

Pharmacokinetics and Pharmacodynamics of Chlorpyrifos and 3,5,6-Trichloro-2-pyridinol in Rat Saliva After Chlorpyrifos Administration

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Sensors have been developed for noninvasive biomonitoring of the organophosphate pesticide chlorpyrifos (CPF), and previous studies have suggested consistent partitioning of 3,5,6-trichloro-2-pyridinol (TCPy), a metabolite of CPF, into saliva after exposure to TCPy. The objective of this study was to quantitatively evaluate *in vivo* pharmacokinetics and pharmacodynamics of CPF and TCPy in saliva after CPF administration. Rats were coadministered CPF (0.5–5 mg/kg) and pilocarpine (~13 mg/kg) *iv*. Saliva and blood were collected, and levels of CPF, TCPy, and cholinesterase (ChE) activity were quantified. Experimental results suggest that CPF is rapidly metabolized after *iv* administration. Formation of TCPy from administered CPF at the low dose (0.5 mg/kg) was slower than from higher CPF doses, potentially due to differences in plasma protein binding to CPF. CPF was measured in saliva only at the first time point sampled (0–15 min), indicating low partitioning and rapid metabolism. After formation, TCPy pharmacokinetics were very similar in blood and saliva. Saliva/blood TCPy concentration ratios were not affected by TCPy concentration in blood, saliva flow rate, or salivary pH and were consistent with previous studies. ChE activity in plasma demonstrated a dose-dependent decrease, and ChE activity in saliva was extremely variable and demonstrated no dose relationship. A physiologically based pharmacokinetic and pharmacodynamic model for CPF was modified and predicted the data reasonably well. It is envisioned that a combination of biomonitoring compounds like TCPy in saliva coupled with computational modeling will form an approach to measure pesticide exposure to susceptible human populations such as agricultural workers.

Key Words: chlorpyrifos; 3,5,6-trichloro-2-pyridinol; trichloro-pyridinol; TCPy; pharmacokinetics; saliva; biomonitoring.

Biomonitoring techniques have become valuable tools for quantitatively evaluating human exposures to chemicals from both occupational and environmental sources. Noninvasive matrices (e.g., saliva) have been advocated over traditional matrices (e.g., blood and urine) in order to ease sample collection, maximize volunteer participation, and potentially reduce cost (Kaufman and Lamster, 2002; Nigg and Wade, 1992).

A number of chemicals and biomarkers of chemicals have been found in saliva, and many of these have been evaluated as for potential noninvasive biomonitoring candidates, including chlorpyrifos (CPF) (Hayashi *et al.*, 1989; Kaufman and Lamster, 2002; Lu *et al.*, 1997a, 2003; Schramm *et al.*, 1992; Timchalk *et al.*, 2007).

CPF is a broad-spectrum organophosphorus (OP) insecticide used in agricultural production of a variety of crops. Once absorbed into the body, CPF can be metabolized by cytochrome P450 enzymes to undergo dearylation (oxidative ester cleavage), forming 3,5,6-trichloro-2-pyridinol (TCPy) and diethylthiophosphate, or oxidative desulfuration, forming CPF-oxon (Fig. 1) (Kamataki *et al.*, 1976). CPF-oxon can bind to the active site of various B-esterase enzymes (e.g., acetylcholinesterase) effectively inhibiting them. Thus, CPF-oxon is widely considered the ultimate toxicant from CPF exposure. Alternatively, CPF-oxon can also be further metabolized by A- and B-esterases, forming TCPy and diethylphosphate (Chanda *et al.*, 1997; Pond *et al.*, 1998). TCPy can be excreted in urine or further metabolized by phase II conjugation and excreted as glucuronide, sulfate, or other conjugates in urine (Bakke *et al.*, 1976; Nolan *et al.*, 1984).

Sensors have been developed for noninvasive biomonitoring of CPF in saliva using several different approaches, including salivary measurements of TCPy, cholinesterase (ChE) activity, and phosphorylated ChE. The original technique developed for TCPy monitoring in saliva is a competitive immunoassay using TCPy antibodies coupled with horseradish peroxidase for electrochemical signal conversion (Liu *et al.*, 2005, 2006). More recently, immunoassays with greater sensitivity have been developed including optimized versions of the horseradish peroxidase sensor (Wang *et al.*, 2011) and a sensor utilizing quantum dot labels for signal propagation (Zou *et al.*, 2010). The most recent sensors allow for detection of 0.1 ng/ml TCPy (Wang *et al.*, 2011). A second salivary CPF biomonitoring approach is based on the measurement of an enzymatic product of ChE activity (Wang *et al.*, 2008b). This technique has been advanced using strategies to account for the requirement of

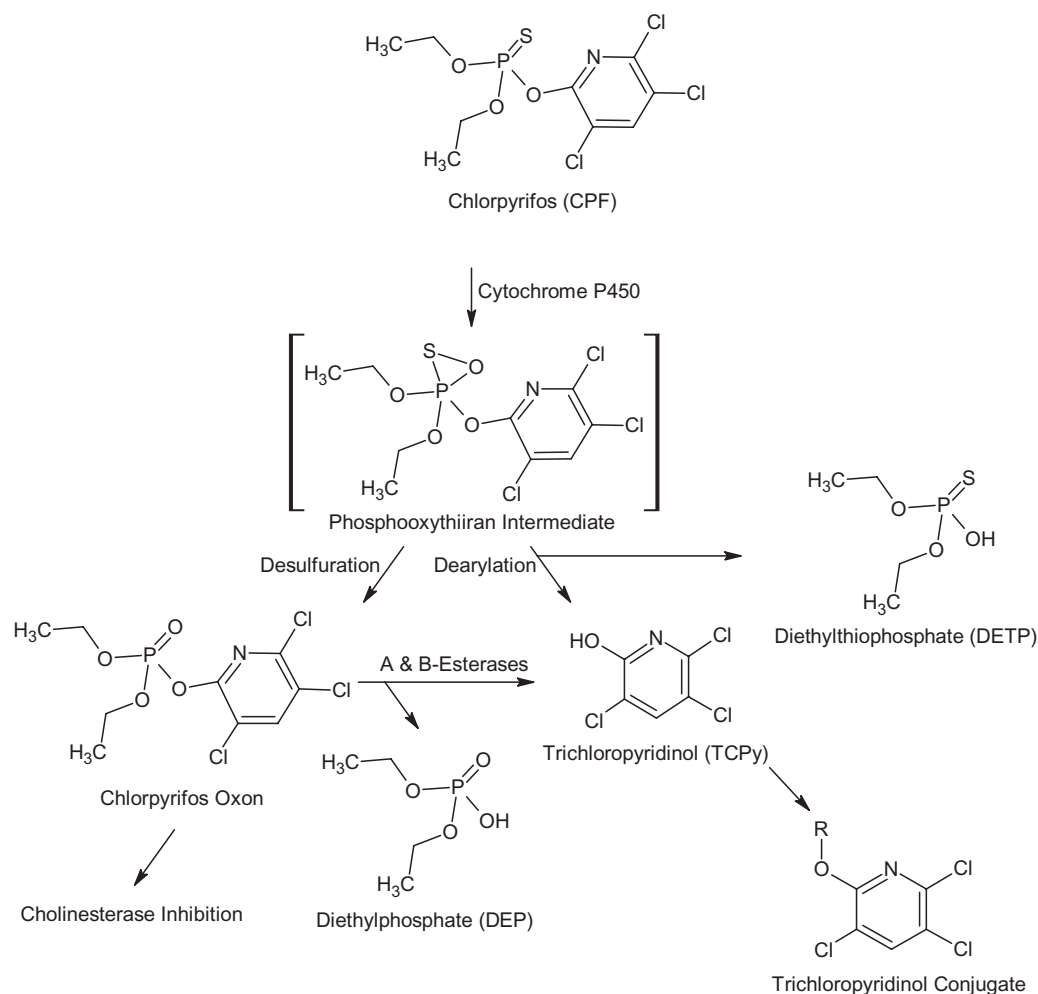


FIG. 1. Enzymatic metabolism of CPF, CPF-oxon, and TCPy.

control ChE samples, including reactivation techniques (Du *et al.*, 2009) as well as simultaneous measurement of activity and amount of enzyme (Du *et al.*, 2011). A final biomonitoring approach is based upon the measurement of phosphorylated ChE as a biomarker of exposure to OP (Liu *et al.*, 2008; Wang *et al.*, 2008a).

In order to make full use of these sensors, there is a critical need for a robust understanding of CPF pharmacokinetics and pharmacodynamics in saliva. Timchalk *et al.* (2007) was the first to measure TCPy in the saliva of rats after CPF exposure and suggested that saliva could be used as a biomonitoring matrix. More recently, Smith *et al.* (2010) demonstrated that after TCPy exposure to the rat, TCPy partitioning from plasma to saliva was relatively consistent over a range of varying conditions, including TCPy concentration in blood and saliva flow rate. Results were integrated within a physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model for CPF and accurately predicted TCPy concentrations in saliva (Smith *et al.*, 2010; Timchalk *et al.*, 2002). Although these studies provide a proof-of-principle and a good quantification

of salivary TCPy clearance, characterization TCPy clearance after CPF exposure has yet to be conducted as well as impact of ChE inhibition in saliva. These characterizations are critical for maximizing potential impact of developing sensors.

The objective of this study was to quantitatively evaluate *in vivo* pharmacokinetics and pharmacodynamics of CPF and TCPy in saliva after CPF administration. Experimental methodology used in this study was similar to our previous work with TCPy, the major metabolite of CPF (Smith *et al.*, 2010), and to what others had previously used for other pesticides, including atrazine (Lu *et al.*, 1997a,b, 1998) and diazinon (Lu *et al.*, 2003). Results from these investigations showed relatively consistent transfer of both parent pesticides and metabolites from blood to saliva, which is promising for potential biomonitoring. Finally, pharmacokinetic data from this study were integrated into a PBPK/PD model of CPF (Smith *et al.*, 2010; Timchalk *et al.*, 2002). It is envisioned that a combination of real-time analysis capability from various developing approaches for CPF biomonitoring in saliva and data extrapolating potential of PBPK/PD models including salivary

clearance will form a novel platform for CPF biomonitoring of susceptible and/or sensitive populations, such as agricultural workers. This complete platform will have advantages over conventional methods of being noninvasive, portable, and real-time, which could all potentially save time and money and protect the public when rapid pesticide exposure assessments are required.

MATERIALS AND METHODS

Chemicals. CPF, TCPy, and TCPy- $^{13}\text{C}_2$ - ^{15}N were kindly provided by the Dow Chemical Company (Midland, MI). The TCPy isotope was stable with the ^{13}C located at the 2 and 6 positions and the ^{15}N located at the 1 position of the pyridinol ring. N-(tert-butylidimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA), ethyl acetate, toluene, and other general laboratory chemicals were purchased from Sigma-Aldrich (St Louis, MO) and were reagent grade or better.

Animals. Adult male Sprague Dawley rats (300–400 g) were purchased from Charles River Laboratories Inc. (Wilmington, MA). Rats were housed in solid bottom cages with hardwood chips under standard laboratory conditions. Water and feed (PMI 5002, Certified Rodent Diet) were provided *ad libitum*. All procedures involving animals were in accordance with protocols established in the NIH/NRC Guide and Use of Laboratory Animals and were reviewed by the Institutional Animal Care and Use Committee of Battelle, Pacific Northwest Division.

In vivo methods. Rats were anesthetized using an inhalation anesthesia machine (VetEquip Inc., Pleasanton, CA) with isoflurane (Baxter Healthcare Corp., Deerfield, IL) mixed with oxygen. Once under anesthesia, femoral and jugular veins were cannulated using PE 50 polyethylene tubing. The trachea was then surgically intubated using PE 240 polyethylene tubing, and tubing was connected to the anesthesia machine to maintain anesthesia. Anesthetized rats were then infused with 1 mg/ml pilocarpine in physiological saline at 3 ml/h, and total saliva was collected gravimetrically from the oral cavity using a $9.52\text{ cm} \times 2.49\text{ mm o.d.} \times 1.42\text{ i.d.}$ glass capillary tube draining into a 2-ml microcentrifuge collection vial. The 3-ml/h infusion rate of physiological saline maintained rat hydration, as it was roughly the equivalent volume of saliva excreted by rats over a 2-h period at this exposure level of pilocarpine (Smith *et al.*, 2010). It has been reported that no major alterations in pharmacokinetics have occurred due to pilocarpine coadministration for different pesticides (Lu *et al.*, 1997b; Smith *et al.*, 2010). Once salivation had been stimulated (appearance of saliva in the oral cavity) and maintained for 7 min, rats were administered an iv bolus dose (1 ml/kg body weight dose volume) of 0.5, 1, 2.5, or 5 mg/kg CPF in saline with 5% Tween 20 via the femoral vein. Total saliva was collected continuously, changing collection vials at 15 min intervals. Blood (~200 μl) was collected from the jugular vein cannula at the midpoint of saliva collection intervals, and sodium heparin was used as an anticoagulant. Aliquots of blood collections (~20 μl) were obtained using hematocrit tubes. Saliva and blood collection continued until 120 min post CPF administration. At this time, the experiment was discontinued, rats were euthanized, and blood was obtained by intracardiac puncture using sodium heparin as an anticoagulant. Blood in hematocrit tubes was centrifuged for 10 min at $1600 \times g$, and plasma was separated from the packed red blood cell fraction. ChE activity was measured using the Ellman assay (Ellman *et al.*, 1961), and protein content of plasma was measured using bicinchoninic acid assay (Smith *et al.*, 1985).

Analytical methods. Levels of CPF and TCPy were quantified in blood and saliva using a method similar to Brzak *et al.* (1998). The stable TCPy isotope was added as an internal standard. Analytes were extracted from the incubation solution three times with 500 μl ethyl acetate. Extracts were dried

with Na_2SO_4 and evaporated under a gentle stream of nitrogen. Residues were reconstituted with toluene and derivatized with MTBSTFA. Calibration curve standards were prepared simultaneously by spiking known amounts of CPF and TCPy into control blood and saliva and extracting those samples using the same method. Samples were analyzed using gas chromatography-mass spectrometry (GC-MS). The instrument used was a Hewlett-Packard (Palo Alto, CA) 5973B mass selective detector in negative ion chemical ionization mode interfaced with Hewlett-Packard model 6890 GC using ChemStation software for programming and data analysis. Separations were achieved using a Restek (Bellefonte, PA) RTX-1701 30 m \times 0.25 mm i.d. \times 0.25 μm df column. Helium carrier gas was maintained at a constant pressure of 10 psi. GC oven temperature program initiated at 150°C for 1 min followed by a 25°C/min ramp to 180°C, a 20°C/min ramp to 250°C, and was held for 12 min. Inlet temperature was 210°C. Selected ion monitoring was used for analysis for increased sensitivity. Ions selected for monitoring included 161 and 166 m/z for the MTBSTFA-TCPy derivative and the MTBSTFA-TCPy internal standard derivative, respectively, as well as 313 m/z for CPF. Contributions of MTBSTFA-TCPy isotope derivative to the 161 m/z primary ion and the MTBSTFA-TCPy derivative to the 166 m/z ion were corrected using ratios of each compounds' contribution determined by analyzing individually (Brzak *et al.*, 1998). The entire method allowed quantification of at least 12 ng/ml for both CPF and TCPy.

Pharmacokinetic analysis/PBPK modeling. To analyze pharmacokinetic data, we utilized a combination of simple compartmental models, noncompartmental modeling techniques, and more complex PBPK/PD models to better understand the underlying biology and subsequently make species extrapolations and predictions from experimental data.

In order to compare pharmacokinetic parameters, time course of CPF concentrations in blood was fit to a one-compartment model (Equations 1 and 2), where A_1 is the amount in compartment 1, k_{e1} the elimination constant, V_1 the compartment volume, and C_1 the concentration of the compartment (Gibaldi and Perrier, 1982). Concentrations of TCPy in blood and saliva were likewise fit to a one-compartment model with first-order formation (combination of several processes, including CPF metabolism, CPF-oxon metabolism, protein binding, partitioning, etc.) (Equations 3 and 4), where k_f is the formation constant and A_a the molar amount of CPF available for metabolism (Gibaldi and Perrier, 1982).

$$\frac{dA_1}{dt} = -A_1 \times k_{e1} \quad (1)$$

$$C_1 = \frac{A_1}{V_1} \quad (2)$$

$$\frac{dA_1}{dt} = A_a \times k_f - A_1 \times k_{e1} \quad (3)$$

$$C_1 = \frac{A_1}{V_1} \quad (4)$$

Noncompartmental analysis was also applied to the data. Area under the curve (AUC) values were calculated from measured CPF and TCPy concentrations in blood and saliva using the trapezoidal rule (Gibaldi and Perrier, 1982).

A PBPK/PD model for CPF that had previously been modified to accommodate TCPy salivary excretion in rats and humans was modified and utilized to model rat data and to make predictions of salivary CPF and TCPy levels in humans after simulated exposures (Smith *et al.*, 2010; Timchalk *et al.*, 2002). In this model, CPF and CPF-oxon were distributed among internal compartments mediated by blood circulation described as a flow-limited process. The fraction of the compound that was not bound to plasma protein (described simply as a fraction bound, where FBC is the fraction of CPF bound to plasma proteins, $\text{CCFP}_{\text{bound}}$ the concentration of CPF bound to plasma proteins, and CCPF_{tot} the total concentration of CPF in blood; Equation 5) was free to partition to various compartments described by partition coefficients (Timchalk *et al.*, 2002). Fractional binding of CPF to serum albumin (0.99) was measured previously using a dialysis system

(Lowe *et al.*, 2009). As CPF-oxon undergoes rapid hydrolysis in the presence of albumin, it was assumed that CPF and CPF-oxon have equivalent fraction binding in plasma. Human metabolic parameters, including CPF dearylation, CPF desulfuration, and CPF-oxon hydrolysis, were updated (Smith *et al.*, 2011). Other human parameters were also updated from a variety of literature sources, including ChE activities, enzyme aging, degradation, reactivation, and turnover rates (Albers *et al.*, 2010; Hojring and Svensmark, 1976; Li *et al.*, 2005; Maxwell *et al.*, 1987; Mortensen *et al.*, 1998; Pope *et al.*, 2005; Sidell and Kaminskis, 1975). In order to better simulate the physiology of rats in this study, the cardiac output was reduced 15% from standard values ($15 \times \text{body weight (kg)}^{0.75}$) (Brown *et al.*, 1997; Conzen *et al.*, 1992; Vollmar *et al.*, 1992). A description of CPF partitioning to saliva was included based on the description of TCPy partitioning to saliva (Smith *et al.*, 2010). Briefly, levels of CPF in saliva were defined as the CPF concentration in blood ($CCPF_{bl}$) multiplied by saliva/blood CPF partitioning coefficient ($P_{sal/bl}$; saliva/blood CPF ratio) (Equation 6), which was determined experimentally using the mean saliva/blood concentration ratio.

$$CCPF_{bound} = CCPF_{bl} \times FBC \quad (5)$$

$$CCPF_{sal} = CCPF_{bl} \times P_{sal/bl} \quad (6)$$

A sensitivity analysis was conducted to identify the most important parameters for estimating levels of CPF and CPF-oxon in blood from an iv dose of 1 mg/kg CPF. Normalized sensitivity coefficients were calculated for a 1% change in a given model parameter when all other parameters were held fixed. Based upon the sensitivity analysis, parameters describing the fraction of CPF and CPF-oxon bound to plasma proteins were optimized to CPF levels in blood and ChE activity in plasma, respectively.

Data analysis. Parameters of compartmental pharmacokinetic models were analyzed using a one-way ANOVA coupled with a Tukey-Kramer *post hoc* test for multiple comparisons and linear regression models. Because TCPy concentration in saliva, TCPy concentration in blood, CPF concentration in blood, and salivary pH were all repeated measures within individual rats, comparisons of saliva/blood TCPy concentration ratios, salivary protein levels, salivary flow rates, and salivary pH values were all accomplished using mixed linear regression models that accounted for fixed effects (due to treatment) and random effects within each rat (Pinheiro and Bates, 2000). Pearson product-moment correlation coefficients were calculated for TCPy concentrations in saliva and blood as well as salivary pH to salivary flow rates and protein levels. All statistical tests used an α value of 0.05. Software used to statistically analyze data was "R: A language and environment for statistical computing" Version 2.13.1 (R Foundation for Statistical Computing, Vienna, Austria). The PBPK model was coded in acslX version 3.02.01 (Aegis Technologies, Huntsville, AL).

RESULTS

Exposure to pilocarpine induced salivation in rats in 2–12 min. Saliva flow rates ranged 0.01–0.14 ml/min at each sampling point. Flow rates were faster near the beginning of the experiment (0.08 ± 0.03 ml/min mean \pm SD at 0 min) and slowed to steady state (0.03 ± 0.02 ml/min mean \pm SD at 82.5 min) near the end of the experiment. Salivary protein levels ranged 1–20 mg/ml and significantly decreased over the course of the experiment (-0.08 mg/ml/min, $p < 0.001$, Fig. 2). Salivary pH (8.8 ± 0.2 , mean \pm SD) increased very slightly over the course of the experiment (0.001 pH/min, $p = 0.002$). Salivary pH was negatively correlated with salivary flow rate ($r = -0.54$, $p < 0.001$) and was not correlated to salivary protein levels ($r = -0.09$, $p = 0.4$).

Following iv administration of CPF, both CPF and TCPy were quantifiable in all blood samples (Fig. 3). TCPy was quantified in all saliva samples that had adequate saliva volume to extract (~ 100 μ l), indicating that CPF is rapidly metabolized, and TCPy partitions rapidly into saliva. CPF was quantified in three saliva samples, and for others, was below the limits of quantitation. Samples that had quantifiable CPF in saliva included two rats dosed with 5 mg/kg CPF and one rat dosed with 2.5 mg/kg CPF, and all three samples were from the first saliva sample taken (0–15 min). Interestingly, these samples were not paired with the three highest overall blood CPF levels but were paired with the three highest blood CPF levels at the first blood sampling time point (7.5 min). The saliva-to-blood CPF concentration ratio was 0.019 ± 0.003 (mean \pm SD). CPF in blood as well as TCPy in both blood and saliva demonstrated AUC linear increases with dose administered (Fig. 4, Tables 1 and 2). Ratios of TCPy AUC increases by dose were equivalent in blood and saliva (e.g., ratios of AUC values measured after a 1-mg/kg dose to that measured after a 0.5-mg/kg dose are 4.3 and 4.2 in blood and saliva, respectively), highlighting parallel pharmacokinetics of TCPy in blood and saliva.

One-compartment models were fit to time course data of each individual rat. One-compartment CPF elimination rate constants in blood for individual rats dosed with 0.5 mg/kg CPF were significantly lower than all other dose groups using a one-way ANOVA ($p < 0.04$, Table 1) and linear regression ($p = 0.03$). All other CPF elimination rate constants did not vary with dose ($p > 0.47$). Formation rates of TCPy in blood and saliva demonstrated a similar pattern as CPF elimination. In blood, TCPy formation rates did not significantly vary with dose when analyzed with a one-way ANOVA ($p = 0.14$, Table 2); however, a linear regression model suggests that formation rates significantly increased with dose (1.8 kg/mg/h, $p = 0.03$). In saliva, TCPy formation rates were significantly higher in 5 mg/kg dose group compared with the 0.5 mg/kg dose group ($p = 0.03$), and no other significant differences among dose groups existed ($p > 0.6$). A linear regression model suggests that formation rates significantly increased with dose (0.9 kg/mg/h, $p = 0.02$).

Formation rates of TCPy in saliva were not significantly different from those in blood ($p = 0.1$). Elimination rates of

TABLE 1
Observed AUC Values and Elimination Rates of CPF
Concentrations in Blood

Dose (mg/kg)	Matrix	AUC (μ mol/l·h)	Elimination rate ^a (h^{-1})
0.5	Blood	7.8	$0.25 \pm 0.27^*$
1	Blood	39.7	1.08 ± 0.15
2.5	Blood	61.0	1.47 ± 0.12
5	Blood	165.5	1.35 ± 0.47

^aMean \pm SD.

*Significantly different from others $p < 0.04$.

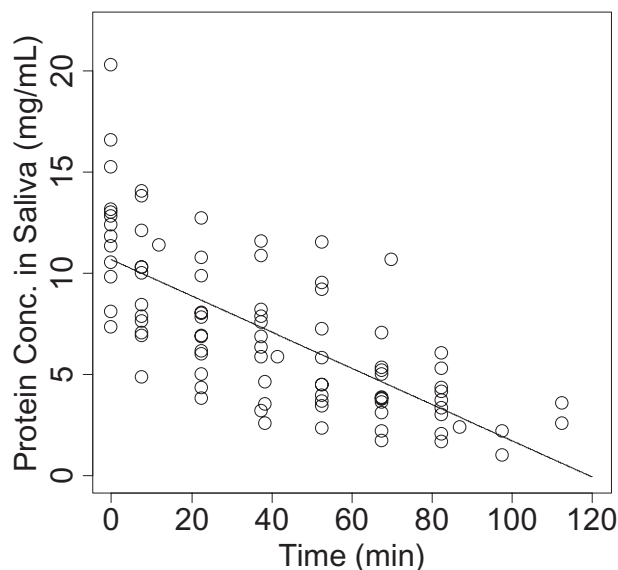


FIG. 2. Concentrations of protein in saliva over time from rats dosed with CPF and pilocarpine. The line represents a linear regression fit to the data.

TABLE 2
Observed AUC Values, Formation, and Elimination Rates of
TCPy Concentrations in Blood and Saliva

Dose (mg/kg)	Matrix	AUC ($\mu\text{mol/l}\cdot\text{h}$)	Formation rate ^a (h^{-1})	Elimination rate ^a (h^{-1})
0.5	Blood	44.11	0.96 ± 0.30	0.54 ± 0.45
1	Blood	187.7	5.80 ± 1.54	0.38 ± 0.25
2.5	Blood	631.9	5.11 ± 4.14	0.30 ± 0.10
5	Blood	1082	11.48 ± 8.49	0.34 ± 0.13
0.5	Saliva	1.848	$0.84 \pm 0.48^*$	0.47 ± 0.27
1	Saliva	7.81	3.47 ± 2.05	0.52 ± 0.74
2.5	Saliva	28.5	1.56 ± 0.79	0.71 ± 0.50
5	Saliva	44.4	$5.65 \pm 2.44^*$	0.44 ± 0.30

^aMean \pm SD.

*Significantly different each other $p = 0.03$.

TCPy also did not vary with dose in both blood and saliva ($p = 0.7$ and 0.9 , respectively) and were not significantly different from one another ($p = 0.3$, Table 2). Overall, evidence suggests that in the lowest dose group (0.5 mg/kg), TCPy was formed slower than other dose groups; yet once formed, TCPy pharmacokinetics were equivalent in saliva and blood.

TCPy concentrations in saliva were highly correlated to TCPy concentrations in blood ($r = 0.94$, $p < 0.001$, Fig. 5). Saliva/blood TCPy concentration ratios (0.049 ± 0.014 [mean \pm SD]) did not vary over TCPy concentrations in blood ($p = 0.08$; Fig. 6A), salivary flow rates ($p = 0.30$; Fig. 6B), and salivary pH values ($p = 0.11$; Fig. 6C). Saliva/blood TCPy ratios significantly decreased with increasing salivary protein levels ($p = 0.01$); however, the effect size was negligible

TABLE 3
Sensitivity Analysis for Selected Parameters of a PBPK/PD
Model for CPF

Parameter	Sensitivity coefficient	
	CPF in blood	CPF-oxon in blood
Fraction of CPF plasma protein binding	143	37
Fraction of CPF-oxon plasma protein binding	0	51
Hepatic CPF partition coefficient	-0.003	-0.001
Fat CPF partition coefficient	-0.08	-0.07
Hepatic CPF-oxon partition coefficient	0	-0.54
Fat CPF-oxon partition coefficient	0	-0.003
CPF dearylation, V_{\max}	-0.0004	-0.09
CPF dearylation, K_m	0.0004	0.09
CPF desulfuration, V_{\max}	-0.002	0.09
CPF desulfuration, K_m	0.002	-0.09
Hepatic CPF-oxon hydrolysis, V_{\max}	0	-0.52
Hepatic CPF-oxon hydrolysis, K_m	0	0.52
CPF-oxon hydrolysis V_{\max} in plasma	0	-0.50
CPF-oxon hydrolysis K_m hepatic	0	0.50
Fat blood flow	0.18	0.16
Hepatic blood flow	0.01	1.1
Cardiac output	-1.2	-0.11

(-0.002 ml/mg). All these measures suggest rapid CPF metabolism and consistent TCPy partitioning from blood to saliva.

ChE activity was measured in plasma and saliva. Due to small volumes of plasma and highly variable ChE activities in saliva, ChE activities were normalized to protein level reducing the coefficient of variability from 47 and 90% to 41 and 63% for plasma and saliva, respectively. Protein normalized plasma ChE activity decreased with CPF dose, and maximal inhibition (~50% of control) occurred after exposure to 5 mg/kg CPF (Fig. 7). Protein normalized ChE activity in saliva was extremely variable (ranged 19–25.6% of control), and no significant changes over time for each dose were observed ($p = 0.13$ –0.82).

A previously developed PBPK/PD model for CPF that includes TCPy partitioning into saliva was used to model the data (Smith *et al.*, 2010; Timchalk *et al.*, 2002). Initial fits overestimated CPF levels in blood (1–2 orders of magnitude), underestimated TCPy levels in blood and saliva (~1 order of magnitude), and overestimated plasma ChE inhibition (22–36%). A sensitivity analysis to CPF levels in blood was conducted, and plasma protein binding of CPF was the most

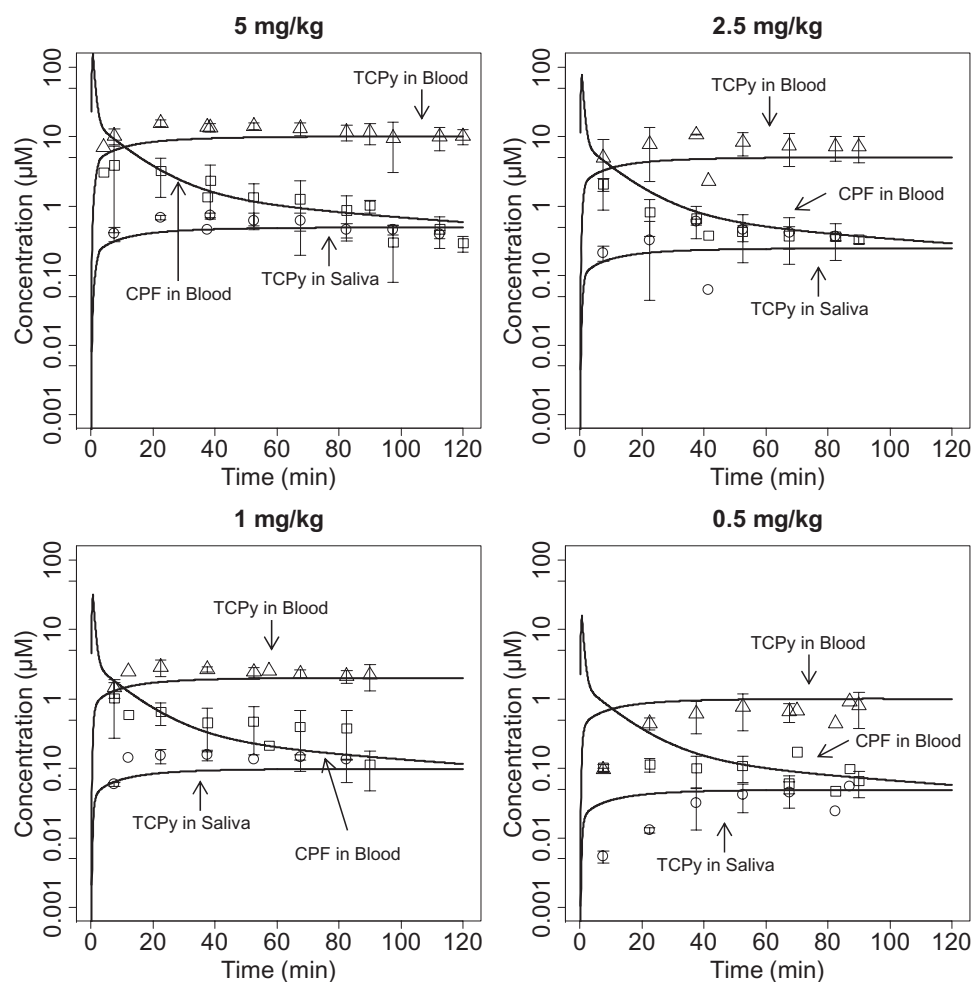


FIG. 3. Mean concentrations with SD error bars of CPF in blood (squares) as well as TCPy in blood (triangles) and saliva (circles) from rats dosed with 0.5–5 mg/kg CPF. Lines are PBPK/PD model fits to the data. Note. y-axis is on a logarithmic scale.

sensitive parameter (Table 3), suggesting that CPF plasma protein binding may be different after oral versus iv doses. Fitting the CPF plasma protein binding parameter to CPF levels in blood (0.65) provided improved PBPK/PD model fits to CPF time course in blood and TCPy time course data in blood and saliva (Fig. 3). The model still slightly over predicts CPF levels in blood at early time point and under predicts TCPy levels in blood at the same time points. This suggests that plasma protein binding could be dynamic and/or have some saturation level. However at steady state, the model predicts experimental data reasonable well. Model predictions of ChE inhibition in plasma were still overestimated after adjusting the level of CPF binding to plasma protein. Thus as previous, a sensitivity analysis to levels of CPF-oxon in blood was conducted, and plasma protein binding of CPF-oxon was the most sensitive parameter, with CPF plasma protein binding as being highly sensitive as well (Table 3). This suggests that there may be some differences in plasma protein binding for CPF-oxon between iv and oral dosing, otherwise ChE activities following CPF administration would have been more accurately simulated. Fitting the

CPF-oxon plasma protein binding parameter to levels of ChE activity in blood (0.92) provided reasonably good fits to the data (Fig. 7). Overall, with new estimates of CPF and CPF-oxon plasma protein binding, the PBPK model fits the data reasonable well at steady-state conditions.

DISCUSSION

In order to make full use of emerging noninvasive biomonitoring strategies (Du *et al.*, 2009, 2011; Liu *et al.*, 2005, 2006, 2008; Wang *et al.*, 2008b, 2011; Zou *et al.*, 2010), pharmacokinetics of CPF and TCPy were quantitatively evaluated in rat saliva *in vivo* following CPF iv administration.

Several compounds were coadministered with CPF in this study. Pilocarpine, a nonselective muscarinic receptor agonist, was coadministered with CPF, as it is the preferred experimental stimulant of salivation in rodent models, and no major alterations in pharmacokinetics have been reported regarding pilocarpine administration (Dawes, 1966; Lu *et al.*, 1997b;

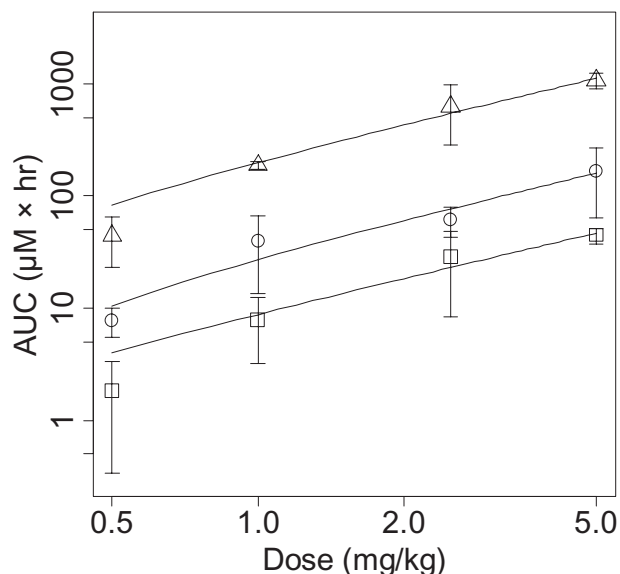


FIG. 4. Mean observed AUC values (with SD) of time course data (0–90 min) of CPF in blood (squares) as well as TCPy in blood (triangles) and saliva (circles) from rats dosed with 0.5–5 mg/kg CPF. Lines are linear regression models fit to the data.

Piraino *et al.*, 1980; Smith *et al.*, 2010). Isoflurane was also administered as an anesthetic. Others have reported a moderate reduction (15–20%) in cardiac output after exposure to isoflurane (Conzen *et al.*, 1992; Vollmar *et al.*, 1992). Cardiac output was sensitive with respect to simulated CPF levels in blood (Table 3); therefore, it was adjusted to account for isoflurane anesthesia. Finally, Tween 20 was administered with CPF as a detergent/emulsifier to solubilize CPF in physiologically based saline.

Stimulated salivation in rats was consistent with what others have previously reported. Salivary flow rates and pH levels were very consistent with previous studies with TCPy (Smith *et al.*, 2010). Here, salivary pH levels were negatively correlated to salivary flow rate, which has been observed prior (Sommer *et al.*, 1975). Salivary pH is positively controlled by the amount of bicarbonate in saliva (Haeckel, 1993), indicating bicarbonate concentrations increase with increasing salivation rate (Sommer *et al.*, 1975).

In this study, faster TCPy formation rates were observed from iv administration of CPF compared with PBPK/PD model simulations, which was developed from oral administrations of CPF. We hypothesize this is due to decreased amounts of CPF binding to plasma proteins after iv compared with oral administration. Decreased levels of CPF plasma protein binding would increase the amount of CPF available to partition into the liver, and thus, increase overall level of TCPy formation. Additionally, a PBPK/PD model of CPF that was developed with oral exposures overestimated CPF levels in blood measured here. Following PBPK/PD model simulations, an empirical description of plasma protein binding was the most sensitive parameter, suggesting that plasma protein binding

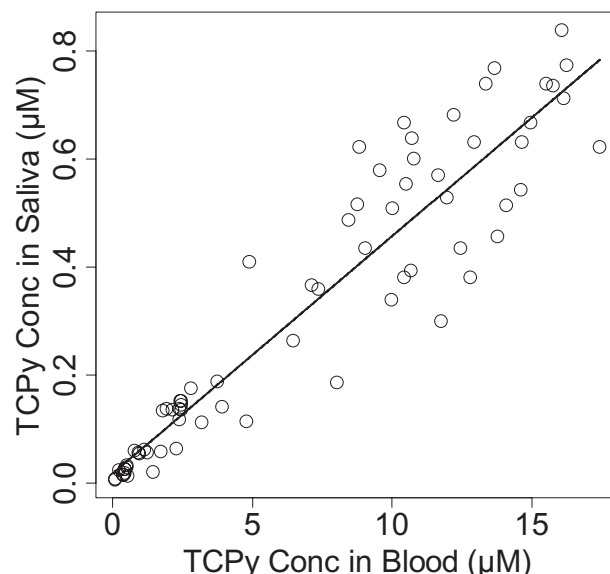


FIG. 5. Relationship of TCPy concentrations in blood and saliva from rats dosed with CPF. The line is a linear regression model fit to the data.

from oral dosing is different than from iv exposures. Reducing the parameter describing CPF binding to plasma protein resulted in increased unbound CPF, increased metabolism, and better predictions of CPF and TCPy disposition.

Specific mechanistic differences in plasma protein binding that could account for differences between the two routes may include saturable plasma protein binding, temporal differences in plasma protein binding, or binding itself may be completely different for iv and oral routes. For example, Roth *et al.* (1993) suggested that for many lipophilic compounds administered orally in oil vehicles (such as routine with CPF, a lipophilic compound), oral absorption is mediated through chylomicron production and transport into the lymphatic system. This type of binding and absorption would not occur after administration of an iv bolus dose of an emulsified chemical, such as used in our experimental design, and thus lead to differences in empirical estimates of protein binding in the model. Tween 20 could also interact with plasma protein binding. It has been reported that Tween 80, a related compound, reduced plasma protein binding of docetaxel (Loos *et al.*, 2003), even though Tween 80 is rapidly metabolized in plasma (van Tellingen *et al.*, 1999). Tween 20 did not alter TCPy pharmacokinetics administered iv versus orally (Busby-Hjerpe *et al.*, 2010; Smith *et al.*, 2010). Temporal relationships of plasma protein binding may exist as CPF partitions from the dosed emulsification, possibly due to rapid metabolism of Tween 20, if it is indeed responsible for altering plasma protein binding. Saturable protein binding in blood would create CPF-dependent levels of plasma protein binding with a threshold near the level of saturation. In this study, we measured differences in TCPy formation rates from low-dose CPF versus other doses. CPF levels in blood measured here are higher than oral administrations due to

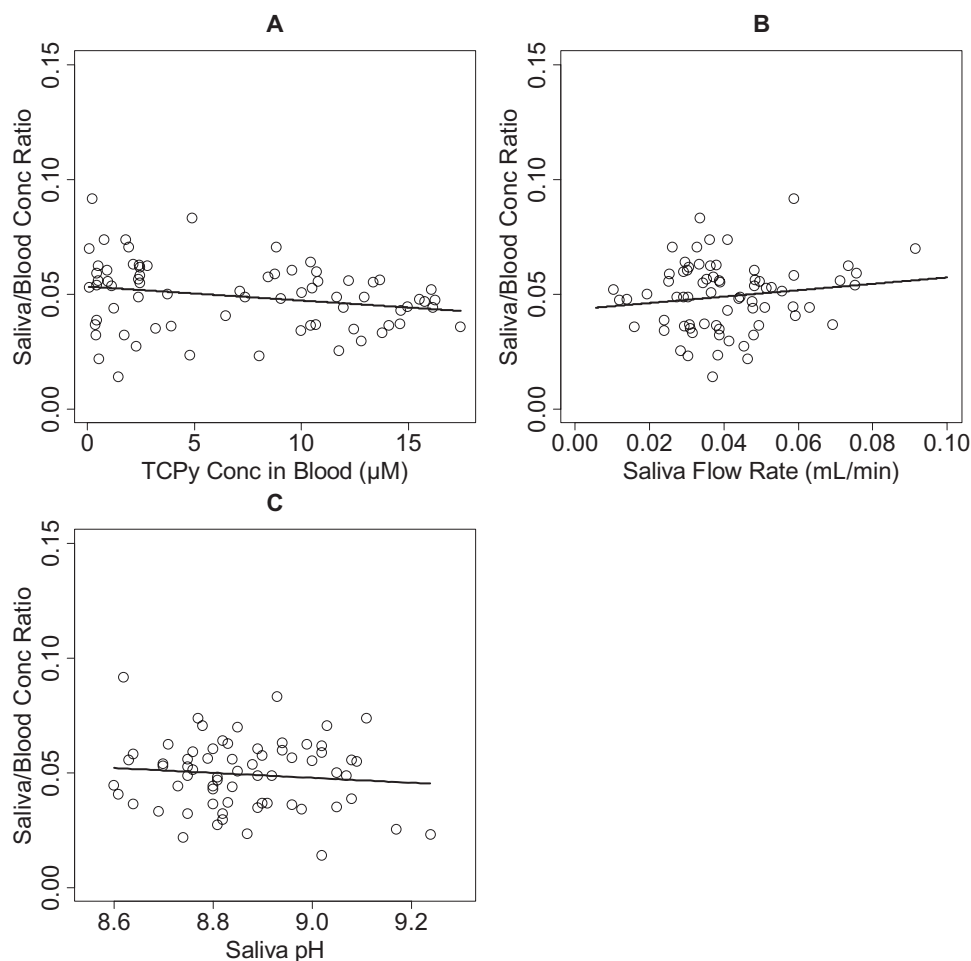


FIG. 6. Saliva/blood TCPy concentration ratios over varying TCPy concentrations in blood (A), salivary flow rates (B), and salivary pH values (C) all from rats dosed with CPF. Lines are linear regression models fit to the data.

slower absorption and rapid first-pass metabolism of orally administered CPF. After a 5-mg/kg dose, the highest CPF levels measured here (3.9 µM at 7.5 min) are much higher than peak level CPF levels in blood after 5 mg/kg orally (0.09 µM at 3 h) (Timchalk *et al.*, 2002) and are nearly double to those receiving 100 mg/kg CPF orally (2.3 µM at 3 h) (Timchalk *et al.*, 2002). CPF plasma protein binding has been measured using varying concentrations of rat and human albumin in buffer (0.04–20 mg/ml) and CPF (0.009 and 0.29 µM), and for high albumin concentrations, it was reported that 99% of CPF was bound at both CPF concentrations tested (Lowe *et al.*, 2009). CPF concentrations from that study were lower than what was measured *in vivo* in this study.

Differences in protein binding may not be limited to CPF. Protein plasma binding to CPF-oxon also had to be reduced to fit the time course of ChE inhibition (Fig. 7). This suggests that CPF-oxon and CPF may occupy at least some of the same binding sites on plasma proteins, and if saturation levels of CPF binding are reached, there would be a reduction of the fraction of CPF-oxon binding. Lowe *et al.* (2009) attempted to measure

CPF-oxon plasma protein binding but had difficulties measuring the parameter due to excessive CPF-oxon hydrolysis. Thus, due to similarities in chemical structure, it has been assumed that CPF-oxon plasma protein binding would be equivalent to that of CPF (Lowe *et al.*, 2009; Timchalk *et al.*, 2002).

A limited number of saliva samples contained quantifiable CPF. These saliva samples were from two high-dose rats (5 mg/kg) and one from the second highest dose rat (2.5 mg/kg), all at the first sampling point (0–15 min). These saliva samples were paired with the three highest CPF levels in blood for the first sampling time point (7.5 min), but not overall CPF levels in blood, as various other samples from other time points had higher CPF levels (e.g., 5-mg/kg dosed rat sampled at 22.5 min vs. 2.5-mg/kg dosed rat sampled at 7.5 min). Generally, passive transcellular diffusion is thought to be the primary method for which most chemicals are transferred from blood to saliva (Haeckel, 1993; Jusko and Milsap, 1993), and it has been hypothesized that pesticides and metabolites of pesticides, including CPF and TCPy, transfer from plasma to saliva via transcellular diffusion (Lu *et al.* 1997a, 1998, 2003, 2008;

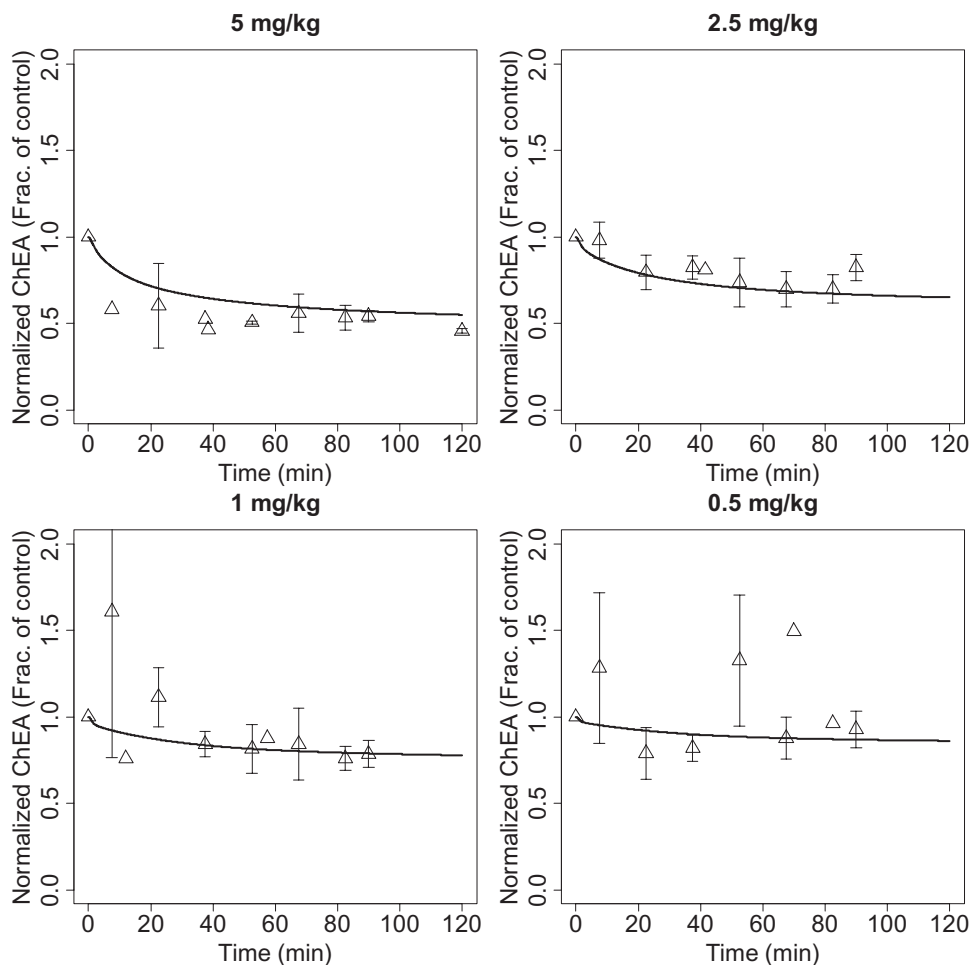


FIG. 7. Mean normalized ChE activities (fraction of control) with SD error bars in plasma over time of rats dosed with 0.5–5 mg/kg CPF. Lines represent PBPK/PD model predictions of the data.

Smith *et al.*, 2010; Timchalk *et al.*, 2007). A number of sources have reported that the chemical concentration in saliva reflects the chemical concentration that is not bound to plasma proteins in blood, as large protein-chemical complexes are too large to transfer from blood to saliva by diffusion (Jusko and Milsap, 1993; Lu *et al.*, 1998, 2003; Smith *et al.*, 2010). Thus, measured CPF in saliva represents unbound CPF in plasma circulation, which was experimentally quantifiable in only the earliest time point of saliva collection. This further supports the hypothesis of dynamic CPF binding to plasma proteins (such as saturable or temporal binding), as the fraction of CPF bound to protein would decrease with increased CPF above the saturation level. This would mean that the steady-state saliva/blood CPF ratio at CPF levels below the saturation point may be lower than what was measured here (0.019), as it represents a window at which unbound CPF is relatively high compared with steady-state levels. Alternatively due to rapid metabolism, CPF levels in saliva could be lower than our limits of quantitation.

After formation from CPF metabolism, TCPy partitioning from blood to saliva in rats was relatively constant over a range

of varying conditions in this study. AUC values of TCPy levels in blood and saliva demonstrated equivalent proportionalities among doses, and TCPy demonstrated similar elimination rates in each matrix and dose (Table 2). Saliva/blood TCPy concentration ratios were not affected by TCPy concentration in blood (Fig. 6A), saliva flow rate (Fig. 6B), and salivary pH (Fig. 6C). This is very consistent with previous studies investigating the pharmacokinetics of TCPy in saliva (Smith *et al.*, 2010), and the measured saliva/blood TCPy concentration ratio for the two studies was equivalent (0.049). This was also consistent with previous studies after oral administration of CPF to rats (0.055) (Timchalk *et al.*, 2007).

The pinnacle of noninvasive biomonitoring is the ability to directly monitor a parent compound of interest or a biological effect of the parent compound of interest using a noninvasive matrix, such as saliva. Here, we demonstrated very low CPF levels in saliva at only the first sampling time point after iv administration, which underscores very rapid CPF metabolism. Previously, we predicted that a daily oral dose of 12 µg/kg CPF to a 70-kg human for 30 days would achieve adequate

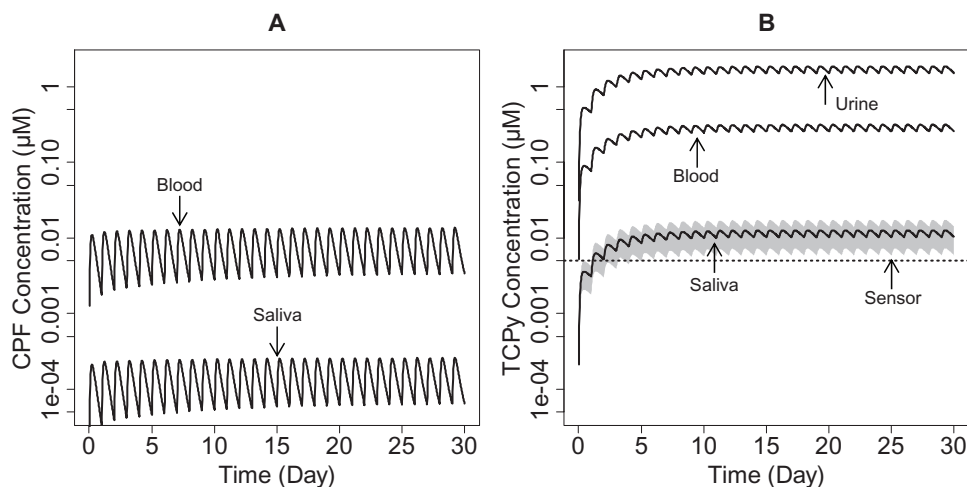


FIG. 8. PBPK/PD model of CPF simulations of a 70-kg human exposed to 12 $\mu\text{g/kg}$ CPF daily for 30 days, including CPF levels in blood and saliva (A) and TCPy levels in urine, blood, and saliva (B). The shaded area indicates TCPy concentrations in saliva over a normal range of salivary pH values (6.2–7.4), and the dotted line indicates the limit of TCPy detection for a TCPy sensor (Wang *et al.*, 2011). Note. In (B), the y-axis is on a logarithmic scale.

TCPy levels in saliva for detection with current sensor technology (Smith *et al.*, 2010; Wang *et al.*, 2011). Using the saliva/blood CPF ratio measured here (even though it may be a high estimate), the PBPK/PD model predicts very low CPF levels in saliva using the same dosing scenario (6×10^{-5} to $2 \times 10^{-4} \mu\text{M}$ CPF at steady state, Fig. 8A). Thus in saliva, rapid metabolism and very low levels of CPF partitioning probably eliminate CPF from being a very useful biomarker without extremely sensitive sensors.

When measuring levels of a parent compound of interest or effects of the parent compound of interest are not feasible, effects of metabolites or metabolite levels are generally the next best candidates for biomarkers. CPF-oxon (the toxic metabolite of CPF) inhibits ChE, which in plasma and red blood cells, has been used extensively to biomonitor for organophosphate exposure (Barr and Angerer, 2006). In this study, ChE activity was measured in saliva after exposure to CPF, and ChE activity levels were extremely variable. Due to the large variability and necessity of a control sample, ChE activity levels in saliva demonstrated no discernible dose relationship. Salivary ChE levels in humans have also been noted to be extremely variable as well (Henn *et al.*, 2006), questioning the utility of salivary ChE as a biomarker because of the necessity of a consistent control sample. New sensitive sensor technologies that use a sample as its own control (by measuring both protein levels and activities) may negate issues of high variability of ChE activity in saliva, potentially rendering salivary ChE as a useful biomarker (Du *et al.*, 2009, 2011); however, more robust research is needed to further develop and validate these methods. Similar TCPy pharmacokinetics in blood and saliva, a moderately strong correlation coefficient of blood and saliva TCPy concentrations, and relatively consistent saliva/blood TCPy concentration ratios in rats are all evidence that indicates TCPy in saliva can be a useful biomonitoring medium. Additionally with current

sensor sensitivity, predicted TCPy levels are sufficient to measure (Fig. 8B), making TCPy the most useful biomarker of those explored in saliva with currently available technologies.

In conclusion, experimental results suggest that CPF is rapidly metabolized after iv administration. After administration of CPF at the low dose (0.5 mg/kg), TCPy was formed at a slower rate than from other CPF doses, potentially due to differences in plasma protein binding to CPF. CPF was measured in a limited number of saliva samples, at only the first time point sampled (0–15 min), indicating low partitioning and rapid metabolism. After formation, TCPy pharmacokinetics were very similar in blood and saliva, and saliva/blood TCPy concentration ratios were not affected by TCPy concentration in blood, saliva flow rate, or salivary pH. ChE activity in plasma demonstrated a dose-dependent decrease, and ChE activity in saliva was extremely variable and demonstrated no dose relationship. A revised CPF PBPK/PD model predicted the data reasonably well. It is envisioned that a combination of biomonitoring technologies and computational modeling will form an approach to measure pesticide exposure to susceptible human populations such as agricultural workers.

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