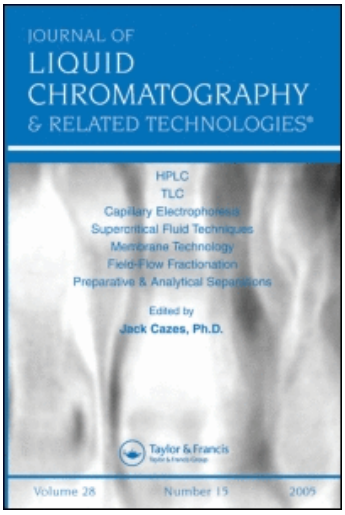

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of Chlorpyrifos and its Metabolites in Rat Blood Using Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry

Leah N. Williamson[a]; Michael G. Bartlett[a]; Alvin V. Terry Jr.[b]

[a] Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, The University of Georgia, Athens, GA, USA [b] Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, GA, USA

To cite this Article Williamson, Leah N. , Bartlett, Michael G. and Terry Jr., Alvin V.(2007) 'Determination of Chlorpyrifos and its Metabolites in Rat Blood Using Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry', Journal of Liquid Chromatography & Related Technologies, 30: 2, 273 — 285

To link to this Article: DOI: 10.1080/10826070601064573

URL: http://dx.doi.org/10.1080/10826070601064573

Journal of Liquid Chromatography & Related Technologies®, 30: 273–285, 2007

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070601064573

Determination of Chlorpyrifos and its Metabolites in Rat Blood Using Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry

Leah N. Williamson and Michael G. Bartlett
Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, The University of Georgia, Athens, GA, USA

Alvin V. Terry, Jr.
Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, GA, USA

Abstract: A method has been developed to quantify chlorpyrifos (O,O-diethyl O-[3,5,6,-trichloro-2-pyridyl] phosphorothionate) and its metabolites chlorpyrifos-oxon (O,O-diethyl-O[3,5,6,trichloro-2-pyridyl] phosphate) and TCP (3,5,6,-trichloro-2-pyridinol) in rat blood by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS). Rat blood was treated by liquid-liquid extraction and the analytes were separated by gradient elution on an Agilent Zorbax Extended-C_8 column (2.0 × 150 mm, 5 μm). Chlorpyrifos and chlorpyrifos-oxon were detected in positive ion mode using multiple reaction monitoring (MRM). TCP was detected in negative ion mode using parent-to-parent transition monitoring.

This method was validated and the specificity, linearity, limit of quantitation (LOQ), precision, accuracy, stability, and recoveries were determined. The method was then applied to determine the level of chlorpyrifos and its metabolites from rats exposed to a subcutaneous injection of 10 and 18 mg/kg.

Keywords: Organophosphate insecticides, HPLC, Tandem mass spectrometry, Chlorpyrifos, Electrospray ionization, Liquid-liquid extraction

Address correspondence to Michael G. Bartlett, Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, The University of Georgia, Athens, GA 30602-2352, USA. E-mail: bartlett@rx.uga.edu

INTRODUCTION

Chlorpyrifos (O,O-diethyl O-[3,5,6,-trichloro-2-pyridyl] phosphorothionate) is an organophosphate insecticide that is widely used agriculturally (Lorsban^{TM}). This insecticide is used because it inhibits acetylcholinesterase, an enzyme that is necessary for the normal function of the nervous system. It is metabolized by Cytochrome P-450 into Chlorpyrifos-oxon (O,O-diethyl-O[3,5,6,trichloro-2-pyridinly] phosphate) and TCP (3,5,6,-trichloro-2-pyrinidol). TCP is also formed by the hydrolysis of chlorpyrifos-oxon. Chlorpyrifos-oxon is about 3,000 times more potent than chlorpyrifos in its inhibition of acetylcholinesterase, which leads to neurotoxicity.[1] TCP is nontoxic and is eliminated by the kidneys. The use of chlorpyrifos has been restricted by the United States Environmental Protection Agency, however, TCP has recently been detected in the urine of 96% of approximately 2,000 samples analyzed from subjects living in the United States.[2]

Methods have been published to detect chlorpyrifos and its metabolites in blood,[3] serum,[4] and brain tissue.[5] The method proposed by Brzak et al.[3] using gas chromatography/mass spectrometry requires isotope-labeled internal standards and derivatizing agents. Because of the high temperature at the GC inlet port, the chlorpyrifos and chlorpyrifos-oxon are hydrolyzed to TCP. However, the internal standards corrected for the low chlorpyrifos and chlorpyrifos-oxon and the high TCP concentrations. Two separate methods were used to quantitate the chlorpyrifos and chlorpyrifos-oxon and the TCP because the derivatizing agent for the TCP caused the chlorpyrifos-oxon to degrade. The method proposed by Sancho et al.[4] detected chlorpyrifos and TCP in serum, however, the chlorpyrifos-oxon metabolite was not detected. This may be due to the fact that the serum was not acidified to stop the chlorpyrifos-oxon hydrolysis. Therefore, if using this method to detect chlorpyrifos and its metabolites in real serum samples, higher TCP concentrations may be reported.

Chlorpyrifos and its metabolites have been detected using high performance liquid chromatography with UV detection.[6–9] A method has also been published to detect chlorpyrifos in serum using liquid chromatography and atmospheric pressure chemical ionization mass spectrometry.[10] These methods reported limit of quantitations (LOQs) of 150–200 ng/mL. Because TCP levels in the urine are a marker for chlorpyrifos exposure, several methods have been published to measure TCP in urine.[4,11–15]

Animal studies have indicated that repeated exposures of chlorpyrifos at sub-threshold levels can lead to persistent impairment in cognitive function.[16] It is important that a sensitive and accurate method be developed to detect the small amounts of chlorpyrifos and its metabolites in the blood to further explore the underlying neurological bases for these cognitive changes. The method we propose detects chlorpyrifos, chlorpyrifos-oxon, and TCP in one injection with a run time of 10 minutes. This method also did not require the use of derivatizing agents or internal standards.

EXPERIMENTAL

Chemicals and Reagents

Chlorpyrifos (Figure 1(a)), chlorpyrifos-oxon (Figure 1(b)), and TCP (Figure 1(c)) were purchased from Chem Service (West Chester, PA, USA). HPLC-grade acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA, USA). Deionized water was generated from a Continental Deionized Water System (Natick, MA, USA). Formic acid was purchased from J.T. Baker (Phillipsburg, NJ, USA). Trichloroacetic acid was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Calibration Standards and Quality Control Standards

Standard stock solutions were made by dissolving chlorpyrifos, chlorpyrifos-oxon, and TCP in acetonitrile (1 mg/mL) and were stored at 4°C. The stock solutions were further diluted with acetonitrile to make working solutions with concentrations of 3.0 and 2.0 μg/mL. Standard calibration samples were spiked in blank rat blood from different lots to yield final concentrations of 1, 5, 15, 25, 50, 75, 100, and 200 ng/mL. Quality control (QC) samples were spiked in rat blood to yield final concentrations of 1, 2, 5, 10, 30, and 150 ng/mL.

Liquid Chromatography

An Agilent 1100 Series HPLC system (Palo Alto, CA, USA) equipped with a degasser, binary pump, autosampler, and thermostated column compartment was used. The column used was an Agilent (Palo Alto, CA, USA) Zorbax

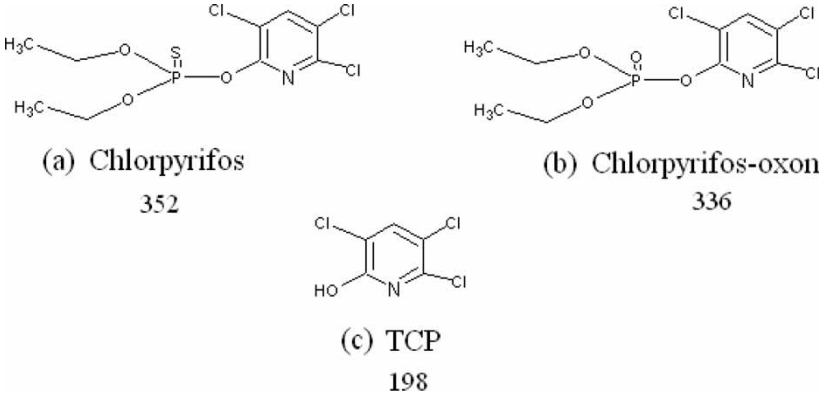

Figure 1. Chemical structures of (a) chlorpyrifos, (b) chlorpyrifos-oxon, and (c) TCP.

Extended-C_8 column (2.0 × 150 mm, 5 μm) equipped with a 4.0 × 2.0 mm Phenomenex (Torrance, CA, USA) Security Guard C_8 guard column. The compounds were separated by gradient elution with mobile phases of deionized water with 0.0025% formic acid (A) and acetonitrile (B) at a flow rate of 0.30 mL/min. The formic acid content of the mobile phase was chosen to be 0.025% because good peak shape and a high signal for the acidic TCP were maintained, as shown in a previous study.[4] The initial conditions were 40% A and 60% B. From 0 to 2 minutes, B was increased from 60% to 80% and held for 3 minutes. From 5 to 6 minutes, B was decreased back to the starting conditions. The column then reequilibrated from 6 to 10 minutes.

Mass Spectrometry

The HPLC system was interfaced to a Quattro micro API tandem mass spectrometer equipped with a Z-spray ion source and syringe pump (Manchester, UK). Nitrogen gas was used as the desolvation gas and was set to a flow rate of 500 L/h with a temperature of 350°C. The cone gas flow was set to 50 L/h. Argon was the collision gas used and the collision cell pressure was 2.2×10^{-3} mbar. TCP was detected in negative ion mode using a capillary voltage of 2.5 kV, a cone voltage of 20 V, a dwell time of 200 ms, and a collision energy of 2 eV. Chlorpyrifos and chlorpyrifos-oxon were detected in positive ion mode with a capillary voltage of 3.5 kV, a cone voltage of 20 V, a dwell time of 200 ms, and a collision energy of 20 eV. The source temperature was 120°C. Samples were acquired in multiple reaction monitoring (MRM) mode and were processed using Masslynx 4.0 software.

Sample Preparation

This method was applied to 18 male albino Wistar rats obtained from Harlan Biosciences (Indianapolis, IN, USA). All procedures were reviewed and approved by the Medical College of Georgia Committee on Animal Use for Research and the Veterans Affairs Medical Center Subcommittee on Animal Use, and were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by US National Institute of Health. The rats were housed in pairs in a temperature controlled room (25°C) with a 12 hour light/dark cycle. The rats were injected with chlorpyrifos every other day for 30 days, followed by a washout period of 14 days where no injections took place. The chlorpyrifos was dissolved in dimethyl sulfoxide (DMSO) and then added to peanut oil. The rats were injected subcutaneously at concentrations of 10 and 18 mg/kg. On the day of sacrifice, the rats were anesthetized with ketamine (40 mg/kg) and xylazine (18 mg/kg). The jugular vein was located and ~500 μL of blood was withdrawn. Trichloroacetic acid solution (1 g/mL) was added to the

blood to decrease the pH to ~4 (75 μL/mL blood). Acidifying the blood prevented the chlorpyrifos-oxon from enzymatically hydrolyzing to TCP. From a previous study, it was found that proteins with A-esterase activity for chlorpyrifos-oxon had maximum activity at pH 4.9–5.2.[17] The blood was transferred to a Microtainer that contained the TCA solution and briefly vortexed. The blood was stored at −70°C until analysis.

For the validation of the method, untreated rat blood was obtained from Harlan Biosciences (Indianapolis, IN, USA). The acidified rat blood (35 μL) was spiked with 5 μL of each analyte. The samples were briefly vortexed and were prepared by liquid-liquid extraction using 200 μL of ethyl acetate/acetonitrile (60:40, v/v). The samples were then vortexed for 5 minutes and centrifuged for 10 minutes at 13,000 rpm. The organic layer was extracted and dried under a gentle stream of nitrogen. The residue was reconstituted in 50 μL of acetonitrile. The samples were sonicated and centrifuged for 10 minutes at 13,000 rpm; 10 μL was used as the injection volume.

Method Validation

Calibration curves were acquired (8 points for chlorpyrifos and chlorpyrifos-oxon and 7 points for TCP) by plotting peak area against the analyte concentration. The curves were acquired daily for 3 days (n = 5 for intra-day and n = 15 for inter-day). Precision and accuracy were determined for the 3 QC points (10, 30, and 150 ng/mL for TCP and 2, 30, and 150 ng/mL for chlorpyrifos and chlorpyrifos-oxon) and the LOQ (5 ng/mL for TCP 1 ng/mL for chlorpyrifos and chlorpyrifos-oxon). The LOQ values were determined by observing where precision and accuracy values did not exceed 20%. Precision was reported as percent relative standard deviation and accuracy was reported as percent error.

Recoveries were also determined. Absolute recovery was determined by dividing the response of the analyte in the blood by the response of the standard solution in acetonitrile. Relative recovery was determined by dividing the response of the analyte in the blood by the response of the analyte in blood that was spiked after the extraction process. The matrix effects were determined using the method reported by Matuszewski et al.,[18] and were calculated to be the response of the homogenate spiked after the extraction divided by the response of the standard solution in acetonitrile.

The stability of the stock solutions were determined at their storage conditions of 4°C. Other stability experiments were determined at concentrations of 10 and 150 ng/mL for TCP and 2 and 150 ng/mL for chlorpyrifos and chlorpyrifos-oxon. Freeze/thaw stability was determined over 3 cycles. Autosampler stability was determined by preparing samples and injecting them at different time intervals over 24 hours. Bench top stability was determined by spiking blank blood and preparing the samples at different time intervals over 24 hours.

RESULTS AND DISCUSSION

Development of the Analytical Method

Full scan and product ion mass spectra were obtained by infusing 100 μg/mL of chlorpyrifos, chlorpyrifos-oxon, and TCP into the electrospray ionization (ESI) probe at a rate of 10 μL/min. TCP ionized in negative ion mode, while chlorpyrifos and chlorpyrifos-oxon ionized in positive ion mode. The collision induced dissociation of TCP yielded an abundant ion at m/z 198, which corresponded to the $[M\text{-}H]^-$ of TCP, and m/z 35 for the chloride ion. Since the abundance of the TCP precursor ion was high relative to the chloride ion, the deprotonated molecular ion was used for quantitation. This same transition was used in an earlier study of TCP.[4] The MRM transitions monitored were: m/z 198 → 198 for TCP, m/z 336 → 280 for chlorpyrifos-oxon, and m/z 352 → 200 for chlorpyrifos. The MRM transitions monitored were selected based on the chromatograms that yielded the highest signal-to-noise ratio.

Method Validation

Specificity

From the MRM chromatograms of blank blood, it can be seen that there are no interfering peaks. In comparing the MRM chromatograms of the spiked blood and the standard solutions (Figure 2), this method was shown to be specific for chlorpyrifos, chlorpyrifos-oxon, and TCP.

Linearity

Standard solutions were made fresh each validation day and a linear calibration curve was generated for each analyte. The linear range was 1 to 200 ng/mL for chlorpyrifos and chlorpyrifos-oxon and 5 to 200 ng/mL for TCP. A weighting factor of $1/x^2$ was used for each curve. The coefficients of variation (r^2) for each compound were: 0.9910 ± 0.0028 for chlorpyrifos, 0.9922 ± 0.0010 for chlorpyrifos-oxon, and 0.9777 ± 0.0039 for TCP.

Accuracy and Precision

Precision and accuracy measurements were acquired for the 3 QC points and the LOQ for each compound. The accuracy and precision data can be seen in Table 1. Values for the intra-day precision and accuracy ranged from 3.23–14.16% and 2.68–10.75%, respectively, for the QC samples and 6.50–15.21% and 4.87–9.48% for the LOQ samples. Inter-day precision and accuracy ranged from 3.23–11.45% and 5.16–11.34%, respectively, for the QC samples and 8.31–11.34% and 7.07–11.28% for the LOQ samples.

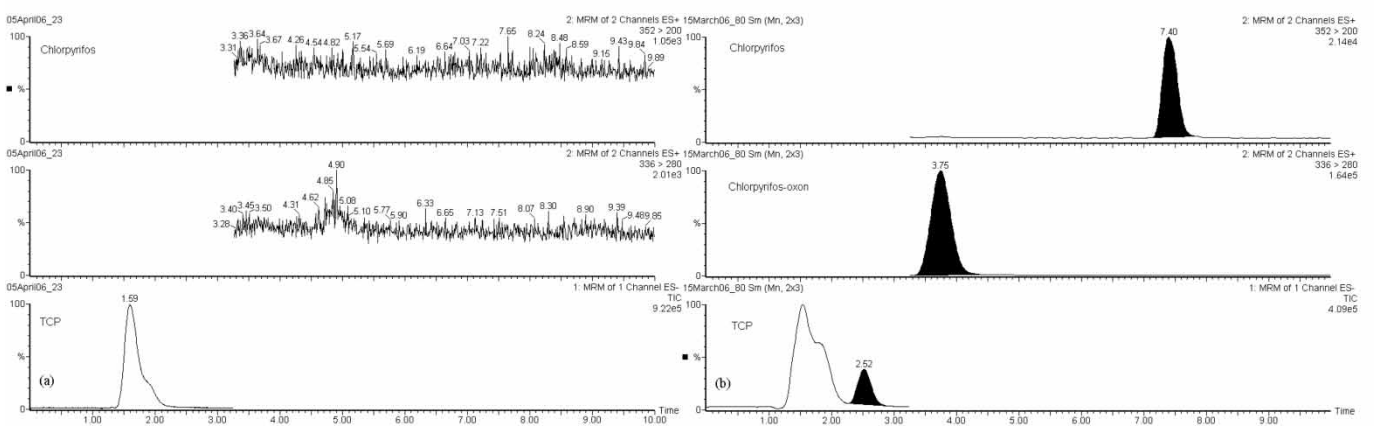

Figure 2. MRM chromatograms of (a) blank blood and (b) blood spiked at 15 ng/mL.

Table 1. Intra- and inter-day precision (%RSD) and accuracy (%Error) of the method in rat blood

Analyte		Theoretical concentration (ng/mL)	Experimental concentration (ng/mL)	RSD (%)	Error (%)
Chlorpyrifos	(n = 5)	1	0.98 ± 0.12	12.74	8.06
		2	1.92 ± 0.17	8.84	7.08
		30	32.71 ± 2.86	8.74	10.07
		150	160.00 ± 9.40	5.86	7.64
	(n = 15)	1	1.05 ± 0.12	11.34	9.63
		2	1.92 ± 0.14	7.52	5.59
		30	32.66 ± 2.01	6.16	9.50
		150	163.00 ± 9.80	6.01	8.93
Chlorpyrifos-oxon	(n = 5)	1	1.03 ± 0.16	15.21	9.48
		2	1.92 ± 0.05	2.42	4.45
		30	33.22 ± 1.22	3.68	10.75
		150	151.35 ± 4.89	3.23	2.68
	(n = 15)	1	1.09 ± 0.10	9.59	11.28
		2	2.05 ± 0.15	7.19	6.15
		30	33.10 ± 1.26	3.81	10.34
		150	150.15 ± 9.56	6.34	5.16
TCP	(n = 5)	5	4.88 ± 0.32	6.50	4.87
		10	10.89 ± 1.54	14.16	9.18
		30	31.77 ± 2.11	6.65	7.57
		150	160.47 ± 17.94	11.18	7.16
	(n = 15)	5	4.76 ± 0.39	8.31	7.07
		10	10.67 ± 1.22	11.45	11.34
		30	32.86 ± 2.06	6.26	10.09
		150	154.84 ± 13.74	8.88	6.28

Recovery and Matrix Effects

Absolute recovery, relative recovery, and matrix effects data are summarized in Table 2. Relative recoveries ranged from 77.93–102.01%, so liquid-liquid extraction was a sufficient method for extracting the compounds from the blood. The TCP signal was greatly suppressed (>35%) at all three concentrations. This certainly contributed to the higher LOQ for TCP. This suppression may be due to the fact that TCP was the first compound to elute. From this, we concluded that residual biological matter competes more effectively for the charge against TCP.

Stability

The stock solutions were stable at the storage conditions (4°C) for 3 months. Spiked samples were found to be stable over 3 freeze/thaw cycles. Prepared

Table 2. Absolute recovery, relative recovery, and matrix effects (mean ± SD) of analytes in rat blood (n = 5)

Analyte	Concentration (ng/mL)	Absolute recovery (%)	Relative recovery (%)	Matrix effect (%)	Type of effect
Chlorpyrifos	2	109.44 ± 6.01	96.73 ± 5.25	113.21	13.21% enhancement
	30	89.17 ± 3.45	77.98 ± 2.35	114.37	14.37% enhancement
	150	89.44 ± 3.70	80.22 ± 5.49	111.70	11.70% enhancement
Chlorpyrifos-oxon	2	107.90 ± 5.09	97.55 ± 5.97	110.70	10.70% enhancement
	30	89.29 ± 1.94	77.93 ± 2.83	114.63	14.63% enhancement
	150	90.03 ± 1.28	79.18 ± 7.93	114.30	14.30% enhancement
TCP	10	50.02 ± 3.69	102.01 ± 10.06	49.18	50.82% suppression
	30	45.08 ± 5.34	84.01 ± 20.30	54.88	45.12% suppression
	150	50.50 ± 3.10	79.18 ± 7.93	64.27	35.73% suppression

Table 3. Concentrations of chlorpyrifos and TCP in rat blood (n = 6) after 5th dose (Day 9) of chlorpyrifos. Reported as mean ± S.D. (%RSD)

Dosage	Chlorpyrifos (ng/mL)	TCP (ng/mL)
10 mg/kg	2.20 ± 0.86 (39.16)	44.23 ± 13.32 (30.11)
18 mg/kg	5.06 ± 0.89 (17.50)	54.80 ± 17.64 (32.19)

samples were stable in the autosampler for up to 24 hours. Spiked blood samples were found to have a change in the peak areas when left at room temperature. At 24 hours, the concentrations of the chlorpyrifos and chlorpyrifos-oxon decreased by as much as 49%, while the concentrations for TCP increased by about 23%. This could be due to the fact that the hydrolysis of chlorpyrifos and chlorpyrifos-oxon could be temperature dependent, as well as pH dependent. There was no change in the concentration of these samples after 4 hours at room temperature. Therefore, it is recommended that the blood samples be prepared directly upon thawing.

Application of the Method

The concentration data for chlorpyrifos and its metabolites are reported in Table 3. The TCP concentrations were shown to reach a maximum value

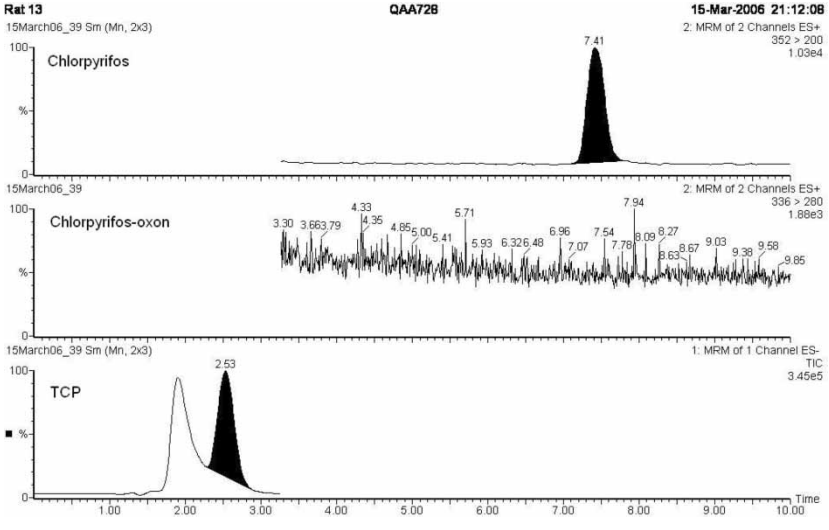

Figure 3. MRM chromatogram of blood from rat dosed with 10 mg/kg chlorpyrifos after 14-day wash-out period.

after the first week of injections, then continued to decrease until the end of the 30 days. The concentration of chlorpyrifos decreased after the 8th injection and then increased after the 12th injection. A MRM chromatogram from a rat dosed with 10 mg/kg of chlorpyrifos after a 14-day wash-out period can be seen in Figure 3. We observed no chlorpyrifos-oxon in the blood. This is consistent with the findings that the chlorpyrifos-oxon levels in rat blood are 50–140 times lower than chlorpyrifos levels.[19] In an earlier study where an oral dose of chlorpyrifos was given at 15 mg/kg, the blood levels of TCP were 10–20 fold higher than chlorpyrifos levels.[20] This is consistent with our findings.

CONCLUSIONS

This validated method was efficient for the determination of chlorpyrifos, chlorpyrifos-oxon, and TCP in rat blood. The liquid-liquid extraction sufficiently recovered all three analytes from the blood. All analytes had high recoveries ($>77\%$) from the blood. The method was accurate and precise, with %RSD and %Error values of $<16\%$ over three validation days.

ACKNOWLEDGMENTS

The authors thank Dr. Debra Gearhart and Dan Beck for providing the blood samples from the dosed rats.

REFERENCES

1. Caldwell, J.; Huston, D.H.; Paulson, G.D. *Intermediary xenobiotic metabolism; Methodolgy, mechanisms, and significanc*; Taylor and Francis: Basingstoke, U.K., 1989; 99–115.
2. Barr, D.B.; Allen, R.; Olsson, A.O.; Bravo, R.; Caltabiano, L.M.; Montesano, A.; Nguyen, J.; Udunka, S.; Walden, D.; Walker, R.D.; Weerasekera, G.; Whitehead, R.D., Jr.; Schober, S.E.; Needham, L.L. Concentrations of selective metabolites of organophosphorus pesticides in the United States population. Environ. Res. **2005**, *99*, 314–326.
3. Brzak, K.A.; Harms, D.W.; Bartels, M.J.; Nolan, R.J. Determination of chlorpyrifos, chlorpyrifos oxon, and 3,5,6-trichloro-2-pyridinol in rat and human blood. J. Anal. Toxicol. **1998**, *22*, 203–210.
4. Sancho, J.V.; Pozo, O.J.; Hernandez, F. Direct determination of chlorpyrifos and its main metabolite 3,5,6-trichloro-2-pyridinol in human serum and urine by coupled-column liquid chromatography/electrospray-tandem mass spectrometry. Rapid Commun. Mass Spectrom. **2000**, *14*, 1485–1490.
5. Williamson, L.N.; Terry, A.V., Jr.; Bartlett, M.G. Determination of chlorpyrifos and its metabolites in rat brain tissue using coupled-column liquid

chromatography/electrospray ionization tandem mass spectrometry. Rapid Commun. Mass Spectrom. **2006**, *20*, 2689–2695.
6. Abu-Qare, A.W.; Abou-Donia, M.B. Development of a high-performance liquid chromatographic method for the quantification of chlorpyrifos, pyridostigmine bromide, N,N-diethyl-m-toluamide and their metabolites in rat plasma and urine. J. Chromatogr. B Biomed. Sci. Appl. **2001**, *754*, 533–538.
7. Abu-Qare, A.W.; Abou-Donia, M.B. Determination of diazinon, chlorpyrifos, and their metabolites in rat plasma and urine by high-performance liquid chromatography. J. Chromatogr. Sci. **2001**, *39*, 200–204.
8. Abu-Qare, A.W.; Abou-Donia, M.B. Simultaneous determination of chlorpyrifos, permethrin, and their metabolites in rat plasma and urine by high-performance liquid chromatography. J. Anal. Toxicol. **2001**, *25*, 275–279.
9. Sultatos, L.G.; Costa, S.D. Murphy determination of organophosphorus insecticides, their oxygen analogs and metabolites by high pressure liquid chromatography. Chromatographia **1982**, *15*, 669–671.
10. Kawasaki, S.; Ueda, H.; Itoh, H.; Tadano, J. Screening of organophosphorus pesticides using liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. J. Chromatogr. **1992**, *595*, 193–202.
11. Bicker, W.; Lammerhofer, M.; Lindner, W. Determination of chlorpyrifos metabolites in human urine by reversed-phase/weak anion exchange liquid chromatography-electrospray ionisation-tandem mass spectrometry. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. **2005**, *822*, 160–169.
12. Bartels, M.J.; Kastl, P.E. Analysis of 3,5,6-trichloropyridinol in human urine using negative-ion chemical ionization gas chromatography-mass spectrometry. J. Chromatogr. **1992**, *575*, 69–74.
13. Hernandez, F.; Sancho, J.V.; Pozo, O.J. An estimation of the exposure to organophosphorus pesticides through the simultaneous determination of their main metabolites in urine by liquid chromatography-tandem mass spectrometry. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. **2004**, *808*, 229–239.
14. Koch, H.M.; Angerer, J. 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. J. Chromatogr. B Biomed. Sci. Appl. **2001**, *759*, 43–49.
15. Aprea, C.; Betta, A.; Catenacci, G.; Lotti, A.; Magnaghi, S.; Barisano, A.; Passini, V.; Pavan, I.; Sciarra, G.; Vitalone, V.; Minoia, C. Reference values of urinary 3,5,6-trichloro-2-pyridinol in the Italian population—validation of analytical method and preliminary results (multicentric study). J. AOAC Intl. **1999**, *82*, 305–312.
16. Terry, A.V., Jr.; Stone, J.D.; Buccafusco, J.J.; Sickles, D.W.; Sood, A.; Prendergast, M.A. Repeated exposures to subthreshold doses of chlorpyrifos in rats: hippocampal damage, impaired axonal transport, and deficits in spatial learning. J. Pharmacol. Exp. Ther. **2003**, *305*, 375–384.
17. Pond, A.L.; Coyne, C.P.; Chambers, H.W.; Chambers, J.E. Identification and isolation of two rat serum proteins with A-esterase activity toward paraoxon and chlorpyrifos-oxon. Biochem. Pharmacol. **1996**, *52*, 363–369.
18. Matuszewski, B.K.; Constanzer, M.L.; Chavez-Eng, C.M. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal. Chem. **2003**, *75*, 3019–3030.

19. Timchalk, C.; Nolan, R.J.; Mendrala, A.L.; Dittenber, D.A.; Brzak, K.A.; Mattsson, J.L. A physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model for the organophosphate insecticide chlorpyrifos in rats and humans. Toxicol. Sci. **2002**, *66*, 34–53.
20. Timchalk, C.; Poet, T.S.; Hinman, M.N.; Busby, A.L.; Kousba, A.A. Pharmacokinetic and pharmacodynamic interaction for a binary mixture of chlorpyrifos and diazinon in the rat. Toxicol. Appl. Pharmacol. **2005**, *205*, 31–42.

Received September 25, 2006
Accepted October 28, 2006
Manuscript 6946