resuspended in buffer and ultracentrifuged at 100,000 × *g* for 1 h at 4 °C. The final purified microsomal pellet was resuspended in buffer, aliquoted, and stored at −80 °C until use. Total protein concentration was determined using a BSA Assay (Pierce Research Products, Rockford, IL).

2.4. Incubation of ^{2}H-labeled and unlabeled CPF with microsomes

Hepatic *in vitro* incubations were conducted using methods previously described (Poet et al., 2003), briefly, 0.5 mg microsomal protein was incubated in 1 mL with 50 μM of either ^{2}H-labeled CPF or unlabeled CPF. This concentration was chosen to be within the linear range of *in vitro* metabolism of CPF to TCPy and CPF-oxon. Heat inactivated protein blanks and EDTA controls (to inhibit PON1 activity) were also incubated at each concentration. All incubations were initiated by an excess of NADPH and conducted in 0.1 sodium phosphate buffer for either 2 or 20 min. Termination of metabolism was achieved via the addition of sodium metabisulfite and HCl saturated with NaCl.

2.5. In vivo

Groups of male Sprague–Dawley rats were orally administered (via gavage) single equal molar doses of ^{13}C-labeled CPF and TCPy (Group I, $n = 10$), or ^{2}H-labeled CPF and DETP (Group II, $n = 4$). A targeted CPF dose of 5 mg/kg (14 μmol/kg) was selected to be within the linear kinetic range, provide adequate analytical sensitivity to quantify key metabolites in blood and urine, yet low enough to avoid acute cholinergic effects (CPF LD_{50} ~92 mg/kg). For each dose group labeled CPF and metabolites were premixed together in corn oil and administered as targeted molar doses of 14 μmol/kg for both the parent insecticide and metabolite in a dosing volume of 5 mL/kg. All dose solutions were prepared the day before use and refrigerated in a closed container until used. The administered doses of labeled CPF, TCPy, and DETP for each treatment group are presented in Table 1.

Rats were housed in glass metabolism cages designed for the separate collection of urine and feces. Rats were fed Purina Certified Rodent Chow® 5002 (Purina Mills, St. Louis, Mo USA) *ad libitum* with the exception that it was withdrawn 12 h prior to dosing and returned 3 h post-dosing. Water was available *ad libitum* throughout the duration of the study. Blood (100–200 μL) was collected through the jugular vein cannula at 0, 5 min, 15 min, and 0.5, 0.75, 1, 2, 3, 6, 8, 12, 24, 48, and 72 h post-dosing and replaced with the appropriate volume of physiological saline. Urine was collected continuously, and sample collections were accumulated for 0–12, 12–24, 24–48 and 48–72 h post-dosing. Rats were humanely euthanized at 72 h post-dosing.

2.6. Analytical methods

Standard curves were prepared alongside each set of samples in control matrices (urine or blood) spiked with TCPy (Group I), DETP (Group II), or DEP (*in vitro* only). Standard curves and samples underwent acidification with 1 M HCl saturated with NaCl, extraction with ethyl acetate, followed by 10 min of strong vortexing, and 30 min of centrifugation at 1500 × *g* at room temp. Each sample was extracted three times and the organic layers for each sample were pooled and dried over anhydrous Na_2SO_4 before being blown down under a gentle stream of inert N_2. To remove inorganic phosphate from urine, 10% (w/v) of calcium hydroxide ($Ca(OH)_2$) was added to samples and removed via centrifugation before extraction (Blair and Roderick, 1976). To prevent oxidation of DETP to DEP during the derivatization process, 10% (w/v) of sodium metabisulfite ($Na_2S_2O_5$) was added to urine and blood samples from the DETP treatment (Group II). Samples and standard curves were reconstituted in varying volumes of toluene to place them in instrumental linear range (0.1–50 μg/mL, coefficient variable range 0.97–0.99), and incubated with 10 μL of MTBSTFA derivatizing agent at 60 °C for 60 min.

2.7. GC/MS analysis

Mass spectral analyses of samples from Group I (^{13}C-CPF and TCPy), Group II (^{2}H-CPF and DETP) and *in vitro* (^{2}H-CPF and CPF) were performed using a HP5973 mass selective detector interfaced with an HP model 6890 GC and HP ChemStation software. Spectra were collected in the electron ionization mode at 70 eV with a mass range scanned from 50 to 500 amu. Separation was achieved in splitless mode (initial inlet temp 250 °C), on a DB5-MS column (30 m × 0.25 mm ID × 0.25 μm (or equivalent): J&W Scientific, Folsom, CA), with helium carrier gas, flow rate 1 mL/min, GC oven program 80 °C ramped at 10 °C/min up to a final temp of 310 °C, and total run time of 24 min. Selected ion monitoring (SIM) was utilized in instances where increased sensitivity was required. Ions selected for quantification included *m/z*

Table 1
Summary of doses of ^{13}C- and ^{2}H-labeled chlorpyrifos (CPF), 3,5,6-trichloro-2-pyridinol (TCPy), and diethylthiophosphate (DETP) orally administered to rats. The following 2-treatment groups were co-exposed to CPF and the specified metabolites: Group I (^{13}C-CPF + TCPy) and Group II (^{2}H-CPF + DETP).

	Group I (TCPy analysis) μmol/kg, $n = 10$		Group II (DETP analysis) μmol/kg, n = 4	
	TCPy dose	^{13}C-CPF dose	DETP dose	^{2}H-CPF dose
^{13}C-CPF	–	13.71 ± 0.59	–	–
^{2}H-CPF	–	–	–	13.26 ± 0.48
TCPy	12.71 ± 0.59	–	–	–
DETP	–	–	18.03 ± 0.67	–
BWT (kg)	0.244 ± 0.036		0.260 ± 0.018	

256 (TCPy) and m/z 261 (^{13}C-labeled TCP metabolized from ^{13}C-labeled CPF, ^{13}C-TCP), m/z 155 (DEP) and m/z 157 (^{2}H-labeled DEP metabolized from ^{2}H-labeled CPF, ^{2}H-DEP), and m/z 227 (DETP) and m/z 237 (^{2}H-labeled DETP metabolized from ^{2}H-labeled CPF, ^{2}H-DETP).

2.8. Pharmacokinetic and non-compartmental analysis

To estimate pharmacokinetic parameters, either a one or two-compartment pharmacokinetic model was fit to blood data from the various *in vivo* studies. Model structures were as follows:

One-compartment pharmacokinetic model (TCPy):

$$A_0 = \text{Dose} \times e^{-k_{a/f} \times t} \quad (1)$$

$$\frac{dA_1}{dt} = (k_{a/f} \times A_0) - (k_e \times A_1) \quad (2)$$

$$CA_1 = \frac{A_1}{V_1} \quad (3)$$

Two-compartment pharmacokinetic model (DETP):

$$A_0 = Dose \times e^{-k_{a/f} \times t} \quad (4)$$

$$\frac{dA_1}{dt} = (k_{a/f} \times A_0) - (k_e \times A_1) - (k_{12} \times A_1) + (k_{21} \times A_2) \quad (5)$$

$$\frac{dA_2}{dt} = (k_{12} \times A_1) - (k_{21} \times A_2) \quad (6)$$

$$CA_1 = \frac{A_1}{V_1} \quad (7)$$

For both TCPy and DETP it was assumed that 100% of the administered doses were absorbed. A_0, A_1, and A_2 represent the amount (μmol) of CPF metabolites in the absorption/formation, central, and peripheral compartments, respectively. Dose is the molar amount of labeled CPF or metabolites (TCPy, or DETP) administered. The rate constants $k_{a/f}$, k_e, k_{12}, and k_{21} are first-order transfer rates (h^{-1}) among compartments. The $k_{a/f}$ is a combined notation for both the absorption (k_a) and formation rates (k_f) which apply to the TCP/DETP and CPF dosing groups, respectively. V_1 represents the volume of distribution (L) for the central compartment. CA_1 is the concentration (μmol/L) of metabolites in the central compartment (blood), and t is time (h). Estimates of model parameters were obtained using the Nelder-Mead algorithm in acslX Version 2.4.2.1 software (AEgis Technologies Group, Inc., Huntsville, AL, USA). In addition, the two-compartment model rapid and slow disposition rates (α and β) and half-life ($t_{1/2}$) were calculated (Gibaldi and Perrier, 1982). Graphpad Prism® 4 was also used to calculate the area-under-the-curve (AUC) for blood using the trapezoidal rule (Gibaldi and Perrier, 1982).

3. Results

3.1. In vitro

Initial *in vitro* rat liver microsomal metabolism studies were conducted to evaluate the potential for a deuterium isotope effect that could modulate the rate of deuterium labeled CPF metabolism relative to an unlabeled substrate. Results from this analysis are presented in Fig. 2. In these studies, hepatic microsomes prepared from naïve male rats were used to quantify the CYP450-mediated metabolism of CPF resulting in the formation of the major degradation metabolites TCPy, DETP and DEP. Equal molar concentrations of either CPF (unlabeled) or ^{2}H-labeled CPF were evaluated. As expected, both forms of CPF were rapidly metabolized and the extent of metabolism was time-dependent with the greater amounts of metabolites being formed during longer incubation times (2 min versus 20 min). Of particular interest were the relative yields of metabolites utilizing the labeled versus unlabeled CPF. The relative yields of TCPy and DETP for the D10-labeled CPF ranged from 85% to 92% and 92% to 100%, respectively relative to the yield from the unlabeled CPF. In contrast, the yield of DEP from the D10-labeled CPF was substantially less (20% and 65% at 2 and 20 min, respectively) than the metabolite yield obtained with unlabeled CPF. These results are consistent with a deuterium kinetic isotope effect modulating D10-CPF metabolism to DEP, and based on these findings the comparative (labeled versus unlabeled CPF) *in vivo* metabolism studies utilizing D10-labeled CPF only focused on quantifying the CPF metabolism to DETP. For the TCPy comparative studies a ^{13}C-labeled CPF was utilized; hence a deuterium isotope effect was not a concern.

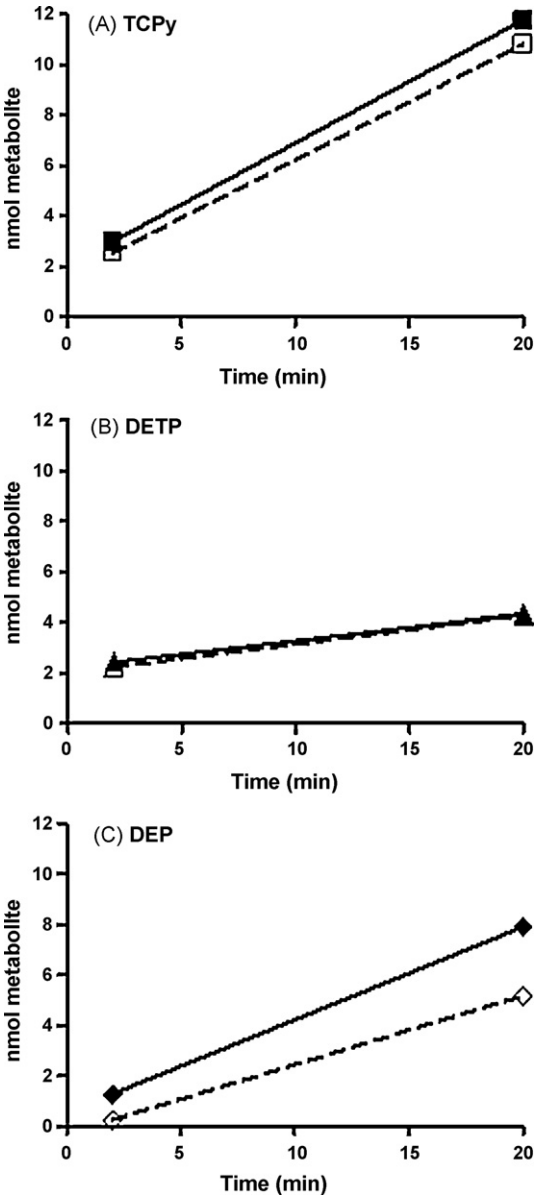

Fig. 2. *In vitro* microsomal metabolism of unlabeled chlorpyrifos (CPF; solid black symbols) or ^{2}H-labeled CPF (open symbols) following a 2 or 20 min incubation. The results are presented as nmol of metabolite formed for: (A) trichloropyridinol (TCPy; square symbol), (B) diethylthiophosphate (DETP; triangle symbol), and (C) diethylphosphate (DEP; diamond symbol). The line between data is included for illustration purposes only.

3.2. In vivo

The administered doses of labeled CPF and metabolites are presented in Table 1. For treatment Group I, and Group II the molar doses of the TCPy, and DETP metabolites were 93%, and 136%, of the corresponding labeled CPF doses, respectively. Although the DETP molar dose was slightly higher (~18 μmol/kg versus 13 μmol/kg) than the co-administered ^{2}H-CPF dose, substantial pharmacokinetic differences were not anticipated. Hence, these molar co-exposures allowed for a direct pharmacokinetic comparison of TCPy and DETP, either dosed individually or resulting from the metabolism of CPF.

3.3. Trichloropyridinol (TCPy) pharmacokinetics (Group I)

The blood TCPy concentrations, interval urinary excretion, blood, and urinary time-course of TCPy following a simultaneous

Table 2
Blood concentration of 3,5,6-trichloro-2-pyridinol, TCPy (Group I), and diethylthiophosphate, DETP (Group II) following equal molar single oral (gavage) doses of CPF or metabolites in rats.

Time post-dosing (h)	Blood metabolites concentration (nmol/mL blood)			
	Group I (TCPy analysis)		Group II (DETP analysis)	
	TCPy dose	^{13}C-CPF dose	DETP dose	^{2}H-CPF dose
0	–	–	–	–
0.08	6.96 ± 6.1 (7)	ND	1.71 ± 0.9 (5)	0.22 ± 0.1 (5)
0.25	15.96 ± 4.2 (8)	0.25 ± 0.2 (3)	2.16 ± 1.2 (5)	0.50 ± 0.2 (5)
0.5	13.76 ± 4.3 (8)	0.42 ± 0.2 (5)	1.65 ± 0.8 (5)	0.52 ± 0.2 (5)
0.75	12.18 ± 5.1 (8)	0.90 ± 0.5 (5)	1.59 ± 1.1 (5)	0.59 ± 0.1 (5)
1	7.22 ± 3.6 (7)	0.67 ± 0.4 (5)	1.30 ± 1.3 (5)	0.62 ± 0.5 (5)
2	5.49 ± 2.5 (8)	1.42 ± 0.4 (5)	1.68 ± 1.0 (5)	1.26 ± 0.7 (5)
3	5.18 ± 3.7 (8)	3.50 ± 2.3 (6)	1.75 ± 1.3 (5)	1.61 ± 0.7 (5)
6	5.45 ± 4.4 (8)	9.24 ± 6.2 (5)	1.10 ± 0.2 (3)	1.10 ± 0.6 (5)
8	3.16 ± 1.7 (7)	5.48 ± 2.6 (7)	0.66 ± 0.4 (4)	0.51 ± 0.3 (5)
12	1.88 ± 1.4 (4)	2.22 ± 2.1 (6)	0.51 ± 0.2 (4)	0.43 ± 0.2 (5)
24	ND	ND	0.47 ± 0.3 (3)	0.16 ± 0.0 (5)
48	ND	ND	0.39 ± 0.2 (3)	0.29 ± 0.2 (5)
72	ND	ND	ND	ND

Data is mean ± SD (*N*). ND = Non-detected.

equal molar dosing of ^{13}C-labeled CPF and TCPy are presented in Tables 2 and 4 and Figs. 3 and 5, respectively. Following oral administration of TCPy, a maximum blood concentration of ~16 ± 4 nmol/mL was obtained within 15 min of dosing; whereas, following a molar equivalent oral dose of CPF the maximum blood TCPy concentration was slightly lower (9 ± 6 nmol/mL), and was not obtained until ~6 h post-dosing. Although the blood TCPy concentrations were substantially different early after administration of the TCPy and CPF doses (Fig. 3, insert), by 3 h post-dosing the measured blood TCPy concentrations were comparable. These blood time-course TCPy data for both the TCPy and CPF doses were both fit to a one-compartment model (Fig. 3 and Table 3). Major pharmacokinetic differences were noted early after dosing (<2 h), which are consistent with the needed metabolic steps (primarily CYP450-mediated dearylation) directly converting CPF to TCPy. Nonetheless, once a substantial amount of the CPF has been metabolized to TCPy (≥3 h post-dosing) the pharmacokinetics for both treatment groups became more similar. Based on the pharmacokinetic model fits of the blood TCPy time-course data for the TCPy and CPF dose groups, the terminal phase half-lives were 3.6 and 6.3 h, respectively (see Table 4). Again, the slightly slower (1.8-fold) half-life for TCPy for CPF relative to direct TCPy dosing is consistent with the needed metabolic step (CPF → TCPy). Nonetheless, by 72 h post-dosing, the TCPy blood AUC's for the TCPy and CPF doses were 57.6 and 53.3 nmol mL^{-1} h^{-1} respectively; which is consistent with the rapid and near complete metabolism of CPF to TCPy (Timchalk et al., 2007a).

The interval amount of urinary TCPy excreted (nmol) and excretion rates (nmol h^{-1}), for free and acid-hydrolyzed samples, following the TCPy or CPF oral dose are presented in Table 4 and

Table 3
Pharmacokinetic parameters of compartmental models fit to blood levels of 3,5,6-trichloro-2-pyridinol, TCPy (Group I), diethylphosphate, DETP (Group II), following equal molar single oral (gavage) doses of CPF or metabolites in rats.

	Pharmacokinetic parameters			
	Group I (TCPy analysis)		Group II (DETP analysis)	
	TCPy dose	^{13}C-CPF dose	DETP dose	^{2}H-CPF dose
Blood $t_{1/2}$ (h)	3.79	6.31	2.57 ($t_{1/2\alpha}$) 37.98 ($t_{1/2\beta}$)	1.63 ($t_{1/2\alpha}$) 33.57 ($t_{1/2\beta}$)
Blood AUC (nmol mL^{-1} h^{-1})	57.6	53.3	29.4	19.4
k_a (h^{-1})	11.9	N/A	13.5	N/A
k_f (h^{-1})	N/A	0.24	N/A	0.42
k_e (h^{-1})	0.18	0.11	0.06	0.12

Note: k_a is the first-order absorption rate constant for metabolites. k_f is the first-order metabolite formation rate. k_e is the first-order elimination rate.

Table 4
Urinary excretion (amount) for of free and total 3,5,6-trichloro-2-pyridinol, TCPy (Group I), diethylthiophosphate, DETP (Group II) following equal molar single oral (gavage) doses of CPF or metabolites in rats.

Collection intervals (h)	Urine metabolites excretion (nmol)					
	Group I (free TCPy analysis)		Group I (total TCPy analysis)		Group II (DETP analysis)	
	TCPy dose	^{13}C-CPF dose	TCPy dose	^{13}C-CPF dose	DETP dose	^{2}H-CPF dose
0–12	324 ± 112	282 ± 109	2067 ± 801	1626 ± 616	468 ± 180	333 ± 120
12–24	237 ± 90	260 ± 117	455 ± 242	634 ± 272	401 ± 291	287 ± 248
24–48	70.6 ± 27.5	100 ± 52	43.3 ± 13.8	74.7 ± 19.1	66.9 ± 44.9	46.0 ± 18.0
48–72	42.1 ± 11.7	43.9 ± 11.8	17.5 ± 10.7	22.6 ± 11.8	34.5 ± 4.39	28.3 ± 2.93
Total	673 ± 196	686 ± 211	2583 ± 820	2358 ± 718	1001 ± 508	750 ± 272
Average recovery	21.7%	20.5%	83.3%	70.5%	21.3%	21.5%

Data is mean ± SD, *n* = 5 animals/treatment group.

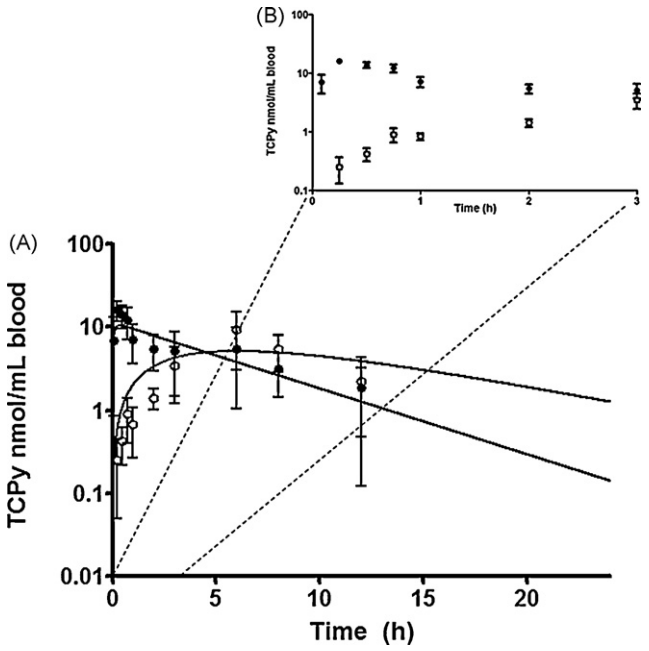

Fig. 3. (A) Time-course of trichlorpyridinol (TCPy) following an equal molar oral dose (~5 mg/kg equivalent) of unlabeled TCPy (solid circle symbol) or ^{13}C-labeled chlorpyrifos (CPF; open circle symbol). Lines represent the pharmacokinetic model fit to the experimental data. (B) Expanded scale between 1 and 3 h post-dosing. Data represents mean ± SD, for n = 3–8.

Fig. 4. These results are likewise consistent with the observed blood pharmacokinetics of TCPy, where the amount of TCPy excreted in the early intervals (0–12 and 12–24 h post-dosing) are slightly greater for the TCPy dose, again most likely due to the CPF metabolism step. Conjugated TCPy (acid-labile) accounted for the majority of the urinary metabolite for the 0–12 and 12–24 h collection intervals, resulting in a total urinary recovery ranging from 70% to 83% of the administered dose; whereas, free TCPy accounted for ~21% of the dose. The urinary excretion rates (nmol h^{-1}; Fig. 4) for both total and free TCPy were likewise very comparable for both the TCPy and CPF doses; suggesting that there is no pharmacokinetic interactions.

3.4. Diethylthiophosphate (DETP) pharmacokinetics (Group II)

Blood DETP concentrations, interval urinary excretion, blood and urinary time-course of DETP following a simultaneous equal molar dosing of ^{2}H-labeled CPF and DETP are presented in Tables 2 and 4 and Figs. 5 and 4, respectively. In the case of DETP, a maximum blood concentration of 2.2 ± 1.2 nmol/mL was obtained within 15 min of dosing. Following a molar equivalent oral dose of CPF the maximum blood DETP concentration was comparable at 1.6 ± 0.7 nmol/mL, but was not obtained until ~3 h post-dosing. As was the case with TCPy, the time-course of DETP were initially quite different for the DETP and CPF doses, but became comparable within 1 h post-dosing (Table 2 and Fig. 5) and were reasonably described using a 2-compartment pharmacokinetic model. The observed early (<1 h) pharmacokinetic differences (Fig. 5 insert) were consistent with the rapid and substantial metabolism of CPF to both TCPy and DETP (see Fig. 1). The 2-compartment models for either the DETP or CPF dosed groups were comparable with the terminal phase (β) half-lives of 38 and 34 h, respectively (Table 3). The DETP blood AUC's for the DETP and CPF doses were 29.4 and 19.4 nmol mL^{-1} h^{-1} respectively; the slightly lower AUC for CPF is consistent with the importance of metabolism and the 34-38 h terminal phase half-life for DETP. The amount of DETP excreted in urine (nmol) and the urinary excretion rates (nmol h^{-1}) following the co-exposures to DETP and CPF are presented in Table 4 and Fig. 4. These results are likewise coherent with observed blood pharmacokinetics of DETP, where the amount of DETP excreted in urine was slightly greater for the DETP dose; however, the overall urinary excretion kinetics were very comparable. Nonetheless, by 72 h post-dosing for both doses (CPF and DETP) urine accounted for

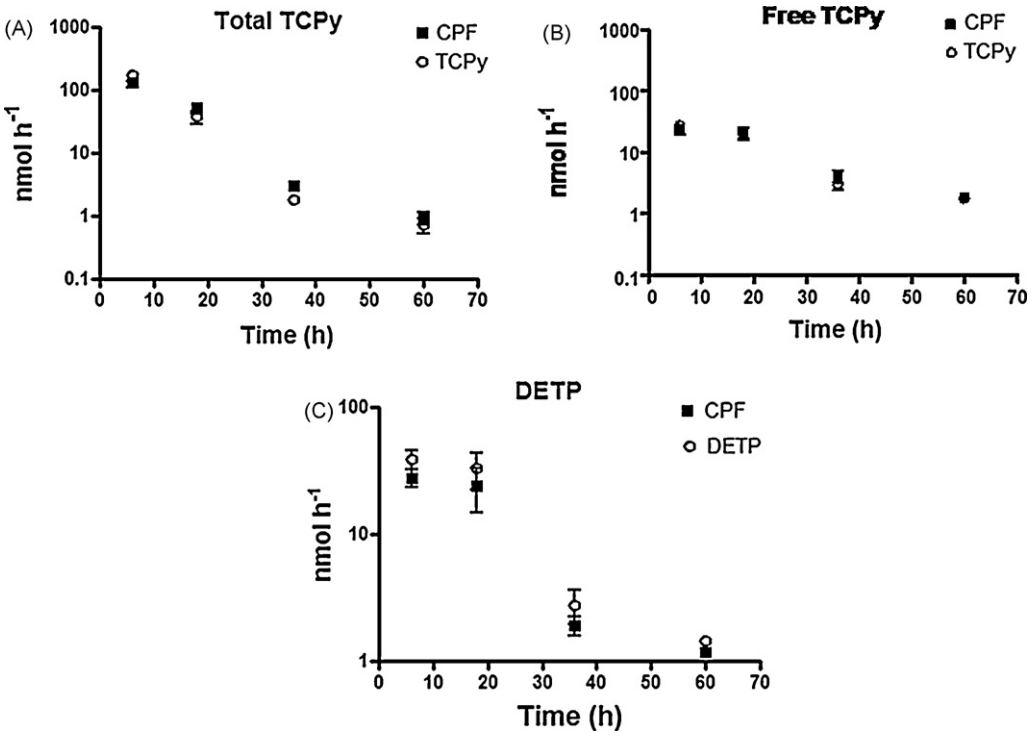

Fig. 4. Urinary excretion rate for (A) total trichlorpyridinol (TCPy), (B) free TCPy, and (C) diethylthiophosphate (DETP) in rats following an equal molar oral dose (~5 mg/kg equivalent) of unlabeled TCPy or DETP (open circles symbols) or 13C-/2H-labeled chlorpyrifos (CPF; solid square symbols). Data represents mean ± SD for n = 5.

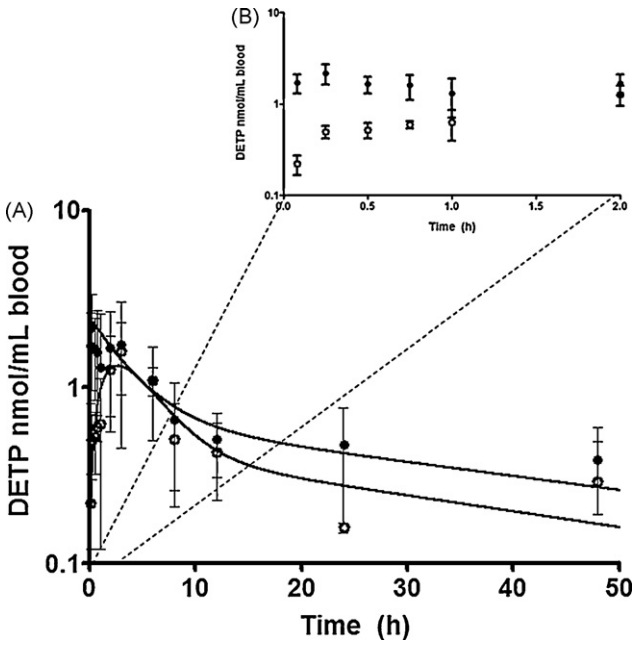

Fig. 5. (A) Time-course of diethylthiophosphate (DETP) following an equal molar oral dose (~5 mg/kg equivalent) of unlabeled DETP (solid circle symbol) or ^{2}H-labeled chlorpyrifos (CPF, open circle symbol). Lines represent the pharmacokinetic model fit to the experimental data. (B) Expanded scale between 1 and 2 h post-dosing. Data represents mean ± SD, for n = 3–5.

~22% of the dose administered as DETP, suggesting that urine is not the major elimination pathway for this metabolite.

4. Discussion

The current study was designed to evaluate the pharmacokinetic implications of co-oral exposure to CPF and its major metabolites, specifically to ascertain whether exposure to low doses of metabolites would impact the pharmacokinetics of CPF. The use of isotopically labeled (^{13}C- and ^{2}H-labeled) CPF enabled discernment of the individual metabolite pharmacokinetics versus those resulting from CPF metabolism. The combined CPF and metabolite doses (14 μmol/kg for each) were selected to be within the linear pharmacokinetic range (Timchalk et al., 2002). The initial focus was on TCPy, DETP and DEP since these represent the major CPF metabolites utilized for biological monitoring (Barr and Angerer, 2006; Duggan et al., 2003; Hardt and Angerer, 2000; Nolan et al., 1984).

As we previously noted, a number of studies have reported the detection of the OP breakdown products (DETP, DEP and TCPy) in fruit juices as well as solid food samples obtained from homes and day care centers, suggesting the potential for alternative metabolite exposure sources (Lu et al., 2005; Morgan et al., 2005; Wilson et al., 2003). Lu et al. (2005), utilizing a computational model, simulated urinary DETP and DEP levels based upon environmental and dietary exposure and noted that urinary levels of these metabolites were consistently higher than model predicted values. In this context, we recently reported that DETP, DEP and TCPy were readily absorbed following oral administration in rats (Timchalk et al., 2007a). Assuming similar human absorption, the excessive amounts of these metabolites in human urine (Lu et al., 2005) are consistent with absorption of these metabolites as residues from food. Hence, the current study evaluated the pharmacokinetic implications of co-exposure to CPF and its major metabolic breakdown products.

In vitro metabolism studies are consistent with a deuterium kinetic isotope effect where the presence of the deuterium label on DEP resulted in substantially less DEP formation (20–65%) than what was observed with unlabeled CPF (Fig. 2); suggesting that CYP450-mediated metabolic activation of CPF to CPF-oxon and DEP (see Fig. 1) was slowed by the presence of diethyl deuteriums. Similar isotope effects have been well documented for the metabolism of a range of chemicals and drugs including CYP450-mediated substrate catalysis (Baillie, 1981; Kim et al., 2006; Krauser and Guengerich, 2005; Mizutani et al., 1983; Swann et al., 1983; Vree et al., 1971a,b; Yun et al., 2005), resulting in altered rates of *in vitro* and *in vivo* metabolism in animals and humans. Hence based on these findings, the *in vivo* study only focused on the metabolites TCPy (^{13}C-labeled) and DETP, since no isotope effects were observed for these metabolites (Fig. 2).

Previous studies have evaluated the pharmacokinetics of TCPy and DETP in the rat following a single oral dose of approximately 50 mg/kg (Timchalk et al., 2007a). In the current study, we likewise evaluated the pharmacokinetics of TCPy and DETP, but as a co-exposure with an equal molar dose of the parent insecticide CPF, and at a substantially lower dose level (~5 mg/kg). The overall results of the current investigation are consistent with these earlier finding, suggesting that although TCPy and DETP were co-administered with CPF, the individual metabolite pharmacokinetics were not substantially altered due to the co-administration.

In the current study, major differences in the pharmacokinetics between CPF and metabolite doses were observed within the first 3 h of exposure, due to the required metabolism of CPF to initially form TCPy and DETP. Although a number of previous pharmacokinetic studies have strongly suggested that CPF is rapidly metabolized (Poet et al., 2003; Smith et al., 2009; Timchalk et al., 2007a, 2002, 2006), this is the first study that characterized the early phase of CPF metabolism to TCPy and DETP and directly compared it to the individual metabolites. Although the pharmacokinetics of TCPy and DETP are clearly different, there are qualitative similarities associated with the CYP450 metabolism of CPF to TCP and DETP. As illustrated in Fig. 1 the metabolism of CPF to TCPy involves both a CYP450-mediated dearylation to form TCPy and DETP and oxidative desulfuration forming CPF-oxon. Hepatic and extra-hepatic A-esterases such as PON-1 and tissue B-esterase (cholinesterase; ChE) effectively metabolize CPF-oxon forming TCPy and diethylthiophosphate (Chambers and Chambers, 1989; Ma and Chambers, 1994). In the current study, both dearylation and desulfuration contributed to the formation of TCPy from CPF; whereas, DETP was exclusively formed via dearylation. Hence, differences in metabolism rates along with individual metabolites pharmacokinetic properties most likely account for any observed differences between TCPy and DETP pharmacokinetics and urinary excretion. Nonetheless, the pharmacokinetics of the individual metabolites were not modified by co-exposure to CPF; this suggests that environmental exposure to low dose mixtures of pesticides and metabolites will not impact the pharmacokinetics of either.

These studies are consistent with previous reports that have raised concerns about the impact of metabolite residues on the determination of OP insecticide dosimetry (Barr et al., 2005; Lu et al., 2005; Morgan et al., 2005) and further support the need to modify the current biomonitoring strategy to more effectively use DEP, DETP and TCPy as quantitative biomarkers for exposure (Timchalk et al., 2007a). The lack of pharmacokinetic interactions between CPF and its degradation metabolites can potentially be exploited to quantitatively assess exposures. In this regard, it may be feasible to utilize our understanding of the stoichiometric relationship between the CPF metabolites to understand the dosimetric contribution of metabolite residues versus the parent insecticide (Timchalk et al., 2007a). As has been suggested, more useful approaches may incorporate both biomonitoring along with environmental media sampling and assessment of dietary contribution (Lu et al., 2005). In addition, as noted by Needham (2005),

biomonitoring for the parent insecticide (CPF) still represents the most relevant biomarker, but quantitation is particularly challenging. Nonetheless, concurrent measurement of parent insecticide and metabolites in both blood and urine of individual subjects would provide the greatest degree of confidence in assessing systemic dosimetry (Timchalk et al., 2007a).

It has been suggested that computational dosimetry models could be exploited to help discern the potential dosimetric contribution of metabolites versus the parent insecticide (Timchalk et al., 2007a). Specifically, these computational models could be used to establish and predict the ratio of major urinary metabolites (i.e. DEP, DETP and TCPy), and subsequently used to discern alternative exposure sources. In this context, a number of pharmacokinetic and pharmacodynamic models for CPF and related OP insecticides have been developed (Poet et al., 2004; Rigas et al., 2001; Timchalk et al., 2007a,b, 2002; Timchalk and Poet, 2008). Finally, additional sensitive and specific OP biomarkers are needed that are only associated with direct exposure to the parent insecticide CPF or its toxic metabolite (i.e. CPF-oxon). Efforts are underway to develop novel immunosensor platforms for the detection of modified ChE enzyme resulting from OP specific phosphorylation (Barry et al., 2009; Liu et al., 2008; Wang et al., 2008). It is envisioned that once validated, these new biomarkers will provide the needed specificity and sensitivity for use as a field deployable biosensor, without any potential for confounding results due to pesticide metabolite that are found both within the environment and as residues on food.

In conclusion, the current study indicates that TCPy and DETP present in the environment can be readily absorbed and eliminated in the urine of rats. Furthermore, co-exposure to these metabolites and CPF at equal molar doses had no substantial impact on the pharmacokinetics of the CPF derived metabolites. It is speculated that there would likewise be a lack of pharmacokinetic interaction for humans that are co-exposed to CPF and metabolites, particularly since the anticipated human exposure levels would be at substantially lower doses than used in the current study. It is anticipated that these results can be further exploited to understand the dosimetric contribution of metabolite residues versus the parent insecticide and computational models can be utilized to predict alternative exposure sources. Nonetheless, based on these and other supporting data care must be taken in the interpretation of CPF biomonitoring results based upon the analysis of TCPy and DAP metabolites in urine.

Conflict of interest

Drs. Charles Timchalk and Torka Poet have received funding from The Dow Chemical Company, the manufacturer of chlorpyrifos, to conduct research. The Dow Chemical Company had no input on the current research.

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