TOXICOLOGICAL SCIENCES **113(2)**, 315–325 (2010)
doi:10.1093/toxsci/kfp283
Advance Access publication November 17, 2009

Pharmacokinetics of the Chlorpyrifos Metabolite 3,5,6-Trichloro-2-Pyridinol (TCPy) in Rat Saliva

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Received September 1, 2009; accepted October 29, 2009

Biological monitoring (biomonitoring) to quantify systemic exposure to the organophosphorus insecticide chlorpyrifos (CPF) has historically focused on the quantitation of major CPF metabolites in urine. Noninvasive techniques are being advocated as novel means of biomonitoring for a variety of potential toxicants, including pesticides (like CPF), and saliva has been suggested as an ideal body fluid. However, in order to be acceptable, there is a need to understand salivary pharmacokinetics of CPF metabolites in order to extrapolate saliva measurements to whole-body exposures. In this context, *in vivo* pharmacokinetics of 3,5,6-trichloro-2-pyridinol (TCPy), the major chemical-specific metabolite of CPF, was quantitatively evaluated in rat saliva. Experimental results suggest that TCPy partitioning from plasma to saliva in rats is relatively constant over a range of varying physiological conditions. TCPy pharmacokinetics was very similar in blood and saliva (area under the curve values were proportional and elimination rates ranged from 0.007 to 0.019 per hour), and saliva/blood TCPy concentration ratios were not affected by TCPy concentration in blood ($p = 0.35$) or saliva flow rate ($p = 0.26$). The TCPy concentration in saliva was highly correlated to the amount of unbound TCPy in plasma ($r = 0.96$), and the amount of TCPy protein binding in plasma was substantial (98.5%). The median saliva/blood concentration ratio (0.049) was integrated as a saliva/blood TCPy partitioning coefficient within an existing physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model for CPF. The model was capable of accurately predicting TCPy concentrations in saliva over a range of blood concentrations. These studies suggest that saliva TCPy concentration can be utilized to ascertain CPF exposure. It is envisioned that the PBPK/PD can likewise be used to estimate CPF dosimetry based on the quantitation of TCPy in spot saliva samples obtained from biomonitoring studies.

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

A critical component of risk assessment and management for any chemical compound is an understanding of human exposure from both occupational and environmental sources. Biological monitoring (biomonitoring) is an essential tool used to quantitatively assess human exposure for a variety of potential toxicants. Historically, biomonitoring has been conducted primarily by utilizing biological matrices such as blood and urine. However, to ease collection of dosimetry data, more noninvasive techniques have been advocated, such as utilizing saliva as a biomonitoring matrix (Nigg and Wade, 1992; Pichini *et al*., 1996). Saliva has been used to evaluate the potential for biomonitoring for a number of chemical compounds and biomarkers of those compounds, including pesticides (Hayashi *et al*., 1989; Kaufman and Lamster, 2002; Lu *et al*., 1997a, 2003; Schramm *et al*., 1992; Timchalk *et al*., 2007).

Chlorpyrifos (CPF) is an organophosphorus (OP) pesticide used for broad-spectrum control of insects in crop agriculture. When ingested, inhaled, or absorbed dermally, CPF can be metabolized by cytochrome P450 enzymes. These enzymes can cause CPF to undergo dearylation (oxidative ester cleavage) forming 3,5,6-trichloro-2-pyridinol (TCPy) and diethylthiophosphate (Kamataki *et al*., 1976). Alternatively, cytochrome P450 enzymes could also cause CPF to undergo oxidative desulfation to form chlorpyrifos-oxon (CPF-oxon). CPF-oxon is an extremely potent inhibitor acetylcholinesterase, an enzyme that degrades neurotransmitters after their release into the synapse, and other cholinesterases (ChEs) as well. 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 conjugated by phase II metabolism and then excreted as glucuronide, sulfate, or other conjugate or as unconjugated TCPy in the urine (Bakke *et al*., 1976; Nolan *et al*., 1984).

Recently, several different approaches have been developed for real-time biomonitoring of CPF in saliva. The first approach is based on the measurement of TCPy in saliva as it has been reported that CPF measurements in saliva are unreliable (Lu *et al*., 2008). The primary technique developed for salivary TCPy monitoring is a competitive immunoassay using TCPy antibodies coupled with horseradish peroxidase for electrochemical signal conversion (Liu *et al*., 2005, 2006). Currently

within our laboratory, efforts are underway for development of an immunoassay with quantum-dot (QD) nanoparticle-based optical/electrochemical signal conversion for TCPy detection. A second salivary CPF biomonitoring approach is based on the measurement of ChE activity in saliva (Wang *et al*., 2008b). This sensor measures an enzymatic product of ChE (from the sample) reacting with acetylthiocholine as the substrate using a carbon nanotube–modified screen-printed carbon electrode. This system is coupled with a microflow injection system that provides advantages of real-time continuous detection and decreased required sample volume. A final biomonitoring approach is based upon the measurement of organophosphorylated ChE as a biomarker of exposure to OPs (Liu *et al*., 2008; Wang *et al*., 2008a). ChE can be inhibited by various OPs via a phosphorylation on a serine amino acid at the active site of the enzyme. This phosphorylated enzyme can be measured with a sandwich OP-specific immunoassay using zirconia nanoparticles as the capturing agent and QD antibodies for specificity and signal conversion.

With the development of these approaches for CPF biomonitoring in saliva, there is a need to have a robust understanding of CPF pharmacokinetics in saliva. That way, a salivary measurement of a pharmacokinetic metabolite (such as TCPy) or a pharmacodynamic effect (inhibition of ChE activity or formation of inhibited ChE) can be directly related to CPF dosimetry. Timchalk *et al.* (2007) conducted a preliminary investigation of CPF pharmacokinetics in saliva. TCPy was detected in blood and saliva of rats following exposure to a range of CPF doses, suggesting that saliva could be used as a biomonitoring matrix. However, further characterization is needed to validate this approach, to better understand the mechanism of salivary TCPy clearance, and to better understand what factors could potentially modify the pharmacokinetics.

The objective of this study was to quantitatively evaluate *in vivo* pharmacokinetics of TCPy in saliva. TCPy was administered rather than CPF to eliminate potential variations associated with CPF metabolism that could complicate interpretation of the data. The experimental methodology used in this study was similar to what others had previously used for other pesticides, including OP compounds similar to CPF. Lu *et al*. investigated pharmacokinetics of both atrazine (Lu *et al*., 1997a,b, 1998) and diazinon (Lu *et al*., 2003) in saliva for potential biomonitoring purposes. Results from these investigations showed relatively consistent transfer of pesticides from blood to saliva and promise for potential biomontioring. Finally, pharmacokinetic data from this study were integrated into a modified physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model of CPF (Timchalk *et al*., 2002). It is envisioned that a fusion of real-time analysis capability from the various developing approaches for CPF biomonitoring in saliva and the data extrapolating potential of PBPK/PD models with salivary clearance will form a novel platform for CPF analysis in 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, money, and protect the public when rapid pesticide exposure assessments are required.

MATERIALS AND METHODS

Chemicals. TCPy (CAS # 6515-38-4) was kindly provided by Dow AgroSciences (Indianapolis, IN). N-(*t*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA), ethyl acetate, and pilocarpine were all purchased from Sigma-Aldrich Chemical Company (Milwaukee, WI). Chemicals used in this study were either reagent grade or better.

Animals. Adult male Sprague-Dawley rats (300–400 g) were purchased from Charles River Laboratories, Inc. (Raleigh, NC). 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 National Research Council Guide for the Care and Use of Laboratory Animals and were reviewed by the Institutional Animal Care and Use Committee of Battelle, Pacific Northwest Division.

Progressively dosed rats. Seven 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 polyethlyene tubing. The trachea was then surgically intubated using PE 240 polyethlyene tubing. The tracheal tube was connected to an anesthesia machine to maintain anesthesia. Once all microsurgical techniques had been achieved, rats were infused with 0.1, 0.5, or 1 mg/ml pilocarpine in physiological saline via the femoral vein cannula at a rate of 3 ml/h using an infusion pump (KD Scientific, Inc., Holliston, MA). Pilocarpine induced salivation, and total saliva was collected gravimetrically from the oral cavity using a 9.52 cm × 2.49 mm od × 1.42 id 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 volume of saliva excreted by rats over a 2-h period (4.7 ± 2.4 ml). Once salivation had been stimulated (appearance of saliva in the oral cavity) and maintained for 5 min, rats were dosed with 1 mg/kg body weight with TCPy in saline with 5% Tween 20 via the femoral vein cannula. Total saliva was collected continuously, changing collection vials at 15-min intervals. Blood (100–200 μl) was collected from the jugular vein cannula at the midpoint of the saliva collection intervals. At 60 min past the first dose, rats were again administered TCPy, this dose at 10 mg/kg body weight. Saliva and blood collection continued at the same intervals until 120 min after the initial administration of TCPy. At this time, the experiment was discontinued and rats were humanely euthanized.

Singularly dosed rats. Eleven additional rats were subjected to a similar experimental procedure as the progressively dosed rats; however, these rats received 1, 10, or 30 mg/kg initial doses of TCPy iv without sequential dosing. Saliva was collected from all these rats as described above; however, only seven of these rats had blood collected concurrently with saliva. Salivary pH was measured, and plasma was collected from these rats for *in vitro* protein-binding determination.

Analytical methods. Biological samples were prepared for analytical analysis as described in Campbell *et al.* (2005) similar to that of Brzak *et al.* (1998). Total blood samples and aliquots (~200 μl) of saliva collections were extracted by first adding 100 μl of 3M HCl saturated with NaCl. Resulting solutions were then extracted three times with 400 μl ethyl acetate. Extracts were dried with Na_2SO_4 and evaporated with a gentle stream of N_2. Residues were reconstituted with toluene and derivatized with MTBSTFA. Standards

TABLE 1
Input Parameters for a Modified Algorithm for Calculating the Saliva/Blood TCPy Partitioning Coefficient (Schmitt, 2008)

Parameter	Value (rat/human)	Source (rat/human)
TCPy physiochemical properties		
Fraction unbound in plasma	0.015	Measured/estimated
pK_a	4.55	Fixed[a]
Log K_{ow} at 7 pH	1.3	Fixed[a]
Log K_{ow} at 3 pH	3.2	Fixed[a]
α	0.013	Calculated[b]
Tissue properties		
Plasma		
Fraction protein	0.073	Fixed[c]
Fraction water	0.915	Fixed[c]
pH	7.8/7.4	Measured/fixed
Saliva		
Fraction cells	0	Estimated[d]
Fraction protein	0.003	Fixed[c]
Fraction water	0.98	Fixed[c]
pH	8.9/6.7	Measured/fixed[c]

[a]Racke (1993) and Shemer *et al*. (2005).

[b]α is defined as the ratio of the partitioning coefficient (K_{ow}) of the ionized form of TCPy and the partitioning coefficient of the nonionized form of TCPy (Schmitt, 2008). Using the Henderson-Hasselbalch equation, at a pH of 7, 99.6% of TCPy is ionized and at a pH of 3, 97.2% of TCPy is nonionized. Thus, α was estimated as a ratio of the K_{ow} values at those respective pH values.

[c]Hold *et al*. (1995) and Ritschel and Tompson (1983).

[d]It was assumed that the cellular fraction of saliva was negligible.

were prepared simultaneously by spiking known amounts of TCPy into control saliva, extracting with ethyl acetate, and derivatizing with MTBSTFA. Samples were analyzed using gas chromatography/mass spectrometry (GC/MS). The instrument used was a Hewlett-Packard (HP; Wilmington, DE) 5973B mass selective detector in electron ionization mode interfaced with HP model 6890 GC using HP ChemStation software for programming and data analysis. Separations were achieved using a Restek (Bellefonte, PA) RTX-5MS w/Intra-Guard 30 m × 0.25 mm id × 0.25-μm df column. Helium carrier gas was maintained at 6 ml/min. GC oven temperature program initiated at 80°C for 3 min followed by a 15°C/min ramp to 310°C and then was held for 3 min. Initial temperature of the inlet was 210°C. Selected ion monitoring was used for analysis for increased sensitivity. Ions selected for monitoring included 254 and 256 *m/z* for derivatized TCPy. This method allowed for a quantification limit of approximately 0.12 μmol/l of TCPy.

Determination of protein binding. For *in vitro* protein-binding assessment, naive rat blood plasma samples were spiked with three different levels of TCPy to form three concentrations of spiked plasma 7, 36, and 77 μmol/l as determined by GC/MS. These samples were incubated for 60 min at 37°C. Aliquots were then transferred into a 10,000 nominal molecular weight limit Ultrafree-MC centrifugal filter device (Millipore, Billerica, MA). For *in vivo* protein-binding assessment, plasma from rats dosed with 1, 10, and 30 mg/kg TCPy iv were collected and aliquots were also transferred to centrifugal filter devices. Samples were centrifuged for 60 min at 5000 × g. Unfiltered plasma and plasma filtrate were quantified with GC/MS. Ratio of the TCPy concentration in plasma filtrate to the TCPy concentration in whole plasma sample was used as the calculation of protein binding.

Pharmacokinetic analysis/PBPK modeling. Area under the curve (AUC) was calculated for TCPy concentrations in blood and saliva using the trapezoidal rule (Gibaldi and Perrier, 1982). Pharmacokinetic elimination rates were calculated for blood and saliva time course data using an exponential regression model (Equation 1).

$$f\left(t\right) = A \times e^{(k \times t)}. \quad (1)$$

A PBPK/PD model for CPF (Timchalk *et al*., 2002) was modified to accommodate TCPy salivary excretion. Since TCPy pharmacokinetics is handled as a one-compartment pharmacokinetic compartment in the CPF PBPK/PD, salivary TCPy concentration ($CTCPy_{sal}$) was defined as the TCPy concentration in blood ($CTCPy_{bl}$) multiplied by saliva/blood TCPy partitioning coefficient ($P_{sal/bl}$: saliva/blood TCPy ratio; Equation 2). For rats, the saliva/blood TCPy partitioning coefficient was determined experimentally using the median saliva/blood concentration ratio. Since the physiological conditions of rats in this study were different than normal physiological values in humans (lower saliva pH), saliva/blood TCPy partitioning coefficients were estimated using a modified algorithm for calculating tissue to plasma partitioning coefficients (Schmitt, 2008). This approach calculates partitioning coefficients based upon the composition of tissues in terms of water, various types of lipid content, phsopholipid content, proteins, and pH differences using chemical compound–specific lipophilicity, pK_a, and plasma-protein binding (Schmitt, 2008). Instead of the commonly used assumption that all ionized compounds are unable to cross membranes, this algorithm assumes that ionized compounds are able to traverse membranes at a much lower rate (usually three orders of magnitude) than the nonionized form and estimates the difference in rates based on the ratio of distribution coefficients for the ionized and nonionized forms (Schmitt, 2008). Inputs into this algorithm included various physiochemical properties of TCPy, as well as physiological properties of rat and human plasma and mixed saliva (Table 1). Additional algorithm assumptions included pH-dependent partitioning to the interstitial space fraction of saliva and negligible cellular fraction of saliva.

$$CTCPy_{sal} = CTCPy_{bl} \times P_{sal/bl}. \quad (2)$$

In order to better simulate the physiology of rats in this study, salivary TCPy elimination was included in the PBPK/PD model for rats. Salivary flow rate (Q_{sal}) was defined as a nonlinear dynamic equation fit to the salivary flow rate of rats receiving 1 mg/ml pilocarpine at 3 ml/h (Equation 3). The TCPy elimination rate in saliva was defined as the TCPy concentration in saliva multiplied by the saliva flow rate (Equation 4). Subsequent human simulations with the PBPK/PD model did not include this route of TCPy elimination.

$$Q_{sal}\left(t\right) = A \times t^B + C. \quad (3)$$

$$\frac{dTCPy_{sal}}{dt} = -CTCPy_{sal} \times Q_{sal}. \quad (4)$$

Statistics. Mean time to salivation was statistically analyzed using a one-way analysis of variance coupled with a Tukey–Kramer *post hoc* test for multiple comparisons. Since TCPy concentration in saliva, TCPy concentration in blood, and salivary pH were all repeated measures within individual rats, comparisons of saliva/blood TCPy concentration ratios 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). A linear regression model was used to compare the percent protein binding of TCPy over varying total concentrations of TCPy in plasma. The Student's *t*-test was used to compare *in vitro* and *in vivo* results of TCPy binding as well as saliva/blood TCPy partitioning coefficient determined experimentally and predicted with the modified Schmitt (2008) algorithm. Pearson product–moment correlation coefficients were calculated for TCPy concentrations in saliva and blood, as well as TCPy concentrations in saliva and unbound TCPy concentrations in plasma. 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 2.9.0 (R Development Core Team, 2009).

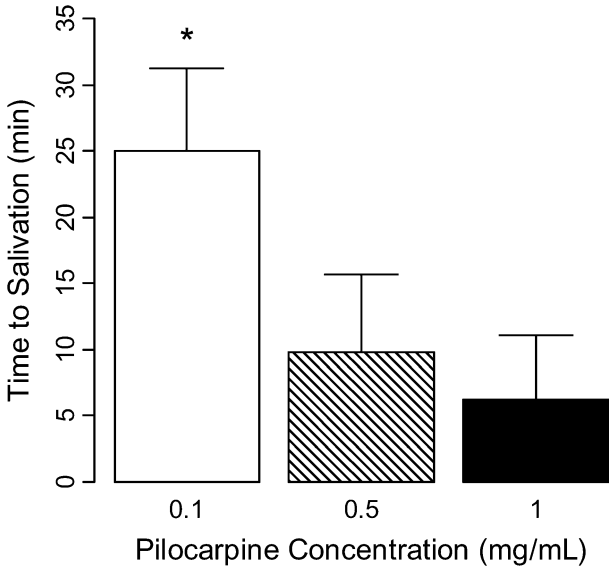

FIG. 1. Mean time to salivation (min) with SD error bars in rats iv infused with varying concentrations of pilocarpine in saline at 3 ml/h. *Indicates statistical groupings.

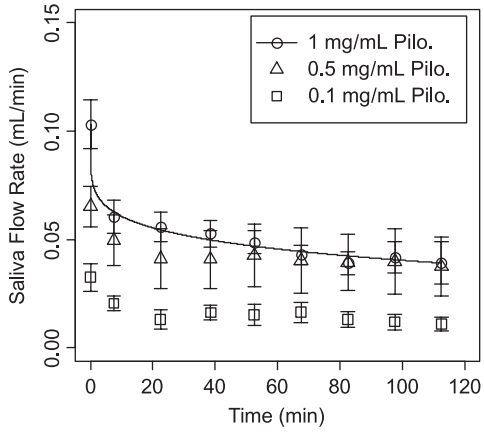

FIG. 2. Mean saliva flow rates (ml/min) with SEM error bars of rats iv infused at 3 ml/h with varying concentrations of pilocarpine. The solid line is a nonlinear regression fit to the saliva flow rate of rats receiving 1 mg/ml of pilocarpine.

RESULTS

To quantitatively evaluate the pharmacokinetics of TCPy in saliva, coexposure to pilocarpine was necessary to induce salivation in the rat and multiple levels of pilocarpine exposure was used to induce differing saliva flow rates. Following pilocarpine administration, mean time to salivation was significantly more rapid in rats infused with 0.5 and 1.0 mg/ml pilocarpine (medium and high pilocarpine exposure levels) compared to rats infused with 0.1 mg/ml pilocarpine ($p \leq 0.005$), with the most rapid mean appearance of saliva occurring at 6 min after dosing (Fig. 1). Saliva flow rate increased with increasing concentrations of pilocarpine infused iv (Fig. 2), and 1 mg/ml pilocarpine caused the highest mean peak saliva flow rate of 0.103 ml/min. Nonlinear regression fit of Equation 3 to the salivary flow rate data from rats infused with 1 mg/ml pilocarpine (Fig. 2) resulted in parameter estimates with SEs of -0.029 ± 0.003 for "A," 0.170 ± 0.022 for "B," and 0.103 ± 0.002 for "C." This equation was used in the calculation for TCPy elimination in the PBPK/PD simulations of rats infused with pilocarpine. Mixed salivary pH from those rats that were measured ranged from 8.5 to 9.5, with a mean value of 8.9 (Fig. 3). These values were consistent over the time course of collection for individual rats ($p = 0.53$).

Following iv administration of TCPy from both the progressive dosing and the singular dosing, TCPy was detected in all blood and saliva samples (Fig. 4). Maximum mean TCPy concentrations in both blood and saliva were obtained at the first sampling time point after each dose (single dose: 7.5 min for blood and 0- to 15-min collection interval for saliva and progressive dose of 10 mg/kg: 67.5 min for blood and 60 to 75-min collection interval for saliva). This indicates that after iv administration, TCPy is distributed quickly throughout the rat and transferred rapidly to saliva. The lack of blood time course data from the 30-mg/kg TCPy dose was due to difficulties with surgery and precluded blood pharmacokinetic calculations from that group. However, for the 1-mg/kg and 10-mg/kg dose groups, blood AUC values were consistently ~25 (23–27) times greater than saliva AUC values (Table 2). Both blood and saliva AUC values increased proportionally to dose (10–12 times increase for 1–10 mg/kg and 4 times increase for 10–30 mg/kg). Elimination rates for blood and saliva in each dose group ranged from 0.0067 to 0.0189 per hour, and the 95% confidence intervals of each group overlapped one another (Table 2), suggesting that no significant differences existed among the elimination rates in saliva and blood for all dose groups. Empirically, it appears that the elimination rate from saliva of rats dosed with 30 mg/kg

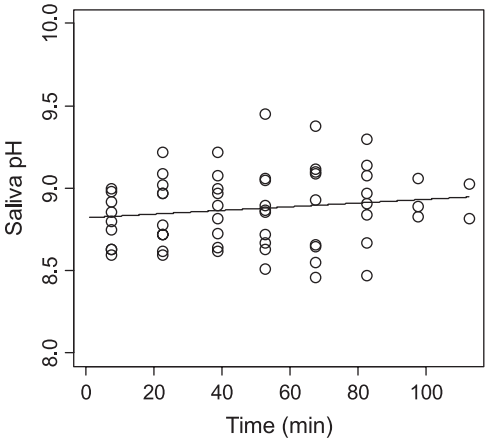

FIG. 3. Salivary pH from rats iv infused with pilocarpine in saline. The line is a linear regression model fits to the data. There was no significant relationship when analyzed using a mixed linear regression model ($p = 0.53$).

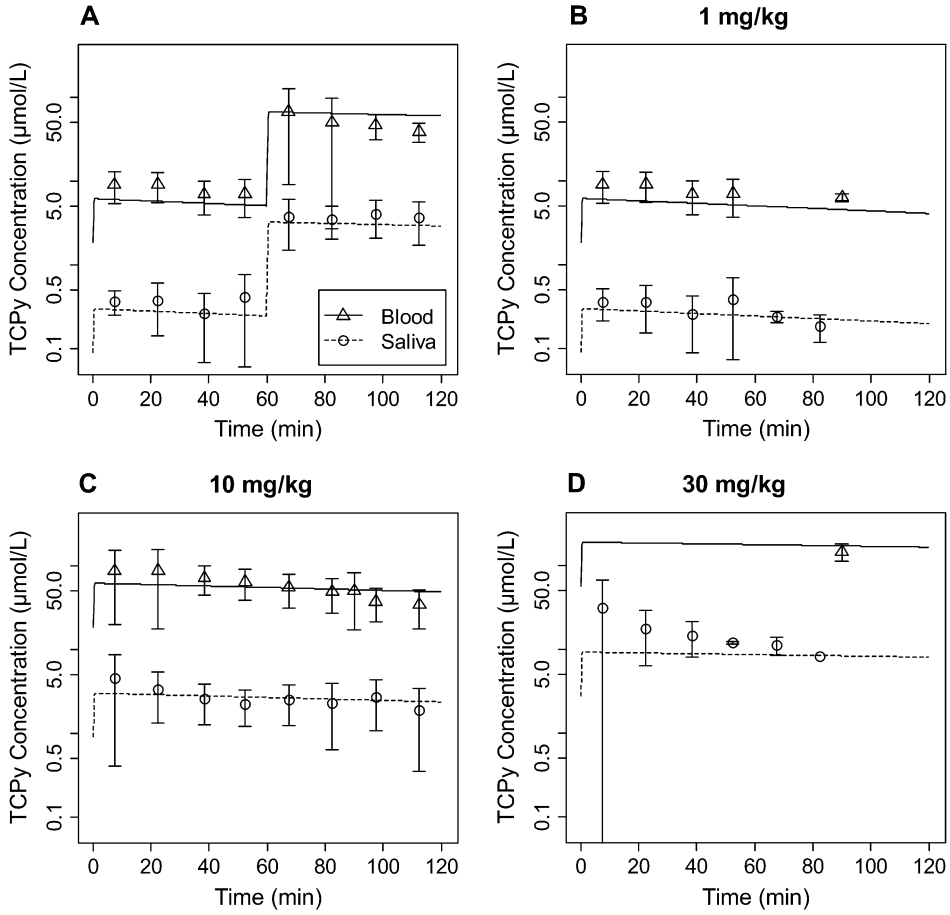

FIG. 4. Mean concentration with SD error bars of TCPy in blood and saliva from rats being progressively dosed with 1 mg/kg at 0 min and 10 mg/kg at 60 min (A) or singularly dosed at 0 min with 1 mg/kg (B), 10 mg/kg (C), or 30 mg/kg (D) with TCPy. (The lack of blood time course data from the 30-mg/kg TCPy dose was due to difficulties with surgery.) Lines are physiologically based PBPK/PD model fits to the data. *Note*. y-Axis is on a logarithmic scale.

TCPy is exceedingly high; however, that elimination rate is driven by the first time points (7.5 min), which are extremely variable (31 ± 36 µmol/l, mean ± SD). Removing those first time points, the elimination rate recalculates to 0.0116 per hour (0.0083–0.0151 per hour 95% confidence level), which is consistent with the other elimination rates, and the 95% confidence intervals overlap those of the other groups. These proportional AUC differences and similar elimination rates indicate that TCPy pharmacokinetics in blood and saliva mirror one another over the TCPy dose range administered. The correlation coefficient of TCPy concentration in saliva to the TCPy concentration in plasma was 0.68 ($p < 0.001$) (Fig. 5). Saliva/blood TCPy concentration ratios were not significantly different when analyzed using a mixed linear regression model over varying TCPy concentrations in blood ($p = 0.35$; Fig. 6), salivary flow rate ($p = 0.26$; Fig. 7), and salivary pH ($p = 0.60$; Fig. 8), suggesting very consistent partitioning of TCPy from blood to saliva.

TCPy plasma-protein binding was determined using ultrafiltration methods from both *in vitro* and *in vivo* derived samples. There was no significant difference between results of the *in vitro* and *in vivo* TCPy plasma protein–binding experiments ($p = 0.78$), and there was no significant difference in the percent of protein binding by TCPy over varying total concentrations of TCPy in plasma ($p = 0.76$). Including both

TABLE 2
The Observed AUC Values and Elimination Rates with 95% CIs of TCPy Concentrations in Blood and Saliva from Rats Dosed iv with 1, 10, and 30 mg/kg of TCPy

Dose (mg/kg)	Matrix	AUC (µmol/l × h)	Elimination rate (per hour)	95% CI of elimination rate (per hour)
1	Blood	615.9	0.0067	−0.0040 to 0.0180
1	Saliva	22.9	0.0068	−0.0024 to 0.0170
10	Blood	6421.0	0.0089	0.0071 to 0.0108
10	Saliva	285.0	0.0074	0.0024 to 0.0129
30	Blood	NA	NA	NA
30	Saliva	1131.1	0.0189	0.0103 to 0.02972

Note. CI, confidence interval; NA, not applicable.

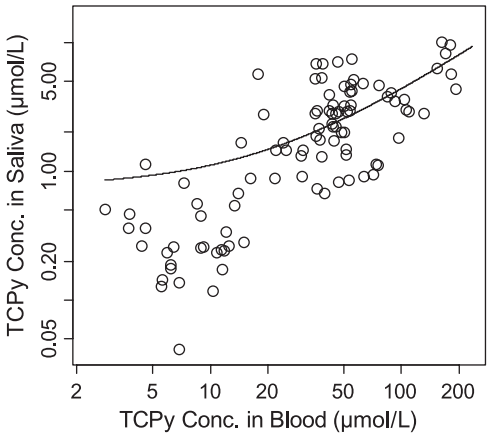

FIG. 5. Relationship of TCPy concentrations in blood and saliva from rats dosed with TCPy. The correlation coefficient of TCPy concentration in saliva to the TCPy concentration in blood was 0.68 ($p < 0.001$). The line is a linear regression model fits to the data. *Note*. Both y- and x-axes are on logarithmic scales.

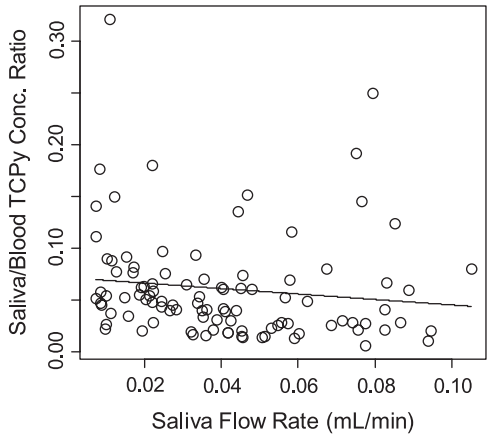

FIG. 7. Saliva/blood TCPy concentration ratios over varying salivary flow rate from rats dosed with TCPy. The line is a linear regression model fits to the data. There was no significant relationship when analyzed using a mixed linear regression model ($p = 0.26$).

in vitro and *in vivo* samples, a mean value of 98.5 ± 1.1% of TCPy was bound to protein in blood plasma (Fig. 9). The correlation coefficient of the unbound TCPy concentration in plasma to the TCPy concentration in saliva was 0.96 ($p = 0.002$).

Since saliva/blood concentration ratios did not change over several varying conditions, the PBPK/PD modeling strategy for TCPy in saliva was based upon the saliva/blood TCPy partitioning coefficient. To prevent bias, the partitioning coefficient was defined as the median saliva/blood concentration ratio (0.049) due to the presence of several high valued outliers that could artificially inflate a mean value. This strategy resulted in reasonably good PBPK/PD model fits to saliva time course data (Fig. 4).

The difference in saliva pH values from humans in the literature and rats from this study was great enough that extrapolation of the TCPy blood to saliva partitioning coefficient from that measured in this study was required to be accurate for human predictions with the absence of human data in this regard. Several approaches were tested, and the modified Schmitt (2008) algorithm resulted in the best fit and a reasonably close estimation of the rat saliva/blood TCPy partitioning coefficient (0.057) as experimentally calculated values versus predicted values showed no significant difference ($p = 0.44$). Thus, this algorithm was used to extrapolate the saliva/blood TCPy partitioning coefficients (Fig. 8B, 0.040 for the mean) for human PBPK/PD simulations using normal human physiological parameters (Table 1).

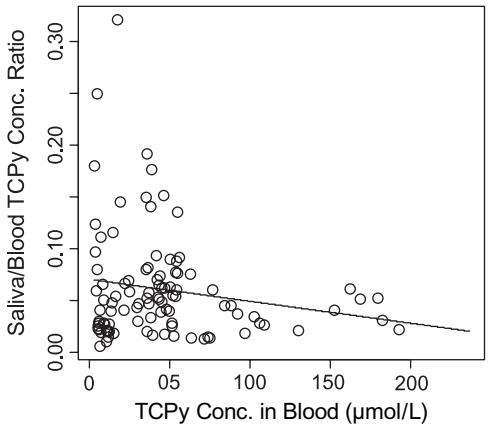

FIG. 6. Saliva/blood TCPy concentration ratios over varying TCPy concentrations in blood from rats dosed with TCPy. The line is a linear regression model fits to the data. There was no significant relationship when analyzed using a mixed linear regression model ($p = 0.35$).

DISCUSSION

A number of strategies have been developed for CPF biomonitoring, and the measurement of urinary TCPy has been the biomarker of choice for assessing CPF exposure due to the rapid metabolism of CPF and the relative ease of TCPy detection versus that of CPF (Barr and Angerer, 2006; Nolan *et al*., 1984; Smith *et al*., 2009; Timchalk *et al*., 2007). Recent efforts in our laboratory have focused on monitoring TCPy levels in saliva (Barry *et al*., 2009; Liu *et al*., 2005, 2006) as a noninvasive approach for CPF biomontioring. However, there is a need to more fully characterize the pharmacokinetics of CPF and key metabolites in saliva to establish if this matrix will be of potential utility for biomonitoring. The objective of this study was to quantitatively evaluate *in vivo* pharmacokinetics of TCPy in saliva and integrate a description of those processes into a modified PBPK/PD model for CPF (Timchalk *et al*., 2002); that way, potential salivary TCPy measurements can be used to predict CPF exposure. *In vivo* administration of TCPy allowed for a focused evaluation of TCPy partitioning from blood to saliva without potential

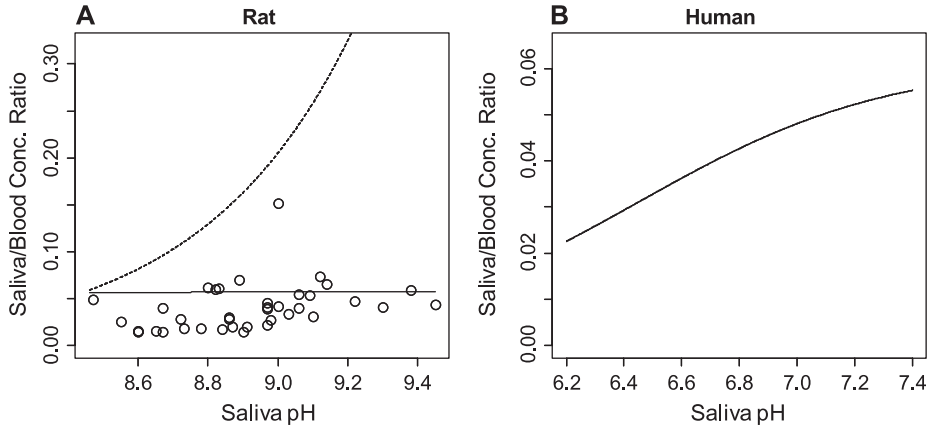

FIG. 8. Saliva/blood TCPy concentration ratios over varying salivary pH from rats dosed with TCPy (A). There was no significant relationship when analyzed using a mixed linear regression model ($p = 0.60$). The dotted line is the predicted saliva/blood TCPy concentration ratio by a modified Henderson-Hasselbalch equation (Matin *et al*., 1974), and the solid line is the same prediction using the modified Schmitt (2008) algorithm. Predicted human saliva/blood TCPy concentration ratios over a relevant range of salivary pH values as predicted by the modified Schmitt (2008) algorithm (B).

confounding processes that occur following a CPF exposure, such as TCPy formation rates from CPF metabolism. Using the PBPK/PD model, mean TCPy concentrations in blood obtained at 90 min after dosing from 1, 10, and 30 mg/kg TCPy doses administered iv extrapolated to oral CPF doses of approximately 4, 29, and 82 mg/kg using peak TCPy concentrations in blood (~4–8 h, depending on the CPF dose). Based upon observed linearity of the dose response across these doses, it is anticipated that pharmacokinetics for lower and more environmentally relevant dose levels will likewise be linear.

A potential limitation of the current study may be the coadministration of pilocarpine, used to induce salivation in rats, and isoflurane, used to induce and maintain anesthesia, along with the chemical of interest, TCPy. Pilocarpine is a nonselective muscarinic receptor agonist and has been the primary agent used to induce salivation in rodent model studies (Dawes, 1966; Piraino *et al*., 1980). Pilocarpine did not have any major effect on TCPy pharmacokinetics, as evidenced by the lack of dose-response effects, and others have reported no major effects of pilocarpine coadministration (Lu *et al*., 1997b). Isoflurane is an inhalable anesthetic, and effects of coadministration of isoflurane with TCPy are unknown. However, due to the reasonably accurate fits of the CPF PBPK/PD model, it can be hypothesized that any isoflurane effect on TCPy pharmacokinetics is minor if existent. Oxygen carrier gas used in conjunction with isoflurane, however, may have been responsible for increasing the salivary pH via induction of alkalosis. A final limitation of this study was measurement of TCPy from mixed saliva. Saliva is a mixture of secretions from three major glands (parotid, submandibular [submaxillary], and sublingual glands) as well as several minor glands. Others have observed a disparity between saliva/plasma chemical concentration ratios from saliva secreted from different glands and mixed saliva (Hayashi *et al*., 1989; Li *et al*., 2002). However, in the interest of compatibility with potential biomonitoring approaches (i.e., measure total saliva), mixed saliva was used as the major end point in this study.

Experimental results from this study suggest that TCPy partitioning from blood to saliva in rats is relatively constant over a range of varying conditions. TCPy pharmacokinetics was very similar in blood and saliva as demonstrated by proportional AUC differences comparing blood to saliva and by both dose group, as well as similar TCPy elimination rates of both blood and saliva (Table 2). Saliva/blood TCPy concentration ratios were not affected by TCPy concentration in blood or saliva flow rate over a range of blood TCPy concentrations (2.8–236.8 μmol/l; Fig. 6) and saliva flow rates (0.01–0.1 ml/min; Fig. 7). Consistent partitioning from blood

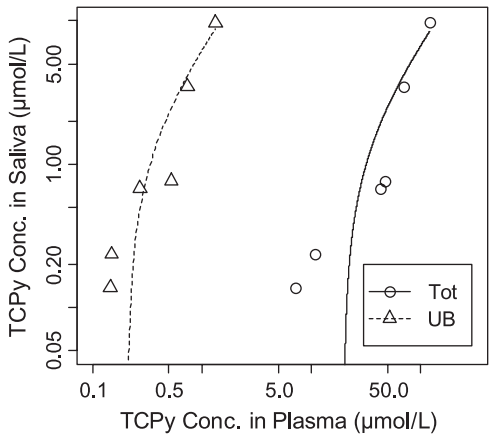

FIG. 9. Concentrations of TCPy in saliva and plasma (total TCPy [Tot] in plasma and TCPy unbound to plasma proteins [UB]) from rats dosed iv with TCPy and infused with pilocarpine. Lines are a linear regression model fits to the data. The correlation coefficient of TCPy concentration in saliva to the unbound TCPy concentration in plasma was 0.96 ($p = 0.002$). *Note*. Both y- and x-axes are on logarithmic scales.

to saliva over a range of administered doses, sampling time, and salivary flow rate was observed by others studying similar pesticides (Lu *et al.*, 1997a,b, 1998, 2003). After orally administering CPF to rats, Timchalk *et al.* (2007) observed consistent TCPy partitioning from plasma to saliva at dose of 1 and 10 mg/kg CPF but lower partitioning at 50 mg/kg CPF at collection times of 3, 6, and 12 h after dosing. However, at the high dose (~50% of LD_{50}), significant cholinergic effects were observed that could have led to nonlinearities. The mean value of saliva/blood TCPy concentration ratio for the two lower dose groups was 0.055, quite similar to the median value reported in this study (0.049).

Chemical compounds can transfer from blood to saliva by passive transcellular diffusion, active transcellular transport, or paracellular ultrafiltration through tight junctions (Haeckel, 1993; Jusko and Milsap, 1993). Generally, passive transcellular diffusion is thought to be the primary method for which most chemicals are transferred from blood to saliva, and the consistent partitioning from higher TCPy concentrations in blood to lower TCPy concentrations in saliva indicates that TCPy is probably transferred through a diffusion process. Lu *et al.* (1997a, 1998, 2003) hypothesized that both atrazine and diazinon transferred from plasma to saliva via transcellular diffusion.

Physicochemical properties that affect the amount of diffusion across a concentration gradient from blood to saliva include molecular size, lipid solubility, the dissociation constant of ionized compounds, and extent of plasma-protein binding (Haeckel, 1993; Jusko and Milsap, 1993). The molecular weight of TCPy is 198.4 g/mol, which supports the hypothesis that TCPy transfers from blood to saliva via diffusion as actively transported compounds are generally small cations or anions, such as lithium or thiocyanate (Gardner *et al.*, 1984; Groth *et al.*, 1974; Jusko and Milsap, 1993). The log octanol/water partitioning coefficient (K_{ow}, a measure of lipid solubility) of TCPy at a pH of 7 is 1.3 (Racke, 1993; Shemer *et al.*, 2005), which compared to other chemicals exhibiting similar K_{ow} values, roughly predicts similar saliva/blood concentration ratios within an order of magnitude (Piraino *et al.*, 1976). The dissociation constant (pK_a) also plays a large role in the transfer process as nonionized compounds can diffuse across membranes much more readily than ionized compounds. The pK_a of TCPy is 4.55 (Racke, 1993; Shemer *et al.*, 2005), which, in physiological conditions (7.4 pH), would mean the majority of TCPy is found in the ionized form and would be less able to diffuse across membranes, creating a saliva/blood TCPy concentration ratio < 1.0, as observed. 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 via diffusion (Jusko and Milsap, 1993; Lu *et al.*, 1998, 2003; Piraino *et al.*, 1980). The amount of TCPy plasma-protein binding measured was substantial (98.5%), and the concentration of unbound TCPy in plasma had a very high correlation coefficient to the concentration of TCPy in saliva (0.96). This suggests that the amount of TCPy binding to plasma proteins is one of the primary driving factors in determining the saliva/blood TCPy concentration ratio.

Physiological conditions such as blood pH, saliva pH, and salivary flow rate can also influence chemical compound diffusion from plasma to saliva (Haeckel, 1993; Jusko and Milsap, 1993). Generally, blood pH is consistently 7.4; however, salivary pH can vary and is primarily controlled by the amount of bicarbonate present in the saliva (Haeckel, 1993). In this study, mixed salivary pH were slightly basic (8.9 ± 0.2), which, using a modified Henderson-Hasselbalch equation (Matin *et al.*, 1974), predicted saliva/blood concentration ratios approximately two to seven times higher than those measured over the same salivary pH range (Fig. 8). Others have noted similar discrepancies between predicted values from this approach and measured values (Li *et al.*, 2002), possibly due to other chemical properties effecting compound diffusion such as lipid solubility. The formation of TCPy zwitterionic species from the nonionized form (Shemer *et al.*, 2005) could also effectively reduce measured saliva/blood TCPy concentration ratios versus theoretical values since those species would have less ability to readily diffuse across membranes. It was because of these other potential factors (TCPy lipid solubility, zwitterions, etc.) that the Schmitt (2008) algorithm was used to extrapolate the human blood to saliva TCPy partitioning coefficient. It has also been noted that pH measurements from mixed saliva may not reflect the pH of saliva when the chemical was transferred from blood to saliva, which brings to question the validity of such measurements (Haeckel, 1993).

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. The use of the blood to saliva TCPy partitioning coefficient in the PBPK/PD modeling strategy resulted in reasonably good model fits to saliva time course data (Fig. 4). Using the PBPK/PD model, other pharmacokinetic and pharmacodynamic measurements have previously been extrapolated from rats to humans (Timchalk *et al.*, 2002); assuming the transfer of TCPy from blood to saliva has the same interspecies mechanism, then predictions from the extrapolated model should be relatively accurate. Because of salivary pH differences between rats from this study (8.9 mean) and published values for humans (6.7 mean, 6.2–7.4 range; Drobitch and Svensson, 1992; Hold *et al.*, 1995; Ritschel and Tompson, 1983) and the relatively accurate prediction of blood to saliva TCPy partitioning coefficient in rats, the Schmitt (2008) algorithm was used to extrapolate the blood to saliva TCPy partitioning coefficient to humans. With physiological differences between rats and humans and the

dearth of human data in this regard, this extrapolation is the best estimate of human TCPy blood to saliva partitioning that can be made at this time. This algorithm predicts variable human blood to saliva TCPy partitioning coefficients over the range of reported human salivary pH values (Fig. 8B). Future experiments should be considered to determine if this prediction is indeed correct. Likewise, additional experiments to determine the level of TCPy binding in human plasma may also be warranted since protein binding is of particular importance in the relationship between plasma and saliva TCPy ratios.

Several states, including Washington and California, have implemented ChE monitoring for agricultural workers who apply pesticides. In the state of Washington, workers who handle pesticides for 30 h in a 30-day period must have their ChE levels monitored. If serum ChE levels drop 40% from an initial preexposure baseline ChE level, the worker is not allowed to continue handling pesticides until their ChE levels have recovered to within 20% inhibition of the baseline level (Chapter 296-307-148 Washington Administrative Code). Assuming that the sole source of plasma ChE inhibition is due to CPF, the PBPK/PD model predicts that a 70-kg human would require a daily oral CPF dose of 12 μg/kg to achieve 40% inhibition of plasma ChE after 30 days (Fig. 10A). PBPK/PD simulations of that same dose predict saliva TCPy levels to approximate 0.01 μmol/l (Fig. 10B) for humans with a salivary pH of 6.7. The developing QD TCPy sensor has a detection limit of 0.005 μmol/l TCPy in plasma, which is a more complex matrix than saliva. This detection limit is below the PBPK/PD predictions of TCPy concentrations in saliva after 30 days.

One potential challenge for using TCPy as a biomonitoring marker is the potential for background human exposure to TCPy from environmental sources. Once in the environment, CPF, chlorpyrifos-methyl (similar insecticide to CPF), and triclopyr (broadleaf herbicide that also has TCPy as a moiety) can all degrade to form environmental sources of TCPy. Since all these pesticides are used in agriculture for pest control, residues of each pesticide may be present in the same dietary food sources where CPF is found (Eaton *et al*., 2008), and each could contribute to the total body burden of TCPy, in turn effecting TCPy levels in saliva. One potential strategy to overcome this problem is to use a combined biomarker approach. Since a number of novel analytical approaches are being or have been developed that use several different biomarkers (Liu *et al*., 2005, 2006, 2008; Wang *et al*., 2008b), combined data from all those approaches integrated into a computational model, such as a PBPK model, would give the most accurate and robust estimates of exposure. In order to bring all these biomarkers together experimentally, future studies aimed at quantifying those other responses (salivary ChE activity) in tandem with TCPy partitioning to saliva over varying conditions are warranted after exposure to CPF.

In conclusion, experimental results suggest that TCPy partitioning from plasma to saliva in rats is relatively constant over a range of varying conditions. TCPy pharmacokinetics was very similar in blood and saliva, and saliva/blood TCPy concentration ratios were not affected by TCPy concentration in blood or saliva flow rate. The concentration of TCPy in saliva was highly correlated to the amount of unbound TCPy in plasma, and a saliva/blood TCPy partitioning coefficient was integrated within a PBPK/PD model for CPF to accurately predict TCPy concentrations in saliva. It is envisioned that this revised PBPK/PD can then be used to estimate CPF exposure from biomonitoring data collected with aforementioned various measurement approaches for real-time monitoring susceptible human populations such as agricultural workers.

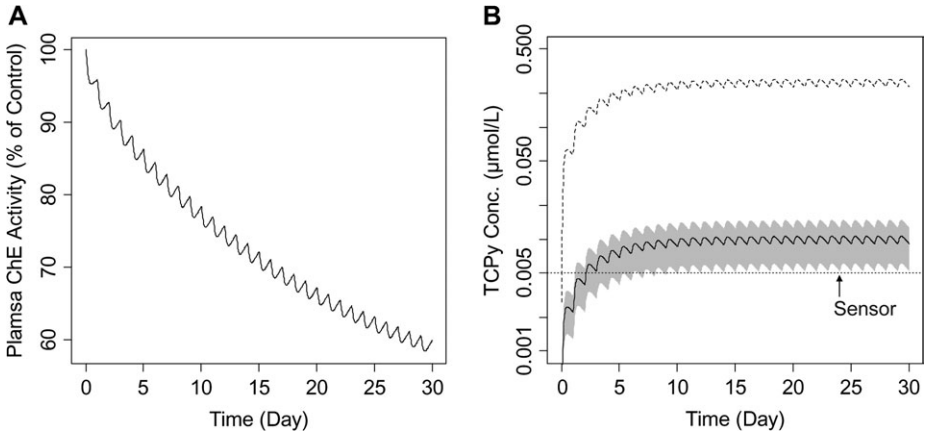

FIG. 10. Physiologically based PBPK/PD model of CPF simulations of a 70-kg human exposed to 12 μg/kg CPF daily for 30 days, the approximate dose to reduce plasma ChE activity to 60% of control (A). In (B), the dashed line indicates TCPy concentrations in blood, the solid line indicates TCPy concentrations in saliva using a saliva pH of 6.7, 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 sensor in plasma currently in development. *Note*. In (B), the y-axis is on a logarithmic scale.

FUNDING

Centers for Disease Control and Prevention/National Institute for Occupational Safety and Health (R01 OH008173 and R01 OH003629).

ACKNOWLEDGMENTS

Findings in this study were those of the authors and do not necessarily reflect the official opinion of Centers for Disease Control and Prevention/National Institute for Occupational Safety and Health. Authors would like to thank E. M. Klohe for technical assistance. The Dow Chemical Company had no input on the current research.

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