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THE IN VIVO QUANTITATION OF DIAZINON, CHLORPYRIFOS, AND THEIR MAJOR METABOLITES IN RAT BLOOD FOR THE REFINEMENT OF A PHYSIOLOGICALLY-BASED PHARMACOKINETIC/PHARMACODYNAMIC MODELS

ANDREA BUSBY, AHMED KOUSBA PH.D, AND CHARLES TIMCHALK, PH.D.

ABSTRACT

Chlorpyrifos (CPF)(O,O-diethyl-O-[3,5,6-trichloro-2-pyridyl]-phosphorothioate, CAS 2921-88-2), and diazinon (DZN)(O,O-diethyl-O-2-isopropyl-4-methyl-6-pyrimidyl thiophosphate, CAS 333-41-5) are commonly encountered organophosphorus insecticides whose oxon metabolites (CPF-oxon and DZN-oxon) have the ability to strongly inhibit acetylcholinesterase, an enzyme responsible for the breakdown of acetylcholine at nerve synapses. Chlorpyrifos-oxon and DZN-oxon are highly unstable compounds that degrade via hepatic, peripheral blood, and intestinal metabolism to the more stable metabolites, TCP (3,5,6-trichloro-2-pyridinol, CAS not assigned) and IMHP (2-isopropyl-6-methyl-4-pyrimidinol, CAS 2814-20-2), respectively. Studies have been performed to understand and model the chronic and acute toxic effects of CPF and DZN individually but little is known about their combined effects. The purpose of this study was to improve physiologically based pharmacokinetic/ pharmacodynamic (PBPK/PD) computational models by quantifying concentrations of CPF and DZN and their metabolites TCP and IMHP in whole rat blood, following exposure to the chemicals individually or as a mixture. Male Sprague-Dawley rats were orally dosed with 60 mg/kg of CPF, DZN, or a mixture of these two pesticides. When administered individually DZN and CPF were seen to reach their maximum concentration at ~3 hours post-dosing. When given as a mixture, both DZN and CPF peak blood concentrations were not achieved until ~6 hours post-dosing and the calculated blood area under the curve (AUC) for both chemicals exceeded those calculated following the single dose. Blood concentrations of IMHP and TCP correlated with these findings. It is proposed that the higher AUC obtained for both CPF and DZN as a mixture resulted from competition for the same metabolic enzyme systems.

INTRODUCTION

Chlorpyrifos (CPF) and diazinon (DZN) are inhibitors of acetylcholinesterase due to the effects of their active oxygen analogs (CPF-oxon and DZN-oxon) (Timchalk et al., 2001). The inhibition of acetylcholinesterase results in a buildup of acetylcholine within the nerve synapses leading to a variety of neurotoxic effects (Mileson et al., 1998). These effects are most clearly seen following acute high dose exposures but they can also be observed in lower dose chronic cases as well.

Chlorpyrifos is the active ingredient in commonly used organophosphorous (OP) insecticides like DURSBAN® and LORSBAN® (Timchalk et. al, 2002). Chlorpyrifos and diazinon are used to eliminate pests in agricultural applications like cotton and fruit crops. Every year globally there are approximately 3 million cases of organophosphate poisoning reported resulting in ~200,000 deaths (Haywood et al., 2000). The public is exposed to these chemicals on a regular basis at chronic low levels from food and water contamination, dermal contact and inhalation. The United States National Health and Nutrition Examination Survey indicated that of approximately 3,600 persons from all 64 NHANES III locations, 70% tested positive for TCP in urine, suggesting exposure to chlorpyrifos (NHANES III, 1994).

The chemical structures of chlorpyrifos, diazinon, and their major metabolites trichlorpyridinol (TCP), and isopropyl-methyl-hydroxypyrimidine (IMHP) are shown in Figure 1. The parent compounds, CPF and DZN, are metabolized to their oxon forms via a desulfuration reaction initiated by cytochrome P450 (CYP) (Poet et al., 2003; Amitai et al., 1998). Competing with the formation of oxon is the detoxification metabolism of CPF to TCP and DZN to IMHP via a dearylation reaction utilizing the same enzymes. A-esterase (PON1) and other B-esterases also contribute to the production of TCP and IMHP through the metabolism of CPF-oxon and DZN-oxon, respectively (Poet et al., 2003; Ma et al., 1994). The ratio between the toxification/detoxification reactions determines the degree of enzyme inhibition and can be used to evaluate metabolism processes (Timchalk et al., 2002)

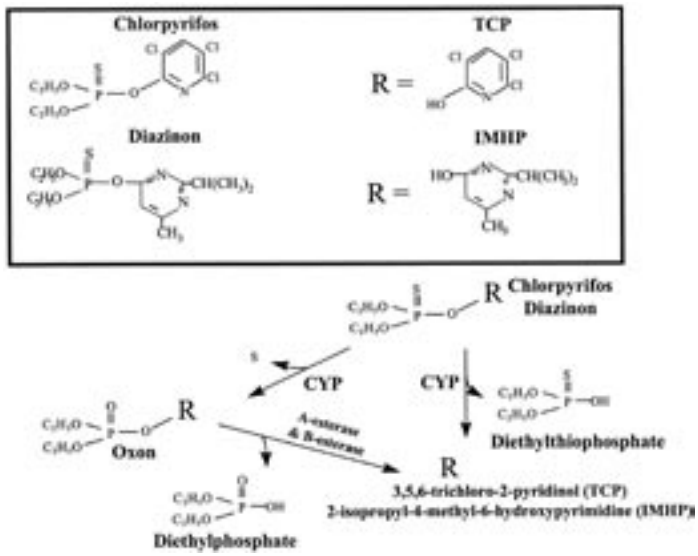

Figure 1. Structures of chlorpyrifos (CPF), diazinon (DZN), trichloropyridinol (TCP), and isopropyl-methyl-hydroxypyrimidine (IMHP) and their associated metabolic scheme (Adapted from Poet et al., 2003).

MATERIALS AND METHODS

Chemicals

Diazinon (*O,O*-diethyl-*O*-2-isopropyl-4-methyl-6-pyrimidyl thiophosphate), IMHP (2-isopropyl-6-methyl-4-pyrimidinol) and methyl-CPF (internal standard) were purchased from Chem Service Inc. (West Chester, PA). Chlorpyrifos (*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). All other chemicals were of reagent grade or better and were purchased from Sigma Chemical Company (St. Louis, MO).

Animals

All procedures using animals were in accordance with protocols established in the NIH/NRC *Guide and Use of Laboratory Animals* and were reviewed by the Institutional Animal Care and Use Committee of Battelle, Pacific Northwest Division. Sprague-Dawley male rats were purchased from Charles River Laboratories (Raleigh, NC) and were 10 weeks old at time of dosing. Prior to their use the animals were housed in solid-bottom cages with hardwood chips under standard laboratory conditions and given free access to water and food (Purina Rodent Chow). Animals were fasted for 12-16 hours prior to their dosing. Food was supplied to the rats 3 hours after dosing.

Sample Collection

Pesticides were dissolved in corn oil and administered to the animals via oral gavage. For each time point, 9 rats were sacrificed by CO_2 asphyxiation and blood was extracted. For each time point, 3 rats were dosed with 60 mg/kg of CPF, 3 were dosed with 60 mg/kg of DZN, and 3 were dosed with a mixture of 60 mg/kg of CPF and 60 mg/kg of DZN. The blood samples were collected via posterior vena cava puncture using a heparinized syringe, processed immediately up to the dried form as indicated below and stored at –80° C. For quantitation of the parent pesticides and metabolites, an *in vitro* standard curve and control rat blood samples were processed as well.

One mL of blood was used for each sample. The internal standard (5 µL methyl-chlorpyrifos: 1mg/mL) was added to the samples and the samples were briefly vortexed. Four hundred µL of an acetic acid solution (2.5 M) saturated with sodium chloride were then added to the samples to halt the

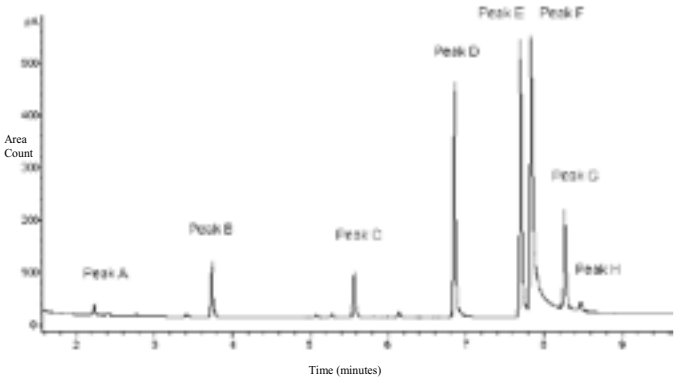

Figure 2. DZN (Peak D), CPF (Peak G), IMHP (Peak B), and TCP (Peak C) following sample derivatization; Peaks A, F, and H represent unidentified peaks only present in derivatized samples; Peak E represents internal standard (M-CPF).

blood metabolism and aid in the extraction process (Brzak et al., 1998).

Extraction of Compounds from Samples

Chemicals were extracted from the samples twice using 2 mL of toluene. The samples were strongly vortexed for 1 minute and centrifuged at 3900 rpm for 15-20 minutes at 15 °C using a Beckman CPR centrifuge. The top organic layer was blown down to dryness (in a form assumed to be stable for at least three weeks) using a light stream of inert nitrogen gas and the samples were frozen at –80 °C until their analysis.

Sample Analysis

The blown down frozen samples were removed from the freezer and reconstituted in 75 µL of toluene. The samples were derivatized using 15 µL of derivatizing agent, incubated for 60 minutes at 60-70 °C, and run immediately on a gas chromatograph (GC).

The samples were run on a Hewlett Packard 6890 GC equipped with a Nitrogen-Phosphorus Detector coupled with a Flame Ionization Detector. Separation was achieved using an organophosphate pesticide column (30m x 0.32 mm id x 0.5 µm df: Restek, Belfont, PA). Helium carrier gas was used with a head pressure of 22 psi. The oven temperature had 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. The retention time of IMHP, TCP, DZN, methyl-CPF (internal standard), and CPF were 3.7, 5.6, 6.8, 7.7, and 8.2 minutes, respectively. Peak areas were obtained for each compound by manual or automated integration.

RESULTS

Preliminary studies without derivatization yielded very broad split IMHP peaks and low analytical sensitivity limits for TCP and IMHP (data not shown). The derivatization process proved to be a simple and powerful method for obtaining clear and quantifiable chromatography peaks for DZN, CPF, IMHP, and TCP. Figure 2 contains a representative chromatogram for the analytical separation of all four compounds following derivatization.

Peak F did not pose a problem in determining the peak area of the internal standard peak (Peak E) because the two peaks were sufficiently split. The unidentified derivatization peaks were attributed to broken down derivatizing agent material. These peaks, also, did not pose a problem because they were very well separated from the peaks of interest as shown in figure 2.

The extraction of the compounds from the samples followed a linear trend at low concentrations. No quantifiable chemical peaks were observed in the control blood samples that were run. A representative standard curve for CPF is shown in Figure 3 in which the linear trend can be seen. Coefficient variables ranged from 0.97 to 0.99 and similar linear responses were seen in the DZN, TCP and IMHP (data not shown).

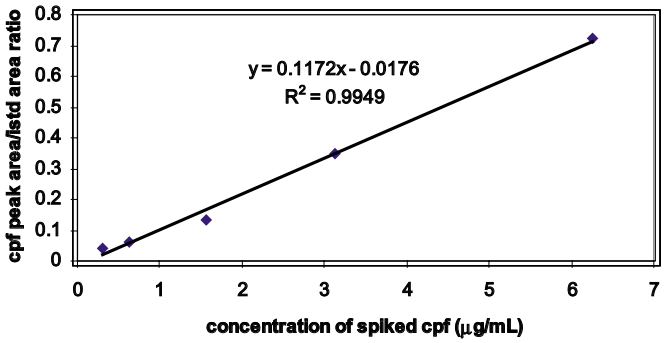

Figure 3. Standard curve showing the linear response of extraction of CPF over the given concentration range in control blood. The y-axis unit is defined as the area of the CPF peak over the internal standard peak area (istd area).

The blood concentration profiles over the 24-hour time period for the four compounds are shown in Figures 4a/b and 5a/b. Each data point represents the average ± SD from three rats.

The y-axis concentration units in figures 4a/b and 5a/b are given as the logarithms of the concentrations for sake of ease of graphing.

When dosed individually, CPF and DZN concentration peaks were achieved at 3 hours followed by a steady concentration decrease (Figures 4a and 5a). When both CPF and DZN were present as a mixture the maximum blood concentration for both compounds shifted slightly to the right attaining a maximum concentration at 6 hours post-dosing. However, the subsequent blood concentrations through 24 hours post-dosing were comparable when both compounds were given singly or as a mixture. At 3 hours post-dosing IMHP and TCP concentrations peaked and then steadily decreased for the duration of the time period in either exposure scenario (single and mix dosing) (Figures 4b and 5b). However, IMHP and TCP had a slightly lower concentration in the mix samples over the whole profile. The maximum blood concentration observed at

AUC (μg*hr/mL)	DZN	CPF	IMHP	TCP
Single	2.32	7.67	69.7	55.9
Mix	4.00	12.0	40.9	43.5

Table 1-AUC calculated for DZN, CPF, IMHP, and TCP using the Trapezoidal Rule. *(DZN-Diazinon, CPF-Chlorpyrifos, IMHP-DZN metabolite, TCP-CPF metabolite)*

3 hours post-dosing in this study with the individual chemicals is consistent with previous studies in which rats were orally administered either chlorpyrifos or diazinon over a very broad dose range (0.5 – 100 mg/kg) (Timchalk et al., 2002 and Poet et al., 2004).

The area under the blood concentration curves (AUC) calculated for the four compounds using the Trapezoidal Rule are shown in Table 1. Diazinon and CPF had higher AUC's for the mix samples whereas IMHP and TCP had higher AUC's for the single samples.

DISCUSSION AND CONCLUSION

The peak blood CPF and DZN concentrations shifted to six hours post-dosing following mixture exposure, whereas when the same compounds were administered individually, peak concentrations were attained by 3-hours post-dosing (Figures 4a and 4b). The delay to achieve peak concentration following mixture exposure could be attributed to metabolic competition between CPF and DZN for CYP active sites. Since CYP metabolism is responsible for the conversion of both CPF and DZN to TCP and IMHP, and is a saturable process (Poet et al., 2003a), it is possible that a binary mixture of the chemicals at the 60 mg/kg dose level overwhelmed the enzymes' metabolic capacity resulting in higher blood concentrations of the parent compounds being initially present. Although the 6 h concentration of CPF and DZN suggests a saturable process, the descriptive kinetic profiles of the later time points (12 and 24 hours post-dosing) no longer indicate saturation. These interpretations were confirmed by the blood time courses of the metabolites, IMHP and TCP following mixture exposure compared with the single exposure (Figures 5a and 5b). The IMHP and TCP concentrations were slightly lower when present as a mixture relative to the single dose exposure. The previous results substantiate the conclusion of the competitive metabolic interaction of the parent compounds in animals exposed to the chemical mixture.

These results are further confirmed by comparing the AUC's for the parent compounds and metabolites (see Table 1). The CPF and DZN total AUC's were greater following mixture exposure than the single exposure and consequently the metabolite AUC's were smaller for the mixture than for the single dose exposure. This observation, along with the shifting of their parent compounds' highest concentrations mentioned previously, substantiate the fact that the nature of the metabolic interaction following mixture exposure is competitive. Diazinon and CPF competition for CYP metabolism may be attributed to their similar chemical structure, and these results also suggest

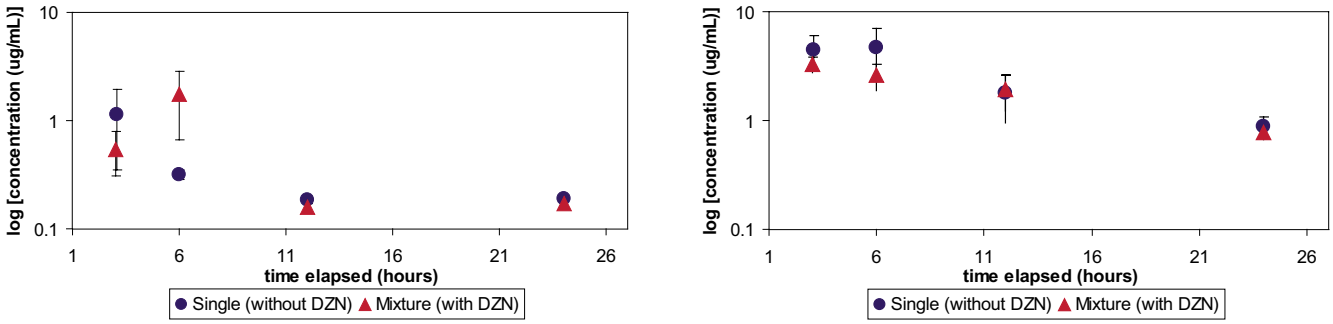

Figure 4. Chlorpyrifos metabolism data, (A) concentration of CPF with respect to time with and without DZN and (B) concentration of TCP with respect to time with and without DZN.

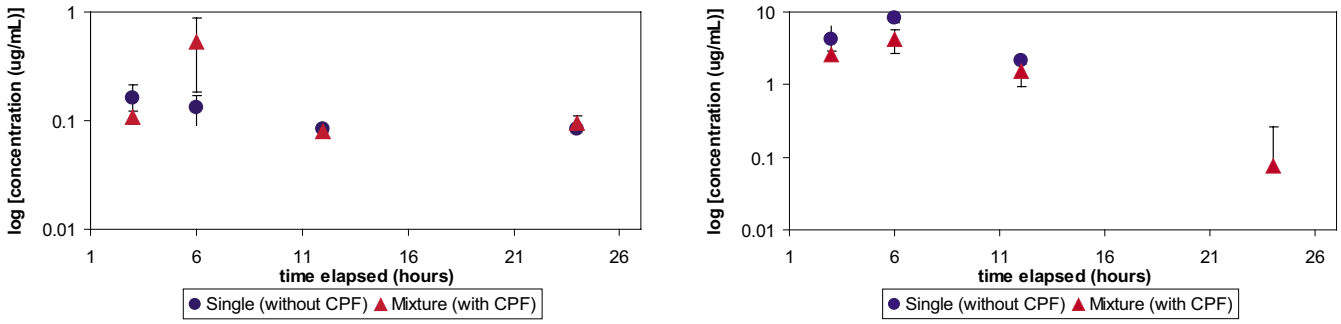

Figure 5. Diazinon metabolism data, (A) concentration of DZN with respect to time with and without CPF and (B) concentration of IMHP with respect to time with and without CPF.

that other structurally related organophosphorous insecticides may exhibit a similar behavior (Timchalk et al., 1989).

Although previous oral *in vivo* studies with CPF or DZN over a broad concentration range did not result in any shifts in the time to peak blood concentration (Timchalk et al., 2002 and Poet et al., 2004) it is conceivable that when present as a mixture they could contribute to a delay in intestinal absorption or metabolism. In this regard, Poet et al. (2003) has reported on the *in vitro* intestinal metabolism of CPF and DZN using microsomes isolated from intestinal enterocytes. Poet et al. suggested that intestinal metabolism could modify *in vivo* bioavailability, however these studies did not evaluate the potential for CPF and DZN interactions.

In vivo analysis of the parent compounds and their corresponding metabolites in rat livers and urine are currently being evaluated following exposure to CPF and DZN over a broad dose range. These studies will further substantiate the degree of mixture interaction and will be used to further develop and refine a binary PBPK/PD model for organophosphorus insecticides.

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