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# Analysis of 3,5,6-Trichloropyridinol in Blood Plasma

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The detection of 3,5,6-trichloropyridinol (3,5,6-TCP), one of the more familiar metabolites of chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate), in urine by gas-liquid chromatography (GLC) and electron capture (EC) detection has been applied to human blood plasma. Human urine samples have been analyzed for 3,5,6-TCP by the Hawaii Epidemiologic Studies Program (HESP), Annual Report No. 10 (1977), using procedures based on a multiresidue method developed for the analysis of rat urine by SHAFIK et al. (1973) for pesticides which biodegrade to halo- and nitro-phenols. This analytical method has been adapted to analyze human blood plasma for the metabolite of chlorpyrifos.

Blood samples were collected from workers who were exposed to chlorpyrifos and other organophosphorous and carbamate pesticides. Samples with normal but low plasma cholinesterase activities were analysed for 3,5,6-TCP. ELIASON et al. (1969) has reported the depression of plasma cholinesterase in individuals occupationally exposed to chlorpyrifos. Blood samples available in the laboratory and which were believed to be free of halo- and nitro- phenolic metabolites and Hyland Chemistry Control Serum-1 were used for controls and for in vitro specimens. Additions and refinements to the analysis of 3,5,6-TCP in human urine and blood plasma are included.

## EXPERIMENTAL

Gas-Liquid Chromatography: An instrument equipped with $^{63}Ni$ EC detectors; 2 borosilicate glass columns, 1.8m x 4.0mm, ID; one column was packed with 4% SE-30/6%OV-210 on 80-100 mesh Gas Chrom Q; 2nd column was packed with 1.5% OV-17/1.95% QF-1 on 80-100 mesh Gas Chrom Q (GLC analyses for each sample were conducted on both columns); operating parameters, temperatures: column, 175-190°C; detectors, 280°C; inlet, 230°C; transfer, 250°C; carrier gas, nitrogen, 60 mL/min.

Blood cholinesterase activities (plasma and whole blood) were measured with a Sargent-Welch Recording pH Stat, S-30240.

Column Chromatography: Chromaflex glass columns (Kontes K-420100, 21 or 22) packed with silica gel Woelm, adsorption

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activity grade I (ICN Pharmaceuticals, W. Germany) or Florisil. Silica gel was prepared according to SHAFIK et al. (1973). The adsorbent was dried at 170°C for 48 h and deactivated in 2 g portions, the amount used per column, with 40 µL benzene extracted distilled $H_2O$ in culture tubes with teflon-lined screw-caps by rotation.

Hydrolysis: A commercial pressure cooker (32cm ID x 27cm depth) was used in the hydrolysis step.

Reagents: Conc. HCl, anhydrous diethyl ether, reagent grade; all the other solvents were of the quality for pesticide analyses; 3,5,6-trichloro-2-pyridinol, 99% purity (Dow), was used for standard solutions; pentachlorophenol (PCP) standard was obtained from the EPA Repository (Research Triangle Park, N.C.); methylating (diazomethane) and ethylating (diazoethane) reagents were prepared respectively from N-methyl- and N-ethyl- N'-nitro-N-nitrosoguanidine (Aldrich Chem. Co. Inc.).

Analysis of Blood Plasma

The determination of 3,5,6-TCP is essentially the same in blood plasma as in urine (HESP laboratory Annnual Report No. 10 1977), with modifications to accommodate the differences in substrates. Briefly, the urinalysis involves: (1) acid (conc. HCl) hydrolysis of the samples, (2) extraction of 3,5,6-TCP, (3) derivatization of the metabolite with diazomethane or diazoethane reagent, (4) chromatographic cleanup and separation of the derivatized (alkylated) metabolite on silica gel, and (5) detection and determination of the derivatized metabolite by EC-GLC.

In the analysis of blood plasma, 3.00 mL samples are placed in culture tubes (16 x 150 mm), with teflon-lined screw caps. About 12 drops, conc. HCl, are added with mixing, and the screw-caps are gently tightened. The samples are then placed on a rack in the pressure cooker for the acid hydrolysis according to the method of BRADWAY (1976). A pressure cooker of this size can easily accommodate 20 samples. Water, 500 - 1,000 mL, is added and the pressure cooker is sealed, placed on a hot plate, and the temperature is increased until a pressure of 15 psi can be maintained for 30 min. The cooker is allowed to cool to room temperature or until the samples can be removed without difficulty, and ca. 2 mL of 0.1 N NaOH, are added to each of the samples. The available 3,5,6-TCP in the hydrolyzed blood plasma is then extracted and separated for derivatization.

A mixture of hexane and anhydrous ethyl ether in a ratio, 1:5 (v/v), is utilized for the extractions. This mixture minimizes emulsion formation during the extraction processes and assists in the better separation of the organic phase from the hydrolyzed plasma. 3 successive 3 ml extractions of a sample are sufficient to ensure removal of all of the 3,5,6-TCP. Each extraction is conducted with 2 min of vigorous shaking or vortexing, followed by separation of the extracting organic phase by centrifugation at 3,000 rpm for about 3 min, and collection of the separated

extractant by means of a disposable pipet. A total of 6-7mL of the combined extractions is collected in a graduated, 15 mL, glass stoppered, centrifuge tube, evaporated down to 4-5 mL with a stream of nitrogen gas, and derivatized (alkylated) with either diazomethane or diazoethane reagent according to SHAFIK et al. (1973). The alkylating reagents are prepared freshly before use by adding the respective nitrosoquanidine reagents to alkaline hexane according to CRANMER & FREAL (1970). Both the preparation of reagents and the derivatization are conducted under a fume hood with adequate draft. Although better results were apparently obtained with diazoethane, the ethyl ether of 3,5,6-TCP having a GLC peak with almost the same retention time as the peak for methylated 3,5,6-TCP but with increased sensitivity, some variation in results was experienced with the ethyl ether derivative, especially with urine samples.

After derivatization, the extract is blown down further to 0.3 mL while rinsing the walls of the centrifuge tube with ca. 2 mL, hexane and chromatographed on a freshly prepared silica gel column according to a modified elution protocol outlined by SHAFIK et al. (1973) which includes a 10 mL hexane prewash and a sequence of elutions with 10 mL fractions of 20, 40, 60 and 80% benzene in hexane mixtures. The concentrated extract is put on the column with two 0.5 mL hexane rinses, and the alkyl ether of 3,5,6-TCP is collected in the first 10 mL fraction of the 20% benzene in hexane elution. An additional 2 mL of the solvent mixture are passed through the column to ensure complete elution. Continuation of the chromatography protocol by SHAFIK et al. (1973) with elution fractions of 40, 60, and 80%, benzene in hexane is not necessary. The concentration of the eluate is then adjusted for analysis by GLC-EC detection.

The analyses of *in vivo* blood plasma samples were initially conducted as described above. However, with continued analyses, the method was simplified by replacing silica gel columns with Florisil columns. Extracts of the derivatized blood plasmas were chromatographed through Florisil columns according to a modification of the analysis of pentachlorophenol (PCP) in urine and blood by the HESP laboratory, Annual Report No. 8 (1975), and then analyzed by GLC. The Florisil columns were prepared by packing chromaflex columns, 20cm x 2.2cm, ID and 50 mL solvent reservoir (Kontes 420100, size 22) with Florisil which was purged overnight in an oven at 135°C and cooled while exposed to ambient laboratory temperature before use. The Florisil was retained in the column with a small wad of glass wool to a height of 100 mm after gentle tapping and topped with 20 mm, anhydrous granular sodium sulfate. The column was prewashed with 10 mL, hexane and the concentrated derivatized extract was put on with rinses not exceeding 2 mL totally. The alkyl ether of 3,5,6-TCP was eluted with 10 mL hexane and collected for GLC analysis. The use of a Florisil column instead of a silica gel column was applicable inasmuch as the analysis was confined to the detection of only one metabolite and did not require the separation of multiresidues of metabolites as in the work by SHAFIK et al. (1973).

The blood plasma analyses of 3,5,6-TCP were monitored with recovery runs. 3.00 mL samples of plasma and analytical control blood serum (Hyland) were fortified with 100 ng of standard 3,5,6-TCP prepared in 0.01N NaOH. With the early trials the recoveries were low (30-60%)largely due to extraction problems caused by emulsions with the acid hydrolyzed blood plasma samples. However, after following the extraction techniques developed for this substrate, recoveries improved to ca. 90% and as high as 97%. The fortified samples along with controls and blanks were processed simultaneously through the entire analytical procedure for 3,5,6-TCP.

Further determinations of 3,5,6-TCP in blood plasma by the described method were conducted with pesticide incident specimens from people suspected of being poisoned through exposures to various commercial formulations containing chlorpyrifos. During these analyses, a GLC peak which had a longer retention time than the one for the ethyl ether of 3,5,6-TCP was recognized as the peak for PCP. The HESP laboratory determines PCP routinely in human blood plasma and urine by GLC and is familiar with this organochlorine pesticide. Analyses therefore were continued with the inclusion of the determination of PCP simultaneously with 3,5,6-TCP. Fortifying standard PCP solutions were prepared in 0.01N NaOH.

Complementary Analyses

Urine, in vivo samples which accompanied human blood plasma samples were also analyzed for 3,5,6-TCP according to the method outlined by the HESP laboratory, Annual Report No. 10 (1977). These determinations were monitored with recoveries of 50 and 100 ng levels of 3,5,6-TCP which were added to control samples of urine. Control urine samples were composites of available urine from subjects believed not to be exposed to chlorpyrifos. Results were improved by extracting an acid hydrolyzed urine sample an additional 3rd time with 3 mL, ethyl ether.

Blood cholinesterase (ChE) activities were measured by the automatic titrimetric pH Stat method (NAAB & WHITFIELD 1967). The HESP laboratory participates in a national interlaboratory, quality control program, EPA, for the blood enzyme analyses conducted by the Medical University of South Carolina.

## RESULTS AND DISCUSSION

The results for the in vivo analyses of 5 individuals from which blood and urine were sampled are shown in Table 1. For each individual the levels of 3,5,6-TCP detected in urine and blood plasma are listed along with the measured cholinesterase activity of blood plasma. The 5 individuals who were selected from a group of 55 had the lowest plasma cholinesterase levels, 2.53-3.78 µm/min/mL (µmole of acetylcholine hydrolyzed per min per mL of plasma, which were close to the lower limit of the normal range of 2.00 µm/min/mL. The average for the group was 4.49 µm/min/mL. All

individuals of the group were apparently healthy men who were being monitored for occupational exposures to chlorpyrifos and other organophosphorous and carbamate pesticides. A typical gas chromatogram for the plasma analyses in these trials is shown in Figure 1.

TABLE 1. Results of the Analyses of *in vivo* Urine and Blood Plasma Samples for 3,5,6-Trichloropyridinol and Measured Plasma Cholinesterase Activities; 5 Individuals

| Individual ID | 3,5,6-TCP in urine, ppb | Plasma ChE, µm/min/mL | 3,5,6-TCP in plasma, ppb |
|---|---|---|---|
| T.L. | 27 | 2.80 | 1.7 |
| T.J. | 16 | 3.24 | 1.7 |
| S.W. | 29 | 3.50 | 1.4 |
| M.T. | 38 | 3.78 | 4.8 |
| M.D. | 12 | 2.53 | 1.7 |

Approximately 4 mos later, more freshly collected blood samples were analyzed. Table 2 lists the results of the continued analyses of blood plasma specimens for 3,5,6-TCP along with plasma cholinesterase activities. The 4 individuals listed in this table were exposed to chlorpyrifos and also belonged to the same work force as the individuals of Table 1. In these trials, Florisil columns were used in place of silica gel columns and diazoethane reagent was used for derivatizations instead of diazomethane reagent. Figure 2 shows quantitative gas chromatograms which are representative for these trials and includes an *in vitro* sample extract for recovery and a reference standard.

TABLE 2. Results of Continued Analyses of 3,5,6-Trichloropyridinol (3,5,6-TCP) in *in vivo* Blood Plasma Samples and Plasma Cholinesterase (ChE) Activities; 4 Individuals

| Individual ID | Plasma ChE m/min/mL | 3,5,6-TCP in plasma, ppb |
|---|---|---|
| V.R. | 4.87 | 7.4 |
| L.W. | 4.77 | 9.7 |
| T.L. | 3.39 | 9.6 |
| M.D. | 3.29 | 7.1 |

From the results of the analyses of *in vivo* samples, Tables 1 and 2, the levels of 3,5,6-TCP found in blood plasma are probably not high enough to show significant depression of cholinesterase activity. Perhaps quantities of chlorpyrifos which would metabolize to levels of 3,5,6-TCP in plasma greater than 10 ppb are required to show indication of enzyme depression. The low though normal plasma cholinesterase activities for the individuals in Table 1 are believed to be caused by exposures to organophosphates other than chlorpyrifos. Moreover, the quantities of 3,5,6-TCP

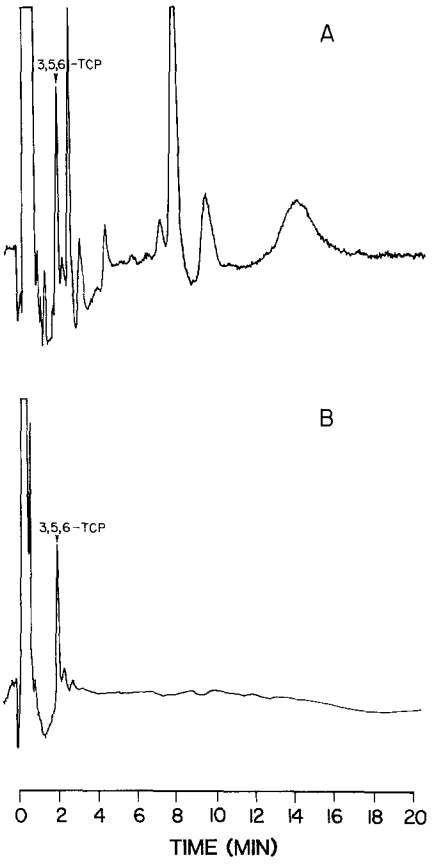


Figure 1. Gas chromatograms for the analysis of 3,5,6-TCP, in vivo blood plasma sample. SE-30/OV-210 Column, temperature, 175°C; derivatization with diazomethane reagent, silica gel cleanup. A. Typical gas chromatogram showing the detection of 3,5,6-TCP in blood plasma sample; injected plasma extract concentrated to 1 ml; 6 µl injection. B. Gas chromatogram of methylated 3,5,6-TCP standard; peak for methyl ether derivative, 60 pg.

detected in urine samples (Table 1) may be considered to be too low to indicate plasma enzyme depressions due to chlorpyrifos exposures. A large number of samples from individuals who are more intensely exposed to chlorpyrifos must be available for analyses to determine the levels of 3,5,6-TCP in blood plasma and urine which could be correlated with measurable depressions of plasma cholinesterase activity. The chronic effects associated with the levels of 3,5,6-TCP detected in plasma and urine in this work are not known. No 3,5,6-TCP was detected in Hyland control samples or in the sera of individuals without occupational exposure to dursban.

The simultaneous analyses of 3,5,6-TCP and PCP in blood plasma and urine specimens received in two appropriate pesticide incidents (suspected poisonings due to pesticide exposures) gave rise to the following results. For one of the cases, a commercial pesticide operator, the results were, blood plasma, trace (<1.0 ppb), 3,5,6-TCP and 390 ppb, PCP; urine, 20 and 190 ppb, same residues respectively. For the second case who was believed to have been exposed to pesticides at residence the results were: chlorpyrifos metabolite in either plasma or urine, in no detectable levels and 180 and 41 ppb levels of PCP in plasma and urine samples respectively. Figure 3 shows representative gas chromatograms for simultaneously determinations of 3,5,6-TCP and PCP blood plasma.

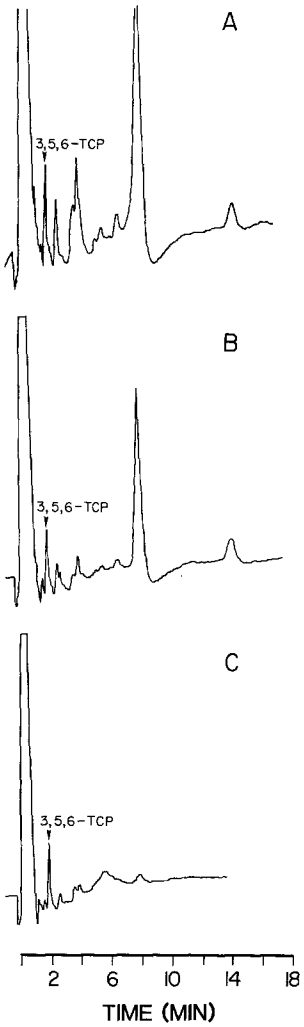


Figure 2

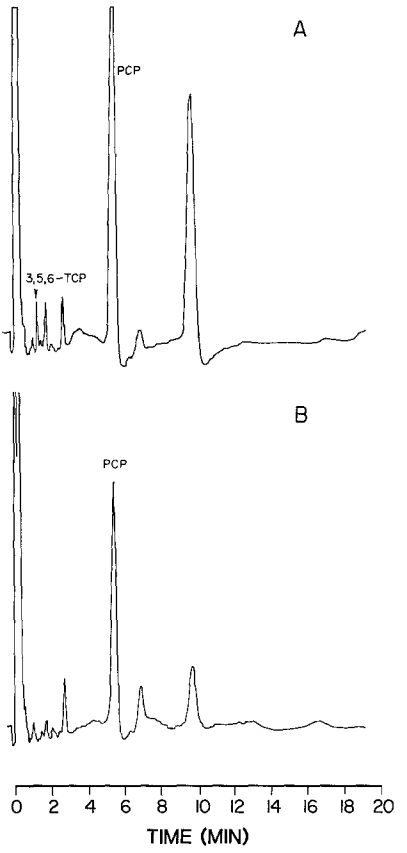


Figure 3

Figure 2. Gas chromatograms for continued analyses of *in vivo* blood plasma samples for 3,5,6-TCP. SE-30/OV-210 column, temperature, 178°C; derivatization with diazoethane reagent, Florisil column cleanup. A. Typical gas chromatogram for an *in vivo* blood plasma sample with detection of 3,5,6-TCP, ethyl ether derivative; volume of plasma extract for GLC, 3 mL; 5 µL injection. B. Gas chromatogram for an extract of *in vitro* blood plasma sample, blood plasma control sample fortified with 100 ng, 3,5,6-TCP standard; volume of plasma extract, 10 mL; 5 µL injection. C. Gas chromatogram for a reference standard, ethylated 3,5,6-TCP; peak for ethyl ether derivative, 50 pg. Standard was prepared by derivatizing 100 ng of 3,5,6-TCP extracted from fortified $H_2O$ blank; 10 mL, volume of reference standard; 5 µL injection.

Figure 3. Gas chromatograms for simultaneous determinations of 3,5,6-TCP and PCP in blood plasma; SE-30/OV-210 column, temperature, 183°C; derivatization with diazoethane reagent, Florisil column cleanup. A. Gas chromatogram for analysis of *in vitro* sample prepared by fortifying control blood plasma with 3,5,6-TCP and PCP standards, 100ng ea. B. Gas chromatogram for control blood plasma. Both plasma extracts injected (A & B) diluted to same volumes; 5 µL injections. Control blood plasma originally contained PCP.

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