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Negative Ion Chemical Ionization Mass Spectrometry for the Analysis of 3,5,6-Trichloro-2-pyridinol in Saliva of Rats Exposed to Chlorpyrifos

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Abstract: Organophosphorus (OP) insecticides (e.g. chlorpyrifos) are widely used in a variety of applications, and the potential exists for significant occupational and environmental exposures. They have been associated with more occupational poisoning cases than any other class of insecticides.

One of the best approaches for accurately assessing human dosimetry and determining risk from both occupational and environmental exposure is biomonitoring. Biological matrices such as blood and urine have been routinely used for biomonitoring; however, other matrices such as saliva represent a simple and readily obtainable fluid. As a result, saliva has been suggested as an alternative biological matrix for the evaluation of a broad range of biomarkers such as environmental contaminants, drugs of abuse, hormones, chemotherapeutics, heavy metals, and pesticides.

Chlorpyrifos (CPF), and its major metabolite, 3,5,6-trichloro-2-pyridinol (TCP), have been quantified in urine and blood as a biomarker for exposure to OP insecticides.

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The purpose of this study was to develop an analytical approach for detecting and quantitating the levels of TCP in saliva obtained from rats exposed to CPF and to evaluate the potential of saliva as a noninvasive biomonitoring matrix for the determination of exposure to OP insecticides.

Adult male rats were administered CPF, and blood and saliva were humanely collected for analysis of TCP and CPF. The TCP was detected and quantitated in saliva using negative ion chemical ionization mass spectrometry with selected ion monitoring. Initial results indicate that saliva potentially may be utilized as a noninvasive biomonitoring matrix to determine exposure to organophosphate insecticides.

Keywords: Chlorpyrifos, 3,5,6-trichloro-2-pyridinol, biomonitoring, saliva, negative ion chemical ionization mass spectrometry, derivatization, gas chromatography, organophosphate insecticides, biological matrices, biomarkers, metabolites

INTRODUCTION

Chlorpyrifos (CPF), [O,O-diethyl-O-(3,5,6-trichloropyridyl) phosphorothioate], is a member of a large class of organophosphorus (OP) insecticides that are widely used in the agricultural industry and in home applications (Aspelin 1992; 1994). As an example, it was reported that in 1997 approximately 5 billion pounds of pesticides were applied in the United States (U.S. EPA). Organophosphorus insecticides are involved in more occupational poisoning cases than are any other single class of insecticide; both intentional (suicide) and accidental human exposure have resulted from in-home utilization (Al-Saleh 1994; Murphy 1986). Approximately 3 million episodes of OP insecticide poisoning a year, resulting in nearly 200,000 deaths (Haywood and Karalliedde 2000), are reported globally. The life-threatening effects exhibited by the OP insecticides are a result of inhibition of acetylcholinesterase (AChE) by their oxon metabolites (Mileson et al. 1998).

Agricultural workers and individuals who are occupationally exposed most often handle concentrated formulations, and therefore, have the potential for higher exposure to these chemical agents. As a result, the state of Washington has established a cholinesterase medical monitoring program for farm workers who work with OP insecticides and carbamates. In the first year (2004), those that work with OP and carbamate insecticides for more than 50 hours in a 30-day period will be given the opportunity to have their blood tested; in the second year the threshold will be lowered to 30 hours. Currently, the program requires annual red blood cell (RBC) and plasma cholinesterase baseline testing by a certified laboratory (Kirk 2003). Lavy et al. (1993) conducted a year-long biomonitoring study in nursery workers who had the potential for multiple pesticide exposure. The results, based on urinary biomonitoring data, showed that approximately 16% of the workers had absorbed low levels of pesticides. Hayes et al. (1980)

performed a biomonitoring study of pest control operators exposed to three insecticides (vapronite, diazinon, and CPF) and reported the presence of pesticide metabolites (alkyl phosphates) in the urine among these same operators. Families of agricultural workers and, particularly children, may also be at increased risk from exposure to these insecticides. Several additional studies have illustrated the potential for children to be exposed. Simcox et al. (1995) found several OP insecticides, azinphos-methyl, CPF, parathion, and phosmet, in 62% of household dust samples that were collected within the homes of agricultural workers. Loewenherz et al. (1997) also conducted a biomonitoring evaluation of OP insecticide exposure among children of agricultural workers in central Washington State. Based on the quantitation of metabolites in urine, they reported that children living in households with pesticide applicators and in proximity to treated orchards had an increased exposure. The general public is also exposed to low levels of OP insecticides through ingestion of residues on food and contaminated water supplies as well as through inhalation and dermal contact. As a result, there is a need to accurately quantify OP exposure, due to the potential for significant exposures. Chester (1993) and Woolen (1993) showed biological monitoring to be a critical tool for evaluation of exposure to a wide range of chemical agents including insecticides.

Biomonitoring offers one of the best approaches for accurately assessing human dosimetry and for determining risk from both occupational and environmental exposure (Friberg and Elinder 1993; Christensen 1995). Biological matrixes such as blood and urine have been utilized primarily for biomonitoring; other matrixes such as saliva represent a simple, readily obtainable fluid not requiring invasive techniques to collect. As a result, saliva has been used to evaluate a broad range of biomarkers, drugs, and environmental contaminants including drugs of abuse, hormones, chemotherapeutics, heavy metals, and pesticides (Joselow, Ruiz, and Goldwater 1968; Hayashi, Watanabe, and Ozeki 1989; Nigg and Wade 1992; Schramm et al. 1992; Lu et al. 1997; 1998). Saliva is also being used to indicate HIV exposure and potential heart disease indicators (Russo 2004; Streckfus et al. 2000).

A high performance liquid chromatography (HPLC) method has been developed for the analysis of CPF, CPF-oxon, and 3,5,6-trichloro-2-pyridinol (Sultatos, Costa, and Murphy 1982) with extraction into ethyl acetate. The quantitation levels reported were 10 to 40 ng per injection. This technique, however, was neither sufficiently sensitive nor selective. Gas chromatography/mass spectrometry with electron ionization also has been reported for the analysis of chlorpyrifos metabolites in serum and urine (Drevenkar et al. 1993). Barr et al. (2002) used isotope dilution high resolution mass spectrometry for the analysis of pesticides in human serum and plasma. Limits of detection were reported to be in the low pg/g range.

Negative ion chemical ionization (NICI) is a technique in which a buffer gas (e.g., isobutane, methane) is used to slow down the electrons in the

electron beam until some of the electrons have the right energy to be captured by analyte molecules. The strengths of NICI include its higher sensitivity for the detection of specific molecules as compared to other ionization processes and its higher selectivity for compounds such as polychlorinated hydrocarbons, polycyclic aromatics, diketones, and trifluoroacetic anhydride derivatives of amino compounds. Campbell et al. (1986) utilized negative ion chemical ionization for the differentiation of isomers of TCP. Bzrak et al. (1998) developed a method for the analysis of CPF, CPF-oxon, and TCP in rat and human blood. For TCP, the method involved extraction and derivatization, with subsequent analysis using NICI with selected ion monitoring to provide additional selectivity and sensitivity.

The structures of CPF and TCP and the metabolic pathway of CPF are illustrated in Fig. 1. The parent compound, CPF, is metabolized to the cholinesterase (ChE)-inhibiting oxon via a desulfuration reaction initiated by CYP450 (CYP) (Sultatos 1994). The detoxification metabolism of CPF to TCP via a dearylation reaction utilizing the same enzymes is in competition with the formation of oxon (Ma and Chambers 1994). In addition, A-esterase (PON1) and other B-esterases contribute to the production of TCP through the metabolism of CPF-oxon (Sultatos and Murphy 1983).

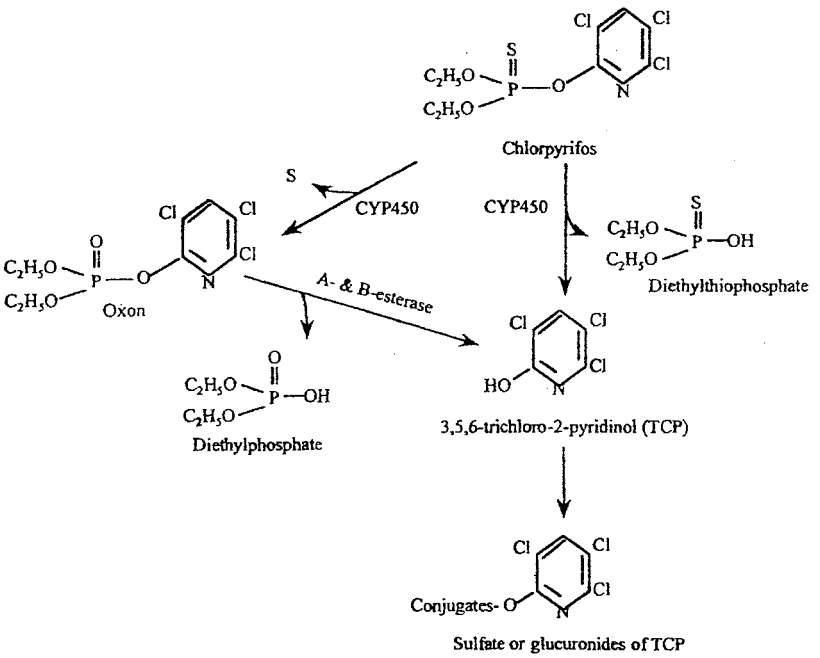

Figure 1. Scheme for the metabolism of CPF to CPF-oxon and TCP.

The objective of this study was to develop a selective and sensitive analytical approach for detecting and quantitating levels of TCP in saliva obtained from rats exposed to CPF and to investigate the utility of saliva as a potential noninvasive biomonitoring matrix. Rats were exposed to CPF, and saliva samples were humanely collected. The TCP was extracted from the saliva, derivatized, and analyzed using NICI with selected ion monitoring. The results indicate that TCP can be detected and quantified, and saliva has the potential for being utilized as a noninvasive biomonitoring matrix for determining exposure to organophosphate insecticides.

EXPERIMENTAL

Chemicals

3,5,6-Trichloro-2-pyridinol and CPF were obtained from Dow AgroSciences. (Indianopolis, IN). N-(t-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) was purchased from Pierce Chemical Company (Rockford, IL). Ethyl acetate was purchased from Aldrich Chemical Company (Milwaukee, WI). The remaining chemicals used in this study were either reagent grade or better.

Animals

Adult male Sprague-Dawley rats (300–350 g) were purchased from Charles River Laboratories Inc. (Raleigh, NC). The 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). All procedures involving animals were in accordance with protocols established in the NIH/NRC *Guide and Use of Laboratory Animals* (NIH/NRC) and were reviewed by the Institutional Animal Care and Use Committee of Battelle, Pacific Northwest Division.

Groups of rats (5/dose level) were orally administered CPF at doses of 1, 10, and 50 mg/kg. Blood and saliva were humanely collected at 3, 6, and 12 h post-dosing for analysis of TCP. To induce salivation 10–15 min prior to starting saliva collection, rats were anesthetized with an ip injection of ketamine: xylazine [87:13 mg/kg and then administered pilocarpine (ip; 1 mg/kg)]. Saliva (0.5–3 mL) was collected over a 30 min period using glass capillary tubes. Samples were stored at −80°C until the time of sample preparation.

Sample Preparation

Saliva samples were prepared in a method similar to one Brzak et al. (1998) applied to analysis of CPF and TCP in blood. To approximately 0.1 mL of

saliva from exposed rats, 1 drop of concentrated HCl (approximately 10 μL) and 0.1 mL of saturated NaCl salt solution were added. The solution was mixed well using a vortex mixer. The solution was extracted three times with 3 mLs of ethyl acetate. The extracts were combined and passed through a column of sodium sulfate to remove water. The extract was then dried under a gentle stream of nitrogen, and the residue was dissolved in toluene. The derivatizing reagent, N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA), was then added, and the solution was heated at 60°C for 1 h prior to analysis by gas chromatography/mass spectrometry (GC/MS).

Standards were prepared by spiking known amounts of TCP ranging from 1–50 ng/mL into control saliva, extracting, derivatizing, and subsequently analyzing it using negative ion chemical ionization mass spectrometry with selected ion monitoring. Extracts were analyzed using a JEOL SX-102/SX-102 four sector mass spectrometer equipped with an HP 5980 GC which employed a splitless inlet. Separations were performed using a DB-5 capillary column (30 m × 0.25 mm i.d. 0.25 μm film thickness). The temperature program was 80°C for 1 min, 80–300°C at 25°/min, and 300°C for 5 min. Negative ion chemical ionization with isobutane as the reagent gas (2×10^{-4} torr.) and selected ion monitoring were utilized for the detection of TCP.

RESULTS AND DISCUSSION

Figure 2 shows a negative ion chemical ionization mass spectrrum of TCP. The major ion of TCP is m/z 161 [M-Cl-Si$(CH_3)_2(CH_3)_3$] under NICI

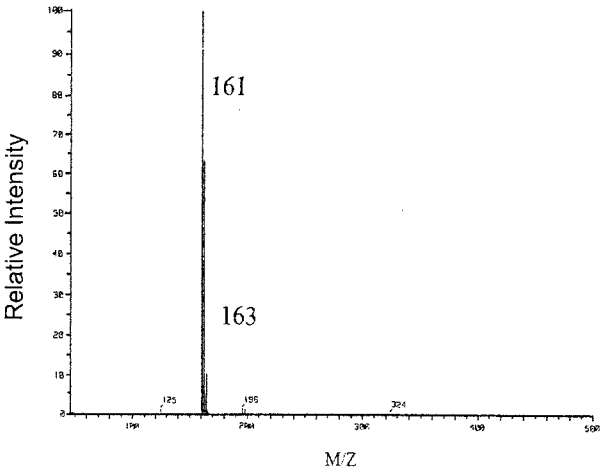

Figure 2. NICI spectrum of TCP.

conditions. As a result, the ions chosen for selected ion monitoring were m/z 161 and 163 for TCP. The ion m/z 163 was also monitored for confirmation of the isotope pattern of 2 Cls.

A calibration curve was generated from TCP spiked in saliva (1 to 50 ng/mL), extracted, derivatized, and subsequently analyzed. The results indicate the response is linear over nearly two orders of magnitude. The limit of quantitation was estimated to be 5 ng/mL. Quantitation would have been more accurate and consistent with isotope dilution; future studies are planned to include the use of labeled TCP for this analysis. Attempts

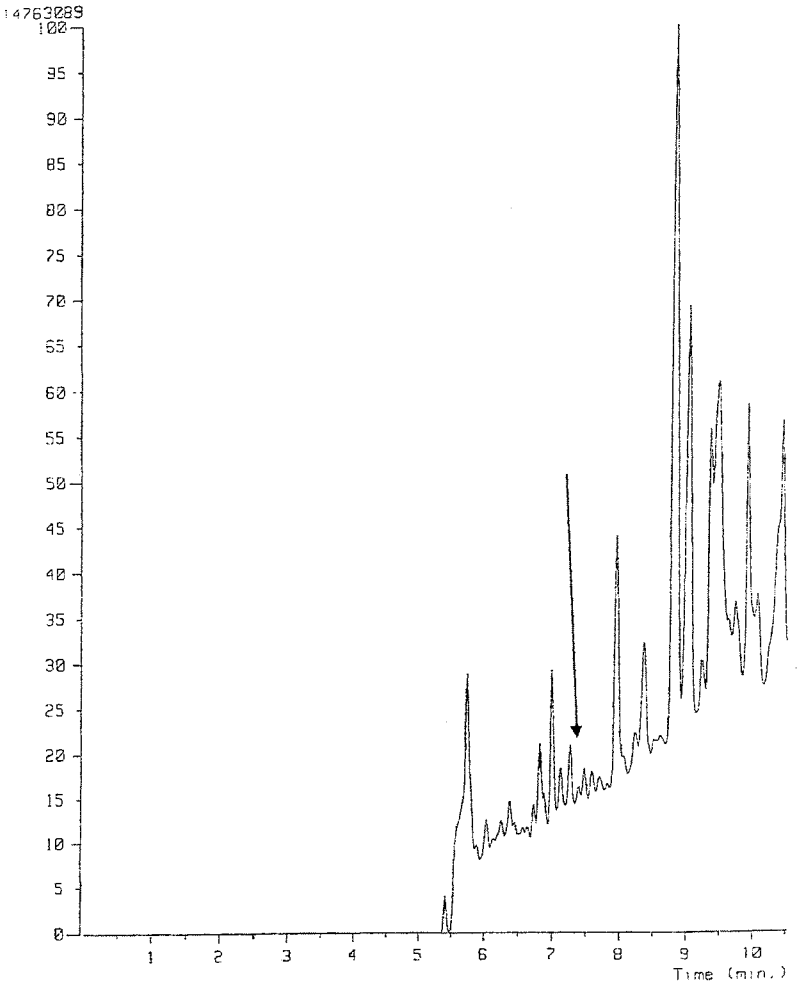

Figure 3. NICI total ion chromatogram of derivatized saliva sample. Arrow indicates elution of TCP.

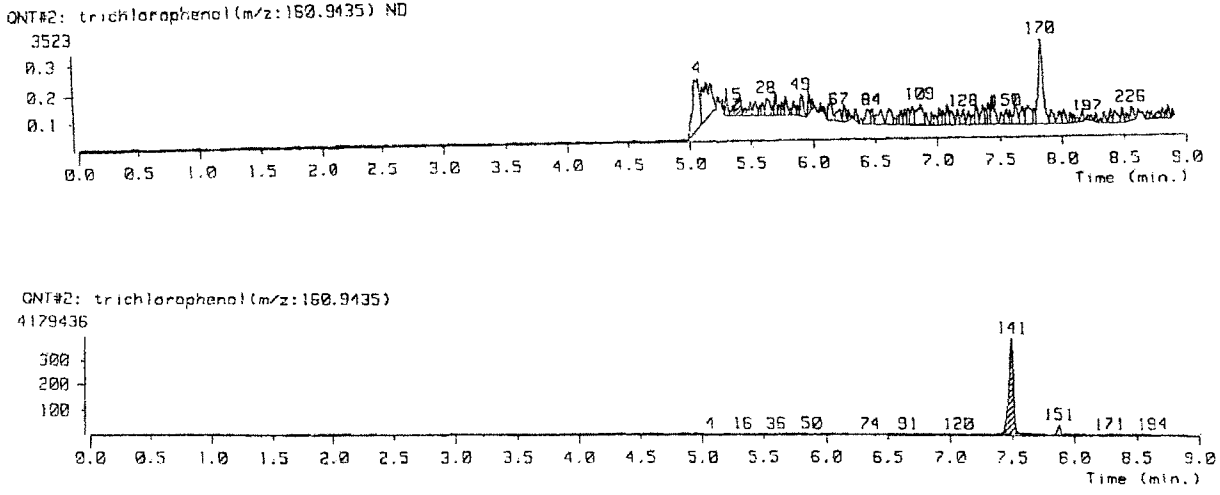

Figure 4. NICI SIM of m/z 161 for blank (above) and sample (below).

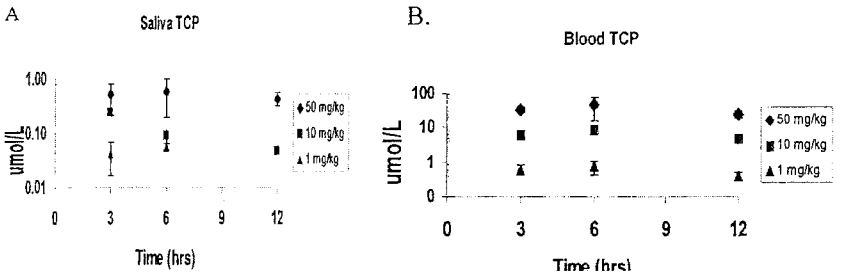

Figure 5. Concentration of TCP in A) saliva and B) blood.

were also made using trichlorophenol as a surrogate compound and pentachlorophenol as an internal standard; however, the results obtained were not consistent.

Figure 3 is a NICI full scan total ion mass spectrum of a derivatized saliva extract. Figure 4 is an NICI-SIM chromatogram of a blank sample and actual derivatized extract from saliva of a rat exposed to CFP. The retention time of TCP is 7.5 min. Compared to the full scan, the NICI-SIM chromatogram is less complex. In the selected ion chromatograms, there was a small degree of interference present at m/z 161 in the control samples. Aside from this interference, the selected ion chromatograms were very clean in the region of interest. As a result, NICI with SIM is very selective and sensitive for the detection of TCP in saliva.

Figure 5A shows the results of TCP in saliva; the results of TCP in blood are shown in Fig. 5B and been reported elsewhere (Timchalk et al. 2004). Trichloropyridinol was detected in the saliva of rats exposed to CPF and the concentrations were less than that observed in blood. The kinetic profile in saliva is similar to the response observed in blood.

Figure 6 illustrates the ratio of TCP concentration in blood/saliva. The concentration of TCP in blood is about two orders of magnitude higher than that in saliva.

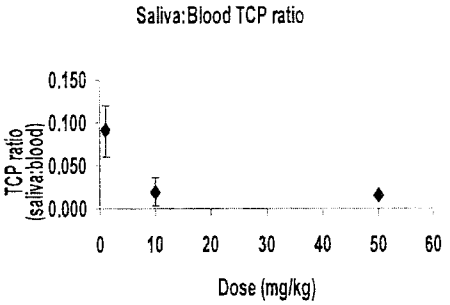

Figure 6. Ratio of TCP in saliva : blood at the dose levels of 1, 10, 50 mg/kg.

CONCLUSIONS

Negative ion chemical ionization mass spectrometry with selected ion monitoring provides a sensitive and selective technique for the analysis of TCP in both blood and saliva. Additional improvement in the detection limit will be necessary to detect low levels of exposure. This may require high resolution mass spectrometry with selected ion monitoring. In addition, further work needs to be performed regarding quantitation. Labeled TCP certainly is an option, but was not pursued in this study.

Preliminary results from this study indicate that saliva may be potentially utilized as a noninvasive biomonitoring matrix for the determination of exposure to OP insecticides and possibly other chemicals. The kinetic profile for TCP is similar in blood and saliva.

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