Determination of Diazinon, Chlorpyrifos, and Their Metabolites in Rat Plasma and Urine by High-Performance Liquid Chromatography

Aqel W. Abu-Qare and **Mohamed B. Abou-Donia***
Department of Pharmacology and Cancer Biology, Duke University Medical Center, P.O. Box 3813, Durham, NC 27710

Abstract

This study reports a simple and rapid high-performance liquid chromatographic (HPLC) method for the determination of the insecticide diazinon (*O,O*-diethyl-*O*[2-isopropyl-6-methylpyridimidinyl] phosphorothioate), its metabolites diazoxon (*O,O*-diethyl-*O*-2-isopropyl-6-methylpyridimidinyl phosphate) and 2-isopropyl-6-methyl-4-pyrimidinol, the insecticide chlorpyrifos (*O,O*-diethyl-*O*[3,5,6-trichloro-2-pyridinyl] phosphorothioate) and its metabolites chlorpyrifos-oxon (*O,O*-diethyl-*O*[3,5,6-trichloro-2-pyridinyl] phosphate), and TCP (3,5,6-trichloro-2-pyridinol) in rat plasma and urine samples. The method is based on using C_{18} Sep-Pak cartridges for solid-phase extraction and HPLC with a reversed-phase C_{18} column and programmed UV detection ranging between 254 and 280 nm. The compounds are separated using a gradient of 1% to 80% acetonitrile in water (pH 3.0) at a flow rate ranging between 1 and 1.5 mL/min in a period of 16 min. The limits of detection ranged between 50 and 150 ng/mL, and the limits of quantitation were 100 to 200 ng/mL. The average percentage recovery of five spiked plasma samples were 86.3 ± 8.6, 77.4 ± 7.0, 82.1 ± 8.2, 81.8 ± 8.7, 73.1 ± 7.4, and 80.3 ± 8.0 and from urine were 81.8 ± 7.6, 76.6 ± 7.1, 81.5 ± 7.9, 81.8 ± 7.1, 73.7 ± 8.6, and 80.7 ± 7.7 for diazinon, diazoxon, 2-isopropyl-6-methyl-4-pyrimidinol, chlorpyrifos, chlorpyrifos-oxon, and TCP, respectively. The relationship between the peak area and concentration was linear over a range of 200 to 2000 ng/mL. This method was applied in order to analyze these chemicals and metabolites following dermal administration in rats.

Introduction

Diazinon and chlorpyrifos are widely used insecticides inside homes and in public places. They are applied to control flying and chewing insects (1–3). Exposure to both compounds results in cholinergic signs attributed to an inhibition of acetycholinesterase enzymes (4). Diazinon has been reported to be absorbed, distributed, and excreted following its application in humans and rats (5–11). Metabolism and elimination of chlorpyrifos has been determined in plasma and urine samples (12–16). Several analytical methods have been used for the identification and quantitation of these chemicals and their metabolites when applied alone. The methods used were high-performance liquid chromatography (HPLC) (17–18), HPLC–mass spectrometry (MS) (19), gas chromatography (GC) (7,20), and GC–MS (15,21). The concentrations of chlorpyrifos and diazinon have been determined in food (22), air (23), and inside offices (24). No published studies have reported on the simultaneous analysis of diazinon, chlorpyrifos, and their metabolites in plasma and urine following combined dermal exposure. We hypothesized that a combined exposure to diazinon and chlorpyrifos could be a result of toxic interactions. In order to study possible toxicokinetic interactions between these compounds, a method was needed to simultaneously determine the parent and metabolites in biological matrices. In this study, we present a reliable method for the simultaneous determination of diazinon, chlorpyrifos, and their metabolites in rat plasma and urine using solid-phase extraction (SPE) coupled with reversed-phase HPLC.

* Author to whom correspondence should be addressed: e-mail donia@acpub.duke.edu.

Experimental

Chemicals and materials

Chlorpyrifos (99% *O,O*-diethyl-*O*[3,5,6-trichloro-2-pyridinyl] phosphorothioate), diazinon (98% *O,O*-diethyl *O*-2-isopropyl-6-methylpyridimidinyl phosphorothioate), diazoxon (98% *O,O*-diethyl *O*-2-isopropyl-6-methylpyridimidinyl phosphate), and 2-isopropyl-6-methyl-4-pyrimidinol were obtained from Chem Service, Inc. (West Chester, PA) (chemical structures shown in Figure 1). Chlorpyrifos-oxon (*O,O*-diethyl-*O*[3,5,6-trichloro-2-pyridinyl] phosphate) was obtained from Dow Chemical Co. (Midland, MI). TCP (95% 3,5,6-trichloro-2-pyridinol) was prepared in our laboratory. Water and acetonitrile (HPLC grade) were obtained from Mallinckrodt Baker, Inc. (Paris, KY). C_{18} Sep-Pak Vac 3-cc (500 mg) cartridges were obtained from Waters Corporation (Milford, MA).

Animal specimens

Rats (Sprague Dawley) were purchased from Zivic Miller (Zelienople, PA). The animals were kept in plastic metabolic cages. Five rats were treated with a single dermal dose of 65 mg/kg diazinon and a single dermal dose of 30 mg/kg chlorpyrifos. Five untreated control rats were treated with a single dermal dose of ethanol. The animals were held in metabolic cages allowing for the collection of urine samples. Urine samples were collected from treated and control rats after 12 h of dosing. The animals were anesthetized with halothane (Halocarbon Laboratories, River Edge, NJ) and exterminated by heart exsanguinations at 12 h. Blood was collected via a heart puncture with a heparinized syringe and centrifuged at 4000 rpm for 15 min at 5°C in order to separate plasma from the extraction. Urine and plasma samples were stored at –20°C prior to analysis by HPLC.

Instrumentation

The liquid chromatographic system (Waters 2690 separation module) consisted of Waters 600E multisolvent delivery system pumps, a Waters Ultra WISP 715 autoinjector, and a Waters 2487 Dual λ absorbance detector. A guard column (2 cm × 4.0 mm, 5 µm) (Supelco Park, Bellefonte, PA) and a reversed-phase µBondapak 125A C_{18} column (10 µm, 3.9 × 300 mm) were used.

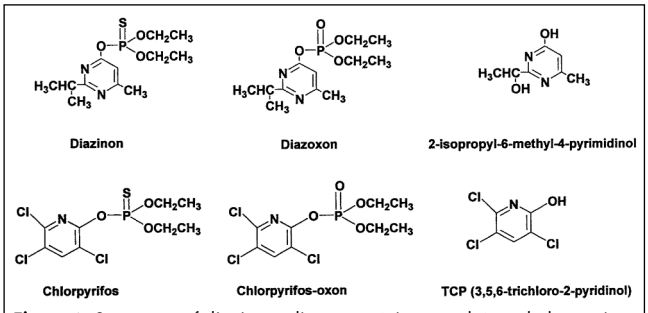

Figure 1. Structures of diazinon, diazoxon, 2-isopropyl-6-methyl-4-pyrimidinol, chlorpyrifos, chlorpyrifos-oxon, and TCP.

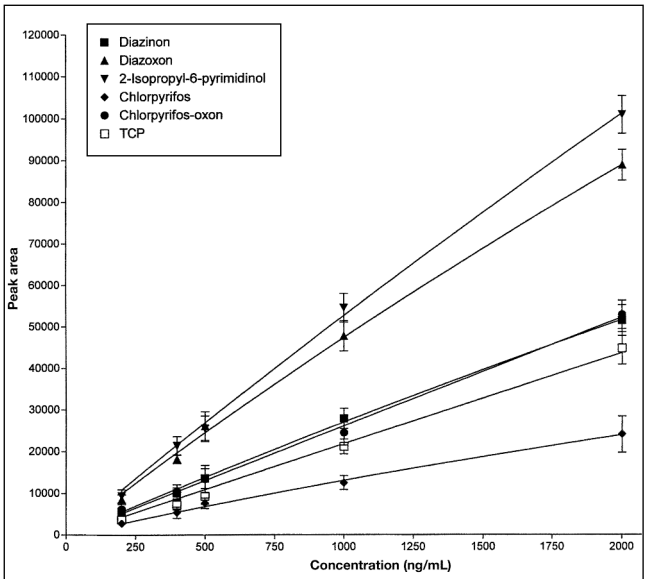

Figure 2. Standard calibration curves for diazinon, diazoxon, 2-isopropyl-6-methyl-4-pyrimidinol, chlorpyrifos, chlorpyrifos-oxon, and TCP.

Sample preparation

Two 0.2-mL plasma and urine samples from untreated rats were spiked with concentrations ranging from 200 to 2000 ng/mL each containing diazinon, diazoxon, 2-isopropyl-6-methyl-4-pyrimidinol, chlorpyrifos, chlorpyrifos-oxon, and TCP. Spiked and treated samples were acidified with 1M acetic acid (pH 5.0). Disposable C_{18} Sep-Pak cartridges were conditioned with 3 mL of acetonitrile then equilibrated using 3 mL of water prior to use. The spiked urine and plasma samples were vortexed for 30 s and centrifuged for 5 min at 1000 rpm. The supernatant was loaded into the disposable cartridges and then washed with 2 mL of water, eluted twice by 2 mL of methanol in a marked small test tube, and reduced to 500 µL using a gentle stream of nitrogen prior to analysis by HPLC.

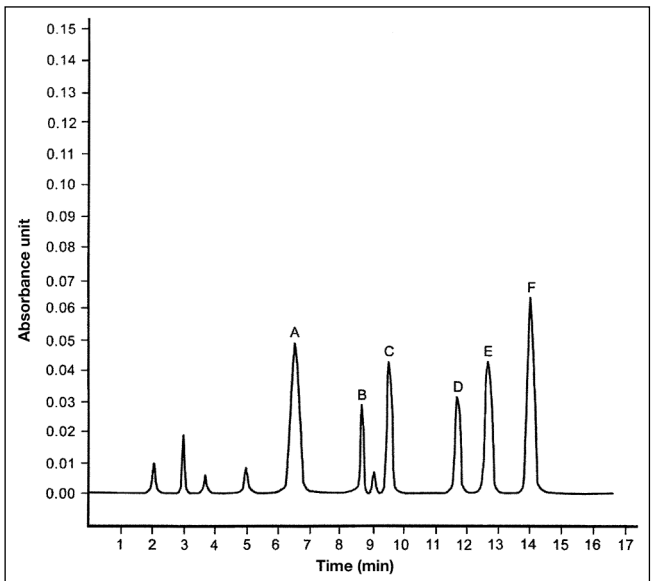

Figure 3. Chromatogram of a spiked plasma sample with (A) 2-isopropyl-6-methyl-4-pyrimidinol, (B) diazoxon, (C) TCP, (D) chlorpyrifos-oxon, (E) diazinon, and (F) chlorpyrifos under established HPLC conditions.

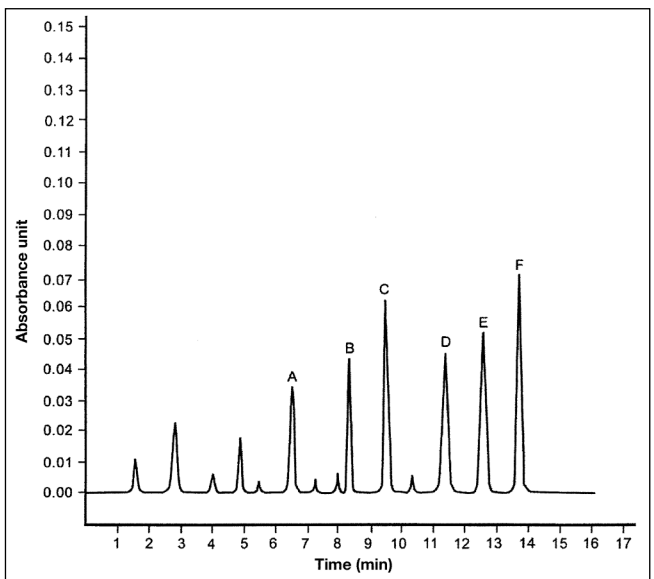

Figure 4. Chromatogram of a spiked urine sample with (A) 2-isopropyl-6-methyl-4-pyrimidinol, (B) diazoxon, (C) TCP, (D) chlorpyrifos-oxon, (E) diazinon, and (F) chlorpyrifos under established HPLC conditions.

Chromatographic conditions

A 10-µL solution of plasma- or urine-concentrated residues was injected into the HPLC. The mobile phase was a water–acetonitrile gradient at a flow rate programmed from 1 to 1.5 mL/min. The water was adjusted to pH 3.0 using 1M acetic acid. The gradient started at 1% acetonitrile, increased to 55% acetonitrile at the 6-min mark, and then increased to 80% acetonitrile at 11 min. The system then returned to 1% acetonitrile at 13 min and was kept under this condition for 3 min in order to re-equilibrate. The eluents were monitored by the UV detection of the wavelengths 254 nm for diazinon, diazoxon, and 2-isopropyl-6-methyl-4-pyrimidinol and 280 nm for chlorpyrifos, chlorpyrifos-oxon, and TCP. The chromatographic analysis was performed at ambient temperature.

Calibration procedures

Five different calibration standards of a mixture of diazinon, diazoxon, 2-isopropyl-6-methyl-4-pyrimidinol, chlorpyrifos, chlorpyrifos-oxon, and TCP were prepared in acetonitrile. Their concentrations ranged from 200 to 2000 ng/mL. Linear calibration curves were obtained by plotting the peak areas of the individual compounds as a function of the concentration using the GraphPad Prism program for Windows (GraphPad Software, Inc., San Diego, CA). The standard curves were used to determine the recovery of the chemicals from the plasma and urine samples.

Limits of detection and limits of quantitation

The limits of detection (LODs) and limits of quantitation (LOQs) were determined at the lowest concentration to be detected or quantitated, taking into consideration a 1:3 and 1:10 ratio of the baseline noise and calibration point, respectively. The LOQ was repeated five times for confirmation.

Results

Standard calibration curves

The standard calibration curves of the peak area versus the concentrations of diazinon, diazoxon, 2-isopropyl-6-methyl-4-pyrimidinol, chlorpyrifos, chlorpyrifos-oxon, and TCP are shown in Figure 2. The calibration plots for all of the analytes were linear over the concentrations range of 200 to 2000 ng/mL, and the correlation coefficient values were between 0.997 and 0.999.

Chromatogram

Chromatographic profiles were obtained for spiked rat plasma and urine samples with a 500-ng/mL concentration of the analytes after SPE using Sep-Pak cartridges under HPLC conditions described previously (Figures 3 and 4). The retention times were 12.5 min for diazinon, 8.2 min for diazoxon, 6.9 min for 2-isopropyl-6-methyl-4-pyrimidinol, 13.4 min for chlorpyrifos, 11.2 min for chlorpyrifos-oxon, and 9.3 min for TCP. The total run time was 16 min. No interference from endogenous substances in the plasma and urine samples were shown in the chromatogram.

Extraction efficiency and recovery

The extraction recoveries of diazinon, diazoxon, 2-isopropyl-6-methyl-4-pyrimidinol, chlorpyrifos, chlorpyrifos-oxon, and TCP from fortified samples were determined at concentrations ranging from 200 to 2000 ng/mL (Tables I and II). Spiked plasma and urine samples were extracted and analyzed for each concentration in five replicates. The average percentage recoveries from plasma were 86.3 ± 8.6, 77.4 ± 7.0, 82.1 ± 8.2, 81.8 ± 8.7, 73.1 ± 7.4, and 80.3 ± 8.0 and from urine were 81.8 ± 7.6, 76.6 ± 7.1, 81.5 ± 7.9, 81.8 ± 7.1, 73.7 ± 8.6, and 80.7 ± 7.7 for diazinon, diazoxon, 2-isopropyl-6-methyl-4-pyrimidinol, chlorpyrifos, chlorpyrifos-oxon, and TCP, respectively.

Table I. Percent Recovery of Diazinon, Chlorpyrifos, and Metabolites from Rat Plasma*

Concentration (ng/mL)	Diazinon	Diazoxon	2-Isopropyl-6-methyl-4-pyrimidinol	Chlorpyrifos	Chlorpyrifos-oxon	TCP
200	84.6 ± 8.9	72.9 ± 10.3	80.5 ± 8.9	80.3 ± 8.0	71.3 ± 5.6	79.3 ± 6.6
400	83.2 ± 6.7	78.1 ± 6.9	81.6 ± 10.3	76.5 ± 9.2	71.9 ± 8.7	78.4 ± 12.1
500	89.1 ± 7.4	76.3 ± 5.2	80.2 ± 6.9	81.2 ± 7.4	75.6 ± 8.9	79.8 ± 5.8
1000	88.5 ± 10.5	80.6 ± 4.7	85.1 ± 6.5	84.2 ± 7.8	72.5 ± 7.2	81.4 ± 7.3
2000	86.2 ± 9.4	79.3 ± 8.1	83.2 ± 8.2	87.0 ± 11.1	74.2 ± 6.8	82.6 ± 8.2

* Values are expressed as the mean ± the standard deviation from five replicates.

Table II. Percent Recovery of Diazinon, Chlorpyrifos, and Metabolites from Rat Urine*

Concentration (ng/mL)	Diazinon	Diazoxon	2-Isopropyl-6-methyl-4-pyrimidinol	Chlorpyrifos	Chlorpyrifos-oxon	TCP
200	81.5 ± 6.3	72.5 ± 8.2	80.6 ± 5.6	77.1 ± 4.2	68.9 ± 5.8	79.2 ± 6.5
400	83.6 ± 6.1	78.6 ± 6.3	82.6 ± 5.2	81.3 ± 8.6	71.4 ± 8.2	83.2 ± 6.3
500	80.1 ± 10.3	74.1 ± 7.5	80.4 ± 9.2	78.9 ± 6.8	75.2 ± 9.4	79.2 ± 8.1
1000	81.0 ± 8.7	80.1 ± 4.5	81.2 ± 10.9	84.2 ± 6.4	77.4 ± 7.8	81.5 ± 8.6
2000	82.6 ± 6.7	77.8 ± 9.1	82.5 ± 8.5	87.6 ± 9.4	75.6 ± 11.6	80.4 ± 9.1

* Values are expressed as the mean ± the standard deviation from five replicates.

LODs and LOQs

Blank plasma and urine samples from untreated rats were used as references for the plasma and urine collections. The LODs and LOQs in the plasma and urine samples were calculated from a peak signal-to-noise ratio of 3:1 and 10:1, respectively (Table III).

Application of the method to biological samples

The method was used for the determination of the parent compounds and their metabolites following a combined dermal administration in rats. The rats were exterminated at 12 h following dosing. Levels of the compounds detected in the plasma and urine samples of treated rats are

shown in Table IV. The results were corrected based on recoveries of the previously mentioned chemicals from the plasma and urine samples.

Discussion

The present study reports the development of an HPLC method for the quantitative analysis of diazinon, chlorpyrifos, and their metabolites in the plasma and urine of treated rats. The chromatogram obtained following SPE and HPLC analysis showed no interference from plasma and urine endogenous substances, indicating that an efficient cleanup method was used. Recoveries of the chemicals and metabolites were suitable for the application of the method used for the analysis of treated samples for parent compounds and their metabolites. In this method, recoveries differed with individual chemicals. Recoveries of the chemicals analyzed in this method were between 73 and 86%.

The LODs reported in this method allowed for the analysis of samples from treated animals following doses resembling real-life exposure. The ability to detect parent compounds and metabolites in plasma after 12 h of dosing exemplifies the method's suitability. 2-Isopropyl-6-methyl-4-pyrimidinol and TCP were detected in urine samples after 12 h of dosing. The failure to detect chlorpyrifos-oxon and TCP in rat plasma might be because of their rapid metabolism and conjugation following absorption (16). The reported LODs in this method were reasonably taken into consideration using HPLC with its applicability for the determination of polar metabolites and the simultaneous determination of the six compounds. In a previous study, the LODs of the chlorpyrifos metabolite TCP in subjects' urine was 1.2 µg/L using GC (21), and the LOD of chlorpyrifos in blood using GC–MS was 0.7 ng/mL (16).

Table III. LODs and LOQs of Diazinon, Chlorpyrifos, and Their Metabolites

Compound	LODs (ng/mL)	LOQs (ng/mL)	
		Plasma	Urine
Diazinon	100		150
200			
Diazoxon	50		
150		150	
2-Isopropyl-6-	50		150
100			
methyl-pyrimidinol			

Table IV. Concentrations of Diazinon, Chlorpyrifos, and Their Metabolites in Rat Plasma and Urine Twelve Hours After Dosing*

Compound	Plasma	Urine
Diazinon	214.5 ± 113	n.d.†
Diazoxon	196.8 ± 15.4	n.d.
2-Isopropyl-6-methyl-4-pyrimidinol	234.5 ± 28.9	492.4 ± 161.6
Chlorpyrifos	426 ± 103	n.d.
Chlorpyrifos-oxon	n.d.	n.d.
TCP	n.d.	218 ± 69

* Values are expressed in nanograms per milliliter as the mean ± the standard deviation from five animals.
† n.d., below the detection limits of the method.

Conclusion

A rapid and simple HPLC method was developed for the separation and residual determination of diazinon, chlorpyrifos, and their metabolites in plasma and urine samples of treated rats with these xenobiotics. The method could be applied routinely for the monitoring of these chemicals in the plasma and urine samples of persons exposed to these combined chemicals. Also, this method could be used in toxicokinetic studies to assess distribution of the parent compounds and metabolites in body tissues and fluids. The main feature of this method is the ability to analyze simultaneously the two chemicals and their metabolites under similar conditions, thus saving time and expenses for sample preparation.

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