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Pharmacokinetic and pharmacodynamic interaction for a binary mixture of chlorpyrifos and diazinon in the rat

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Abstract

Chlorpyrifos (CPF) and diazinon (DZN) are two commonly used organophosphorus (OP) insecticides and a potential exists for concurrent exposures. The primary neurotoxic effects from OP pesticide exposures result from the inhibition of acetylcholinesterase (AChE). The pharmacokinetic and pharmacodynamic impact of acute binary exposures of rats to CPF and DZN was evaluated in this study. Rats were orally administered CPF, DZN, or a CPF/DZN mixture (0, 15, 30, or 60 mg/kg) and blood (plasma and RBC), and brain were collected at 0, 3, 6, 12, and 24 h postdosing, urine was also collected at 24 h. Chlorpyrifos, DZN, and their respective metabolites, 3,5,6-trichloro-2-pyridinol (TCP) and 2-isopropyl-4-methyl-6-hydroxypyrimidine (IMHP), were quantified in blood and/or urine and cholinesterase (ChE) inhibition was measured in brain, RBC, and plasma. Coexposure to CPF/DZN at the low dose of 15/15 mg/kg did not alter the pharmacokinetics of CPF, DZN, or their metabolites in blood. A high binary dose of 60/60 mg/kg increased the C_{max} and AUC and decreased the clearance for both parent compounds, likely due to competition between CPF and DZN for CYP450 metabolism. At lower doses, most likely to be encountered in occupational or environmental exposures, the pharmacokinetics were linear. A dose-dependent inhibition of ChE was noted in tissues for both the single and coexposures, and the extent of inhibition was plasma > RBC ≥ brain. The overall relative potency for ChE inhibition was CPF/DZN > CPF > DZN. A comparison of the ChE response at the low binary dose (15/15 mg/kg), where there were no apparent pharmacokinetic interactions, suggested that the overall ChE response was additive. These experiments represent important data concerning the potential pharmacokinetic and pharmacodynamic interactions for pesticide mixtures and will provide needed insight for assessing the potential cumulative risk associated with occupational or environmental exposures to these insecticides.

Keywords: Cholinesterase; Organophosphorus insecticide; Chlorpyrifos; Diazinon; Mixtures

Introduction

Organophosphorus (OP) insecticides like chlorpyrifos (CPF) (*O*,*O*-diethyl-*O*-[3,5,6-trichloro-2-pyridyl]-phosphorothioate) and diazinon (DZN) (*O*,*O*-diethyl-*O*-[2-isopropyl-4-methyl-6-pyrimidinyl]-phosphorothioate) are widely utilized for the control of agricultural and household pest. Hence, exposure to these insecticides may involve a large segment of the population, which includes agriculture workers and their families, those living in proximity to farms/orchards, and the general population who may be exposed through home application of pesticides or via residues on food (Bradman et al., 2003; Lu et al., 2000; Quandt et al., 2004). Due to the variety of pesticides used, both occupational and environmental exposures are primarily to mixtures; however, only a few studies have begun to characterize the toxicological effects of exposures to concurrent or sequential OP pesticide mixtures (Karanth et al., 2001, 2004; Marinovich et al., 1996; Richardson et al., 2001).

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The primary mechanism of action and the most acutely life-threatening effect of these OP insecticides are related to the accumulation of acetylcholine within the cholinergic synapses resulting from the inhibition of acetylcholinesterase (AChE) by active oxon metabolites. Based upon a shared common mechanism of toxicity via the inhibition of AChE (Mileson et al., 1998), the U.S. Environmental Protection Agency (EPA) under The Food Quality Protection Act (FQPA, 1996) is required to consider the cumulative risk associated with OP pesticide mixture exposures.

A combined exposure of rats to malathion (an OP pesticide) and carbaryl (a carbamate pesticide) modified the pharmacokinetics of the individual pesticides, which was suggested to explain some of the observed toxicity for this mixture (Waldron-Lechner and Abdel-Rahman, 1986). Richardson et al. (2001) compared the in vitro interactions of a binary mixture of CPF–oxon and azinphos–methyl–oxon on cholinesterase (ChE) activity in brain and serum from rats. They reported that at low concentrations of these ChE inhibiting OP metabolites, the interaction appeared to be additive in nature but noted that at higher concentrations there was a departure from additivity. They suggested that the presence of ChE-mediated detoxification pathways may have contributed to the lack of additivity. Karanth et al. (2001, 2004) evaluated the in vivo interactions (acute toxicity and ChE inhibition) between CPF and parathion as well as CPF and methyl–parathion following sequential and concurrent exposures in rats. They concluded that the sequence of exposure could markedly impact the toxicity and suggested that this resulted from the depletion of key detoxification esterases not directly associated with neural function. Collectively, these studies point to the important roles that both metabolic activation and detoxification play in contributing to pesticide interactions.

The metabolism of OP insecticides has been well studied (for a review, see Chambers et al., 2001). In the case of CPF and DZN, metabolism to their active oxon metabolites involves CYP450-mediated oxidative desulfuration to CPF–oxon or DZN–oxon, whereas detoxification via CYP450-mediated dearylation produces 3,5,6-trichloro-2-pyridinol (TCP) or 2-isopropyl-4-methyl-6-hydroxypyrimidine (IMHP), respectively (Fig. 1) (Amitai et al., 1998; Ma and Chambers, 1994, 1995; Poet et al., 2003; Sams et al., 2000; Sultatos and Murphy, 1983). The balance between the rates of desulfuration and dearylation can result in very different levels of AChE inhibition (Timchalk, 2001). Subsequent phase II conjugation of TCP and additional phase I oxidative metabolism of IMHP have also been reported in both humans and rodents exposed to CPF and DZN (Mücke et al., 1970; Nolan et al., 1984). Although several studies describe both the pharmacokinetic and pharmacodynamic responses of CPF or DZN in both rodents and humans (Poet et al., in press; Timchalk et al., 2002), we are currently unaware of any published reports describing the in vivo pharmacokinetic and pharmacodynamic response for binary mixtures of these insecticides.

Based upon similar pharmacokinetics and mode of action properties, a potential for interactions between mixtures of

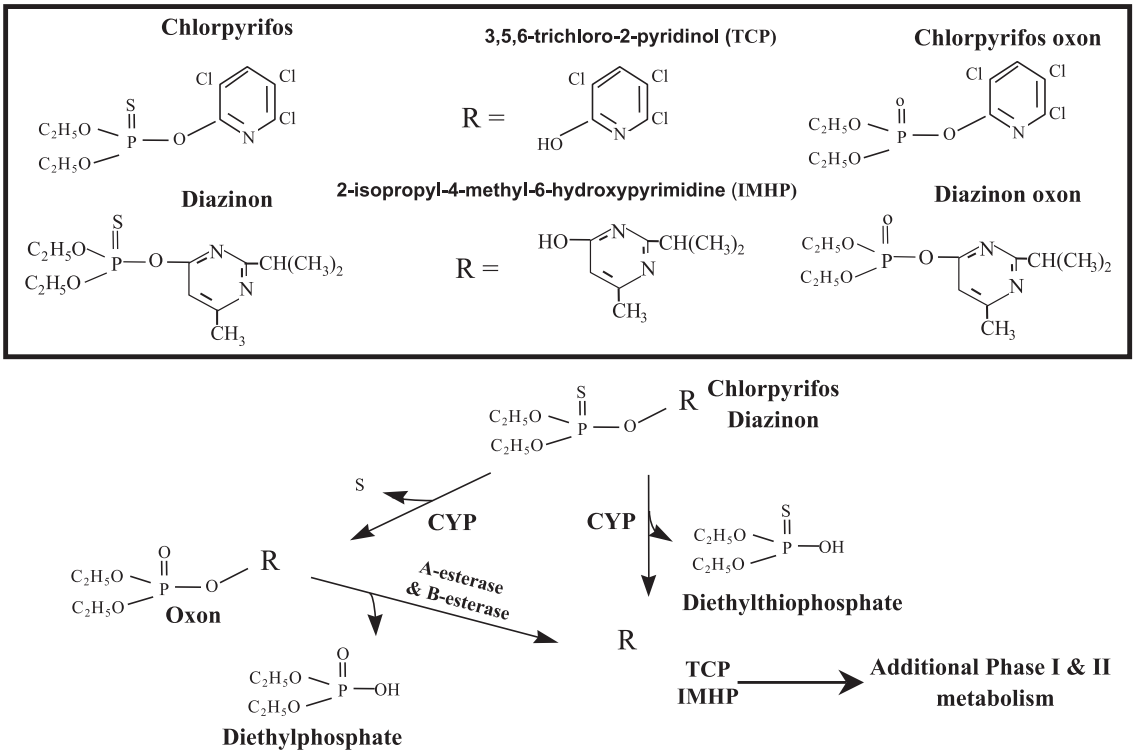

Fig. 1. Metabolic scheme for diazinon (DZN), chlorpyrifos (CPF), and their major metabolites (diazinon-oxon, 2-isopropyl-4-methyl-6-hydroxypyrimidine, chlorpyrifos-oxon, 3,5,6-trichloro-2-pyridinol, diethylphosphate, and diethylthiophospate) showing the similarity in the metabolic profiles for both compounds.

OP insecticides is hypothesized. The objectives of the current study were to evaluate the pharmacokinetic and pharmacodynamic response following in vivo single and binary oral exposures to DZN and CPF in rats. The oral route of exposure was utilized since it is an important exposure pathway, particularly for children (Lu et al., 2000), and to facilitate direct comparison to previous oral pharmacokinetic studies in rats with CPF and DZN (Poet et al., in press; Timchalk et al., 2002). The pharmacokinetic portion of the study focused on quantifying CPF and DZN, and their major metabolites, TCP and IMHP, respectively, in blood and/or urine. No attempt was made to quantify the amount of CPF–oxon or DZN–oxon since previous studies indicated that quantifying oxon in blood following in vivo exposure is problematic due to rapid esterase metabolism (Poet et al., in press; Timchalk et al., 2002). However, it should be noted that the extent of ChE inhibition is an indirect determinant of the amount of oxon formed. The pharmacodynamic studies described herein focused on the impact of binary OP exposures on ChE (acetyl- and butyryl-cholinesterase) activity in the brain and blood (RBC and plasma). Brain AChE inhibition is a critical target that correlates well with observed toxicological response in laboratory animals, while plasma and RBC ChE activities are important as biomarkers for assessing exposure (Heath and Vale, 1992). Mixture exposures over the acute high dose range utilized in this study were adequate to observe both pharmacokinetic and pharmacodynamic interactions.

Materials and methods

Chemicals. Diazinon (molecular wt. 304; 98.5% pure) (*O*,*O*-diethyl-*O*-2-isopropyl-4-methyl-6-pyrimidyl thiophosphate), IMHP (99% pure) (2-isopropyl-4-methyl-6-hydroxypyrimidine), and methyl–CPF (99.7% pure) were purchased from Chem Service Inc. (West Chester, PA). 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). The derivatizing agent (*N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide) was purchased from Sigma-Aldrich (Milwaukee, WI). Acetylthiocholine chloride (ATC) and 5,5,-dithio-bis 2-nitrobenzoic acid (DTNB) were purchased from Sigma (St Louis, MO). Dithionicotinoadenoic acid was purchased from TCI America, Inc. (Portland, OR) and toluene (99.8% pure) was purchased from Burdick and Jackson (Muskegon, MI). 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 (250–300 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 DZN, CPF, and binary mixtures of CPF and DZN were prepared in corn oil. Three groups of rats (four rats/time point), were orally gavaged (5 ml/kg) with DZN, CPF, or a mixture of CPF/DZN solution at dose levels of 0, 15, 30, or 60 mg/kg. Food was withheld for approximately 12 h before and 3 h postdosing and water was available ad libitum. Animals were sacrificed by carbon dioxide asphyxiation at 0, 3, 6, 12, and 24 h postdosing. Rats assigned to the 24-h groups were held in individual glass metabolism cages designed for the collection of excreta and a 0- to 24-h urine sample was collected for the analysis of IMHP and TCP (free and conjugated).

Dose levels and postexposure time selection. The administered dose levels of CPF and DZN were comparable on a mg/kg of body weight basis; however, it should be noted that when compared on a molar basis (μmol/kg), the DZN dose was slightly greater (approximately 14%) than CPF for all treatment groups (see Table 1). The dose range for the study

Table 1
Pharmacokinetic parameters determined using noncompartmental analysis of the time course of diazinon (DZN), chlorpyrifos (CPF), and their major metabolite 3,5,6-trichloro-2-pyridinol (TCP) and 2-isopropyl-4-methyl-6-hydroxypyrimidine (IMHP) following oral gavage administration of 15 and 60 mg/kg DZN, CPF as single doses or as a binary mixture

Treatment	Dose (mg/kg)	Dose (μmol/kg)	$AUC_{0-24\ h}$ (μg ml^{-1} h^{-1})				C_{max} (μg ml^{-1})				Cl (ml h^{-1})	
			CPF	TCP	DZN	IMHP	CPF	TCP	DZN	IMHP	CPF	DZN
CPF	15	43	1.0	26	–	–	0.1	1.91	–	–	3018	–
DZN	15	49	–	–	0.49	15	–	–	0.04	1.43	–	4523
CPF/DZN	15/15	92[a]	0.8	20	0.52	15	0.09	1.39	0.04	1.25	4274	4346
CPF	60	171	4.8	56	–	–	1.14	4.72	–	–	1923	–
DZN	60	197	–	–	1.9	80	–	–	0.18	9.33	–	6628
CPF/DZN	60/60	368[a]	11	46	5.3	83	1.76	3.29	0.86	7.30	1317	2406

AUC—area under the blood concentration curve; C_{max}—maximum blood concentration; Cl—clearance.
[a] Total μmol of OP mixture (CPF + DZN).

(0–60 mg/kg) was selected based upon the individual chemical's acute toxicity profiles. The blood kinetics for CPF and DZN were not evaluated at a dose of 30 mg/kg due to the loss of these samples during the analytical process; however, the ChE dynamics were evaluated at all doses (0, 15, 30, and 60 mg/kg). The low dose (15 mg/kg) was chosen based on several factors including anticipated linearity of kinetic response, mild AChE inhibition (Poet et al., in press; Timchalk et al., 2002), and good analytical sensitivity for quantitation of parent compounds and metabolites (TCP and IMHP) in blood and urine. The single chemical high dose (60 mg/kg) approached approximately 20% and 50% of the LD_{50} for DZN and CPF, respectively. Based upon previous pharmacokinetic and pharmacodynamic studies with CPF and DZN, it was determined that the kinetic and dynamic response in rats would be adequately characterized by 24 h postdosing (Nolan et al., 1984; Poet et al., 2003; Timchalk et al., 2002; Wu et al., 1996).

Tissue preparation. After rats were euthanized by CO_2 asphyxiation, blood was drawn from the posterior vena cava using a heparinized syringe and approximately 1 ml of whole blood was used for analysis of the parent compounds and their metabolites. The remainder of the blood was centrifuged for 20 min at 2000 rpm to separate plasma and RBC for ChE activity determination. Immediately after blood withdrawal, the brain was removed, dry blotted, and homogenized in 9× phosphate buffer, all tissues were stored at −80 °C until analyzed.

Blood analysis and gas chromatography (GC) conditions. For analysis of parent chemicals and metabolites, an internal standard (5 μl methyl–CPF: 1 mg/ml) was added to approximately 1 ml of a weighted blood sample and briefly vortexed. Four hundred microliters of an acetic acid solution (2.5 M) saturated with sodium chloride was then added to halt metabolism and aid in the extraction process (Brzak et al., 1998). Chemicals were extracted from the samples twice using toluene (approximately 1 ml/extraction). The samples were strongly vortexed for 1 min and centrifuged at 3900 rpm for 15 min at 15 °C. The top organic layer was removed, blown down to dryness using a light stream of inert nitrogen gas, and the samples were frozen at −80 °C until analysis (within 2 weeks).

Each sample was reconstituted in 75 μl toluene to place the GC response within the linear range of the calibration curve, 15 μl of the derivatizing agent was added to each sample, and the samples were incubated for 60 min at 60–70 °C. The samples were analyzed using a Hewlett Packard 6890 Gas Chromatograph (GC) equipped with a Nitrogen–Phosphorus Detector (NPD) and a Flame Ionization Detector (FID). To increase analytical sensitivity for the samples obtained at the low dose (15 mg/kg), an Agilent Technologies (6890 N) GC/Mass Spectrometer (MS) with Negative Chemical Ionization (NCI) mode was employed. The analysis used the same sample preparation methods as described above. Helium carrier gas was used with a head pressure of 22 psi. The oven temperature program was ramped with an initial temperature of 100 °C, an initial ramp of 15 °C/min up to 220 °C, and then a final ramp of 40 °C/min up to 300 °C. The injection port and detector temperatures were 275 °C. For both the GC and GC/MS analysis, chemical separation was achieved using an OP pesticide column (30 m × 0.32 mm id × 0.5 m df; Restek, Belfont, PA). The retention times of IMHP, TCP, DZN, methyl–CPF, and CPF were 3.7, 5.6, 6.8, 7.7, and 8.2 min, respectively. Standard curves were constructed utilizing various concentrations of compounds to calculate the individual chemical concentration in rat blood and urine. The standard curves were linear over a concentration range of 0.05–20 μg/ml for TCP CPF, DZN, and IMHP using the GC–NPD and a range of 0.01–6.5 μg/ml for CPF, DZN, and 0.01–0.4 μg/ml for TCP using the GC/MS–NCI.

Urine metabolite analysis. The amount of conjugated TCP excreted in urine was determined according to the method of MacKenzie et al. (2000). Briefly, frozen urine samples were thawed, brought to 35 °C to dissolve any solid content. A 1-ml aliquot of the sample was spiked with 5 μl of neat methyl–CPF internal standard and 100 μl hydrochloric acid (12 N) was added to each sample. The samples were put in sealed vials, vortexed for 30 s, and then hydrolyzed at 80 °C for 60 min. After hydrolysis the samples were brought to room temperature and then processed as described above and the concentration of metabolites within the samples were calculated based on the standard curve developed in a urine matrix. In the current study, IMHP appeared to be acid labile and was degraded by the acid treatment during analytical separation of the conjugated TCP metabolites (data not shown); therefore, for the IMHP analysis, another urine sample not subjected to the hydrolysis step was utilized and processed as described above.

Cholinesterase (ChE) activity determination. Brain homogenates, plasma, and RBC samples were thawed at room temperature and diluted in buffer to be within the linear range to determine total ChE activity. For brain, plasma, and RBC, the final dilutions were approximately 1375-, 110-, and 250-fold, respectively. Total ChE activity in diluted tissue homogenates was determined using the Ellman assay (Ellman et al., 1961), as modified for a 96-well plate reader (Mortensen et al., 1996; Nostrandt et al., 1993), using an automated microplate spectrophotometer ELx808 equipped with a KC4 software package (Bio-Tek Instruments, Inc. Winooski, Vermont). Wells contained 0.25 ml diluted tissue homogenate with Ellman reagent (5,5′-dithiobis-nitrobenzoic acid) and acetylthiocholine at final concentrations of 0.1 and 0.4 mM, respectively, and a final total volume of 300 μl. The absorbance at 412 nm was monitored over 30–40 min and the slope of the increasing absorbance with time was used for the measurement of uninhibited ChE activity. To determine RBC AChE activity, 6,6'-dithionicotinic acid

was used as a coupling agent rather than the Ellman reagent and the absorbance was measured at 340 nm to avoid the hemoglobin interference (Brownson and Watts, 1973; Grassetti et al., 1969; Hakathorn et al., 1983).

Data analysis. The time course of parent compounds (CPF and DZN) and their metabolites (TCP and IMHP) were analyzed using noncompartmental methods. Peak concentration of parent and metabolite in blood (C_{max}) was determined by a visual analysis of the individual observed concentration time data. The area under the concentration time curve from 0 to 24 h (AUC) was determined using linear trapezoidal methods, and total clearance (Cl; ml h^{-1}) was estimated by dividing the administered dose (μg) by the AUC (μg ml^{-1} h^{-1}) for the corresponding compounds. Other than the calculation of mean ± S.D. for the chemical blood concentrations, amount in urine, and ChE inhibition time courses, no additional statistical evaluations were conducted.

Results

Pharmacokinetics

The time course for DZN, CPF, and their metabolites IMHP and TCP in blood following oral administration of either individual chemicals or as a binary mixture at doses of 15 and 60 mg/kg are shown in Figs. 2 and 3, and the noncompartmental parameter estimates are presented in Table 1. At 15 mg/kg, peak blood concentrations of DZN and CPF were attained by 6 and ≤ 3 h postdosing, respectively, and the C_{max} (0.1 vs. 0.04 μg ml^{-1}) and AUC (1.0 vs. 0.49 μg ml^{-1} h^{-1}) for CPF were 2- to 2.5-fold greater than for DZN (see Fig. 2). For the metabolites (TCP and IMHP), peak blood levels following a single chemical exposure at 15 mg/kg were achieved by approximately 6 h postdosing, and the C_{max} (1.91 vs. 1.43 μg ml^{-1}) and AUC (26 vs. 15 μg ml^{-1} h^{-1}) for TCP were only slightly greater (1.3- to 1.7-fold) than for IMHP. The overall clearance rate (Cl) for DZN was 1.5-fold faster than for CPF and was consistent with a lower AUC and C_{max} for DZN.

A comparison of the dose-dependent pharmacokinetics for DZN (see Figs. 2A and B, 3A and B, and Table 1) indicates that a fourfold increase in the dose (15–60 mg/kg) resulted in a 4.5-fold increase in the C_{max} (0.04–0.18 μg/ml), a 3.9-fold increase in the AUC (0.49–1.9 μg ml^{-1} h^{-1}), and the overall Cl for DZN increased 1.5-fold (4523–6628 ml h^{-1}). For these same animals, a fourfold increase in DZN dose resulted in blood IMHP C_{max} and AUC increasing 6.5-fold (1.43–9.33 μg/ml) and 5.3-fold (15–80 μg ml^{-1} h^{-1}), respectively. These results suggest that the DZN kinetics are reasonably proportional over this dose range.

In contrast, for CPF (see Figs. 2C and D, 3C and D, and Table 1), a fourfold increase in the dose (15–60 mg/kg) resulted in an 11.4-fold increase in blood CPF C_{max} (0.1–1.14 μg/ml) and a 4.8-fold increase in AUC (1.0–4.8 μg ml^{-1} h^{-1}); whereas the TCP C_{max} and AUC only increased 2.5-fold (1.91–4.72 μg/ml) and 2.2-fold (26–56 μg ml^{-1} h^{-1}), respectively. The overall Cl rates for CPF at 15 and 60 mg/kg were 4125 and 3437 ml h^{-1}, respectively. This suggests that with an increasing dose of CPF, there may be a disproportionate increase in the CPF C_{max}. However, the increase in AUC appeared to be less pronounced, but the

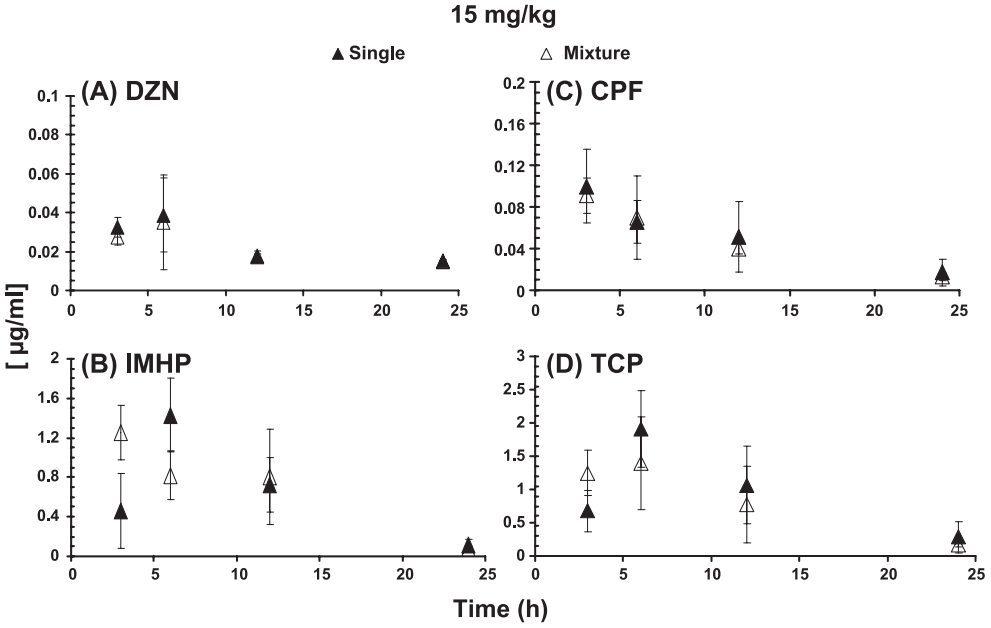

Fig. 2. The concentration of DZN (A), IMHP (B), CPF (C), and TCP (D) in the blood of Sprague–Dawley rats following an oral gavage dose of 15 mg/kg DZN or CPF as single chemicals (filed triangles) or as a binary mixture (15/15 mg/kg) of DZN/CPF (open triangles). The data are expressed as concentration (μg/ml) over time (h) and represent the mean ± S.D. of four animals per time point.

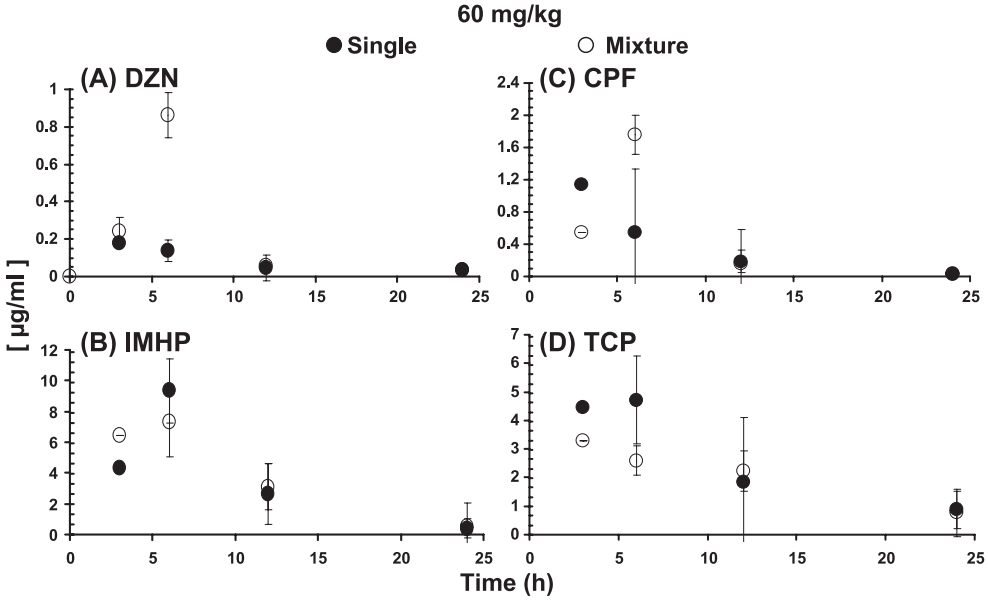

Fig. 3. The concentration of DZN (A), IMHP (B), CPF (C), and TCP (D) in the blood of Sprague–Dawley rats following an oral gavage dose of 60 mg/kg DZN or CPF as single chemicals (filed circle) or as a binary mixture (60/60 mg/kg) of DZN/CPF (open circle). The data are expressed as concentration (µg/ml) over time (h) and represent the mean ± S.D. of four animals per time point.

overall Cl rate was decreased approximately 64%. Likewise, with increasing dose of CPF the C_{max} and AUC for TCP demonstrated a less than proportional increase, which is consistent with less CPF being metabolized to TCP.

To evaluate the potential kinetic and dynamic interactions associated with a binary OP pesticide exposure, rats were given a mixture of CPF/DZN by gavage at doses of either 15/15 mg/kg (Fig. 2) or 60/60 mg/kg (Fig. 3), and the kinetics of CPF, DZN, and their metabolites were evaluated. Based on a comparison of the kinetic profiles, AUC and Cl rates, coadministration of CPF and DZN at the low dose (15/15 mg/kg), had no impact on the overall pharmacokinetic response of CPF, DZN, or their metabolites (Table 1). In contrast, a binary dose of CPF/DZN at 60/60 mg/kg did result in appreciable alterations in the observed pharmacokinetics of both parent compounds. As illustrated in Figs. 3A and C, the primary impact was to substantially increase the measured C_{max} for both DZN (0.18–0.86 µg/ml) and CPF (1.14–1.76 µg/ml). Likewise, high dose coexposure increased the AUC for CPF 2.3-fold (4.8–11 µg ml^{-1} h^{-1}) and 2.9-fold (1.9–5.3 µg ml^{-1} h^{-1}) for DZN and was likewise reflected by comparable decreases in the overall Cl rates. The impact of the coexposure on the kinetics of the metabolites IMHP and TCP was less dramatic. For TCP, the AUC was slightly decreased (1.2-fold; 56–46 µg ml^{-1} h^{-1}), whereas for IMHP the AUC was unchanged (80–83 µg ml^{-1} h^{-1}). It was of interest to note that although the time points and maximal values for the measured C_{max} for the parent compounds were impacted by the coexposure, the remaining kinetic profile for the parent compounds in the blood were very comparable to that observed for the single chemicals.

The total amount of TCP (conjugated plus free) and IMHP excreted in the urine by 24 h postdosing is presented in Fig. 4. No CPF or DZN was detectable in the urine samples. The total amount of TCP (free + conjugated) following either CPF only or CPF/DZN binary doses of 15/15 and 60/60 mg/kg was proportional to the administered dose (see Fig. 4A). At both dose levels, coadministration of DZN with CPF resulted in a reduction of the total urinary TCP by approximately 20–50%, for both the 15 mg/kg (2364 ± 1264 vs. 1248 ± 18 µg) and 60 mg/kg (9530 ± 4569 vs. 5817 ± 1103 µg) doses, respectively. However, the total amount of free TCP did not increase proportionally with the dose of CPF when administered either as a single dose or as a binary mixture with DZN (see Fig. 4B). At the 15 mg/kg dose, free TCP accounted for 13–20% of the total TCP pool, whereas at 60 mg/kg the free TCP only accounted for 3–5% of the total.

The total IMHP excreted in the urine following the DZN only doses of 15 and 60 mg/kg ranged from 669 ± 332 to 2698 ± 642 µg, respectively (see Fig. 4C). Whereas, the average IMHP following the binary doses were 818 ± 169 and 2295 ± 1239 µg for the 15 and 60 mg/kg groups, respectively, and were reasonably proportional (approximately three- to fourfold increase). The binary exposure did not modify the extent of metabolism of DZN to IMHP as reflected by the comparable amounts of IMHP excreted in the urine.

Pharmacodynamics

To characterize the potential pharmacodynamic interaction with a binary mixture of CPF and DZN, the ChE activities in brain, RBC, and plasma were assessed in rats (Figs. 5–7). Animals orally administered 60 mg CPF/kg of body weight alone, or as a binary mixture with DZN (60/60

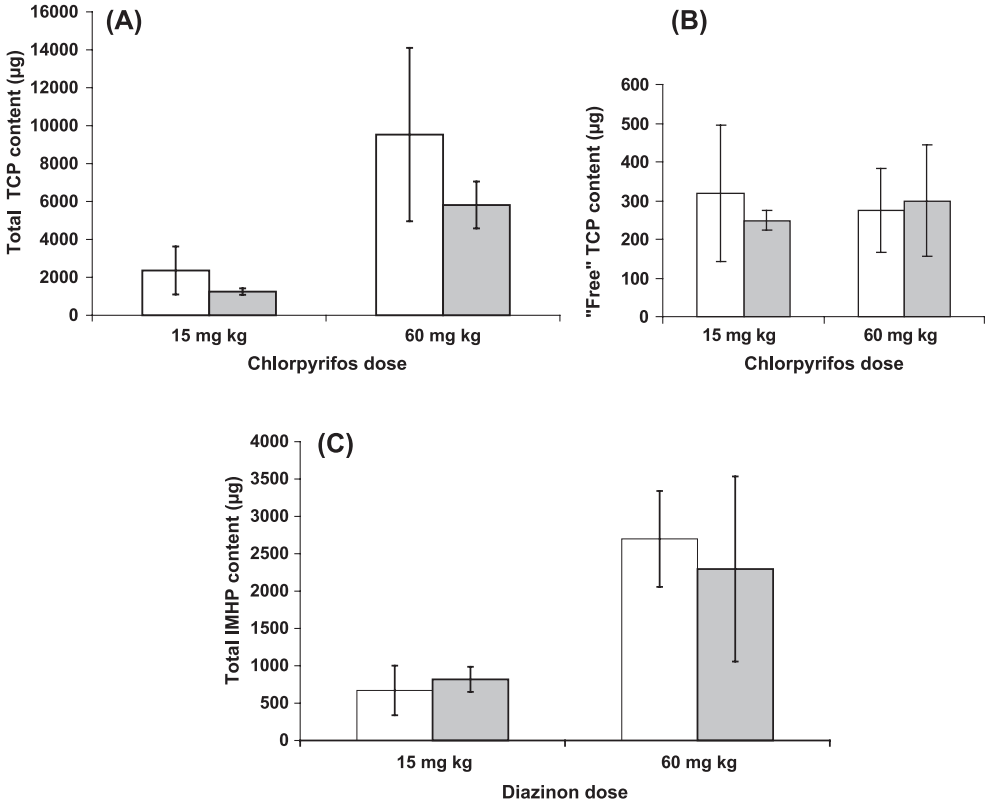

Fig. 4. The total (free + conjugated) amount of TCP (A), free TCP (B), and IMHP (C), excreted in the urine (0–24 h) in Sprague–Dawley rats following oral gavage doses of 15 and 60 mg/kg DZN and CPF as individual chemicals (white bars) and as a binary mixture of CPF/DZN (gray bars). The data are expressed as total amount (μg) and represent the mean ± S.D. of four animals per treatment time point.

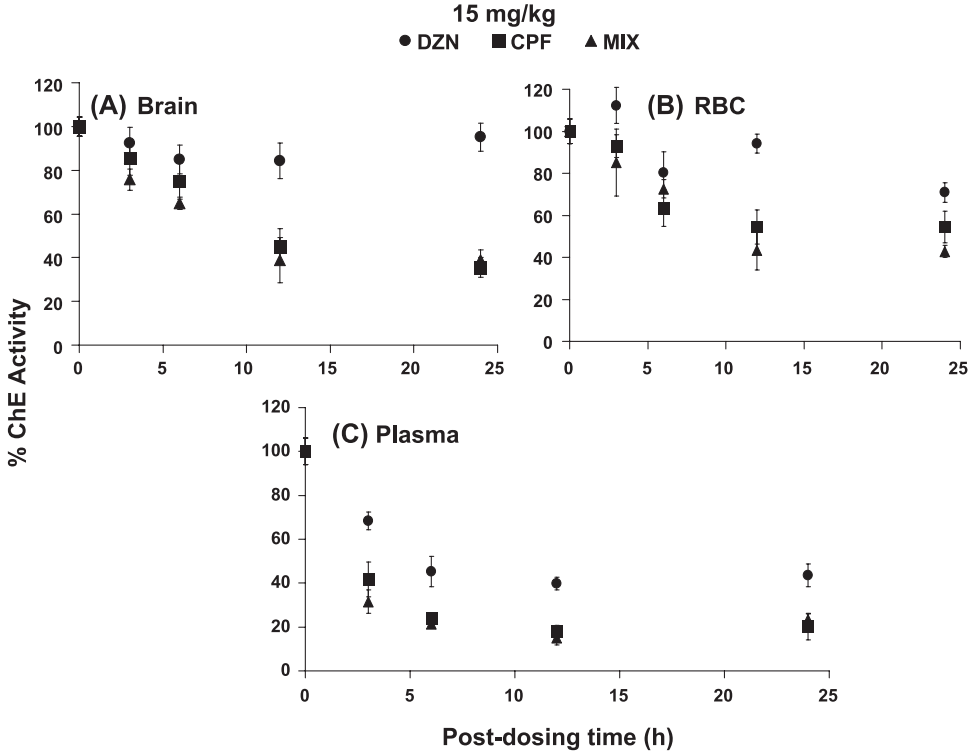

Fig. 5. Experimental data for the brain (A), RBC (B), and plasma (C), cholinesterase activity in Sprague–Dawley rats following a 15 mg/kg oral gavage dose of DZN (circles), CPF (squares), and their binary mixture (triangles). The data are expressed as percentage of total ChE activity as a function of time (h) and represent the mean ± S.D. of four animals per treatment time point.

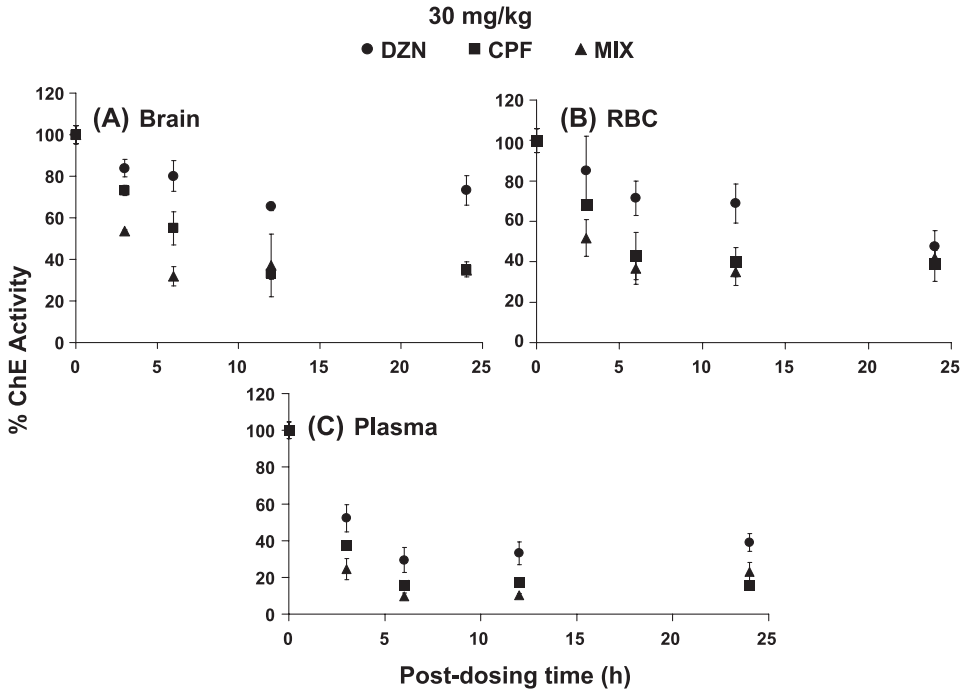

Fig. 6. Experimental data for the brain (A), RBC (B), and plasma (C), cholinesterase activity in Sprague–Dawley rats following a 30 mg/kg oral gavage dose of DZN (circles), CPF (squares), and their binary mixture (triangles). The data are expressed as percentage of total ChE activity as a function of time (h) and represent the mean ± S.D. of four animals per treatment time point.

mg/kg), exhibited moderate signs of clinical toxicity 3–6 h postdosing, in the form of diarrhea and muscle fasciculation, whereas there was no observable acute toxicity noted in animals administered CPF or DZN as single or binary doses of 15/15 or 30/30 mg/kg (data not shown). The degree of ChE inhibition was generally dose dependent, the extent of inhibition followed plasma > RBC ≥ brain, and the relative potency was CPF/DZN > CPF > DZN.

At doses of 15 and 30 mg/kg (see Figs. 5 and 6), peak inhibition was attained by 6–12 h postdosing in all tissues

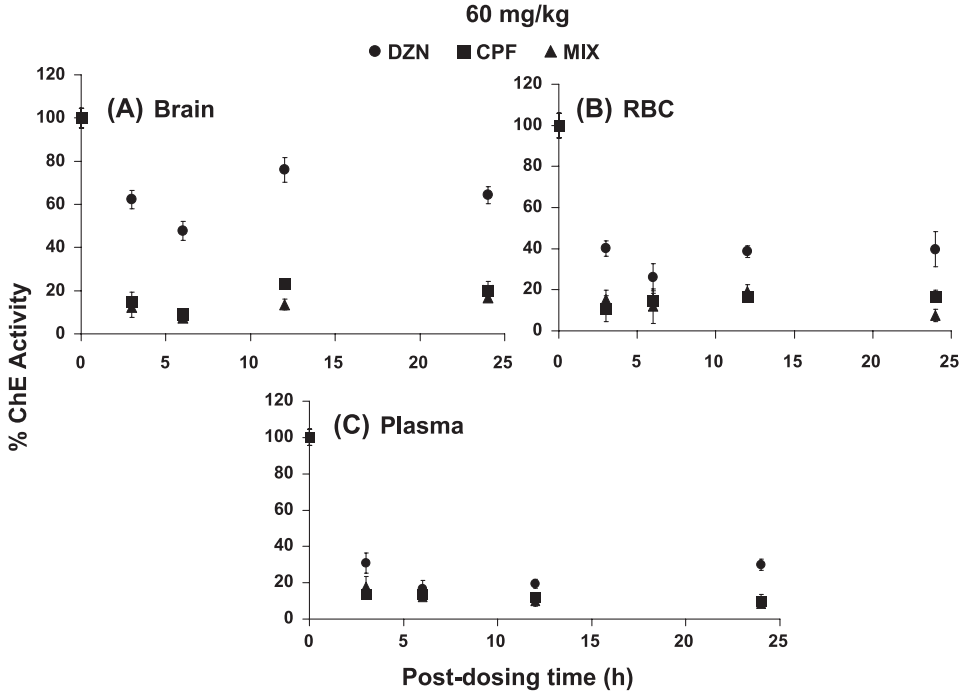

Fig. 7. Experimental data for the brain (A), RBC (B), and plasma (C), cholinesterase activity in Sprague–Dawley rats following a 60 mg/kg oral gavage dose of DZN (circles), CPF (squares), and their binary mixture (triangles). The data are expressed as percentage of total ChE activity as a function of time (h) and represent the mean ± S.D. of four animals per treatment group.

and the extent of inhibition was similar in brain and RBC, while plasma exhibited a greater inhibition. Specifically, in brain and RBC, ChE was maximally inhibited to 9–26%, 51–61%, and 53–64% of control at both dose levels following exposure to DZN, CPF, or a binary mixture, respectively; whereas, in plasma, DZN, CPF, and a binary mixture maximally inhibited the tissue ChE activity by 60–70%, 82–84%, and 85–90%, respectively. Overall, the extent of inhibition for the 30 mg/kg dose was slightly greater than at the lower dose.

Fig. 7 illustrates the time course for ChE activity in brain, RBC, and plasma following the 60 mg/kg doses of CPF, DZN, or a binary mixture. Consistent with the observed acute toxicity, ChE activity was severely inhibited in all tissues for the CPF or CPF/DZN exposures. Likewise, for the DZN-only treatment group, maximum RBC and plasma ChE were substantially inhibited (approximately 70% and 80%, respectively), but brain ChE activity was only maximally inhibited to 50% by 6 h postdosing. For all groups, peak ChE inhibition was observed between 3 and 6 h in all tissues, compared to peak inhibition at 6–12 h postdosing at the lower doses (see Figs. 5 and 6). These results suggest a potential shift towards shorter times to achieve maximum inhibition with increasing doses of CPF and DZN.

Discussion

Organophosphorus insecticides are routinely used to control pests in virtually all crop and commercial applications (California Environmental Protection Agency (Cal EPA), 1998); therefore, it is entirely feasible to anticipate that professional applicators, agricultural field workers, and the general population may be exposed to insecticide mixtures. A number of biomonitoring studies have measured the presence of several OP metabolites in urine and reported the inhibition of plasma ChE in these same workers (Hayes et al., 1980; Lavy et al., 1993). Additionally, children of agricultural families and families that live in proximity to farms and orchards may be at greater risk from mixed chemical exposure due to the higher use of these chemical agents in and around their residence. In this regard, Simcox et al. (1995) noted the presence of azinphos–methyl, chlorpyrifos, parathion, and phosmet in 62% of dust samples collected from the households of children of agricultural workers, suggesting the potential for both occupational and nonoccupational exposure to insecticide mixtures. Therefore, it is important to understand the toxicological implications for exposures to these insecticide mixtures.

Chlorpyrifos and DZN are structurally similar phosphorothionate insecticides that share common biotransformation pathways (see Fig. 1), a common mode of toxicological action (i.e., ChE inhibition), yet quantitatively differ with regard to the extent of metabolism (Poet et al., 2003) and the degree of ChE inhibition (Poet et al., in press; Timchalk et al., 2002). Based upon this common mode of action, there is a need to better understand the potential for cumulative toxicity (Mileson et al., 1998). Hence, the objective of the current study was to evaluate, in the rat, the in vivo pharmacokinetic and pharmacodynamic interactions for a mixture of CPF and DZN.

Overall, the findings indicate that both CPF and DZN were readily absorbed and extensively metabolized following oral administration as reflected from the resultant dosimetry (blood and urine) and tissue ChE inhibition as seen previously (Nolan et al., 1984; Poet et al., in press; Timchalk et al., 2002; Wu et al., 1996). Over the dose range (15–60 mg/kg) evaluated the kinetics of both CPF and DZN were reasonably proportional, although there is some suggestion that at 60 mg/kg, CPF metabolism may be approaching saturation. This is consistent with previous in vitro studies that reported CPF having a high affinity and lower capacity for CYP450 metabolism than DZN (Poet et al., 2003).

Although coexposure at the low dose (15/15 mg/kg) did not appreciably impacted the pharmacokinetics or metabolism of either pesticide, the high (60/60 mg/kg) binary dose of CPF/DZN resulted in a disproportionate increase in C_{max} and AUC for both CPF and DZN. The increase in C_{max} at the high dose could be related to the saturation of the initial CYP450 metabolism and/or shifts in the extent of oral absorption. Ongoing research suggests, that in rats, CPF can inhibit DZN microsomal metabolism in vitro (Wu et al., 2004). Specifically, CPF competitively inhibited the metabolism of DZN to IMHP, whereas the metabolism of DZN to DZN–oxon was uncompetitively inhibited, suggesting a potential enzyme suicide inhibition, which has been previously reported for similar OP insecticides (Halpert et al., 1980). The relatively high inhibitory constant obtained in the in vitro experiments suggest that in vivo kinetic interactions would only be observed at high acute binary doses, consistent with the results observed in the current study. A number of studies have compared the in vitro CYP450 metabolism of both CPF and DZN in human and rodent microsomal and cellular systems expressing human CYPs (Buratti et al., 2002, 2003; Fabrizi et al., 1999; Sams et al., 2000; Tang et al., 2001). These studies indicate that the CYP450 metabolism of CPF and DZN do share a number of common isoenzymes.

Although no attempt was made to measure the levels of CPF–oxon or DZN–oxon in blood, it is possible that the metabolism of CPF and DZN to CPF–oxon and DZN–oxon, respectively, could be inhibited at higher dose levels. Verification of this inhibition will be aided by additional in vivo metabolic studies focused on quantifying oxon metabolites.

It is well established that oral absorption of a chemical or drug can be modified by both intestinal and liver metabolism (Wacher et al., 2001; Zhang and Benet, 2001),

modifying a chemical's bioavailability. A shift in the amount and rate of oral absorption could result in the observed alterations in the time course of both parent compounds resulting in an increase and delay in achieving C_{max}. Poet et al. (2003) evaluated the in vitro intestinal (enterocytes) metabolism of both CPF and DZN and reported that intestinal CYP450 and A-esterase activity could impact the metabolism of CPF and DZN and subsequently modify in vivo bioavailability. Since enterocyte CYP450 activity is substantially lower than in liver, a dose-dependent saturation of the intestinal CYP450 based metabolism of CPF and DZN is feasible and could likewise contribute to greater bioavailability than would be encountered at lower doses. However, these results also suggest that at lower, more environmentally relevant doses, the kinetics of CPF and DZN would not be appreciably impacted by coexposure to these insecticides.

For OP insecticides, the extent of ChE inhibition in blood and tissues correlates with the amount of oxon formed since the oxon is directly responsible for the enzyme inhibition (Timchalk, 2001). However, the inhibitory potency for individual OP insecticides varies based upon differences in oxon binding affinities for ChE (Kousba et al., 2004). The results from the current study demonstrate a clear dose- and time-dependent inhibition of brain, RBC, and plasma ChE activity and the extent of in vivo tissue sensitivity followed the order: plasma > RBC ≥ brain for both CPF and DZN and are quantitatively similar to previous findings with CPF and DZN in the rat (Poet et al., in press; Timchalk et al., 2002). The greater ChE inhibition in the blood (plasma and RBC) relative to brain is partially explained by differences in dosimetry since PBPK/PD model simulations predict substantially higher oxon AUC (two orders of magnitude) in the blood relative to the brain (Timchalk et al., 2002).

It is of interest to note that the plasma ChE response was particularly sensitive to inhibition by both DZN and CPF, as evidenced from the near maximal inhibition at a dose as low as 15 mg/kg either individually for CPF or as a binary mixture of CPF/DZN (see Fig. 5). Rodent plasma ChE is the sum of both AChE and BuChE (50:50); whereas in brain and RBC, ChE is nearly all AChE (approximately 95–100%; Kousba et al., 2003; Maxwell et al., 1987; Traina and Serpietri, 1984). In addition, based upon in vitro determinations of ChE inhibition potency and PBPK/PD model simulations of in vivo inhibition dynamics, BuChE is more sensitive to the inhibitory effects of both CPF–oxon and DZN–oxon than is AChE (Amitai et al., 1998; Kousba et al., 2003; Poet et al., in press; Timchalk et al., 2002). Therefore, the greater sensitivity of the plasma ChE to inhibition, relative to the brain and RBC AChE response, could be partially attributed to the BuChE present in the rat plasma.

The overall potency for ChE inhibition was greater for CPF than DZN and the binary mixture response appeared to be strongly influenced by CPF at all doses (see Figs. 5–7). This is particularly interesting since the rats actually received slightly higher molar doses of DZN (approximately 14%; see Table 1), yet CPF was clearly more potent. This greater in vivo potency for CPF relative to DZN is consistent with the findings of Poet et al. (2003) who compared the in vitro hepatic CYP450 metabolism of both CPF and DZN to their respective oxon's and reported V_{max}/K_m ratios of 18 and 0.75, respectively, which suggested that CPF was more rapidly metabolized to CPF–oxon (approximately 24×) relative to DZN. Additionally, it was reported that the rate of A-esterase detoxification for both CPF–oxon and DZN–oxon were similar (V_{max}/K_m 3700 and 4400, respectively). Based upon these comparisons, the differences in CYP450 metabolic capacity may partially contribute to the observed differences in in vivo ChE inhibition dynamics.

It is also possible that other reasonable mechanisms such as differences in the affinity of the oxon metabolites for ChE enzymes or the depletion of important detoxification enzymes could contribute to observed differences in OP insecticide potency. Richardson et al. (2001) suggested that the in vitro interactions of CPF–oxon and azinphos–methyl–oxon with brain AChE are best characterized using a dose additive model since the extent of AChE inhibition is dependent upon the relative potency of each oxon for the enzyme. Further complicating our understanding of OP mixture interactions, Karanth et al. (2001, 2004) reported that the sequence of exposure to OP insecticides dramatically influence the toxicological response. They reported that preexposure to CPF resulted in greater toxicity and ChE inhibition with sequential doses of parathion and methyl parathion and concluded that CPF pretreatment effectively blocked the hepatic detoxification of paraoxon and methyl paraoxon (due to the depletion of carboxylesterase), whereas the reverse (pretreatment with parathion and methyl parathion) had a minimal effect on the detoxification (A-esterase-mediated metabolism) of CPF–oxon. These complex interactions for OP mixtures highlights the importance of chemical specific detoxification and suggest that a quantitative understanding of metabolic interactions can provide important insight into mixture interactions.

The results in the current study point out the need for additional research to more fully ascertain the in vivo mechanism(s) for the binary interactions between CPF and DZN. In particular, additional studies are needed that focus on the early kinetics (<3 h postdosing) to provide greater insight into the potential importance of intestinal first-pass metabolism for OP mixtures. Secondly, it has been previously suggested (El-Masri et al., 1997) that PBPK modeling can be used as a potential integration tool for quantitative evaluation of chemical mixture interactions. In this regard, future efforts will focus on development of a binary PBPK/PD model based on the model structures already developed and validated for CPF and DZN (Poet et al., in press; Timchalk et al., 2002).

In conclusion, the pharmacokinetic (dosimetry) and pharmacodynamic (ChE inhibition) impact of acute binary

exposures to CPF and DZN was evaluated in rats. Coexposure at a high dose (60/60 mg/kg) modified the blood kinetics of both parent compounds and is most likely associated with competition between CPF and DZN for CYP450 metabolism, although modifications in oral bioavailability cannot be excluded. However, at lower doses, most likely to be encountered in both occupational- and environmental-related exposures, the pharmacokinetics are expected to be linear. A dose-dependent inhibition of ChE activity was also noted in plasma, RBC, and brain for both the single and coexposures to CPF and DZN. In all tissues, the overall potency for ChE inhibition was greater for CPF than DZN and the binary mixture response appeared to be strongly influenced by CPF at all doses. A comparison of the ChE response at the low dose (15 mg/kg) where there are no pharmacokinetic interactions for the binary mixture suggests that the overall ChE response is additive. These experiments provide needed insight for evaluating interactions for binary pesticide mixtures and can be used for assessing the potential cumulative risk associated with occupational or environmental exposures to these insecticides.

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