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Analysis of 3,5,6-trichloro-2-pyridinol in urine samples from the general population using gas chromatography–mass spectrometry after steam distillation and solid-phase extraction

Holger M. Koch, Jürgen Angerer*

Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine, University of Erlangen–Nuremberg, Schillerstr. 25, D-91054 Erlangen, Germany

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Abstract

We have developed a new method for the quantitative trace determination of 3,5,6-trichloro-2-pyridinol (TCPyr). TCPyr is a urinary metabolite specific to the organophosphorus pesticides chlorpyrifos and chlorpyrifos-methyl. After hydrolysis and separation of TCPyr from the urinary matrix using semi-automated steam distillation and solid-phase extraction on a new polystyrol–divinylbenzene copolymer (Isolute™ 101) the analyte was converted into its tert-butyldimethylsilyl derivative by *N*-tert-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA). Separation and quantitative analysis were carried out by capillary gas chromatography and mass selective detection in selected ion monitoring mode. 2,6-Dibromophenol (DBP) was used as the internal standard. The detection limit was 0.05 μg/l; the limit of quantification was 0.1 μg/l urine. The relative standard deviation of the within-series imprecision was 4.2% at a concentration of 3.5 μg/l. The relative recovery was 104%. The new method was used to analyse the urine samples of 12 persons from the general population without known exposure to the above-mentioned pesticides. TCPyr concentrations between 0.27 and 6.6 μg/l urine were detected in all urine samples. This indicates that there is a baseline excretion of TCPyr in the general population. Four urine samples collected from workers who had applied chlorpyrifos were also analysed. In these samples TCPyr was found in concentrations from 4.7 to 7.9 μg/l.

Keywords: 3,5,6-Trichloro-2-pyridinol; Chlorpyrifos; Chlorpyrifos-methyl

1. Introduction

Chlorpyrifos [*O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridinyl)phosphorothioate] and chlorpyrifos-methyl [*O*,*O*-dimethyl-*O*-(3,5,6-trichloro-2-pyridinyl)phosphorothioate] are broad-spectrum organophosphate insecticides. They are widely used in agriculture and for urban pest control [1,2]. As a result of their widespread use, exposure of the general population can occur. 3,5,6-Trichloro-2-pyridinol (TCPyr) is the specific urinary metabolite of chlorpyrifos and chlorpyrifos-methyl, and is excreted in the urine mainly as the glucuronide [3,4]. The human metabolic pathway of chlorpyrifos and chlorpyrifos-methyl is shown in Fig. 1. About 70% of orally administered chlorpyrifos is excreted in urine as TCPyr with a half-time of 27 h [3]. Exposure of pesticide sprayers and the

*Corresponding author. Tel.: +49-9131-852-2374; fax: +49-9131-856-126.

E-mail address: juergen.angerer@ipasum.imed.uni-erlangen.de (J. Angerer).

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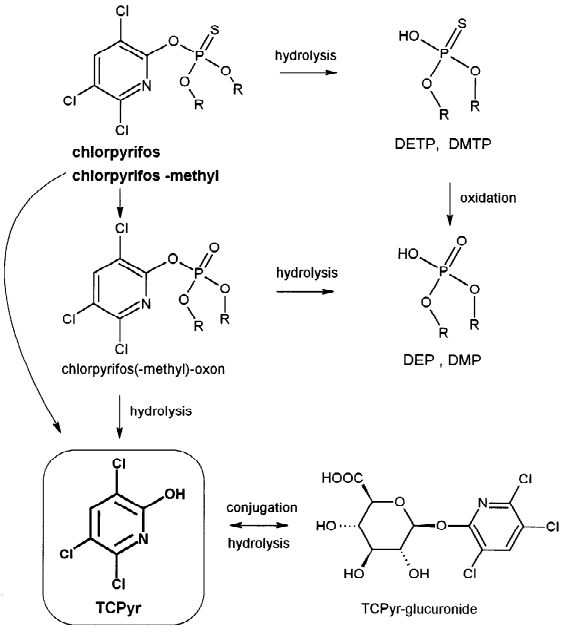

Fig. 1. Metabolic pathway of chlorpyrifos and chlorpyrifos-methyl (TCPyr, 3,5,6-trichloro-2-pyridinol; DETP, diethylthiophosphate; DMTP, dimethylthiophosphate; DEP, diethylphosphate; DMP, dimethylphosphate; R = $-CH_3$ for chlorpyrifos-methyl, DMP and DMTP, R = $-CH_2CH_3$ for chlorpyrifos, DEP and DETP).

general population to the organophosphorus insecticides mentioned above can be assessed by quantifying this metabolite [5,6]. The quantification limit of existing methods for the analysis of TCPyr was 1 μg/l [7], with detection limits between 0.2 and 1.2 μg/l urine [7–10]. The aim of this study was to develop a practical and still more sensitive method suitable for investigating the urinary excretion of TCPyr in the general population.

2. Experimental

2.1. Chemicals

3,5,6-Trichloro-2-pyridinol (TCPyr, 10 ng/μl in acetonitrile, certified) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). 2,6-Dibromophenol (DBP, 99%) was obtained from Aldrich (Steinheim, Germany). *N*-tert-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA, >98%), 25% analytical grade hydrochloric acid, acetonitrile SeccoSolv™ (max. 0.005% H_2O), toluene SupraSolv™ (for organic trace analysis), analytical grade methanol, analytical grade sodium sulphate and highly purified water were obtained from Merck (Darmstadt, Germany). Isolute™ 101 (100 mg/3 ml) SPE columns were purchased from IST (Grenzach-Wyhlen, Germany).

2.2. Solution of the internal standard

The stock solution for the internal standard (I.S.) was prepared by dissolving 25 mg 2,6-dibromophenol (DBP) in 50 ml acetonitrile (500 mg/l). Five ml of this stock solution were placed in a 50-ml glass volumetric flask and diluted to the mark with acetonitrile (50 mg/l). Two ml of this intermediate standard solution were then transferred to a 100-ml glass volumetric flask and diluted to the mark with water. The resulting DBP standard solution (1 mg/l) was used for spiking urine samples during sample preparation (see Section 2.3).

2.3. Sample preparation

Frozen urine samples were allowed to equilibrate to room temperature. The samples were vortexed and aliquots of 10 ml were then transferred to 25-ml glass screw-cap vials. One ml hydrochloric acid (25%) was added to the urine sample and the mixture was spiked with 250 μl of the I.S. solution. The samples were heated for 1.5 h at 80°C in a drying oven. After hydrolysis each sample underwent steam distillation in a Gerhard Vapodest Vap 20 apparatus (vapour pressure intensity 50%, distillation time 250 s). Volumes of 49 ml of the distillate were collected in 50-ml volumetric flasks containing 1 ml of 25% hydrochloric acid. To avoid contamination of the apparatus, each distillation was followed by an additional distillation step using pure water (pressure intensity 50% and distillation time 60 s). The distillate was then passed through a preconditioned (2 ml methanol, 2 ml ultra pure water and 2 ml 0.1 mol/l hydrochloric acid) polystyrol–divinylbenzene copolymer phase (Isolute® 101) and washed with 5 ml 0.1 mol/l hydrochloric acid and 4 ml highly purified water. The column was dried by centrifugation for 5 min at 7000 *g* and by applying vacuum on the solid-phase for 45 min. The analytes were then

desorbed with four portions of acetonitrile, each of 0.25 ml. The solvent was allowed to equilibrate with the solid-phase for 2 min prior to elution. Seventy-five μl of toluene and 50 mg of sodium sulphate were added to the eluate. The eluate was shaken for 5 min and then centrifuged. The solvent mixture was then transferred to a 1.8-ml glass vial, the sodium sulphate was washed with 200 μl of acetonitrile and the washing solution was added to the vial. The combined phases were concentrated to a volume of 150 μl using a vacuum centrifuge. For derivatisation 15 μl of MTBSTFA were added, the mixture was transferred to a 250-μl micro insert and put into the vial. The vial was capped and heated for 45 min at 70°C in an oven. One μl of the sample was then injected into the GC–MS system for quantitative analysis.

Urinary creatinine concentrations were determined according to Larsen [11].

2.4. Calibration procedure and quality control

A stock solution was prepared containing 10 mg TCPyr per litre acetonitrile.

From this standard stock solution five calibration standards were prepared by diluting the solution with pooled urine or water. The solutions were spiked with concentrations of TCPyr in the range from 0.13 to 15 μg/l for the calibration standards prepared with urine and 0.23 to 22.5 μg/l for those prepared with water. Unspiked samples of pooled urine and water were used as blanks. The standards were processed as described in Section 2.3. Linear calibration curves were obtained by plotting the quotients of the peak areas of TCPyr and the I.S. as a function of the concentrations used. Both water and pooled urine standards produced the same linear slope of regression. The coefficients of correlation were higher than 0.99. These graphs were used to ascertain the unknown concentrations of TCPyr in urine samples.

As there was no quality control material commercially available it had to be prepared in the laboratory. We spiked pooled urine from laboratory personnel with 2.5 μg TCPyr per litre after freezing and filtering the urine. Due to the background levels of TCPyr this resulted in a concentration of ~3.5 μg/l. The pool was divided into aliquots and stored at −18°C. For quality assurance one control sample was included in each analytical series.

Recovery was calculated by analysing the spiked and unspiked pooled urine as described and comparing the results with the spiked amount of TCPyr.

Within-series imprecision was determined by analysing the quality control urine eight times in a row. Furthermore, imprecision was determined using five individual urine samples from different people not previously exposed to chlorpyrifos. Spiked specimens (spiked concentration 1.8 μg/l) and the same specimens without the addition of TCPyr were analysed. The TCPyr content of the unspiked specimens was in the range between 0.44 and 8.2 μg/l. Between-day imprecision was determined by analysing the quality control sample on 7 different days.

The efficiency of extraction by steam distillation and SPE up to the derivatisation step was calculated as follows. A solution of TCPyr in 150 μl toluene–acetonitrile 1:1 (v/v) was derivatised directly and analysed by GC–MS. The results were compared with those obtained from a processed urine sample spiked with the same amount of TCPyr (including the background level of 0.98 μg/l).

2.5. GC–MS analysis

2.5.1. Gas chromatography

Analysis was carried out with a Hewlett-Packard HP 5890 Series II plus gas chromatograph fitted with a Hewlett-Packard HP 7673 autosampler and a split/splitless injector operating in splitless mode. The operating temperature of the injector was 280°C. Chromatographic separation was performed using a HP35 capillary column (crosslinked 35% diphenyl-dimethylpolysiloxane, 60 m×0.25 mm internal diameter (I.D.), 0.25 μm film thickness) purchased from GGA (Moers, Germany). The conditions were as follows: helium 5.0 with an initial inlet pressure of 1.45 bar, constant for 5 min, raised at a rate of 0.035 bar/min to 2.0 bar and raised further at a rate of 0.07 bar/min to a final pressure of 2.25 bar. The initial column temperature was 90°C with an initial time of 1 min; it was then raised at a rate of 25°C/min to 125°C and kept isothermal for 1 min. Afterwards the temperature was raised at a rate of 6°C/min to 230°C and held for 2 min. Finally it was raised at a rate of

30°C/min to 280°C and this temperature was held for 12 min. The injection volume was 1 μl.

The retention times for the derivatised analyte and I.S. observed under the described conditions were 18.5 min (TCPyr) and 21.5 min (I.S.), respectively.

2.5.2. Mass spectrometry

A Hewlett-Packard HP MSD 5972 mass spectrometer fitted with a quadrupole mass filter was used in electron impact (EI) mode. EI mass spectra were obtained at an ionisation energy level of 70 eV and the electron multiplier voltage was 2300 V. The MSD transfer line temperature was maintained at 290°C. For the quantitative analysis of TCPyr, selected ion monitoring was used and the tert-butyldimethylsilyl derivatives of TCPyr and the I.S. were registered using three masses. The fragment ions monitored correspond to the derivatised molecules after the loss of the tert-butyl moiety (ions for TCPyr *m/z* 254, 256, and 258) (Fig. 2). The intensities according to the isotope ratios M/M+2 for molecules containing three chloride atoms were checked. The ion *m/z* 256 was used for quantification. The ions of I.S. were *m/z* 307, 309, and 311 (Fig. 2).

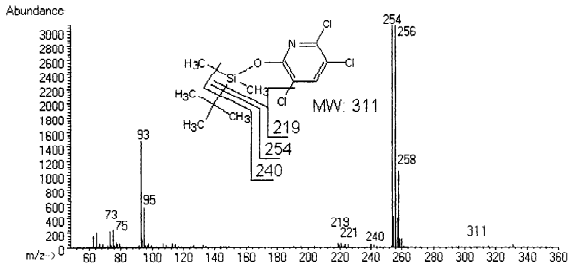

Fig. 3. Mass spectrum of derivatised 3,5,6-trichloro-2-pyridinol (TCPyr-butyldimethylsilyl) *m/z* 254 (+2,+4): M^+-butyl, *m/z* 240 (+2,+4): M^+-butyl-methyl, *m/z* 219 (+2,+4): M^+-butyl-Cl.

Their intensities were checked according to the isotope ratios M/M+2 for molecules containing two bromide atoms. The ion *m/z* 309 was used for quantification. The mass-spectrum of TCPyr-tert-butyldimethylsilyl is shown in Fig. 3.

2.6. Study subjects

We investigated 12 spot urine samples from persons not occupationally exposed to any organophosphorus pesticide. In addition, 24-h urine speci-

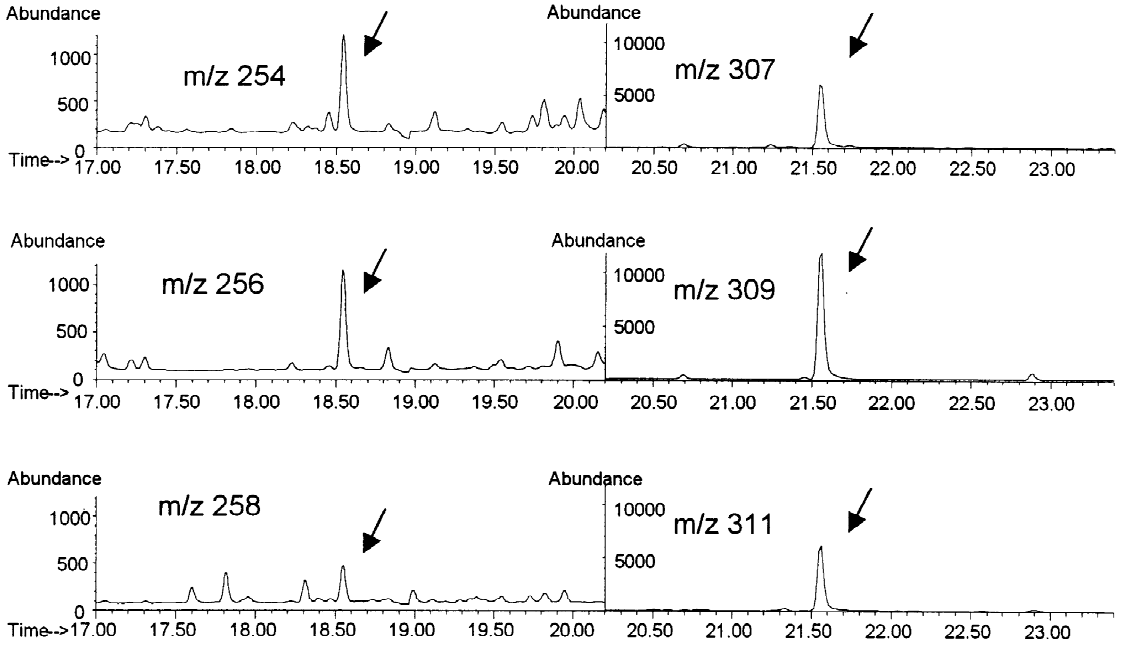

Fig. 2. SIM chromatogram of a processed urine sample from a person not exposed to organophosporus pesticides (TCPyr: 2.42 μg/l, creatinine: 1.09 g/l). The concentration of I.S. was 25 μg/l. The quantifier ion registered for TCPyr was *m/z* 256, for I.S. *m/z* 309.

mens from four agricultural workers were analysed after they had applied the insecticide formulations CD forte and Empire 20 (both containing chlorpyrifos as active ingredient) for 0.25 to 0.5 h. Protective clothing was worn by each person. All urine samples were stored at −18°C until they were processed. Creatinine levels were found to be in the range from 0.47 to 2.9 g/l.

3. Results and discussion

In order to achieve a detection limit well below 100 ng/l without the method being too time-consuming, the apparatus too complicated and the chemicals too expensive, we combined different clean up techniques with mass spectrometry for detection. The techniques used were known to be very efficient for the analysis of environmental phenols. Steam distillation has proved to be very effective for separating phenols from the biological matrix [12]. Furthermore we chose a new SPE material (Isolute™ 101), a polystyrene-based polymer, with ideal characteristics for fast and effective extraction and pre-concentration of non-polar organic substances in aqueous solution.

3.1. Clean up and derivatisation

Steam distillation is a proven tool for separating phenols and chlorophenols from the complex urine matrix [12,13]. A further advantage of steam distillation is that the partly sulfate-bound and glucuronide-bound phenolic substances are hydrolysed by mineral acids simultaneously with steam distillation. The steam distillation procedure was tested for TCPyr and proved to be suitable.

Subsequent solid-phase extraction is ideal as a further fast and effective clean-up step. The Isolute™ 101 columns can be loaded at flow-rates of up to 10 ml/min. Unlike C_{18} material, the 101 material does not require preconditioning, is allowed to run dry during the process and can be completely dried afterwards, which is a prerequisite for the silylating procedure with MTBSTFA. The elution volume can be kept at 1 ml, which facilitates the final concentration step.

MTBSTFA has been used as the derivatisation reagent for TCPyr before [8,10] and has been found to produce good and reproducible results in GC–MS analysis.

In contrast to previous methods, which used electrochemical detectors [10] or MS in negative chemical ionization mode [8], we used electron-impact ionisation mode. This was found to produce satisfactory results and a detection limit below 0.1 μg/l.

3.2. GC–MS analysis

The tert-butyldimethylsilyl derivatives of TCPyr and the I.S. were registered each with three masses. All three fragment ions were still detectable at the limit of detection. The mass ratio of m/z 256 (analyte) and m/z 309 (I.S.) was used for quantification. For further peak verification we calculated the mass ratios of the registered masses within each molecule. A threefold standard deviation of the mass ratios calculated from the results of the processed aqueous calibration standards was regarded as acceptable.

3.3. Calibration graphs

The calibration graphs were linear for the range investigated. Aqueous calibration standards (0.23 to 22.5 μg/l) yielded correlation coefficients higher than 0.99. No TCPyr was detectable in the water blank. No pooled urine free of TCPyr was obtainable. The urine used was found to contain TCPyr in a concentration of 0.98 μg/l. Thus the urinary calibration standards resulted in calibration curves in the range from 1.11 to 15.98 μg/l. The calibration curves intersected in the positive range. The associated correlation coefficients were higher than 0.99. Calibration curves generated from both urinary and aqueous standard solutions produced the same slope of regression.

3.4. Reliability of the method

3.4.1. Precision and accuracy

In order to assess the within-series imprecision, pooled urine with a creatinine content of 1.1 g/l was spiked with TCPyr and analysed eight times. The relative standard deviation was 4.2%. With a spiked

Table 1
Precision and accuracy data (accuracy given as the average relative recovery)

	Within-series		Between-day
	Pooled urine $n=8$	Individual urine $n=5$	Pooled urine $n=7$
Spiked concentration (μg/l)	2.5	1.8	2.5
Imprecision (%)	4.2	7.1	2.0
Accuracy (%)	104	94	102

amount of 2.5 μg/l and a background level equal to 0.98 μg/l, the relative recovery was calculated to be 104%.

The imprecision was also calculated based on five individual urine samples reflecting a broad range of creatinine content (creatinine: 0.79–4.7 g/l). The relative standard deviation was 7.1%. The relative recovery at the given spiked concentration (1.8 μg/l) was 94%. The results were not found to depend on the level of creatinine and the different matrices did not affect recovery.

The relative standard deviation of the between-day imprecision was determined to be 2.0% and the relative recovery 102%. The precision and accuracy data are presented in Table 1.

Losses during the whole analytical procedure could not be determined because no tert-butyldimethylsilyl derivatives of TCPyr were available as standards. The losses up to the derivatisation step due to steam distillation and liquid solid extraction were calculated to be between 45% and 49% in urine.

3.4.2. Detection limit

TCPyr could not be detected in purified water processed as described in Section 2.3. The limit of detection, defined as a signal-to-noise ratio of three for the registered ion m/z 256, was 0.05 μg/l in water. At this concentration all three registered mass fragments of TCPyr could be detected. The LOD in urine was estimated to be 0.05 μg/l, the same as in water. The limit of quantification, defined as a signal-to-noise ratio of six, was estimated to be 0.1 μg/l.

3.4.3. Sources of error

The dryness of the final extract prior to derivatisation with MTBSTFA is crucial. Therefore, the drying step was retained despite the fact that a parallel sample clean-up step without the addition of Na_2SO_4 after elution from the solid-phase yielded the same results.

3.5. Results of biological monitoring

The results of the biomonitoring of the 12 persons not occupationally exposed to pesticides and the four occupationally exposed persons are summarised in Table 2. All urine samples were found to contain TCPyr. Fig. 2 shows the chromatogram of a processed spot urine sample from a person not occupationally exposed. The results suggest the exposure of the general population to chlorpyrifos is not negligible. This exposure might be caused by dietary intake and the household application of pesticides [14,15]. Two recent studies also showed there to be a

Table 2
Results of biological monitoring: 3,5,6-trichloro-2-pyridinol in human urine

	General population ($n=12$)		Exposed persons ($n=4$)	
	Concentration (μg/l)	Concentration (μg/g creatinine)	Concentration (μg/l)	Concentration (μg/g creatinine)
Average	2.8	2.6	5.6	3.7
Median	2.2	1.3	5.3	3.6
Range	0.27–6.6	0.16–10.5	4.7–7.9	2.3–4.7
95th percentile	6.4	8.0	–	–

baseline concentration for the excretion of TCPyr. In an American study [9], which investigated the general population using an analytical procedure with a detection limit of 1 μg/l, TCPyr was detected in 82% of the urine samples. The median value was 3.0 μg/l and the 95th percentile 13 μg/l. Another study, conducted in Italy, produced similar results [10].

Whether the intake of chlorpyrifos, chlorpyrifos-methyl or the metabolite TCPyr itself causes the body burden remains speculative.

The TCPyr values of the pesticide users tended to be higher than the TCPyr values of the persons not occupationally exposed but the difference was not significant. This can be partly explained by the fact that the level of exposure to chlorpyrifos of the users was relatively low. The duration of application was short (0.25 to 0.5 h) and protective suits and gloves were worn.

4. Conclusions

We have developed a reliable analytical procedure for the determination of TCPyr in human urine samples. TCPyr is a metabolite specific to chlorpyrifos and chlorpyrifos-methyl. Within-day and between-day imprecision is very good, even at low concentrations, as is the relative recovery. The clean-up procedures are very efficient so that there is no effect of the matrix on the analytical results. As a consequence the analytical background interference is very low yielding 0.05 μg/l as a limit of detection. The method is therefore perfectly suited for determining low levels of TCPyr. This is necessary in the field of environmental medicine for assessing the body burden of the general population. Using this method we were able for the first time to detect TCPyr in every urine sample of the general population. This proved that there is background exposure to these pesticides in the general population, probably resulting from dietary intake.

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