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Analysis of 3,5,6-trichloropyridinol in human urine using negative-ion chemical ionization gas chromatography–mass spectrometry

M. J. Bartels* and P. E. Kastl

Departments of Health and Environmental Sciences and Analytical Sciences, The Dow Chemical Company, Midland, MI 48674 (USA)*

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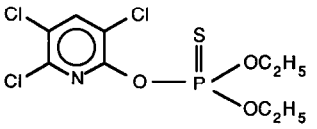
ABSTRACT

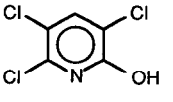
A sensitive gas chromatographic–negative-ion chemical ionization mass spectrometric (GC–NCI-MS) method was developed to measure levels of the chlorpyrifos metabolite 3,5,6-trichloropyridinol (3,5,6-TCP) in human urine. The metabolic 3,5,6-TCP was isolated from urine by acid hydrolysis of urine aliquots, followed by diethyl ether extraction. The residues of ether extraction were taken up in *o*-xylene and derivatized with N-(*tert.*-butyldimethylsilyl)-N-methyltrifluoroacetamide (overall five-fold concentration). The structural isomer 3,4,5-trichloropyridinol was used as an internal standard in this analysis. This method was found to be linear for the determination of 3,5,6-TCP over the range 0.8–792 ng/ml. The limit of detection for 3,5,6-TCP in human urine was estimated to be 0.5 ng/ml. Low levels of 3,5,6-TCP (1–18 ng/ml of urine) were identified in control human urine samples. Structural confirmation of the background 3,5,6-TCP was obtained via GC–NCIMS–MS analysis.

INTRODUCTION

Quantitation of 3,5,6-trichloropyridinol (3,5,6-TCP) in human urine is necessary to support biomonitoring studies with the organophosphate insecticide chlorpyrifos [O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl)phosphorotioate] (Fig. 1). Previous work with chlorpyrifos has shown that 3,5,6-TCP is a major urinary metabolite in man [1]. Existing methods for the analysis of 3,5,6-TCP call for acid hydrolysis of urine samples followed by organic extraction, derivatization, prep-column clean-up and gas chromatographic (GC) analysis [1–5]. The detection limit reported in the existing methods is 10 ng 3,5,6-TCP per ml urine [2,5]. The current work was performed to develop a simpler method for 3,5,6-TCP analysis using gas chromatography–mass spectrometry (GC–MS), which would have enhanced selectivity and sensitivity for the analysis of 3,5,6-TCP in human urine. Details on the choice of derivatization reagent and ionization conditions used are also discussed.



CHLORPYRIFOS



3,5,6-TRICHLORO-2-PYRIDINOL

Fig. 1. Structures for chlorpyrifos and 3,5,6-trichloropyridinol.

EXPERIMENTAL

Chemicals

3,5,6-TCP and 3,4,5-trichloropyridinol (3,4,5-TCP) were obtained from DowElanco (Midland, MI, USA). N-(*tert.*-Butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) was obtained from Aldrich (Milwaukee, WI, USA). All other compounds and solvents were reagent grade or better.

Sample extraction and derivatization

Control urine samples (5.0-ml aliquots of pooled male control urine) were fortified with 3,5,6-TCP (0–649 ng/ml) and/or 3,4,5-TCP (0.5–1.0 μg). These samples were then acidified (0.5 ml concentrated hydrochloric acid) and heated at 80°C for 2 h. Upon cooling to room temperature, the samples were extracted with diethyl ether (1 × 5 ml). The ether extracts were taken to dryness (nitrogen stream). The residues were then taken up in 1.0 ml *o*-xylene and derivatized with 100 μl MTBSTFA (60°C for 1 h).

Portions (100 ml) of pooled control urine were fortified with 0–169 ng 3,5,6-TCP per ml urine. These samples were then stored at 0°C for up to twelve weeks. Aliquots (5.0 ml) were then fortified with 3,4,5-TCP and worked up as above for determination of the stability of 3,5,6-TCP in human urine.

External standard solutions were also prepared, containing 3,5,6-TCP (0–649 ng/ml) and/or 3,4,5-TCP (0.5–1.0 μg). These samples were derivatized with MTBSTFA, as above, for use as quantitative standard solutions.

Gas chromatographic–mass spectrometric analysis

GC–MS analyses for the quantitation of the 3,5,6-TCP present in urine extracts were performed with a Finnigan TSQ-70 GC–MS system. Chromatographic conditions for these analyses were: a J&W DB-5 fused-silica capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA); helium carrier gas at 1–2 ml/min (0.2 min splitless injection); GC oven temperature program: 175°C for 5 min, 25°C min to 275°C; injector and capillary transfer line at 280°C. Mass spectral conditions [negative-ion chemical ionization (NCI), Q3 (quadrupole No. 3)] for quantitation were: ion source temperature, 150°C; ionizing current, 0.20 mA; electron energy, 70 eV; methane reagent gas source pressure, 0.5–1 Torr. Quantitative data for the 3,5,6-TCP and 3,4,5-TCP were obtained by selected-ion monitoring of the dichloropyridinol fragment ions (*m/z* 161 and 163; 0.125 s/scan).

Mass spectral conditions for daughter-ion analysis of background 3,5,6-TCP were as above with the following additions: argon collision cell gas pressure, 0.6 Torr; collision cell offset voltage, 20 eV. Analyses were performed both in the full daughter-scan mode (*m/z* 60–180 for daughters of *m/z* 161 and 163) and selected-ion mode (*m/z* 161 → 125 and *m/z* 163 → 127).

Mass spectral conditions for the positive ion chemical ionization (Q3-MS) analysis were the same as for NCI analysis above. Selected-ion monitoring was performed by monitoring the MH^+ ions (*m/z* 312 and 314; 0.125 s/scan).

Mass spectral conditions for the electron-impact (Q3-MS) analysis were the same as for NCI analysis above, with the exception of no methane reagent gas. Selected-ion monitoring was performed by monitoring the fragment ions corresponding to the loss of the *tert.*-butyl moiety (*m/z* 254 and 256; 0.125 s/scan).

Experimental calculations

The GC–MS peak areas for the derivatized trichloropyridinol isomers were obtained by integration of the dichloropyridinol fragment ion peak areas using the data system of the mass spectrometer. A linear least-squares standard curve was obtained form the 3,5,6-TCP/3,4,5-TCP peak-area ratios of the fortified control urine standards. Concentrations of 3,5,6-TCP in the fortified urine samples were then calculated using the experimentally derived standard curve.

RESULTS AND DISCUSSION

Sample preparation

The urine samples were first fortified with the 0.5–1 μg of the internal standard 3,4,5-TCP. This

compound was initially added as 10–25 μl of a methanol solution. However, fewer chromatographic interferences were observed if the compound was added in an acetonitrile solution.

The samples were then acid-hydrolyzed to liberate the urinary metabolite, 3,5,6-TCP. The acid hydrolysis used in this method involved acidification with concentrated hydrochloric acid acid–urine, 10:90, v/v) and heating at 80°C for 2 h. The previous method of Nolan *et al.* [1] involved hydrolysis with concentrated sulfuric acid (acid–urine, 20:80, v/v; 90°C for 1 h). Samples of human urine hydrolyzed via the current method were found to contain 104 ± 9% (n = 3) of the 3,5,6-TCP present in the same samples hydrolyzed via the method of Nolan *et al.* [1]. The samples were then extracted once with an equal volume (5 ml) of diethyl ether. The resulting extracts were taken to dryness with a nitrogen stream to afford a dark-colored residue. This residue was found to be soluble in the *o*-xylene and MTBSTFA upon heating.

Previous methods for 3,5,6-TCP analysis utilized trimethylsilyl derivatives of the pyridinol [1,4,5]. Although preliminary work with this derivative was successful, the *tert.*-butyldimethylsilyl derivative was finally chosen for the current method. This choice was made based on the expected increase in hydrolytic stability for the *tert.*-butyldimethylsilyl derivative over the trimethylsilyl analog [6]. The resulting derivatives were found to be stable for at least one month at room temperature (data not shown).

Chromatography

The GC–MS method used in this analysis was found to be sufficiently selective and sensitive for the determination of 3,5,6-TCP in human urine. A baseline chromatographic separation was obtained between 3,5,6-TCP and the 3,4,5-TCP isomer used as an internal standard in this analysis (Fig. 2). No interferences were observed to coelute with the 3,4,5-TCP derivative in the blank urine extracts. No cross-contamination was found between the 3,5,6-TCP and 3,4,5-TCP standard compounds used in this analysis.

A minor interference was observed in the

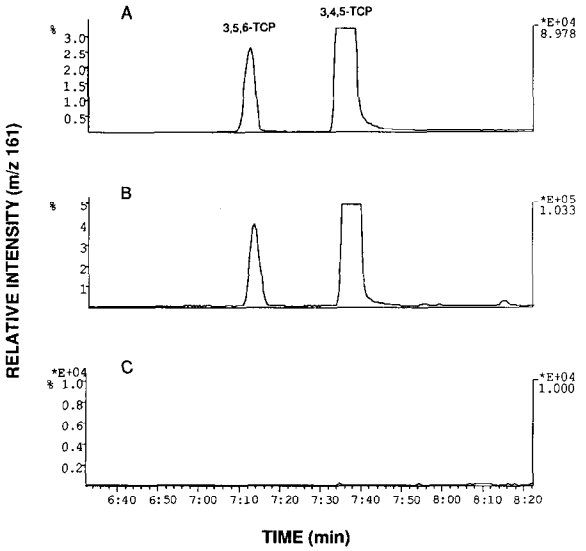


Fig. 2. Selected-ion chromatograms for the 3,5,6-TCP and 3,4,5-TCP isomers, as *tert.*-butyldimethylsilyl derivatives (NCI). (A) Solvent standard containing 24 ng 3,5,6-TCP and 1007 ng 3,4,5-TCP per ml; (B) extract of urine sample containing 7 ng 3,5,6-TCP and 202 ng 3,4,5-TCP per ml urine; (C) reagent blank standard.

pooled control urine sample that had the same retention time as the derivatized 3,5,6-TCP. This background material was present at a level equal to approximately 5 ng/ml 3,5,6-TCP. Attempts to avoid this interference by altering GC columns or derivatizing reagents were unsuccessful. Analysis of reagent blank samples verified that the interference did arise from the urine samples and not contaminated reagents or solvents. GC–MS–MS analysis of the control urine extracts indicated that the interference was 3,5,6-TCP (Fig. 3). Indicative fragment ions seen correspond to successive losses of HCl from derivatized 3,5,6-TCP. The M/M+2 isotope ratio for the 125/127 daughter ion, corresponding to loss of HCl from the m/z 161/163 ions, was also found to be consistent with a standard of 3,5,6-TCP (data not shown).

Detection

A number of ionization conditions were examined for the analysis of 3,5,6-TCP via GC–MS. As shown in Fig. 4, significant interferences were

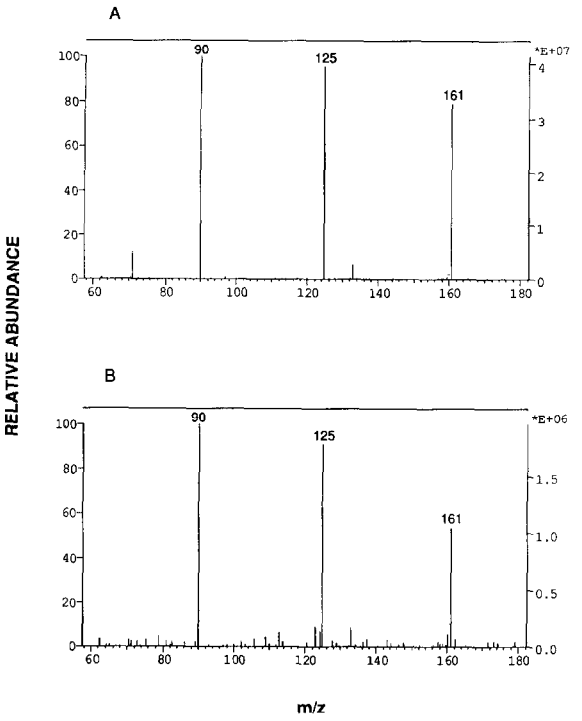


Fig. 3. NCI daughter ion spectra of 3,5,6-TCP as the *tert.*-butyldimethylsilyl derivative (daughters of *m/z* 161). (A) Solvent standard of 3,5,6-TCP (811 ng/ml); (B) urine extract containing 5 ng/ml 3,5,6-TCP.

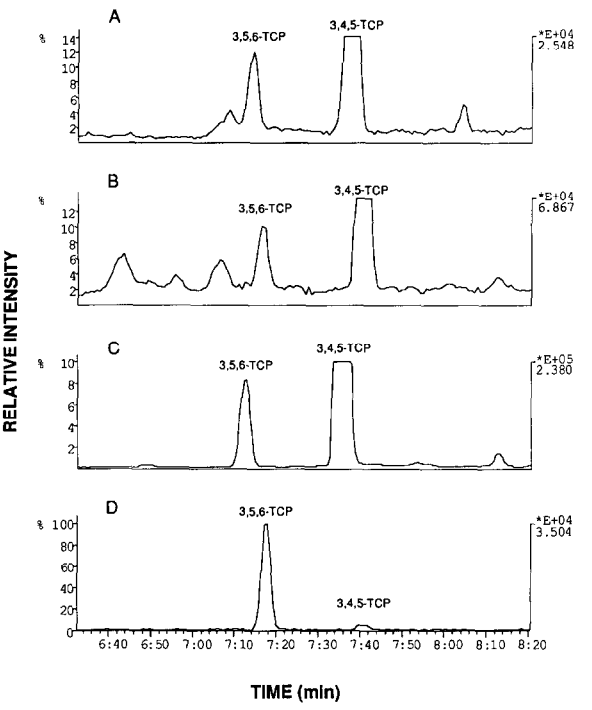


Fig. 4. Selected-ion chromatograms for a fortified urine sample containing 3,5,6-TCP (15 ng/ml) and 3,4,5-TCP (202 ng/ml), as *tert.*-butyldimethylsilyl derivatives. (A) Electron-impact ionization (M − *tert.*-butyl fragment, *m/z* 254); (B) positive-ion chemical ionization (MH^+, *m/z* 312); (C) NCI (dichloropyridinol fragment, *m/z* 161); (D) NCI for daughter ions of *m/z* 161 (chloropyridinol daughter, *m/z* 125).

observed for electron-impact and positive-ion chemical ionization analysis. NCI analysis had more chromatographic peaks in the retention time range of the two pyridinol derivatives than did the NCI-MS–MS chromatogram. However, the NCI-MS technique had approximately 10 and 100 times greater sensitivity for 3,5,6-TCP and 3,4,5-TCP, respectively, than did the MS–MS technique. Based on these results, NCI-MS ionization was chosen as the ionization technique for this method.

The dichloropyridinol fragment ions of 3,5,6-TCP and 3,4,5-TCP were monitored for quantitative analysis of the pyridinols. These were the only significant ions observed in the NCI spectra of these two derivatives (Fig. 5).

The mass spectral response was quite sensitive for the detection of the trichloropyridinol isomers. The chromatographic results for the control urine extracts indicate that this method is sufficient for the detection of 3,5,6-TCP in urine at concentrations down to 0.5 ng/ml (limit of detection = 3× signal-to-noise ratio). The mass spectral response for 3,5,6-TCP/3,4,5-TCP was linear over the concentration range of 0.8–792 ng/ml 3,5,6-TCP (r^2 for 0.8–238 ng/ml = 0.99992; r^2 for 0.8–792 ng/ml = 0.99956).

Quantitative analysis

The relative recovery for 3,5,6-TCP from fortified urine samples was found to range from 80.6 to 89.9% over the concentration range 4.1–411 ng/ml of urine (Table I). The absolute recoveries for 3,5,6-TCP were not calculated (due to mass spectral response variation) but were estimated to be 80–100%.

The day-to-day variability for this method was found to be quite low. Urine samples fortified

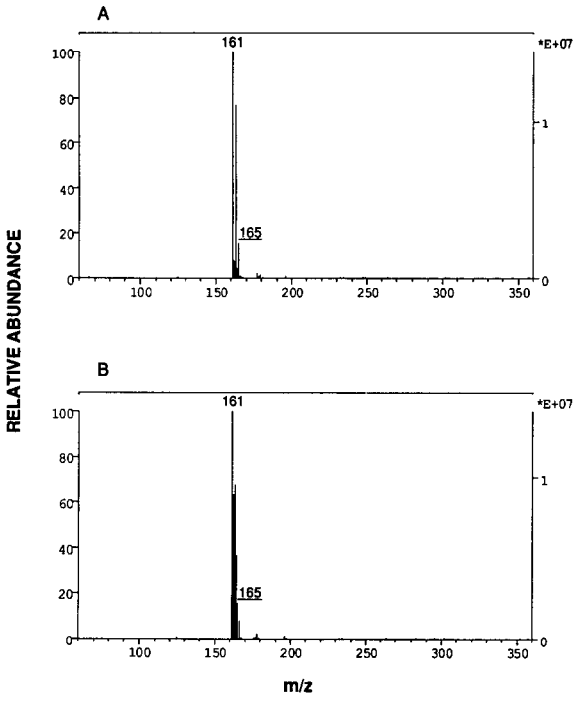


Fig. 5. NCI mass spectra. (A) 3,5,6-TCP as the *tert.*-butyldimethylsilyl derivative; (B) 3,4,5-TCP as the *tert.*-butyldimethylsilyl derivative.

with 16 and 79 ng/ml were analyzed via this method on subsequent days. The calculated concentrations for these samples were found to vary less than 3% between analyses (Table II).

3,5,6-TCP was found to be stable in fortified urine for up to twelve weeks. Table III shows the recovery of the analyte from urine samples stored at 0°C. The recovery of the pyridinol averaged

TABLE I

RECOVERY OF 3,5,6-TCP FROM FORTIFIED HUMAN URINE SAMPLES (n = 3)

Concentration (ng/ml)	Relative recovery (mean ± S.D.) (%)
4.1	80.6 ± 2.7
12.3	82.7 ± 1.8
41.1	85.2 ± 0.5
123.2	87.6 ± 0.3
411.0	89.9 ± 1.4

TABLE II

DAY-TO-DAY VARIABILITY FOR THE DETERMINATION OF 3,5,6-TCP IN HUMAN URINE

Concentration (ng/ml)	Recovery (%)		Day-to-day variability (%)
	Day 1	Day 2	
16	93.1	95.8	2.7
16	91.6	97.2	5.6
16	97.8	96.4	1.4
16	97.8	97.4	0.4
16	101.3	101.8	0.5
Mean			2.1
S.D.			2.2
79	96.3	96.9	0.6
79	90.8	93.3	2.5
79	93.1	96.2	3.1
79	91.6	93.8	2.2
79	91.3	92.7	1.4
Mean			2.0
S.D.			1.0

TABLE III

STABILITY OF 3,5,6-TCP IN HUMAN URINE (TWELVE WEEKS STORAGE AT 0°C)

Concentration added (ng/ml)	Recovery (%)
55	120
61	96
64	98
111	107
114	115
115	92
132	113
141	120
147	99
147	99
147	99
147	99
151	86
169	98
Mean	103
S.D.	10

103% for samples containing 55–169 ng/ml of urine.

CONCLUSIONS

A selective and sensitive GC–MS method has been developed for the determination of 3,5,6-TCP in human urine samples. The sample preparation method is simple, involving diethyl ether extraction of the acid-labile conjugates of 3,5,6-TCP, followed by derivatization with MTBSTFA. This procedure eliminates the prep-column clean-up previously required in other assays [1–4].

The samples are then analyzed via selected-ion GC–NCI-MS, using the structural isomer, 3,4,5-TCP, as a quantitative internal standard. This mass spectral method offers more selectivity than the existing GC methods. Monitoring several chlorine isotope ions for 3,5,6-TCP affords additional structural confirmation beyond retention time matching with a reference standard. In addition, further specificity for the determination of 3,5,6-TCP can be obtained via daughter-ion MS–MS analysis.

Finally, the limit of detection for this method (0.5 ng/ml) is significantly lower than those of previously reported methods. This was found to be useful in the determination of background levels of 3,5,6-TCP in human urine (above). It may also be useful in the support of future pharmacokinetic studies with compounds that yield low levels of 3,5,6-TCP as a urinary metabolite.

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