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Biological Monitoring of Exposure to Chlorpyrifos by High Performance Liquid Chromatography

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Chlorpyrifos (O,O-diethyl O-[3,5,6-trichloro-2-pyridyl])phosphorothioate is the active ingredient in the formulated products, Dursban and Lorsban insecticides. Chlorpyrifos (CPF) is currently registered in the United States for crops and livestock, ornamental plants, turf, household pests, and mosquito control. The principal manifestations of CPF toxicity in mammals are due to the inhibition of cholinesterase (ChE) (Sultatos et al. 1982a, Hodgson et al. 1986, Costa et al. 1990, Pope et al. 1991). Plasma ChE (the pseudo form) and red blood cell (RBC) ChE are depressed by smaller doses of CPF than inhibit brain ChE or produce signs of toxicity (McCollister et al. 1974).

Due to its heavy use in household pest-control, the US National Academy of Sciences had recommended in 1982, an interim guideline level of 10 µg/m3 for continuous exposure to CPF (NAS 1982). In the working environment, the time-weighted-average Threshold Limit Value (TLV) has been set at 0.2 mg/m3 for an 8- to 12-hr work day or 40-hr work week by the American Conference of Governmental Industrial Hygienists (ACGIH 1994). The acceptable daily intake (ADI), established by the Joint FAO/WHO Meeting on Pesticide Residues, is 0.01 mg/kg body wt. (JMPR 1993).

Conventionally, an inhibition of plasma or RBC ChE and/or a measurement of urinary diethyl (thio)phosphate have been used for the evaluation of an individual's exposure to CPF (Lores et al. 1978, Nolan et al. 1984, Hodgson et al. 1986, Sunaga et al. 1989, Vasilic et al. 1992, Fillmore and Lessenger 1993). Both approaches are not CPF-specific. An analysis of blood or urinary 3,5,6-trichloro-2-pyridinol (TCP) was used to confirm a CPF exposure (Lores et al.

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1978). Recently, several reports have tried to use the analysis of urinary TCP as a means of evaluation of an exposure to CPF (Fenske and Elkner 1990, Gibbons et al. 1993).

The analysis of TCP in biological fluids has always been tedious and time consuming. However, it showed great sensitivity. It mainly involves (i) an acid hydrolysis to deconjugate the glucuronide conjugation, (ii) derivatization with N,O-bis-(trimethylsilyl) acetamide to mask the hydroxyl functional group, (iii) extraction with an organic solvent, (iv) evaporation to dryness, (v) re-dissolution into a smaller volume of a proper solvent, and (vi) analysis with gas chromatography (GC) coupled with electron-capture detector (Guinivan et al. 1982, Nolan et al. 1984) or mass spectrometer (Allender and Keegan 1991, Bartels and Kastl 1992) or with high performance liquid chromatography (HPLC) (Sultatos et al. 1982b, Sunaga et al. 1989).

In this report, a modified HPLC method with a much simplified sample preparation scheme was characterized and evaluated using a rat study of low-dose exposure to CPF. The exposure levels evaluated ranged from less than 1% LD_{50} to 6%. The oral LD_{50} of CPF in rats is 145 mg/kg (Merck Index 1983). The method was further verified for its feasibility by the analysis of human urines collected from a small group of the professional pest-control sprayers.

MATERIALS AND METHODS

Male Wistar rats, 5 per dose group, were given an oral dose of 5 mL/kg of Mazola corn oil in which was dissolved CPF (99%, provided by DowElanco, Taiwan) at 0, 1.1, 2.2, 4.4, and 8.7 mg/kg. Prior to the intubation of CPF, rats were housed one per cage in metabolic cages (Nalge, Rochester, NY) and provided with Purina rat chow and tap water *ad libitum*. The light cycle of the animal room was kept from 6 am to 6 pm. 24-Hour urine samples from each rat were collected separately for one-day before and one-day after the exposure. Samples were collected in polypropylene tubes that were kept at room temperature and in the dark. Samples were then stored at -30°C until analyzed. The volume of each urine sample was recorded (Chang and Lin 1995).

Urinary creatinine was determined by the Jaffe reaction. The sample preparation for the analysis of urinary TCP included (i) quick thaw

and centrifugation (10,000 x g for 20 min at 4°C) to remove particulate matter, (ii) precipitation at 4°C of the macromolecules with perchloric acid (final concentration of 5%) and a brief centrifugation to remove the precipitate, (iii) acid hydrolysis of the cleared supernatant at 85°C for 2 hr to release TCP from its glucuronide and other conjugates, and (iv) addition of the internal standard (IS), o-nitrophenol, prior to HPLC analysis (Chang and Lin 1995). The TCP used (99.7% purity) to construct the standard curve was kindly provided by the Chemistry Laboratory of DowElanco (Indianapolis, IN).

An Eldex 9600 HPLC coupled with an Alcott 738 autosampler and a Linear UVIS 205 at 300 nm was used. The column (15 cm x 4.6 mm i.d.) was packed with a reversed-phase packing, 5 pm Chemcosorb ODS (Chemco Co., Osaka, Japan). The mobile phase consisted of a mixture of acetonitrile and water containing 1% acetic acid (44:56 by volume). The flow rate was 0.5 mL/min. Injection volume was 20 µL. A 1 to 25 dilution of urine samples, with mobile phase, was made for the rat study. A working standard curve, which consisted of 6 spiked urine samples, ranging from 0.22 to 7.11 µg TCP per mL urine (Table 1, B), was included in every batch of a determination. The percent relative error of each spiked urine standard was kept at no greater than ± 10%.

A recovery study was performed with a pooled control urine. As presented in Table 1, two series of parallel spiked standards were prepared in mobile phase as well as the pooled control urine and analyzed simultaneously. A linear regression equation was computed separately for each series. The recovery was calculated as the ratio of the slope (Chang and Lin 1995).

The feasibility of the method to be used in the biological monitoring of an occupational exposure to CPF was verified with an analysis of urine samples collected from 16 professional pest-control sprayers and 2 office workers. Individual urine sample was collected within 3 hours post-shift. No dilution of human urine sample was involved in the analysis of TCP. Five spiked standards, ranging from 0.2 to 4.0 µg/mL, were used in the human study.

RESULTS AND DISCUSSION

As shown in Table 1, the method demonstrated a good linearity ($r^2 >$ 0.99), good accuracy (all of the percent relative errors < ±10%), and good reproducibility. The interday variation, CV, was < 10% at all

levels except the lowest spiked mobile phase standard, which was ~12%. The percent recovery was 102 ± 15%, n = 6. Based on another series of 5 spiked mobile phase standards (without internal standard), ranging from 0.11 to 1.78 µg/mL, a detection limit of 2.2 ng per 20 µL injection volume was obtained.

Table 1-A. Recovery and linearity studies of the 3,5,6-trichloro-2-pyridinol (TCP) spiked control rat urine

	TCP [µg/mL]	mobile phase std. $\overline{\text{pk ht ratio}}^a \pm$ SD	CV, % n = 6	% rel. error
A.	0.22	0.3006 ± 0.0353	11.7	-4.0
	0.44	0.5580 ± 0.0180	3.2	-3.2
	0.89	1.1086 ± 0.0443	4.0	-0.5
	1.78	2.2076 ± 0.0824	3.7	+1.2
	3.56	4.3359 ± 0.1467	3.4	+0.5
	7.11	8.5544 ± 0.3552	4.2	-1.3
	TCP [µg/mL]	spiked urine std. $\overline{\text{pk ht ratio}}^a \pm$ SD	CV, % n = 6	% rel. error
B.	0.22	0.2706 ± 0.0164	6.1	+9.2
	0.44	0.5591 ± 0.0447	8.0	+8.2
	0.89	1.0520 ± 0.0686	6.5	-1.2
	1.78	2.1879 ± 0.1937	8.8	+1.6
	3.56	4.1719 ± 0.3935	9.4	-3.7
	7.11	8.7430 ± 0.5948	6.8	+0.8

a. $\overline{\text{Pk ht ratio}}$ stands for the mean peak height ratio, the Int. Std. is o-nitrophenol at 0.70 µg/mL. The linear regression equation is y = 1.199 x + 0.047 (A) and y = 1.223 x - 0.023 (B), $r^2 > 0.99$ for both.

The results of the detection limit study had the same degree of precision and accuracy as those shown in Table 1. The detection limit was not as low as those accomplished by the methods of GC/ECD (Nolan et al. 1984) and GC/MS (Bartels and Kastll 1992). However, it was about 5 to 20 times more sensitive than the HPLC study reported by Sultatos et al. (1982b), of which an ethyl acetate extraction and evaporation were also included in the sample preparation.

The relationships between the administered dose vs. urinary TCP,

either expressed as µg/mL or µg/mg creatinine are presented in Table 2. A linear dose-response relationship was clearly seen for the urinary TCP both in µg/mL and µg/mg creatinine. The urinary TCP was determined after an acid hydrolysis (perchloric acid at a final concentration of 0.5 N, 85°C for 2 hr). In a pilot study, we have found that with the acid hydrolysis, used to deconjugate the phenolic metabolite, a significant increase in urinary TCP was observed for all dose levels evaluated. According to Bakke et al. (1976) and Nolan et al. (1984), the major conjugation of TCP is to β-glucuronic acid both in rats and humans.

Table 2. Dose-response relationship of low dose chlorpyrifos vs. acid-released urinary 3,5,6-trichloro-2-pyridinol (TCP) in rats[a]

	mean ± SD, n = 5	
Dose (mg/kg)	µg TCP/mL	µg TCP/mg creatinine
0	0.0 ± 0.0	0.0 ± 0.0
1.1	6.0 ± 4.1	5.1 ± 3.9
2.2	8.7 ± 5.5	9.6 ± 9.3
4.4	23.1 ± 10.3	19.0 ± 9.1
8.7	52.7± 23.9	41.2 ± 22.3
r^2	0.99	1.00

Wistar rats, 5 per dose group, were gavaged with a single dose of chlorpyrifos in corn oil. From each rat, 24-hour urine samples were collected at room temperature and protected from light.
a. With 0.5 N perchloric acid at 85°C for 2 hr.

The human urine samples, collected from a small group of professional pest-control workers, were analyzed by the same method with a calibration curve ranging from 0.2 to 4.0 µg TCP per mL. The standards were prepared with a pooled control human urine. A linearity of $r^2 > 0.99$ was obtained for the human standard curve, with all of the percent relative errors < ± 10% except the lowest standard, which had a percent relative error of +16.5%.

Summarized in Table 3 are data of the analysis of 16 pest-control sprayers who handled CPF on the day of the sample collection. Urine samples were collected at 2 to 3 hours immediately after

Table 3. The use of chlorpyrifos (CPF) by the professional pest-control workers and their urinary concentration of 3,5,6-trichloro 2-pyridinol (TCP)

ID	spray time (min)	vol used (L)	CPF used (kg)	urine TCP (μg/mL)	urine TCP (μg/mg creatinine)
A20	170	3.0	1.22	0.79	0.31
A21	170	1.0	0.41	0.40	0.16
A22	170	3.0	1.22	0.25	0.25
A23[a]	170	1.0	0.41	0.60	0.18
A25	170	3.0	1.22	(0.19)	(0.18)
B01	60	2.4	0.82	0.22	0.11
B02	60	2.4	0.82	(0.12)	(0.07)
B03	30	1.0	0.30	0.38	0.17
C01	90	2.0	0.60	0.38	0.18
C02[a]	90	2.0	0.60	0.28	0.31
C03[a]	90	2.0	0.60	1.67	0.81
D01	50	0.7	0.21	0.56	0.27
D02[a]	50	0.7	0.21	0.84	0.62
D03[a]	100	0.8	0.24	(0.13)	(0.04)
D04	100	1.0	0.30	(0.05)	(0.15)
D05	100	1.0	0.30	0.29	0.10
C04[a]	0	0	0	ND	0
C05[a]	0	0	0	ND	0

Numbers in parenthese represent each datum was below the lowest standard. ND stands for not detected.
a. Blood sample was taken, at the preshift hour on the next day, for the determination of plasma ChE.

spraying. Also shown at the bottom of Table 3 are the analysis results of 2 office workers, who did not handle CPF, no TCP was detected in their urine. The total acid-released TCP, analyzed in duplicate, was determined for each human sample. Of the 16 sprayers, 4 had a level of lower than the lowest standard. No correlation was found between the measured TCP vs the amount (kg) of CPF used.

In conclusion, a simple and sensitive isocratic HPLC/UV method was developed to quantify urinary TCP, which is the chemical specific

urinary metabolite of CPF. The straightforward sample preparation included (i) an ice-cold acid denaturation of the urinary macromolecules, (ii) a low speed centrifugation to remove the denatured precipitates, and (iii) an acid hydrolysis of the TCP-conjugates. The feasibility of the method, to be used as a non-invasive chemical specific biological monitoring of the occupational exposure to CPF, was adequately demonstrated with urine samples collected from professional pest-control sprayers.

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