Available online at www.sciencedirect.com
ScienceDirect
Toxicology and Applied Pharmacology 219 (2007) 217–225
Toxicology and Applied Pharmacology
www.elsevier.com/locate/ytaap

Development of a non-invasive biomonitoring approach to determine exposure to the organophosphorus insecticide chlorpyrifos in rat saliva

Charles Timchalk [a,*], James A. Campbell [a], Guodong Liu [a], Yuehe Lin [a], Ahmed A. Kousba [b]

[a] *Pacific Northwest National Laboratory, 902 Battelle, Blvd., Richland, WA 99352, USA*
[b] *TargeGen Inc., 9380 Judicial Dr., San Diego, CA 92121, USA*

Received 30 August 2006; revised 2 October 2006; accepted 3 October 2006
Available online 6 October 2006

Abstract

Non-invasive biomonitoring approaches are being developed using reliable portable analytical systems to quantify dosimetry utilizing readily obtainable body fluids, such as saliva. In the current study, rats were given single oral gavage doses (1, 10, or 50 mg/kg) of the insecticide chlorpyrifos (CPF). Saliva and blood were then collected from groups of animals (4/time-point) at 3, 6, and 12 h post-dosing, and were analyzed for the CPF metabolite trichloropyridinol (TCP). Trichloropyridinol was detected in both blood and saliva at all doses and the TCP concentration in blood exceeded saliva, although the kinetics in blood and saliva were comparable. A physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model for CPF incorporated a compartment model to describe the time-course of TCP in blood and saliva. The model adequately simulated the experimental results over the dose ranges evaluated. A rapid and sensitive sequential injection (SI) electrochemical immunoassay was developed to monitor TCP, and the reported detection limit for TCP was 6 ng/L (in water). Computer model simulation in the range of the Allowable Daily Intake (ADI) or Reference Dose (RfD) for CPF (0.01–0.003 mg/kg/day) suggests that the electrochemical immunoassay has adequate sensitivity to detect and quantify TCP in saliva at these low exposure levels. However, to validate this approach, further studies are needed to more fully understand the pharmacokinetics of CPF and TCP excretion in saliva. These initial findings suggest that the utilization of saliva as a biomonitoring matrix, coupled to real-time quantitation and PBPK/PD modeling represents a novel approach with broad application for evaluating both occupational and environmental exposures to CPF.

Keywords: Chlorpyrifos; Saliva; Non-invasive; Biomonitoring; PBPK/PD

Introduction

In light of the potential for human exposures to a broad range of chemical contaminants, there is a need to develop rapid approaches for assessing internal exposure (dosimetry) and the resulting health hazards. A strategy has been developed that effectively links non-invasive biomonitoring approaches that will utilize portable micro-analytical systems for real-time analysis. To help interpret the non-invasive measurements, pharmacokinetic models are then used to estimate dosimetry (Timchalk et al., 2001).

Organophosphorus insecticides, like chlorpyrifos (CPF), constitute a large class of chemical insecticides that are widely used in the agricultural industry and historically in home applications (Aspelin, 1992, 1994). As a result, they are involved in more occupational poisoning cases than any other single class of insecticide, and in-home use has resulted in both intentional (suicide) and accidental human exposure (Al-Saleh, 1994). The toxic effects of organophosphorus insecticides are associated with the capacity of the parent chemical, or an active metabolite, to inhibit acetylcholinesterase (AChE) enzyme activity within nerve tissue (Murphy, 1986; Sultatos, 1994). The biochemical interactions between organophosphates and AChE and the toxicological implications of AChE inhibition are well understood. In general, phosphorothionates like CPF, lack the capacity to directly inhibit AChE, and must first be metabolized to the corresponding oxygen analog (CPF-oxon). The

* Corresponding author. DABT, Center for Biological Monitoring and Modeling, Pacific Northwest National Laboratory, 902 Battelle, Blvd., PO Box 999, MSIN: P7-59, Richland, WA 99352, USA. Fax: +1 509 376 9064.
E-mail address: charles.timchalk@pnl.gov (C. Timchalk).

0041-008X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2006.10.002

metabolic scheme for the thionophosphate insecticide CPF (#1) is presented in Fig. 1. Activation to CPF-oxon (#2) is mediated by CYP mixed function oxidases primarily in the liver. In addition, oxidative dearylation of CPF to trichloropyridinol (TCP) and diethylthiophosphate represents a competing detoxification pathway, which is likewise mediated by hepatic CYP (Ma and Chambers, 1994). A-Esterases (i.e. PON-1) can also effectively metabolize CPF-oxon to TCP without inactivating the enzyme (Pond et al., 1998); whereas, B-esterases (B-EST), such as carboxylesterases (CaE) and butyrylcholinesterases (BuChE), also detoxify CPF-oxon to TCP. However, these B-EST become stoichiometrically inhibited by the oxon (Chanda et al., 1997; Clement, 1984). Studies in humans and rodents indicate that TCP (#3) represents the primary urinary metabolite of CPF although glucuronide and sulfate conjugates of TCP have also been observed (Nolan et al., 1984; Bakke et al., 1976, Timchalk et al., 2005).

Biomonitoring offers one of the best approaches for accurately assessing human dosimetry and for determining risk from both occupational and environmental exposure to xenobiotics (Friberg and Elinder, 1993; Christensen, 1995). In this regard, organophosphorus insecticide biomonitoring has primarily focused on the assessment of ChE activity in blood or the quantification of metabolites in urine (Peoples and Knaak, 1982; Chester, 1993). As such, TCP has been used as an important analyte for biomonitoring CPF exposure (Nolan et al., 1984). Geer et al. (2004) reviewed a number of pesticide registrant studies that employed a combination of whole body dosimetry and biological monitoring to assess exposure to CPF. In these occupational studies, doses of CPF were determined by the measurement of urinary TCP, and based on these analyses the CPF dose was estimated to range from 97 to 275 μg/applicant (~1.3–4 μg/kg). With respect to non-occupational exposures, biological monitoring studies conducted in children prior to the EPA phase out of CPF for indoor residence use (EPA, 2000), also reported CPF dosimetry based on TCP biological monitoring. In a study that evaluated exposure 2 weeks after crack-and-crevice application of CPF, average post-application doses of CPF in children, again based on urinary TCP, were reported to range from 0.02–4.8 μg CPF/kg/day (Hore et al., 2005). Likewise, several studies have focused on non-occupational exposures (i.e. dietary or home use of insecticides), evaluated both aggregate chemical exposures (i.e. range of organic pesticides and persistent pollutants) as well as specific exposure to CPF in children. The authors reported median CPF doses in pre-school children ranging from 3 to 30 ng/kg/day (Wilson et al., 2003; Morgan et al., 2005). Since these exposures to CPF occurred prior to the EPA halting the use of CPF-based products for indoor and home applications, it is anticipated that they most likely represent upper limit dosimetry estimates.

To facilitate biomonitoring, there is a need to develop reliable, portable, and cost-effective analytical instrumentation for on-site personal monitoring (Timchalk et al., 2004). For the insecticide CPF, immunoassays have been developed for analysis of the parent compound or metabolite residues in food, agrochemicals, and environmental samples (Brun et al.,

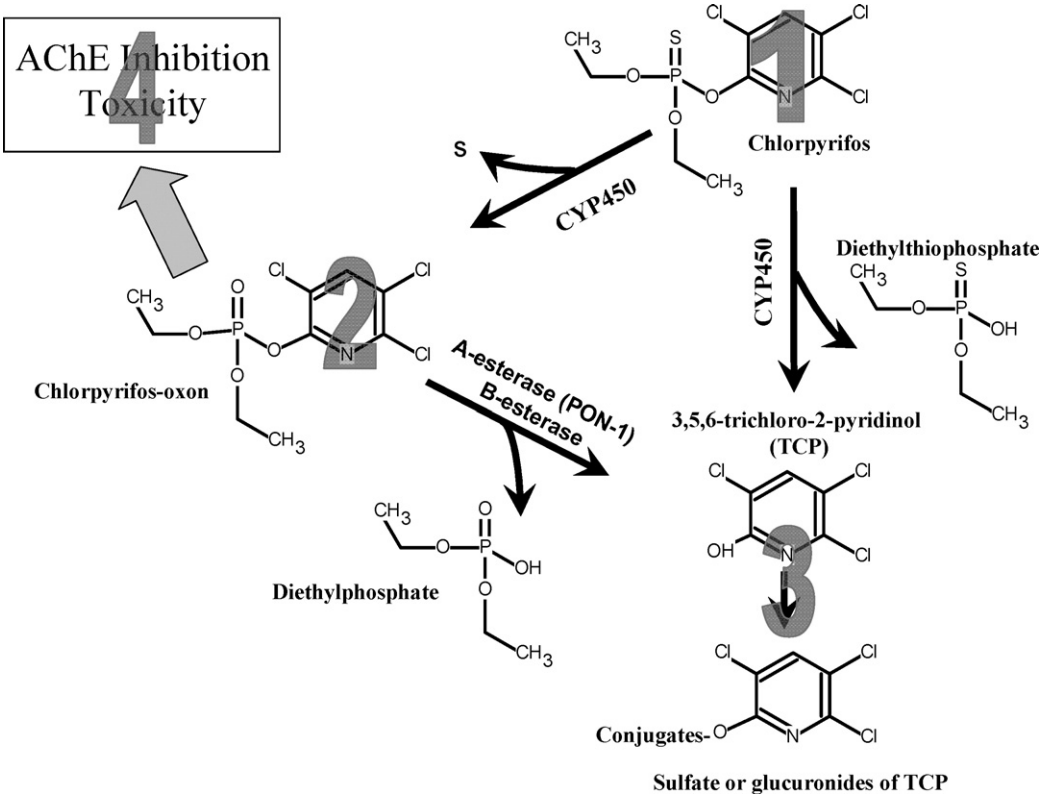


Fig. 1. Metabolic scheme for (#1) chlorpyrifos (CPF), and the major metabolites (#2) chlorpyrifos-oxon (CPF-oxon), (#3) trichloropyridinol (TCP), diethyl phosphate, and diethylthiophosphate.

2005; Banks et al., 2005; Galve et al., 2002). Similar approaches can be readily applied to quantify the insecticide or metabolites in readily obtainable human biological samples. Although biomonitoring has been conducted primarily by utilizing biological matrices such as blood and urine, other matrices such as saliva represent a simple and readily obtainable fluid. Although currently not extensively used as a biomonitoring matrix, saliva has been used to evaluate a number of biomarkers, drugs, and environmental contaminants including drugs of abuse, hormones, chemotherapeutics, heavy metals, and pesticides (Joselow et al., 1968; Hayashi et al., 1989; Nigg and Wade, 1992; Schramm et al., 1992; Lu et al., 1997, 1998). To reliably estimate internal dosimetry from a "spot" saliva sample requires a good understanding of the pharmacokinetics of the chemical and the relationship between the chemical concentration in the saliva and blood. Nigg and Wade (1992) summarized a number of early studies that attempted to correlate saliva levels of drugs, hormones, and chemicals with plasma levels to accurately estimate internal dose. In experimental animal models such as the rat, there are a number of studies evaluating the kinetics of saliva clearance for environmental contaminants (Borzelleca and Skalsky, 1980; Mobarak and P'an, 1984; Bratt et al., 1995; Lu et al., 1997, 1998; Timchalk et al., 2002; Kosuba et al., 2003). With increasing interest in the use of non-invasive monitoring for drug testing and for the evaluation of chemical exposures, it is anticipated that many more agents will be included in future lists. To facilitate understanding of the kinetics of drug and/or chemical clearance from blood into saliva, a fundamental understanding of salivary gland physiology and biochemistry is essential. Höld et al. (2000) provide a detailed review of human salivary gland anatomy, physiology as well as the mechanisms associated with drug transfer from blood to saliva. Although a fundamental understanding of saliva gland physiology exists, comparative cross-species (i.e. rodent vs. human) pharmacokinetic studies are lacking and are needed to further develop and refine dosimetry models. The current study serves to provide such models and support the utilization of saliva for pharmacokinetic and pharmacodynamic evaluation of chemical exposure.

The importance that physiologically based pharmacokinetic (PBPK) modeling can play in facilitating biomonitoring has been suggested (Mason and Wilson, 1999). These models are used to calculate target tissue dosimetry, facilitate extrapolation to low-doses, and enable cross-species extrapolation (Andersen, 2003). These models integrate standard values for organ volumes, and blood flows with chemical-specific parameters (e.g., tissue solubility, metabolic rate constants) to determine tissue dosimetry and pharmacodynamics. The PBPK models also provide a biologically based approach for assessing aggregate and cumulative risk associated with environmental exposures to a range of chemical agents. A physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model for the organophosphorus insecticide CPF has been developed (Timchalk et al., 2002), and is being modified to accommodate salivary clearance. An illustration of the PBPK/PD model structure developed for CPF is presented in Fig. 2. This model is comprised of four sub-models describing the pharmacokinetics of CPF (#1), CPF-oxon (#2), TCP (#3), and the pharmacodynamics of ChE inhibition (#2 and #4) in both rats and humans (for a more detailed discussion of the CPF PBPK/PD model see Timchalk et al., 2002). As previously noted, the application of these models has been recognized as an important tool for equating exposure to target tissue dosimetry (Andersen, 2003) and biological response.

The objectives of the current study were: (1) to evaluate the pharmacokinetics of the major CPF metabolite TCP in blood and saliva; (2) to modify the PBPK/PD model to accommodate TCP salivary clearance; and (3) to highlight current approaches for developing an electrochemical immunoassay for quantifying TCP in biological samples. These initial accomplishments are being used to facilitate additional model refinements, design of *in vivo* clearance studies, and sensor platform validation studies over a realistic range of concentrations.

Materials and methods

Chemicals. Chlorpyrifos (molecular wt. 350; 99% pure) (*O,O*-diethyl-*O*-[3,5,6-trichloro-2-pyridyl]-phosphorothioate) and TCP (3,5,6-trichloro-2-pyridinol) were kindly provided by Dow AgroSciences (Indianapolis, IN). Methyl-CPF (99.7% pure) was purchased from Chem Service Inc. (West Chester, PA). The derivatizing agent (*N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide) was purchased from Sigma Aldrich (Milwaukee, WI). The remaining chemicals used in this study were reagent grade or better and were purchased from Sigma Chemical Company (St. Louis, MO).

Animals. Male Sprague–Dawley rats (~250 g) were purchased from Charles River Lab Inc. (Raleigh, NC). Prior to use, animals were housed in solid-bottom cages with hardwood chips under standard laboratory conditions and given free access to water and food (PMI 5002, Certified Rodent Diet; Animal Specialties, Inc., Hubbard, OR). 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 at Battelle, Pacific Northwest Division.

Dose formulation and administration. The oral dose solutions for CPF were prepared in corn oil. Four groups of rats (4 rats/time-point) were orally gavaged (5 mL/kg) with CPF at dose levels of 1, 10, or 50 mg/kg of body weight. Food was withheld for ~12 h before and 3 h post-dosing and water was available *ad libitum*. At each time-point, saliva was collected from groups of rats (4 rats/dose) as previously described (Timchalk et al., 2001; Kosuba et al., 2003). In brief, rats were anesthetized with an ip injection of ketamine (87 mg/kg):xylazine (13 mg/kg), then given an ip injection of the cholinergic agonist pilocarpine (1 mg/kg) to induce salivation. Saliva was then collected, using a glass capillary tube, for approximately 30 min (~1–2 mL saliva) at which time the rats were exsanguinated by cardiac puncture. Whole blood and saliva specimens were weighed and stored frozen (−80 °C) until the TCP analysis was conducted.

Blood and saliva analysis. The concentration of TCP in blood and saliva was determined by negative ion chemical ionization mass spectrometry as previously described by Campbell et al. (2005) and similar to the approach developed by Brzak et al. (1998) for analysis of CPF and TCP in blood. The limit of TCP quantitation was reported to be 5 ng/mL.

Pharmacokinetic analysis. The time-course of TCP in blood and saliva was described using a one-compartment pharmacokinetic model that was originally used to describe the formation and elimination of TCP in a PBPK/PD model (see Fig. 2) for CPF (Timchalk et al., 2002). The equations used to describe the time-course of TCP are presented in Fig. 3. In this model the total amount of TCP formed from the metabolism of CPF and CPF-oxon in the PBPK/PD model is summed and the elimination of TCP in urine (Eq. (3) in Fig. 3) is described using a 1st order-rate (Ke); whereas, the clearance and excretion of TCP in saliva were

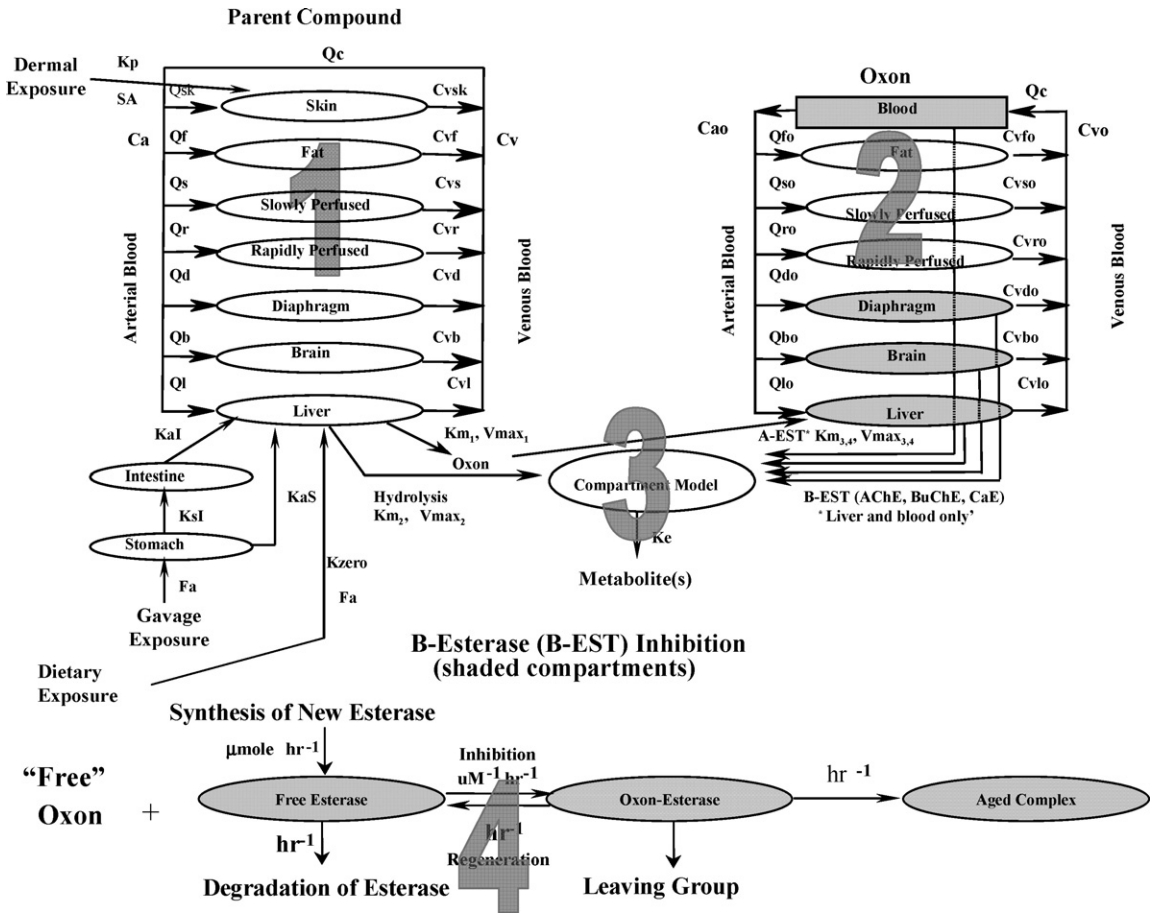


Fig. 2. Physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model used to describe the disposition of (#1) chlorpyrifos (CPF), (#2) chlorpyrifos-oxon (CPF-oxon), (#3) trichloropyridinol (TCP), and (#4) B-esterase inhibition in rats and humans following oral (gavage and dietary), and dermal exposures. The shaded tissue compartments indicate organs in which B-esterases (AChE, butyrylcholinesterase (BuChE), and carboxylesterase (CarbE)) enzyme activity is described. Model parameter definitions: QC cardiac output (L/h); Q_i, blood flow to "i" tissue (L/h); Ca, arterial blood concentration (μmol/L); Cao, arterial blood concentration of oxon (μmol/L); Cv, pooled venous blood concentration (μmol/L); Cv_i, venous blood concentration draining "i" tissue (μmol/L); Cv_io, venous blood concentration of oxon draining "i" tissue (μmol/L); SA, surface area of skin exposed (cm^2); KP, skin permeability coefficient (cm/h); K_{zero}, zero (μmol/h) rate of absorption from diet; Fa, fractional absorption (%); KaS and KaI, 1st-order rate constants for absorption from compartments 1 and 2 (per hour); KsI, 1st-order rate constant for transfer from compartment 1 and 2 (per hour); Ke, 1st-order rate constant for elimination of metabolite from compartment 3; $K_{m(1-4)}$, Michaelis constant for saturable processes (μmol/L); $V_{max(1-4)}$, maximum velocity for saturable process (μmol/h).

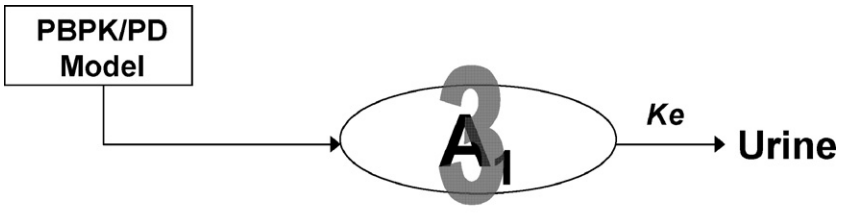
best described as a non-linear process using a Michaelis–Menten equation (Eq. (5) in Fig. 3). The concentration of TCP in blood (Cb_{tcp}) was defined as total amount of TCP in the compartment (A_1TCP) divided by the volume of distribution (V) for the compartment (Eq. (4) in Fig. 3). The concentration of TCP in saliva (c_{sal}TCP) was calculated by dividing the rate of TCP excretion in saliva $\left(\frac{dTCP_{sal}}{dt}\right)$ by the saliva flow rate (Ksl) (Eq. (7) in Fig. 3).

The pharmacokinetic model was coded using the computer program SIMUSOLV®, which contains a numerical integration, optimization and graphical routine, and is based on the FORTRAN-based software ACLS (Advanced Continuous Simulation Language). Specific values for model parameters used in the saliva compartmental model are presented in Table 1, other PBPK/PD model parameters are as previously described (Timchalk et al., 2002).

Results

The time-course of TCP in both blood and saliva following single oral gavage exposure to CPF over a range of doses (1, 10, and 50 mg/kg) is illustrated in Fig. 4. For all the dose groups, blood TCP was readily detectable at all sampling time points with peak blood TCP concentrations being attained by 6 h post-dosing. The time-course of TCP in the blood of rats demonstrated a linear response with increasing dose and was comparable to previously observed kinetics in humans (Timchalk et al., 2002). Trichloropyridinol was also readily detected in the saliva and peak TCP concentrations were likewise attained at 6 h post-dosing although the concentrations were ~1–2 orders of magnitude less than that observed in blood. At 6 h post-dosing peak saliva TCP concentration following 1, 10, or 50 mg CPF/kg was 0.07±0.03, 0.11±0.05, and 0.60±0.40 μmol/L, respectively. With increasing dose of CPF, there was a less than proportional increase in the TCP saliva concentration. Nonetheless, the saliva TCP kinetic profiles reasonably paralleled the blood TCP kinetics over the 12 h post-dosing sampling period.

To further compare the relationship between the blood and saliva TCP pharmacokinetics, for each dose group the TCP time-course for the blood and saliva concentrations was plotted and the results are presented in Fig. 5. Within each dose group the blood and saliva TCP concentrations are reasonably parallel to each other. As previously noted, although the blood TCP



$$\frac{dA_1TCP}{dt} = \left(\sum \frac{dA_1TCP(i)}{dt}\right) - (A_1TCP * K_e) - \frac{dTCP_{sal}}{dt} \quad (1)$$

$$A_1TCP = \int_0^t dA_1TCPdt \quad (2)$$

$$\frac{dTCP_{exc}}{dt} = A_1TCP * K_e \quad (3)$$

$$Cb_{TCP} = \frac{A_1TCP}{V} \quad (4)$$

$$\frac{dTCP_{sal}}{dt} = \frac{Cb_{TCP} * Fsl * VM_{TCP}}{(Cb_{TCP} * Fsl + Km_{TCP})} \quad (5)$$

$$TCP_{sal} = \int_0^t dTCP_{sal}dt \quad (6)$$

$$c_{sal}TCP = \frac{\frac{dTCP_{sal}}{dt}}{Ksl} \quad (7)$$

Fig. 3. Single compartment model and related equations used to describe the blood, urine, and saliva concentration time-course of trichloropyridinol (TCP). The compartmental model is capable of describing the TCP pharmacokinetics resulting from the metabolism of chlorpyrifos (CPF), as a sub-compartment within the CPF PBPK/PD model.

concentrations do increase proportionally to dose, the saliva concentration does not. Hence, the results suggest that with increasing CPF dose the TCP $\left(\frac{[\text{blood}]}{[\text{saliva}]}\right)$ ratio increases.

The PBPK/PD model that was developed for CPF incorporated a 1-compartment model to accommodate TCP formation and excretion (Timchalk et al., 2002). The TCP compartment model (see Fig. 3) was modified to accommodate saliva, and the model parameters are presented in Table 1. The apparent volume of distribution (V) and elimination rate (Ke) were optimized to fit the TCP blood time-course concentrations. The saliva:plasma ratio (Fsl) was calculated for the high dose (50 mg/kg) and to reasonably fit the non-linear saliva TCP concentrations blood/saliva clearance was described using a Michaelis–Menten equation where the apparent K_m and V_{max} parameters were determined by optimization against the experimental data. The saliva flow rate (Ksl) was determined based on the saliva volume obtained following the pilocarpine injection, and as such is an overestimate of normal saliva flow rates. The pharmacokinetic model was simultaneously fit to all 3 data sets and the resulting model fit is illustrated in Fig. 4. Overall, the model accurately simulated both the blood and saliva TCP time-course results through 12 h post-dosing.

Table 1
Parameter estimates for a TCP saliva compartment using a physiologically based pharmacokinetic and pharmacodynamic model for CPF

Parameter	Value	Estimation method[a]
Volume distribution, V (L)	0.62	Fit
Saliva: plasma ratio, Fsl	$1.5E^{-2}$	Fixed
TCP elimination rate, Ke (h^{-1})	0.07	Fit
TCP_{sal}, V_{max} (μmol h^{-1})	$9.1E^{-4}$	Fit
TCP_{sal}, K_m (μmol L^{-1})	$4.6E^{-2}$	Fit
Saliva flow rate, Ksl (mL h^{-1})	2	Measured

[a] The model parameters were estimated independently and held fixed (fixed), experimentally determined (measured), or estimated by fitting the model to the data (fit).

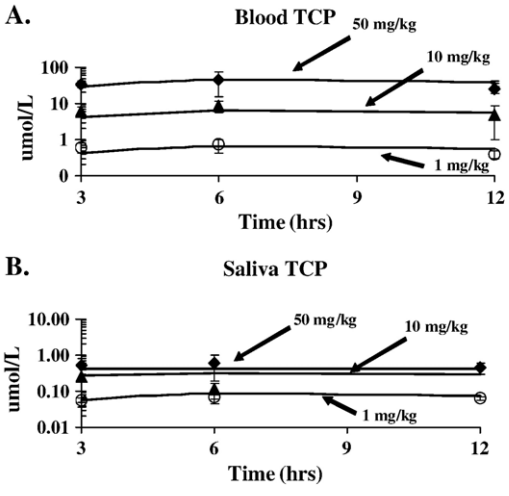


Fig. 4. Concentration time-course of trichloropyridinol (TCP) in blood (A) and saliva (B) following oral administration of chlorpyrifos (CPF) to rats at doses ranging from 1 to 50 mg/kg of body weight. The data points are the mean ± SD of TCP concentration for 4 animals/treatment/time-point. The line is the pharmacokinetic model fit to the experimental data (note: Saliva 12 h post-dosing sample from 10 mg/kg dose group not included, due to analytical error).

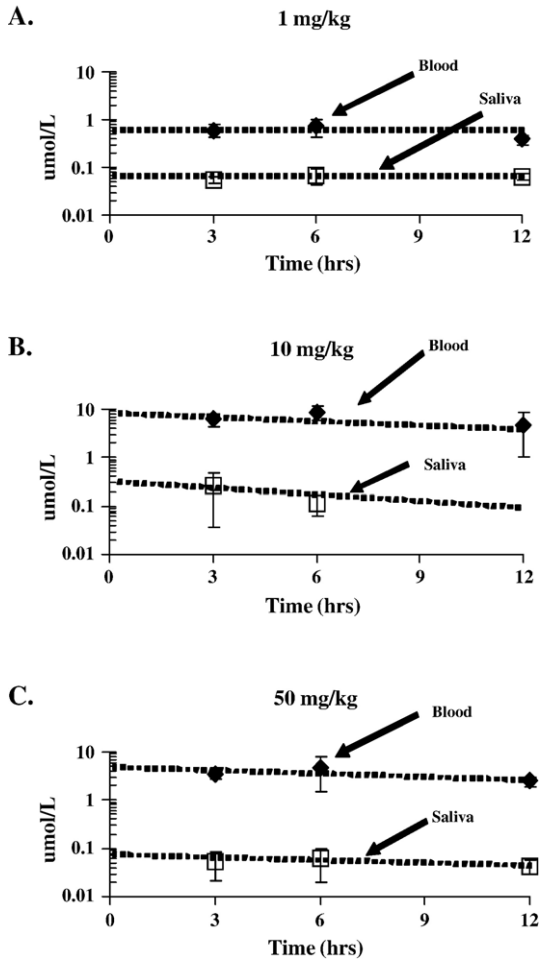


Fig. 5. Concentration time-course of trichloropyridinol (TCP) in blood and saliva following oral administration of chlorpyrifos to rats at doses of (A) 1 mg/kg, (B) 10 mg/kg, and (C) 50 mg/kg. The data points are the mean ± SD of TCP concentration for 4 animals/treatment/time-point. The dashed line was included to illustrate the magnitude of difference between blood and saliva TCP concentrations as a function of increasing dose.

Recent research efforts within our laboratory have focused on the initial development of an automated and sensitive sequential injection electrochemical immunoassay for the detection and quantitation of TCP in environmental and eventually biological samples (Liu et al., 2005). A schematic diagram of the sequential injection competitive immunoassay previously described by Liu et al. (2005) is presented in Fig. 6. The assay works by first immobilizing a TCP antibody-coated magnetic bead (MB) to the internal wall of a reactor tube with a magnet (A). After the beads are washed (B), the sample solution containing the TCP analyte and TCP-horseradish peroxidase (TCP-HRP) is introduced for a competitive immunoreaction (C). The beads are again washed (D) and a substrate solution of tetramethylbenzidine dihydrochloride and hydrogen peroxide ($TMB + H_2O_2$) is introduced to initiate an enzymatic reaction (E). The enzymatic product is then introduced into a thin-layer flow cell for electrochemical quantitation.

The electrochemical signal of the enzymatic product is directly proportional to the amount of formed immunocomplex (TCP-AB-MB/TCP-HRP) and is inversely proportional to the initial concentration of TCP (Liu et al., 2005). A typical electrochemical response and associated calibration curve resulting from increasing TCP concentrations are illustrated in Fig. 7. The detection limit was reported as 6.0 ng TCP/L. Preliminary results evaluating the response of the electrochemical immunoassay system with a commercially available ELISA immunoassays are reported in Table 2 (Liu et al., 2005), where water samples were spiked with known concentrations of TCP. The results with the electrochemical immunoassay are very comparable with those of the ELISA methods.

To help ascertain whether the electrochemical immunoassay would be adequately sensitive to detect TCP in saliva and blood at relevant environmental exposure levels, the PBPK model was used to simulate saliva and blood TCP concentration and blood CPF concentrations in humans. The model was used to simulate repeated dietary exposure over a 72 h period. To more reasonably simulate human behavior, continuous dietary exposures were simulated for 12 h, followed by a 12 h period of no exposure. Simulations were conducted at a dose of 0.01 mg/kg/day corresponding to an Allowable Daily Intake (ADI) or the EPA Reference Dose (RfD) of 0.003 mg/kg/day (Lu, 1995; EPA, 1994). The results from these simulations and the reported detection limits for a commercial TCP ELISA and the electrochemical immunoassay are presented in Fig. 8. In conducting this evaluation, the detection limits for both assays were based on the quantitation of TCP in water; and for the purpose of the current comparison it was assumed that similar detection limits would be achieved in blood or saliva. It is important to note that it is highly probable that the detection limits in biological fluids, like blood and saliva, will be somewhat lower than reported here. However, the comparison is still valuable since it can be used as an initial evaluation to determine a starting point for analytical sensitivity. Based on the current analyses, the commercial ELISA-based detection system would have limited sensitivity to detect saliva TCP at the ADI, and would not be able to detect TCP at an RfD of 3 μg/kg/day. In contrast, the electrochemical immunoassay theoretically has more than adequate sensitivity to detect TCP in both blood and saliva at the ADI or RfD levels (see Fig. 8).

Discussion

Biological monitoring is an important approach for determining absorbed dose since it incorporates all potential exposure routes (Mason and Wilson, 1999). However, the application of biological monitoring is hampered by a number of factors including the need for complicated and/or expensive analytical instrumentation, difficulty in obtaining biological specimens, and the lack of understanding of the pharmacokinetic properties of a chemical; all of which are needed to accurately estimate internal dose (Timchalk et al., 2001, 2004).

The current study demonstrated the ability to detect TCP in both blood and saliva following exposure to CPF at single oral doses ranging from 1 to 50 mg/kg. At all dose levels, the concentration of TCP in the blood exceeded the saliva and the magnitude of the blood:saliva difference increased over the dose range tested, ranging from 11- to 67-fold. These results

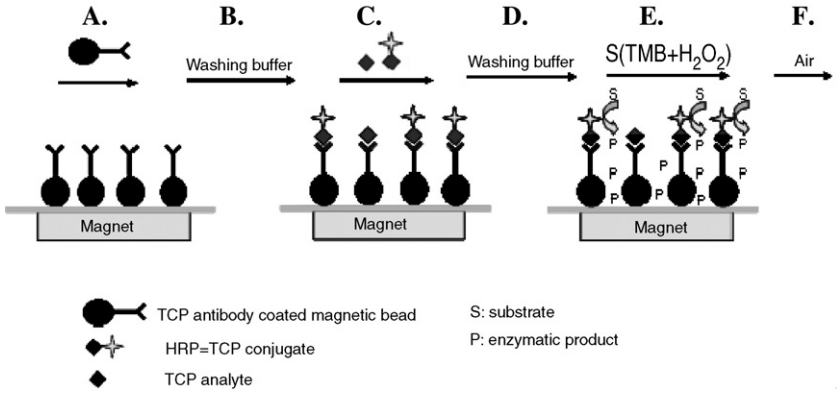


Fig. 6. Diagram illustrating competitive immunoassay in sequential injection system for determining trichloropyridinol (TCP) concentration. Figure adapted from Liu et al. (2005) with permission.

with TCP are generally consistent with results observed with other insecticides. Borzelleca and Skalsky (1980) reported that both the organochlorine insecticide Kepone and the carbamate insecticide carbaryl were detectable in saliva within the first 24 h post-dosing. In the case of carbaryl (most similar to CPF), the plasma concentration exceeded the saliva carbaryl concentration and the uptake (*in vitro*) of carbaryl into the submaxillary gland appeared to be via a passive (non-energy dependent) process. The authors also noted that carbaryl metabolites were excreted in saliva in a parallel fashion to plasma with a corresponding dose-dependent inhibition of saliva ChE (Borzelleca and Skalsky, 1980). Similarly, our research group has characterized salivary ChE activity *in vitro* with CPF-oxon (Kosuba et al., 2003), and has previously reported a dose-dependent inhibition of plasma and saliva ChE following CPF exposure in rats (Timchalk et al., 2004). Therefore the results of both studies suggest that saliva may be a good biological matrix for assessing organophosphorus insecticide exposure since both dosimetry and ChE inhibition are detectable.

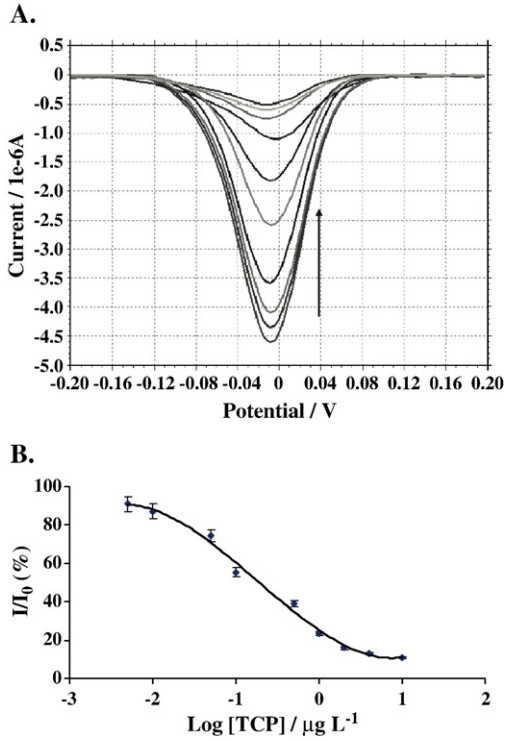


Fig. 7. (A) Typical sensor responses of increasing trichloropyridinol (TCP) concentration in incubation solution. From bottom to top, the concentrations of TCP in incubation solution are 0, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 2, 8, and 10 μg/L, respectively; (B) the resulting calibration curve for TCP. Figure adapted from Liu et al. (2005) with permission.

Although the current study and computational model demonstrates the ability to detect and model TCP in both blood and saliva, initial results suggest that the response is non-linear over the dose levels evaluated. It is important to acknowledge that the dose range used in the current study substantially inhibited plasma and saliva ChE activity (Timchalk et al., 2004) consistent with the high dose representing ~50% of an LD_{50}. These high doses were initially utilized to optimize the ability to detect TCP; however, it is also acknowledged that these doses could adversely impacted salivary clearance, particularly since excessive salivation is clearly associated with cholinergic crisis. In this regard, additional studies similar to those described by Lu et al. (1997) to characterize the salivary clearance of atrazine are needed to characterize TCP clearance at relevant occupational and environmental exposure levels.

Although the use of saliva as a biomonitoring matrix is appealing, a clear understanding of its limitations is needed before it can be used quantitatively to assess chemical exposure (Nigg and Wade, 1992). In general the ease of collection makes

Table 2
Recovery of trichloropyridinol (TCP) from spiked samples of tap water and river water

Recovery % mean±SD[a]			
Sample origin	TCP added (μg/L)	Electrochemical immunoassay	ELISA (spectrometer)
Tap water	0.006	107±7	104±9
Tap water	2	95±6	96±5
Columbia river	0.006	102±8	106±6
Columbia river	2	98±4	100±4

Table adapted from Liu et al. (2005) with permission.
[a] Standard deviation calculated from the average of three measurements.

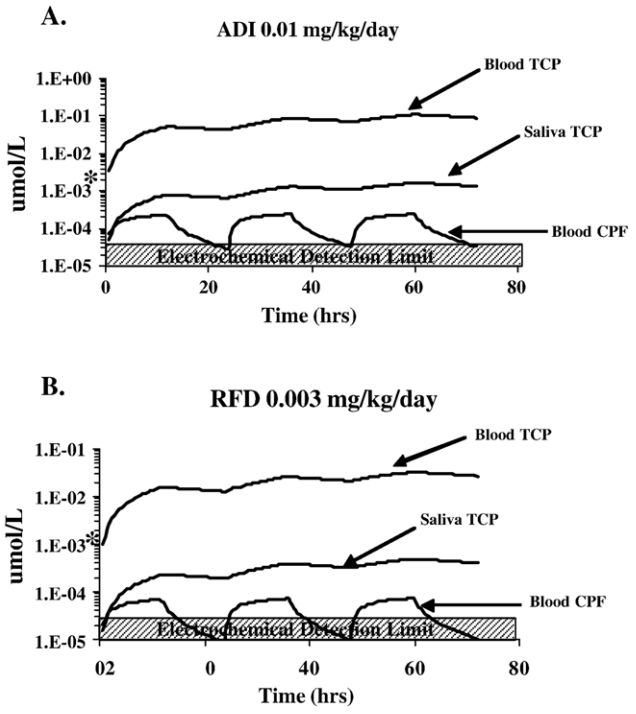


Fig. 8. Model simulation of TCP blood and saliva concentrations and blood CPF concentration following a repeated (3-day) dietary exposure (12 h/day) to (A) 0.01 mg/kg/day (ADI) or (B) 0.003 mg/kg/day (RfD). The detection limit (*) for the ELISA assay is based on the value reported by the manufacturer of the TCP RaPID Assay® kit (0.25 μg/L or $1E^{-3}$ μmol/L).

saliva ideally suited as a non-invasive approach and should result in excellent compliance, which is of critical importance for meaningful interpretation of biomonitoring data. However, to enable utilization of saliva for chemical biomonitoring requires the utilization of very sensitive and specific analytical methods for quantitation and the relationship between chemical concentration in blood and saliva must be established (Timchalk et al., 2004). As previously reported by Liu et al. (2005) and highlighted in this manuscript, a sequential injection electrochemical immunoassay has been developed for the quantitation of TCP, and has a reported detection limit for TCP of 6 ng/L in water. Using the PBPK model to simulate a theoretical blood and saliva TCP concentration resulting from CPF exposure at the ADI or RfD dose levels, suggests that the electrochemical immunoassay could quantify TCP in both blood and saliva specimens. Although this is encouraging, it will be critically important to evaluate the detection limits for TCP in biological fluids. For example, in developing a microanalytical system for the detection of lead (Pb) in blood or saliva, protein in the sample produced some electrode fouling resulting in a reduced responsiveness and irreproducible quantitation (Yantasee et al., 2005). However, optimization of sample pretreatment has resulted in excellent reproducibility and reliability of the sensor using biological fluids.

For saliva to be a useful quantitative biomonitoring matrix for assessing CPF exposure, it is imperative to understand the pharmacokinetic relationship between saliva concentration of TCP and CPF dose. To this end, PBPK modeling has been useful as a quantitative tool (Andersen, 1995). With regard to CPF, a PBPK/PD model has been previously developed and included a compartmental model to handle the formation and excretion of TCP in urine (Timchalk et al., 2002). To facilitate modeling of saliva TCP, the TCP compartmental sub-model within the CPF PBPK/PD model has been modified to accommodate saliva excretion. It is important to recognize that this model was developed with a limited data set; nonetheless the capability of the model to predict blood and saliva TCP concentration following CPF exposure is encouraging. Ongoing efforts are focused at including a saliva compartment within the pharmacodynamic model to enable simulation of salivary ChE inhibition kinetics.

To achieve a goal of employing saliva as a non-invasive quantitative tool for assessing CPF exposure in humans, a number of improvements in the understanding of CPF and TCP saliva kinetics are needed. These include: (1) basic understanding of how TCP is transported from the blood to salivary gland and secreted into saliva, (2) impact of changes in saliva secretion rate on salivary TCP clearance, (3) consequences of non-linear processes, such as TCP binding with blood or saliva proteins, and (4) more extensive *in vivo* data to compare TCP pharmacokinetics in blood and saliva following chronic and acute exposures over a wide dose range.

In summary, the current study demonstrated that the major CPF metabolite, TCP, is detectable in saliva and the kinetics parallel the TCP blood kinetics over the range evaluated. A sequential injection electrochemical immunoassay was developed to monitor for TCP, and theoretical model simulations suggest that the detection limits (based on TCP analysis in water) may be in the range to detect TCP in blood and saliva at relevant exposure levels. The results presented are encouraging and suggest that once fully developed and validated, a field deployable immunosensor platform coupled to PBPK/PD modeling may enable real-time biomonitoring for organophosphorus insecticides following occupational or environmental exposures.

Acknowledgments

This publication was partially supported by grants R01 OH008173-01 and R01 OH003629-03 from Centers for Disease Control and Prevention (CDC) and partially by the US Environmental Protection Agency's STAR program through grant R828608. Its contents are solely the responsibility of the authors and have not been subject to any review by CDC or EPA and therefore do not necessarily represent the official view of CDC or EPA, and no official endorsement should be inferred.

References

Al-Saleh, I.A., 1994. Pesticides: a review article. J. Environ. Pathol. Toxicol. Oncol. 13, 151–161.

Andersen, M.E., 1995. Development of physiologically based pharmacokinetic and physiologically based pharmacodynamic models for applications in toxicology and risk assessment. Toxicol. Lett. 79, 35–44.

Andersen, M.E., 2003. Toxicokinetic modeling and its applications in chemical risk assessment. Toxicol. Lett. 138, 9–27.

Aspelin, A.L., 1992. Pesticide Industry Sales and Usage: 1990 and 1991 Market Estimates. Office of Pesticide Programs, US Environmental Protection Agency, EPA 733-K-92-001, Washington, DC.

Aspelin, A.L., 1994. Pesticide Industry Sales and Usage: 1992 and 1993 Market Estimates. Office of Pesticide Programs, US Environmental Protection Agency, EPA 733-K-94-001, Washington, DC.

Bakke, J.E., Feil, V.J., Price, C.E., 1976. Rat urinary metabolites of *O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphorothioate. J. Environ. Sci. Health Bull. 3, 225–230.

Banks, K.E., Hunter, D.H., Wachal, D.J., 2005. Chlorpyrifos in surface waters before and after a federally mandated ban. Environ. Int. 31 (3), 351–356.

Borzelleca, J.F., Skalsky, H.L., 1980. The excretion of pesticides in saliva and its value in assessing exposure. J. Environ. Sci. Health 15, 843–866.

Bratt, P., Johansson, I., Linder, J., Ericson, T., 1995. Function of the rat salivary glands after exposure to inorganic mercury. Sci. Total Environ. 172, 47–55.

Brun, E.M., Garces-Garcia, M., Puchades, R., Maquieira, A., 2005. Highly sensitive enzyme-linked immunosorbent assay for chlorpyrifos. Application to olive oil analysis. J. Agric. Food Chem. 53 (24), 9352–9360.

Brzak, K.H., Harms, D.W., Bartels, M.J., Nolan, R.J., 1998. Determination of chlorpyrifos, chlorpyrifos oxon, and 3,5,6-trichloro-2-pyridinol in rat and human blood. J. Anal. Toxicol. 22 (3), 203–210.

Campbell, J.A., Timchalk, C., Kousba, A.A., Wu, H., Valenzuela, B.R., Hoope, E.W., 2005. Negative ion chemical ionization mass spectrometry of 3,5,6-trichloro-2-pyridinol in saliva of rats exposed to chlorpyrifos. Anal. Lett. 38, 939–949.

Chanda, S.M., Mortensen, S.R., Moser, V.C., Padilla, S., 1997. Tissue-specific effects of chlorpyrifos on carboxylesterase and choinesterase activity in adults rats: an in vitro and in vivo comparison. Fundam. Appl. Toxicol. 38, 148–157.

Clement, J.G., 1984. Role of aliesterase in organophosphate poisoning. Fundam. Appl. Toxicol. 4, S96–S105.

Chester, G., 1993. Evaluation of agricultural worker exposure to and absorption of pesticides. Occup. Hyg. 37, 509–523.

Christensen, J.M., 1995. Human exposure to toxic metals: factors influencing interpretation of biomonitoring results. Sci. Total Environ. 166, 89–135.

EPA, 2000. U. S. Environmental Protection Agency. Revised Human Health Risk Assessment for Chlorpyrifos and Agreement with Registrants. (Available at: http://www.epa.gov/pesticides/op/chlorpyrifos.htm).

EPA, 1994. U.S. Environmental Protection Agency. Integrated Risk Information System Database, pp. 5–54 (Washington, DC).

Friberg, L., Elinder, C.G., 1993. Biological monitoring of toxic metals. Scand. J. Work, Environ. Health 19 (Suppl. 1), 7–13.

Galve, R., Nichkova, M., Camps, F., Sanchez-Baeza, F., Marco, M.P., 2002. Development and evaluation of an immunoassay for biological monitoring chlroophenols in urine as potential indicators of occupational exposure. Anal. Chem. 74 (2), 468–478.

Geer, L.A., Cardello, N., Dellarco, M.J., Leighton, T.J., Zendzian, R.P., Roberts, J.D., Buckley, T.J., 2004. Comparative analysis of passive dosimetry and biomonitoring for assessing chlorpyrifos exposure in pesticide workers. Ann. Occup. Hyg. 48 (8), 683–695.

Hayashi, Y., Watanabe, J., Ozeki, S., 1989. Salivary excretion of 4-fluorouracil (5-FU): IV. Dependency of saliva/plasma concentration ratio and salivary clearance on plasma concentration of 5-FU during constant-rate intravenous infusion in rats. J. Pharmacobio-Dyn. 12, 137–144.

Höld, K.M., De Boer, D., Zuidema, J., Maes, R.A.A., 2000. Saliva as an analytical tool in toxicology. Inter. J. Drug Testing 1, 1–33.

Hore, P., Robson, M., Freeman, N., Zhang, J., Wartenberg, D., Özkayanak, H., Tulve, N., Sheldon, L., Needham, L., Barr, D., Lioy, P., 2005. Chlorpyrifos accumulation patterns for child-assessible surfaces and objects and urinary metabolite excretion by children for 2-weeks after crack-and-crevice application. Environ. Health Perspect. 113 (20), 211–219.

Joselow, M.M., Ruiz, R., Goldwater, L.J., 1968. Absorption and excretion of mercury in man: XIV. Salivary excretion of mercury and its relationship to blood and urine mercury. Arch. Environ. Health 17, 39–43.

Kosuba, A.A., Poet, T.S., Timchalk, C., 2003. Characterization of the in vitro kinetic interaction of chlorpyrifos-oxon with rat salivary cholinesterase: a potential biomonitoring matrix. Toxicology 188, 219–232.

Liu, G., Riechers, S.L., Timchalk, C., Lin, Y., 2005. Sequential injection/electrochemical immunoassay for quantifying the pesticide metabolite 3,5,6-trichoro-2-pyridinol. Electrochem. Comm. 7, 1463–1470.

Lu, F.C., 1995. A review of the acceptable daily intakes of pesticides assessed by the World Health Organization. Regul. Toxicol. Pharmacol. 21, 351–364.

Lu, C., Anderson, L.C., Morgan, M.S., Fenske, R.A., 1997. Correspondence of salivary and plasma concentrations of atrazine in rats under variable salivary flow rate and plasma concentration. J. Toxicol. Environ. Health 52, 317–329.

Lu, C., Anderson, L.C., Morgan, M.S., Fenske, R.A., 1998. Salivary concentrations of atrazine reflect free atrazine plasma levels in rats. J. Toxicol. Environ. Health 53, 283–292.

Ma, T., Chambers, J.E., 1994. Kinetic parameters of desulfuration and dearylation of parathion and chlorpyrifos by rat liver microsomes. Food Chem. Toxicol. 32, 763–767.

Mason, H., Wilson, K., 1999. Biological monitoring: the role of toxicokinetics and physiologically based pharmacokinetic modeling. Am. Ind. Hyg. Assoc. J. 60, 237–242.

Mobarak, N., P'an, A.Y.S., 1984. Lead distribution in the saliva and blood fractions of rats after intraperitoneal injections. Toxicology 32, 67–74.

Morgan, M.K., Sheldon, L.S., Croghan, C.W., Jones, P.A., Robertson, G.L., Chuang, J.C., Wilson, N.K., Lyu, C.W., 2005. Exposures of preschool children to chlorpyrifos and its degradation product 3,5,6-trichloro-2-pyridinol in their everyday environments. J. Exp. Anal. Environ. Epidemiol. 15, 297–309.

Murphy, S.D., 1986. Toxic effects of pesticides, In: Klaassen, C.D., Amdur, M.O., Doull, J. (Eds.), Casarett and Doull's Toxicology, The Basic Science of Poison, 3rd ed. MacMillam Publishers, New York, N.Y, pp. 519–581.

Nigg, H.N., Wade, S.E., 1992. Saliva as a monitoring medium for chemicals. Rev. Environ. Contam. Toxicol. 129, 95–119.

Nolan, R.J., Rick, D.L., Freshour, N.L., Saunders, J.H., 1984. Chlorpyrifos: pharmacokinetics in human volunteers. Toxicol. Appl. Pharmacol. 73, 8–15.

Peoples, S.A., Knaak, J., 1982. Monitoring pesticide blood cholinesterase and analyzing blood and urine for pesticides and their metabolites. In: Plimmer, J.R. (Ed.), Pesticide Residues and Exposure, Am. Chem. Soc. Symp. Series No. 182. American Chemical Society, Washington, DC, pp. 41–57.

Pond, A.L., Chambers, H.W., Coyne, C.P., Chambers, J.E., 1998. Purification of two rat hepatic proteins with A-esterase activity toward chlorpyrifos-oxon and paraoxon. J. Pharmacol. Exp. Ther. 286, 1404–1411.

Schramm, W., Smith, R.H., Craig, P.A., Kidwell, D.A., 1992. Drugs of abuse in saliva: a review. J. Anal. Toxicol. 16, 1–9.

Sultatos, L.G., 1994. Mammalian toxicology of organophosphorus pesticides. J. Toxicol. Environ. Health 43, 271–289.

Timchalk, C., Poet, T.S., Lin, Y., Weitz, K.K., Zhao, R., Thrall, K.D., 2001. Development of an integrated microanalytical system for analysis of lead in saliva and linkage to a physiologically based pharmacokinetic model describing lead saliva secretion. Am. Ind. Hyg. Assoc. J. 62, 295–302.

Timchalk, C., Nolan, R.J., Mendrala, A.L., Dittenber, D.A., Brzak, K.A., Mattsson, J.L., 2002. A physiologically based pharmacokinetic and pharmacodynamic model for the organophosphate insecticide chlorpyrifos in rats and humans. Toxicol. Sci. 66, 34–53.

Timchalk, C., Poet, T.S., Kousba, A.A., Campbell, J.A., Lin, Y., 2004. Noninvasive biomonitoring approaches to determine dosimetry and risk following acute chemical exposure: analysis of lead or organophosphate insecticide in saliva. J. Toxicol. Environ. Health, Part A 67, 635–650.

Timchalk, C., Poet, T.S., Hinman, M.N., Busby, A.L., Kousba, A.A, 2005. Pharmacokinetic and pharmacodynamic interaction for a binary mixture of chlorpyrifos and diazinon in the rat. Toxicol. Appl. Pharmacol. 205 (1), 21–32.

Wilson, N.K., Chuang, J.C., Lyu, C., Menton, R., Morgan, M.K., 2003. Aggregate exposure of nine preschool children to persistent organic pollutants at day care and at home. J. Exp. Anal. Environ. Epidemiol. 13, 187–202.

Yantasee, W., Timchalk, C., Weitz, K.K., Moore, D.A., Lin, Y., 2005. Optimization of a portable microanalytical system to reduce electrode fouling from proteins associated with biomonitoring of lead (Pb) in saliva. Talanta 67, 617–624.