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Journal of Toxicology and Environmental Health, Part A
Current Issues

ISSN: 1528-7394 (Print) 1087-2620 (Online) Journal homepage: https://www.tandfonline.com/loi/uteh20

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To cite this article: S. Lee , T. S. Poet , J. N. Smith , A. L. Hjerpe , R. Gunawan & C. Timchalk (2011) Impact of Repeated Nicotine and Alcohol Coexposure on in Vitro and in Vivo Chlorpyrifos Dosimetry and Cholinesterase Inhibition, Journal of Toxicology and Environmental Health, Part A, 74:20, 1334-1350, DOI: 10.1080/15287394.2011.567958

To link to this article: https://doi.org/10.1080/15287394.2011.567958

Journal of Toxicology and Environmental Health, Part A, 74:1334–1350, 2011

ISSN: 1528-7394 print / 1087-2620 online
DOI: 10.1080/15287394.2011.567958

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IMPACT OF REPEATED NICOTINE AND ALCOHOL COEXPOSURE ON IN VITRO AND IN VIVO CHLORPYRIFOS DOSIMETRY AND CHOLINESTERASE INHIBITION

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Chlorpyrifos (CPF) is an organophosphorus insecticide, and neurotoxicity results from inhibition of acetylcholinesterase (AChE) by its metabolite, chlorpyrifos-oxon. Routine consumption of alcohol and tobacco modifies metabolic and physiological processes impacting the metabolism and pharmacokinetics of other xenobiotics, including pesticides. This study evaluated the influence of repeated ethanol and nicotine coexposure on in vivo CPF dosimetry and cholinesterase (ChE) response (ChE- includes AChE and/or butyrylcholinesterase (BuChE)). Hepatic microsomes were prepared from groups of naive, ethanol-only (1 g/kg/d, 7 d, po), and ethanol + nicotine (1 mg/kg/d 7 d, sc)-treated rats, and the in vitro metabolism of CPF was evaluated. For in vivo studies, rats were treated with saline or ethanol (1 g/kg/d, po) + nicotine (1 mg/kg/d, sc) in addition to CPF (1 or 5 mg/kg/d, po) for 7 d. The major CPF metabolite, 3,5,6-trichloro-2-pyridinol (TCPy), in blood and urine and the plasma ChE and brain acetylcholinesterase (AChE) activities were measured in rats. There were differences in pharmacokinetics, with higher TCPy peak concentrations and increased blood TCPy AUC in ethanol + nicotine groups compared to CPF only (approximately 1.8- and 3.8-fold at 1 and 5 mg CPF doses, respectively). Brain AChE activities after ethanol + nicotine treatments showed significantly less inhibition following repeated 5 mg CPF/kg dosing compared to CPF only (96 ± 13 and 66 ± 7% of naive at 4 h post last CPF dosing, respectively). Although brain AChE activity was minimal inhibited for the 1-mg CPF/kg/d groups, the ethanol + nicotine pretreatment resulted in a similar trend (i.e., slightly less inhibition). No marked differences were observed in plasma ChE activities due to the alcohol + nicotine treatments. In vitro, CPF metabolism was not markedly affected by repeated ethanol or both ethanol + nicotine exposures. Compared with a previous study of nicotine and CPF exposure, there were no apparent additional exacerbating effects due to ethanol coexposure.

Chlorpyrifos (CPF) is a widely utilized organophosphorus (OP) pesticide. Although CPF has been banned from residential use within the last decade, exposures to CPF via dietary, occupational, and environmental pathways remain an important health concern (Lu et al. 2006; Morgan et al. 2004; Clegg and van Gemert 1999). The predominant mechanism of acute CPF toxicity involves acetylcholinesterase (AChE) activity inhibition

Received 29 October 2010; accepted 6 January 2011.

Although Drs. Timchalk and Poet have received funding from the Dow Chemical Company, the manufacturer of chlorpyrifos, to conduct research, the Dow Chemical Company did not have any involvement in the study design, analysis, and interpretation of data, in the writing of the report, or in the decision to submit this paper for publication. We gratefully acknowledge Dr. J. Campbell for valuable discussions. Although the research described in this article has been funded by the Centers for Disease Control and Prevention (CDC)/National Institute for Occupational Safety and Health (NIOSH) [CDC/NIOSH grant (R01-OH003629) to Dr. Timchalk], it has not been subject to any CDC/NIOSH review and therefore does not necessarily reflect the official views of the agencies; its contents are solely the responsibility of the authors and no public endorsement should be inferred.

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by its neurotoxic metabolite, chlorpyrifos-oxon (CPF-oxon), resulting in acetylcholine (ACh) accumulation at the synapses producing cholinergic signs in the peripheral and central nervous systems (Richardson 1995; Sultatos 1994; Pope 1999). A broad range of age, environmental, genetic, and personal lifestyle factors might influence and alter both the dosimetry and the biological responses associated with exposure to CPF because the balance between bioactivation and detoxification is an important determinant for toxicity (Chen et al. 2003; Faustman et al. 2000).

Alcohol and nicotine (mostly from cigarette smoking) are two of the most commonly co-used and co-abused drugs (Littleton et al. 2007). Among alcoholics approximately 90% smoke cigarettes, compared to 30% of nonalcoholics (Batel et al. 1995), and an increased likelihood to drink alcohol was demonstrated in rats treated with nicotine (Blomqvist et al. 1996). Many studies consistently show that alcohol promotes the use of tobacco and vice versa, where biological mechanisms play a role in the strong correlation between alcohol and nicotine use (Dohrman and Reiter 2003). Alcohol and nicotine alone and in combination are known to affect the metabolism of each other, mostly via the induction of a number of cytochrome P-450 enzymes (CYP450s) in different tissues (Howard et al. 2002). In this regard, Schoedel and Tyndale (2003) suggested that nicotine may increase the elimination of ethanol, and ethanol use may enhance the elimination of nicotine via metabolic cross-tolerance at behaviorally relevant doses. Effects from combined exposures to both ethanol and nicotine on hepatic and brain CYP450s were previously investigated (Yue et al. 2009; Howard et al. 2001), and some of these CYP450s are known to participate in the metabolism of CPF and are inducible by ethanol and/or nicotine. For example, desulfuration of CPF to CPF-oxon (bioactivation) is mediated by CYP1A2, 2B1 (rat), 2B6 (human), and 2D6 (human) at low, environmentally relevant concentrations of CPF, and by CYP3A4/5 and 2B1/6 at higher concentrations (Buratti et al. 2003; Mutch and Williams 2006). In addition, CYP2C, 2D6, and 3A4 were reported to participate in dearylation of CPF to 3,5,6-trichloro-2-pyridinol (TCPy), a major detoxification metabolite of CPF (Tang et al. 2001). Therefore, both nicotine and ethanol at pharmacologically relevant doses might influence key metabolic parameters associated with CPF metabolism, thereby modifying target tissue dosimetry.

While alcohol is known to modify a broad range of brain neurochemistry and physiology, changes in the expression of nicotinic acetylcholine receptors (nAChRs) are a consequence of prior neurochemical changes elicited by nicotine (Buisson and Bertrand 2001). Both alcohol and nicotine act on the mesolimbic dopamine system in several brain regions (Funk et al. 2006). Alcohol and nicotine also exert additive and antagonistic effects against each other. For example, nicotine may exacerbate alcohol-induced cerebrovascular effects (Li et al. 2007), or stimulatory effects by nicotine may alleviate some acute central nervous system (CNS) effects of alcohol (Söderpalm et al. 2000). However, there is only limited literature available demonstrating direct influence either by ethanol (O'Shaughnessy and Sultatos 1995) or nicotine on cholinesterase (ChE; includes AChE and/or butyrylcholinesterase [BuChE]) activity modifications resulting from organophosphorus insecticide exposure in any tissues including the brain.

Previously, the influences of nicotine on both in vitro metabolism and in vivo dosimetry and ChE inhibition of CPF was reported (Lee et al. 2009; 2010). Repeated nicotine treatments resulted in a significant increased (by twofold) in vitro metabolism of CPF to TCPy in rat hepatic microsomes (Lee et al. 2009), and the same dosing regimen resulted in a significantly elevated level of TCPy in blood and less inhibition of brain AChE activity in rats that were repeatedly treated in vivo with both nicotine and CPF compared to animals dosed with CPF only (Lee et al. 2010). It was also demonstrated that repeated oral CPF exposure exerted no significant impact on CYP450-mediated metabolism of CPF (Cometa et al. 2007; Lee et al. 2010).

The current study is part of ongoing research efforts to evaluate the impact that smoking and alcohol consumption may have on agricultural workers who are also routinely exposed to insecticides, given that these workers may face different risks from exposure to other chemical mixtures (Quandt et al. 2004). For example, Tabershaw and Cooper (1966) reported that 60% of agricultural workers who suffered acute OP insecticide intoxication developed intolerance to nicotine and alcoholic beverages. The objective of the current study was to evaluate whether the repeated coexposures to ethanol and nicotine might influence in vitro and in vivo CPF metabolism in male Sprague-Dawley rats.

In the present study, the animals were repeatedly coexposed to ethanol, nicotine, and CPF, and CPF pharmacokinetics along with ChE activity were evaluated. Specifically the pharmacokinetic analysis of the key metabolite, TCPy, in blood and urine, and inhibition profiles of ChE activities in plasma and brain AChE were determined. Furthermore, to investigate the role that metabolism plays in modifying CPF dosimetry, the formation of CPF-oxon and TCPy were also evaluated in vitro using hepatic microsomes from animals that were repeatedly exposed to ethanol or both ethanol and nicotine. A Monte Carlo simulation of the existing CPF physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) model (Timchalk et al. 2002b; Timchalk and Poet 2008) was utilized to help explain the results from these in vitro and in vivo studies.

MATERIALS AND METHODS

Chemicals

Chlorpyrifos (CPF, 99% pure) and 3,5,6-trichloro-2-pyridinol (TCPy, 99% pure) were kindly provided by Dow AgroSciences (Indianapolis, IN). Chlorpyrifos-oxon (CPF-oxon, 98% pure) was purchased from Chem Service, Inc. (West Chester, PA). Nicotine [as (−)-1-methyl-2-(3-pyridyl)pyrrolidine (+)-bitartrate salt], *N-tert*-butyldimethylsilyl-*N*-methyl-trifluoro-acetamide (MTBSTFA, the derivatizing agent for TCPy), acetylthiocholine chloride, and 5,5′-dithiobis(2-nitrobenzoic acid) were purchased from Sigma-Aldrich Corp (St Louis, MO). Ethanol (100%) was purchased from Gold Shield Chemical Co. (Hayward, CA). The remaining chemicals and other solvents were reagent grade or better and were purchased from Sigma-Aldrich.

Animals

Adult male Sprague-Dawley rats (200–225 g, approximately 7 WK old) were purchased from Charles River Laboratories, Inc. (Raleigh, NC). All procedures described in the present study were conducted in accordance with the NIH/NRC Guide and Use of Laboratory Animals, and were approved by the Institutional Animal and Care Use Committee (IACUC) of Battelle, Pacific Northwest Division. Prior to use, animals were housed in solid-bottom cages with hardwood chips and acclimated for 1 wk in a humidity- and temperature-controlled room with a 12-h light/dark cycle. Rodent feed (PMI Certified Rodent Diet # 5002) and water were provided ad libitum.

Dose Selection

The selection of doses and routes of administration for CPF and nicotine was based upon the previous mixture study with nicotine and CPF (Lee et al. 2010), in addition to the ample literature regarding each of these chemicals. There were two considerations in selecting CPF doses: (1) that major metabolites could be quantified in plasma, and (2) that ChE inhibition could be measured in both blood and brain while not resulting in extreme systemic cholinergic signs. Based upon the previous results and these criteria the CPF doses were 1 and 5 mg/kg/d po for 7 d.

There is a large body of literature describing the impact of ethanol on CYP2E1 induction, especially in liver (Rubin and Lieber 1968; Lieber 1997; Tsutsumi et al. 1993). The dose and route for ethanol administration were 1 g/kg/d, po as neat material for 7 d, based

on the previous study by Howard et al. (2001), who reported an approximate twofold increase in hepatic CYP2E1 after rats were treated daily with 0.3 → 3 g ethanol/kg po for 7 d. The oral dose of 1 g ethanol/kg to rats was considered in range with plasma ethanol concentrations observed in humans after 4 standard drinks (Sadler et al. 1996), or similar to plasma concentrations within 1 h of ethanol ingestion in humans (Schlorff et al. 1999).

The dose and route for the repeated nicotine administration, 1 mg/kg/d for 7 d sc, were based on the previous CYP450 induction studies (Lee et al. 2009). The pharmacological impacts of nicotine in animal models compared with cigarette smoking in humans were extensively reviewed (Tricker 2003; Benowitz 2009). In addition, doses of 1 mg nicotine/kg/d in rats were shown to produce similar CNS effects found in smokers (Trauth et al. 2000). The dose of nicotine used in the current study leads to blood nicotine concentrations similar to those those of human smokers who smoke approximately 10 cigarettes per day (Le Houezec et al. 1993), given that humans are slower in metabolizing nicotine than rats (Matta et al. 2007).

Animal Treatments for In Vivo Experiments

Lee et al. (2010) previously reported that brain AChE and plasma ChE activities were not markedly affected by repeated treatments with 1 mg nicotine/kg for 7 d, nor was there any literature identified suggesting that ethanol directly influences ChE activities. Thus, inhibition profiles of plasma ChE and brain AChE activities from CPF-dosed animals coexposed to saline or ethanol + nicotine were compared to those from naive (neither CPF- nor ethanol/nicotine-dosed) groups. Animals were randomly assigned to either the naive group ($n = 4$) or CPF-treated groups ($n = 4$–5 per time point) with a serial sacrifice design (1, 4, 8, 12, or 24 h post last CPF dosing). Table 1 outlines the dosing groups. Rats in both ethanol and nicotine treatment groups were dosed first with 1 mg nicotine/kg (in sterile saline) subcutaneously (sc) at the nape of the neck in a dose volume of 1 ml/kg body weight, followed by the oral gavage administration of 1 g (1.27 ml) neat ethanol/kg/d and 1 or 5 mg CPF/kg/d within 5 min. The CPF dose was prepared in a corn oil vehicle and administered at a dose volume of 5 ml/kg body weight. Animals in the 24-h sacrifice groups were individually housed in plastic metabolism cages (Thermo Fisher Scientific, Rochester, NY) 48 h prior to the last CPF dosing for acclimation. Urine was collected continuously at 12-h intervals post CPF treatment. Rats were euthanized by CO_2 asphyxiation at the specified time points post last CPF dosing, and blood was collected via cardiac puncture into heparinized syringes. Plasma from each animal was prepared by centrifugation of blood at 1100 × g for 10 min. Immediately after blood collection, the brain was removed, dry blotted, and homogenized with 9 volumes of ice-cold 0.1 *M* phosphate buffer (pH 7.4). All samples were stored at –80°C until analyzed. The same volume of blood/urine from each individual animal was treated with 250 μl of NaCl-saturated 3 *M* HCl for the analysis. For analysis of total TCPy in urine sample, urine aliquots were hydrolyzed by adding concentrated HCl and heated at 80°C for 1 h prior to extraction.

Animal Treatments for In Vitro Experiments

To study the potential for ethanol and/or nicotine to alter CPF metabolism in liver, animals were randomly assigned to naive or nicotine- and/or ethanol-treatment groups ($n = 4$ per group) (Table 1). Animals in ethanol treated groups orally (gavage) received 1 g/kg ethanol daily for 7 d. Animals in both ethanol + nicotine-treated groups were first dosed sc with nicotine (in sterile saline) at the nape of the neck (dose volume of 1 ml/kg body weight [bw]), followed by oral gavage dosing of 1 g/kg ethanol within 5 min. Rats were euthanized by CO_2 asphyxiation 4 or 24 h after the last dosing of ethanol. The liver was excised from animals immediately after blood

TABLE 1. Different Ethanol, Nicotine, and CPF Treatment Groups for In Vivo and In Vitro Studies

Groups			Time of sacrifice (post last dosing)
	Treatments, in vivo		
I	Saline, 7 d	5 mg CPF/kg/d, 7 d	
II	Ethanol + nicotine, 7 d	5 mg CPF/kg/d, 7 d	1, 4, 8, 12, or 24 h
III	Saline, 7 d	1 mg CPF/kg/d, 7 d	
IV	Ethanol + nicotine, 7 d	1 mg CPF/kg/d, 7 d	
	Treatements, in vitro		
V	Naive (saline)		
VI	Ethanol (1 g/kg, po), 7 d		4 h
VII	Ethanol (1 g/kg, po), 7 d		24 h
VIII	Ethanol (1 g/kg, po) and nicotine (1 mg/kg, sc), 7 d		24 h

Note. For in vivo study, male Sprague-Dawley rats were dosed with 1 or 5 mg CPF/kg/d, po (corn oil vehicle), in combination with saline or both 1 g ethanol/kg/d, po (neat), and 1 mg nicotine/kg/d, sc (saline vehicle), for 7 d. For in vitro experiments, rats were administered with 1 g ethanol/kg, po (neat), once a day for 7 d, or were dosed daily for 7 d with both 1 g ethanol/kg/d, po (neat), and 1 mg nicotine/kg/d, sc(in saline).

collection and rinsed with ice-cold 1.15% KCl (w/v). Hepatic microsomes from each animal were prepared by centrifugation at 9500 × g for 30 min and then supernatants were centrifuged twice at 105,000 × g for 60 min (Guengerich 1994). Microsomes were aliquoted and stored at –80°C until analysis.

Microsomal CPF Incubation and Enzyme Activity Assays

Protein concentrations and total microsomal CYP450 content were determined using the bicinchoninic acid (BCA) method and reduced CO difference spectra, respectively (Omura and Sato 1964). The activities of ethoxyresorufin *O*-dealkylase (EROD) or pentoxyresorufin *O*-dealkylase (PROD) were spectrophotometrically assayed using the modified method described by Pohl and Fouts (1980), and the activity of 4-nitrophenol (PNP) hydroxylase was determined using the modified method of Reinke and Moyer (1985). The selection of CYP450s was based upon previous literature that showed that (1) these were inducible either by ethanol or nicotine, and/or (2) they participated in CPF metabolism (Mutch and Williams 2006; Sams et al. 2000; Buratti et al. 2003; Tang et al. 2001).

Different concentrations (5–500 μ*M*) of CPF (initially dissolved in methanol and serially diluted in water) were incubated in a total volume of 0.5 ml containing 50 m*M* HEPES buffer, 15 m*M* $MgCl_2$, and 1 m*M* ethylenediamine tetraacetic acid (EDTA) (pH 7.4) with 2 mg hepatic microsomal protein per sample. Metabolism blanks, with 250 μl NaCl-saturated 2.5 *M* acetic acid, were run for comparison for nonspecific breakdown of CPF to TCPy and/or CPF-oxon. For quantification, the matrices for standard curves with the same volume were prepared using microsomes from naive rats by spiking with a series of CPF-oxon and TCPy concentrations, and analyzed alongside each set of samples. Our previous studies verified that 1 m*M* EDTA was sufficient to block any paraoxonase (PON1)-mediated metabolism of CPF-oxon, and the metabolism blanks were identical, regardless of whether the acid was added first or whether NADPH was not added (Poet et al. 2003; Lee et al. 2009). The samples were preincubated at 37°C for 2 min and 1 m*M* NADPH was added to initiate the reaction, which was terminated after 10 min by the addition of 250 μl NaCl-saturated 2.5 *M* acetic acid.

GC/MS Analysis

Samples of blood, urine, or microsomal CPF incubation were extracted (3×) with 0.75 ml ethyl acetate, vortexed in a shaking incubator for 10 min, and centrifuged to separate layers at 1100 × g for 20 min. Three subsequent organic layers were combined, the solvent was evaporated by blowing down

under a gentle stream of N_2, and residues were reconstituted in 0.15 ml toluene. For the analysis of TCPy in blood and urine, MTBSTFA (10 μl) was added and heated at 60°C for 1 h for derivatization of TCPy to its silylated form (Brzak et al. 1998). Half of the reconstituted samples from the in vitro incubation study were used for TCPy analysis, and the remaining solution was transferred to the other set of glass gas chromatography (GC)vials with glass inserts for CPF-oxon analysis. Gas chromatography/mass spectrometry (GC/MS) analyses were performed using an Agilent 5975B Inert XL EI/CI mass-selective detector (MSD), interfaced with an 7683B injector, a G2614A autosampler, and an Agilent 6890N GC equipped with ChemStation software for programming and data analysis (Agilent Technologies, Inc., Santa Clara, CA). Separation was achieved in splitless mode using a Restek RTX 1701 column (30 m × 0.25 mm ID × 1 μm df, Restek Co., Bellefonte, PA). The GC/MS conditions were utilized by following methods described in Brzak et al. (1998). The level of quantification for TCPy analysis with the present method was 0.012 and 0.051 μg/ml in urine and blood, respectively. The background level of TCPy in urine from naive animals was below the level of quantification with the method employed, although the background level of TCPy was determined in other studies using different analytical methods (Campbell et al. 2005).

Determination of ChE Activities in Plasma and Brain

Cholinesterase (ChE) activities in plasma and brain were determined spectrophotometrically using dithiodinicotinic acid (DTNB) as the chromagen. Acetylthiocholine was used as a substrate for both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), while butyrylthiocholine was employed as the substrate only for BuChE (Ellman et al. 1961). Plasma and brain homogenates were diluted 1/200 and 1/1750, respectively, to achieve optimal experimental conditions to place the optical density (OD)/min in the linear range (Kousba et al. 2007; Lee et al. 2010). Slopes from each tissue and group of animals were compared with those from naive animals ($n = 4$) and expressed as percent of naive group ChE activities.

Variability Analysis (Monte Carlo Simulations) of CPF PBPK/PD Model

A physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model for CPF, described by Timchalk and coworkers (Timchalk et al. 2002b; Timchalk and Poet 2008), was used to assess the impact of the variability in CPF brain metabolism on AChE activity in brain with acslX: 2.5.0.6 (AEgis Technologies Group, Inc., Huntsville, AL). Monte Carlo simulations with 1000 iterations were performed by random sampling of V_{max} for the metabolism of CPF to CPF-oxon or CPF to TCPy in brain to establish the enzyme activity distribution, and the data were then used as input parameters for the PBPK/PD model simulation of the oral administration of 5 mg CPF/kg/d for 7 d (Timchalk et al. 2002a). The probability distributions for both V_{max} values were presumed to follow normal distribution based on their means and 3× standard deviations, respectively (Gelman et al. 1996). While there were no available data regarding $V_{max}C$ values of CPF metabolism in brain, means (0.0084 and 0.36 μmol/h/L for CPF-oxon and TCPy, respectively) were based on the ratio of these activities between liver and brain in the rat (Chambers and Chambers 1989) and from preliminary studies (unpublished data). The coefficient of variation along with lower and upper bounds allowed a degree of variability without severely restricting the model, while maintaining physiological plausibility (Table 3). These assumptions were found reasonable because the CPF PBPK/PD model accurately simulated PK and PD responses including esterase inhibition in both rat and human over the wide range of CPF doses. The outputs from Monte Carlo simulations of brain AChE activity were summarized in the form of mean and 95% confidence intervals of brain AChE activity.

Data Analysis

Means and standard deviations of TCPy concentrations in blood, urinary TCPy amount, and ChE activities in plasma and brain AChE were obtained from individual animals in each group. All in vitro CPF incubations were conducted with hepatic microsomal samples prepared from individual animals. The in vitro metabolic rate constants for formation of CPF-oxon and TCPy, V_{max} and K_{mapp} (apparent affinity constants), were calculated by fitting the data to the Michaelis–Menten equation with least-squares fit method using Prism 5 (Graphpad Software, Inc., La Jolla, CA). The statistical difference were tested by one-way analysis of variance (ANOVA), followed by post hoc Dunnett's multiple comparison test, where p values less than .05 were considered to be significant.

RESULTS

General Observations from In Vivo Study

Initially (d 1), animals in the ethanol + nicotine treatment groups manifested acute cholinergic effects, including salivation and tremors, mostly due to nicotine. However, animals showed only transient or negligible cholinergic effects after repeated treatments. The CPF doses (1 and 5 mg/kg/d) were sufficiently low that animals repeatedly dosed with saline and CPF did not exhibit any observable acute cholinergic effects by CPF.

Pharmacokinetics

The time-course profiles of TCPy concentrations in blood after repeated administration with either 1 or 5 mg CPF/kg/d are presented in Figure 1. Peak TCPy concentrations in blood measured at 4 h post last dosing of CPF were higher in ethanol and nicotine coexposed groups than those from animals dosed with CPF only. Ethanol and nicotine co-treatments increased blood TCPy concentrations at 4 h (approximately threefold) following the repeated 1-mg CPF/kg doses; however, these differences were not statistically significant across 5 mg CPF/kg dosing groups. Compared to controls (CPF only), the areas under the curve of blood TCPy through 12 h post last dosing ($AUC_{0\text{-}12h}$) for the 1-mg/kg CPF groups were also increased after ethanol + nicotine coexposure (3.3 and 7.3 μmol-h/L, respectively), whereas in the 5-mg/kg CPF treated groups, $AUC_{0\text{-}24h}$ of blood TCPy increased by approximately fourfold in ethanol + nicotine co-treated groups compared to controls (66.2 and 15.8 μmol-h/L, respectively). When the different doses of 1 and 5 mg CPF/kg/d were compared, AUCs of blood TCPy in the saline and CPF groups were dose-linear.

The amounts of TCPy excreted in the urine, measured after acid hydrolysis of urine samples to quantify total (free and conjugated) TCPy, are presented in Figure 2. Ethanol + nicotine pretreatments exerted no significant impact on the overall urinary TCPy excretion profiles during the 24 h post last dosing period. Total urinary excretion amounts of TCPy from 1 mg CPF groups were 0.6 ± 0.1 and 0.5 ± 0.1 μmol in ethanol + nicotine co-treated and saline groups, respectively, and 4.8 ± 1.1 and 3.8 ± 0.5 μmol of urinary TCPy were recovered after repeated 5-mg CPF/kg/d administration.

Pharmacodynamics

Butyrylcholinesterase (BuChE) and AChE activities were both measured in plasma and AChE activities were measured in brain, given that AChE and BuChE have almost equal contributions to ChE responses in rat plasma, while AChE is the major form of ChE in brain (Chatonnet and Lockridge 1989). The ChE activities in plasma from animals exposed to repeated treatments with both ethanol + nicotine in addition to 1 or 5 mg CPF/kg/d were not significantly different from those animals repeatedly treated with CPF only (Figures 3, A and B). Plasma ChE activities were substantially inhibited after 7 daily repeated doses of 5 mg CPF, whether they were dosed with ethanol + nicotine or just saline (24 ± 5% and 22 ± 6% of those from naive

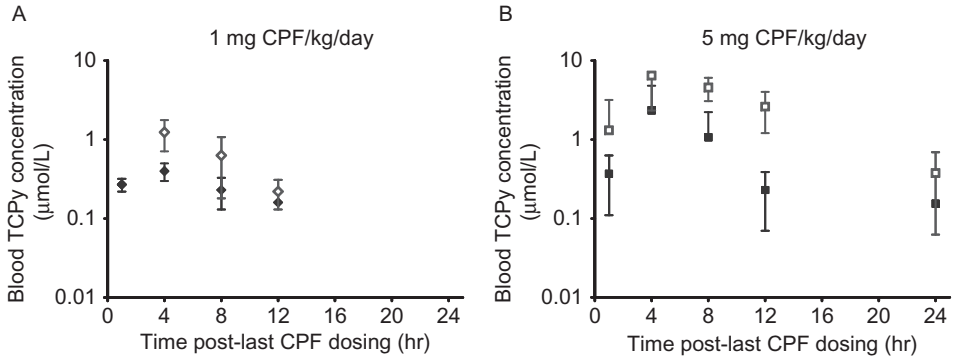

FIGURE 1. Time-course TCPy concentration profiles in blood (μmol/L) after repeated administration of (A) 1 mg CPF/kg/d (in corn oil, po), or (B) repeated 5 mg CPF/kg/d, po for 7 d, combined with saline, sc, or both 1 g ethanol/kg/d, po and 1 mg nicotine/kg/d, sc treatments for 7 d. The animals were euthanized at 1, 4, 8, 12, or 24 h post last dosing of CPF. Values represent mean TCPy concentration ± SD ($n = 4$–5 per group per time point). Filled and open symbols represent saline- and both ethanol + nicotine-treated groups, respectively. *Note*. Blood TCPy concentrations of ethanol, nicotine, and 1 mg CPF group at 1-h time point, as well as those of 24-h time point from all 1-mg CPF-dosed animals, were below the detection limit. In ethanol-, nicotine-, and 5-mg CPF-dosed animals ($n = 4$), blood TCPy concentrations at 24-h time point from 2 animals were below the detection limit.

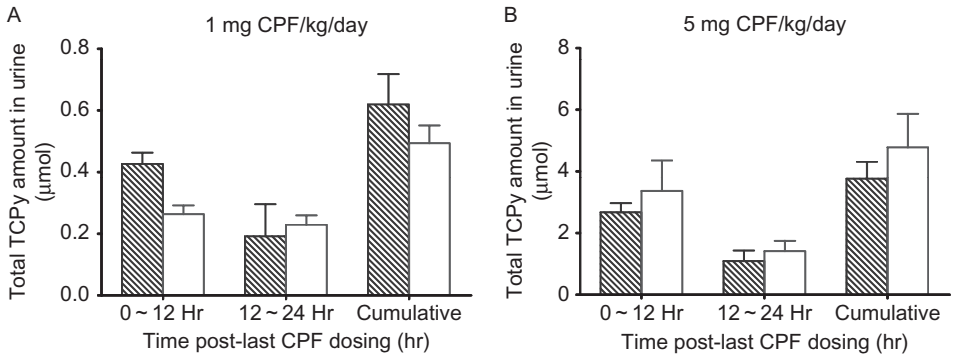

FIGURE 2. Amounts (μmol) of TCPy excreted in urine over 24 hr after the last dosing of (A) 1 mg CPF/kg/d, po, or (B) 5 mg CPF/kg/d, po, for 7 d combined with saline or both 1 g ethanol/kg/d, po, and 1 mg nicotine/kg/d, sc, treatments for 7 d. Bars represent means ± SD ($n = 4$–5 per group). Filled and open bars represent saline- and ethanol + nicotine-treated groups, respectively. Note different *y*-axis scales. Urine was collected (1) before CPF administration, (2) from 0 to 12 h, and (3) 12–24 h after the last CPF dosing.

animals, respectively) (Figure 3A). Following repeated 1-mg CPF doses, maximum inhibition of plasma ChE (AChE + BuChE) activities measured were at 39 ± 7% and 48 ± 4% from ethanol + nicotine coexposed or saline groups, respectively (Figure 3B). Similar to ChE activity inhibition profiles, BuChE activities in plasma was not significantly different due to ethanol + nicotine coexposure (Figure 3, C and D). The measured maximum inhibition of BuChE activities were 19 ± 4% and 18 ± 5% in ethanol + nicotine versus saline groups following a 5-mg/kg/d CPF dose, and 21 ± 5% and 34 ± 8% for 1-mg/kg/d CPF treatments, respectively.

The most significant impact of repeated ethanol + nicotine treatments was observed in the inhibition profiles of brain AChE activity (Figure 4). CPF-only groups for both doses followed the expected inhibition profiles of brain AChE activities (maximum inhibition at 85 ± 15% and 65 ± 7% for 1- and 5-mg/kg/d CPF dose groups, respectively). However, animals that were coexposed to both ethanol and nicotine in addition to CPF had substantially less brain AChE inhibition due to CPF than rats dosed with saline and CPF. After repeated treatments with 1 mg/kg CPF, only the 4 h post last CPF dosing time point exhibited a significant difference (approximately

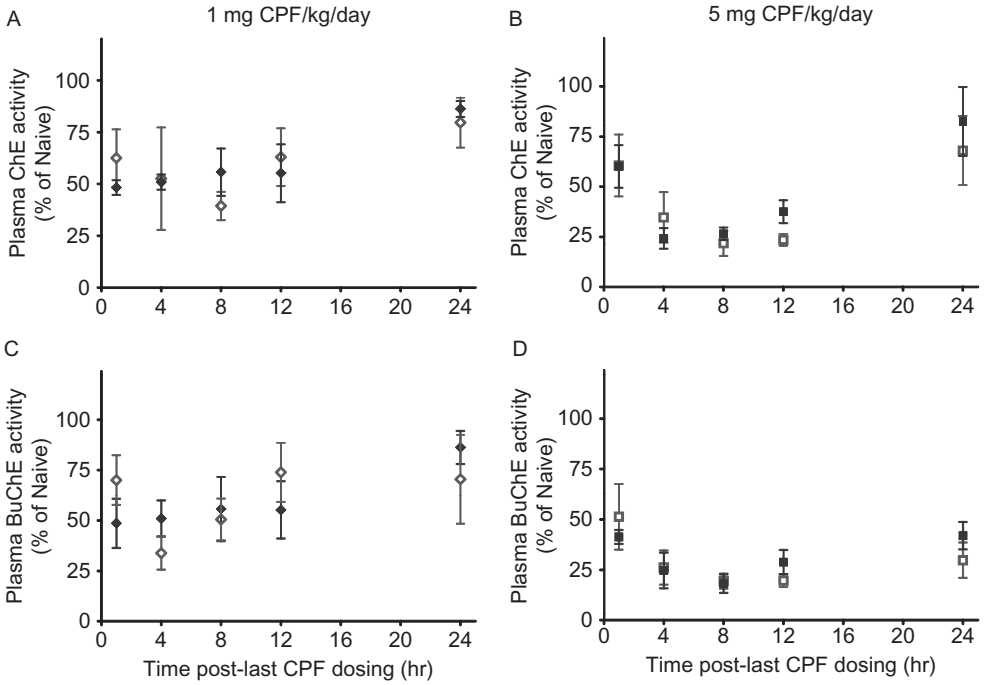

FIGURE 3. Inhibition profiles of ChE activities (A and B) and BuChE activities (C and D) in plasma during 24-h period after last dosing of 1 mg CPF/kg/d, po (A and C), or 5 mg CPF/kg/d, po (B and D), for 7 d, combined with saline or ethanol + nicotine treatments. Symbols represent the means (± SD, $n = 4–5$) as percent of activities of naive animals. Filled or open symbols denote saline- or ethanol + nicotine-treated groups, respectively.

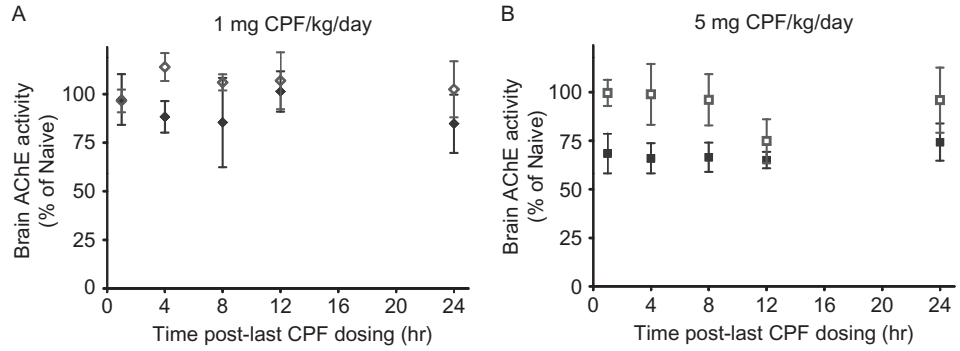

FIGURE 4. Inhibition profiles of AChE activities in brain during 24-h period after last dosing of (A) 1 mg CPF/kg/d, po, or (b) 5 mg CPF/kg/d, po, for 7 d, combined with saline and ethanol + nicotine treatments. Symbols represent the means (± SD, $n = 4–5$) as percent of those activities from naive animals. Filled and open symbols denote saline- or ethanol + nicotine-treated groups, respectively.

10%,) between saline versus ethanol + nicotine coexposed animals (Figure 4A). However, ethanol + nicotine coexposed animals that received a 5-mg/kg/d CPF dose retained higher ChE activities than those from CPF only groups (up to approximately 30% in differences when compared with those from naive animals) (Figure 4B).

CYP450-Mediated In Vitro Metabolism of CPF

In vitro CYP450-mediated CPF metabolism was studied to determine whether the results seen in vivo were associated with changes in hepatic metabolism. Results of CPF metabolism to CPF-oxon and/or TCPy across the different treatment groups are summarized in Table 2

TABLE 2. Comparison of the Kinetic Parameters for In Vitro CYP450 Metabolism of CPF in Hepatic Microsomes After Ethanol or Ethanol and Nicotine Treatments

	CPF-oxon			TCPy		
Groups	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m
V (naive)	0.98 ± 0.13	111.2 ± 12.5	8.6 ± 2.6	3.23 ± 0.64	79.9 ± 8.4	45.7 ± 15.1
VI	0.81 ± 0.20	96.8 ± 16.5	8.4 ± 1.6	3.09 ± 0.52	67.1 ± 6.3	45.9 ± 5.1
VII	1.15 ± 0.20	127.4 ± 15.9	9.7 ± 1.8	3.09 ± 0.63	76.3 ± 39.4	44.7 ± 11.7
VIII	0.71 ± 0.13	88.6 ± 24.7	8.4 ± 2.2	2.37 ± 0.44	49.6 ± 4.9	47.5 ± 5.3

Note. Male Sprague-Dawley rats were treated daily with saline, 1 g ethanol/kg, po, or both 1 g ethanol/kg, po, and 1 mg nicotine/kg, sc, for 7 d. Hepatic microsomes were prepared from individual animals, killed either at 4 or 24 h post last dosing (Table 1). Values for V_{max} (nmol/min/mg), K_{mapp} (μM), or V_{max}/K_m (min^{-1}) are expressed as means ± SD for $n = 4$ determinations.

TABLE 3. Parameters for Variability Analysis of CPF Metabolism in Brain Using Monte Carlo Simulation

	Distribution	Mean (μmol/h/L)	Standard deviation	Lower bound	Upper bound
Desulfation (CPF → CPF-oxon)	Normal	0.0084	0.01	0.0001	0.03
Dearylation (CPF → TCPy)	Normal	0.36	0.7	0.01	3.0

Note. The probability distributions for both $V_{max}C$ values of CPF to CPF-oxon or to TCPy in brain were presumed to follow normal distribution based on their means and the coefficient of variation of 2. The initial values were based on the ratio of activities between liver and brain in the rat. To maintain physiological plausibility, lower bounds for $V_{max}C$ values of CPF-oxon and TCPy were set at 0.0001 and 0.01, respectively, while three standard deviations were used for the upper bound. These bounds also were set large enough to represent all plausible metabolic combinations.

and depicted in Figure 5. The kinetic parameters for CPF metabolism in the naive group are comparable to those reported previously (Poet et al. 2003; Lee et al. 2009). The overall impact on metabolic rates of in vitro CPF metabolism due to repeated administrations with prior in vivo ethanol or ethanol + nicotine treatments were minimal. Animals pretreated with ethanol did not show any significant differences in either CPF-oxon or TCPy formation. Animals exposed to repeated ethanol and evaluated at 24 h post last dosing showed a modest 1.2-fold (not significant) increase in CPF-oxon V_{max}, while there were no observed differences in TCPy formation (Figures 5A and B). The combined pretreatments with ethanol + nicotine suggested no significant impact on the TCPy V_{max}. Furthermore, there were no statistically identified differences among K_{mapp} and V_{max}/K_m values

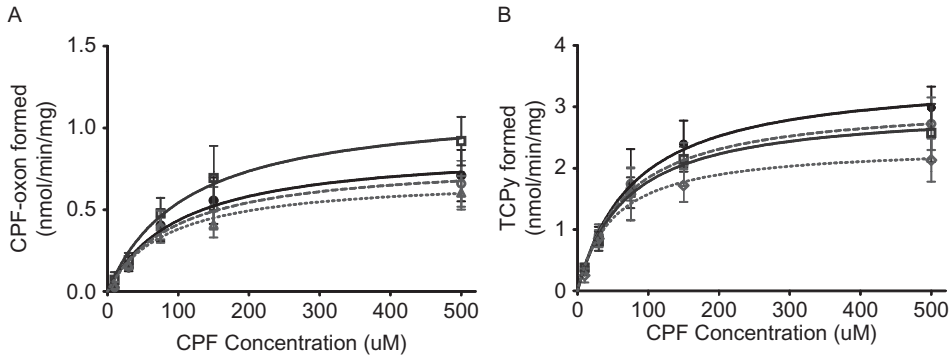

FIGURE 5. The CYP-mediated in vitro metabolism of CPF to CPF-oxon and/or TCPy in hepatic microsomes of male Sprague-Dawley rats after different ethanol or ethanol + nicotine treatments (Naive: filled circles; 7 × EtOH + 4 h: open circles; 7 × EtOH + 24 h: open squares; 7 × EtOH + nicotine + 24 h: open triangles). Values represent means (± SD) of metabolites formed (nmol/min/mg protein), and lines denote nonlinear fits of data with Michaelis–Menten kinetics for each group. Note different scales in *y*-axes.

of CPF-oxon and TCPy between naive and ethanol- or ethanol + nicotine-treated groups. CYP450 marker substrate activities of hepatic CYP1A and 2B were not significantly impacted by either ethanol or ethanol + nicotine treatments. However, animals in repeated ethanol treatment groups showed CYP2E1 induction (1.5- and 1.4-fold increases over naive groups for 4- and 24-h time points, respectively), while ethanol + nicotine-treated groups did not show any marked differences (data not shown).

DISCUSSION

Mixed chemical exposures are common (Carpenter et al. 2002; Feron et al. 2002; Feron and Groten 2002), and individual drug/chemical metabolic clearance may be significantly modulated by high-dose mixture interactions (Fuhr 2000). Hence, exposures to relatively high pharmacologically active doses of alcohol and nicotine are a major concern for these drug–chemical interaction scenarios (De Leon et al. 2007; Bien and Burge 1990), and are of particular interest to understand the impact of these coexposures on the metabolism, disposition, and biological response following exposure to commonly encountered insecticides. In this context, both ethanol and nicotine were found to antagonize the acute toxicity (i.e., less ChE inhibition) of the organophosphorus insecticides CPF and parathion, respectively (O'Shaughnessy and Sultatos 1995; Lee et al. 2010). To address these concerns, the current study was designed to investigate interactions for a binary mixture of ethanol and nicotine on CPF dosimetry and response (i.e., ChE) using an in vitro and in vivo rat model. The current pharmacokinetic analysis focused on TCPy, because previous studies have showed that CPF and CPF-oxon are difficult to detect in blood at lower doses due to rapid metabolism (Timchalk et al. 2007; Nolan et al. 1984).

There are a number of studies demonstrating complex interactions between ethanol and nicotine. In rats coexposed to tobacco smoke and a single dose of ethanol, the elimination rate of cotinine, the major metabolite of nicotine, was enhanced (Florek et al. 2008), whereas chronic exposure (i.e., daily doses for several weeks) to both ethanol and nicotine resulted in a significant depletion of liver glutathione (GSH), which could significantly impact phase II metabolism (Husain et al. 2001). A study by Bachtell and Ryabinin (2001) noted in mice that coexposure to ethanol and nicotine counteracted several inducible transcription factors in brain regions effectively antagonizing the individual drug response. However, no literature was identified that evaluated the effects from alcohol and nicotine coexposures on the dosimetry or biological response of pesticides.

In previous in vivo and in vitro studies, Lee et al. (2009; 2010) reported that repeated nicotine treatments modulated the metabolism of CPF to TCPy (twofold increase) and in vivo brain AChE activity was less (by up to 28%) inhibited (Lee et al. 2010). Based on these results and computational model simulations it was concluded that nicotine co-treatment enhanced CYP450 mediated dearylation (CPF → TCPy), resulting in quantitatively greater detoxification and subsequently less inhibition of brain AChE activity. In the current study, a similar experimental approach was adopted to characterize the impact of ethanol + nicotine coexposure on in vitro and in vivo CPF dosimetry and ChE activity. The influence of the coexposure was pronounced, as evidenced by increases in the peak concentrations and AUC in blood TCPy at both CPF doses (Figure 1); however, there was no observable difference in the urinary elimination of TCPy due to the coexposure (Figure 2). Because TCPy is the end product from both CYP450-mediated dearylation (CPF → TCPy) and desulfation (CPF → CPF-oxon → TCPy) of CPF, overall shifts in the total amount of urinary TCPy excretion would not be observable; however, differences in blood TCPy concentrations would be reflective of metabolic shifts in the overall rate of CPF metabolism (Timchalk et al. 2007). In this regard, these results are similar to those from a previous study with nicotine and CPF where nicotine treatment modulated the blood

pharmacokinetics with no observable shifts in the urinary excretion of TCPy (Lee et al. 2010).

Consistent with these observations, after repeated co-treatments with ethanol and nicotine, the V_{max} and K_m values for in vitro metabolism of CPF to either CPF-oxon or TCPy were proportionally decreased (approximately 20–38%), yet the V_{max}/K_m ratios were not impacted (Table 2). Hence, the observed in vivo rise in TCPy blood concentrations for the coexposures is not explainable by shifts in hepatic CYP450-mediated metabolism as was the case for nicotine only treatments (Lee et al. 2009). When these in vitro data are compared with the previous nicotine study, the combined treatments with ethanol seemed to negate the effects previously seen due to nicotine alone on CPF dearylation to TCPy, which was increased by approximately twofold (Lee et al. 2010). As previously noted, in rats coexposed to ethanol and nicotine there was a significant depletion (by approximately 40%) of glutathione (GSH) content in liver (Husain et al. 2001). Given that TCPy does undergo phase II conjugation with GSH it is conceivable that GSH depletion could modify the extent of TCPy phase II conjugation resulting in different TCPy blood kinetics. This hypothesis was tested using the CPF PBPK/PD model by simulating the impacts of glutathione depletion on TCPy pharmacokinetics in rats (Bakke et al. 1976). When V_{max} for TCPy glucuronidation was decreased by 40% based upon Husain et al. (2001), TCPy C_{max} in blood was increased approximately 23% following repeated 5-mg CPF/kg/d dosing for 7 d (Figure 6), with minimal changes in the urinary elimination profiles of TCPy (simulation not shown). These simulations were consistent with the observed rise in blood TCPy concentration (compare Figures 6 and 1B), yet did not impact overall ChE activities. Hence, in vivo modulation of phase II metabolism may play an important role and contribute to observed differences in TCPy pharmacokinetics. Clearly additional studies are warranted to further evaluate this possibility.

An important observation in the current study concerned changes in brain AChE activities resulting from CPF exposure, manifested by less inhibition at both CPF doses (greater impact at 5 mg/kg/d) due to the combined treatments with ethanol and nicotine (Figure 4), whereas ChE activity in plasma was not impacted by these treatments (Figure 3). Again, these results are consistent with brain AChE and plasma ChE responses seen following repeated nicotine and CPF exposure (Lee et al. 2010). Smith et al. (2009) recently suggested that localized low-level CYP450 brain metabolism may contribute to brain CPF-oxon dosimetry and associated AChE inhibition. The modulation of brain CYP450s has great implications, because brain CYP450s show unique regional expression and induction patterns by diverse inducers, although their expression levels are often low (Miksys and Tyndale 2002). Of particular relevance, Yue et al. (2008) demonstrated that the repeated nicotine treatments modified CYP2D expression (of relevance for CPF metabolism) in different regions of rat brain, especially frontal cortex, hippocampus, and striatum, that might lead to localized shifts (up or down) in CPF brain metabolism. Therefore, it was postulated that coexposure to ethanol and nicotine may modulate brain CYP450 activity altering localized brain CPF metabolism, thereby impacting the extent of brain AChE inhibition, a key endpoint of CPF toxicity.

To evaluate this hypothesis, Monte Carlo simulations were conducted using the CPF PBPK/PD model to evaluate the impact of variability in CYP450-mediated brain metabolism of CPF. For these simulations, the V_{max} values for CYP450 desulfation and dearylation in the brain were set as a fraction (approximately 3%) of liver activity, based upon in vitro hepatic and preliminary in vitro brain metabolism studies (data not shown). The results from the Monte Carlo simulations for the effects of the variability in brain CPF metabolism are presented in Figure 7. When the metabolic rate (V_{max}) of CPF metabolism to CPF-oxon in brain was varied (normal distribution), the effects on brain AChE activity were pronounced, demonstrating a 30% variance (95% confidence interval) following a repeated oral dose of CPF (5 mg/kg/d, 7 d) (Figure 7A).

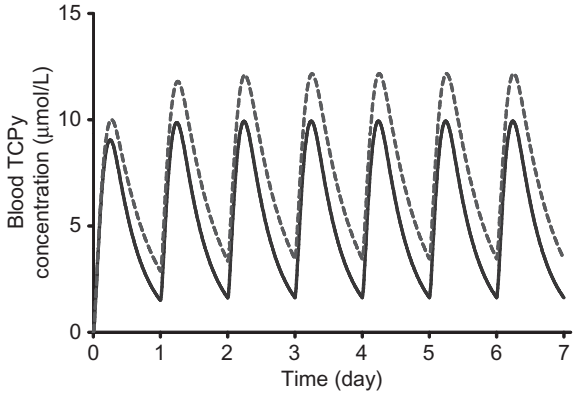

FIGURE 6. PBPK/PD model simulation of the impacts by the different levels of glutathione on the blood TCPy concentration time-course profile, following repeated 5-mg CPF/kg/d dosing for 7 d. The lines represent the simulation with two different values for V_{max} of phase II conjugation (solid: default, and dashed: 40% decreased, respectively).

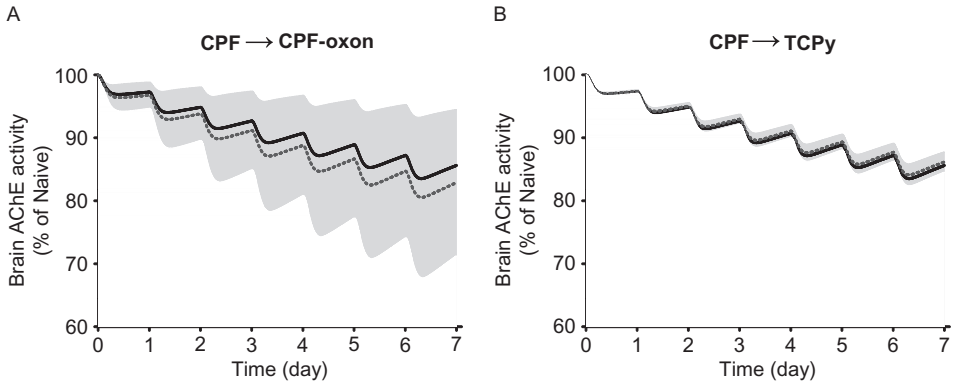

FIGURE 7. PBPK/PD model predictions of brain AChE activity profiles after Monte Carlo simulations (1000 iterations) with varied values of V_{max} in brain of (A) CPF → CPF-oxon or (B) CPF → TCPy, following repeated 5-mg CPF/kg/d dosing for 7 d. The output of mean (dotted lines) and 95% confidence intervals (shaded areas) of brain AChE activity after Monte Carlo simulations are shown with those from control group (thick solid line). Note the greater variations in brain AChE activity due to V_{max} changes of CPF-oxon than those of TCPy in brain (the scale of the *y* axis is expanded for clarity).

When a similar simulation was conducted for the metabolism of CPF to TCPy, there was little observed variability in brain AChE activities (less than 5%), although the V_{max} of TCPy was approximately 40-fold greater than that used for CPF-oxon metabolism in brain (Figure 7B). Overall the outputs from these simulations were comparable with results from the current study (compare Figures 7A and 4B), suggesting that brain AChE activity is influenced by variability in the localized metabolism of CPF to CPF-oxon. Furthermore, the results from Monte Carlo simulations were comparable to the previous PBPK/PD simulations of effects due to the repeated nicotine treatments (twofold increase in V_{max} of CPF to TCPy in liver), which also predicted less brain AChE inhibition. These computational model predictions provide strong support for the stated hypothesis that small modulations in brain CPF-oxon metabolism may have significant implication for CPF toxicity. However, further studies are warranted to experimentally test this hypothesis.

In summary, this study was undertaken to investigate the potential impact of a binary exposure of ethanol and nicotine on the dosimetry and biological response for the insecticide CPF in the rat. As noted, the coexposures did result in higher blood concentrations of the CPF metabolite TCPy and less brain AChE inhibition than observed in naive rats. The better

description of underlying mechanism of how combined exposures to nicotine and ethanol affect dosimetry and biological response for CPF will facilitate understanding highly complex mixture interactions and their potential impact on the health of agricultural workers and the general public.

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