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Short communication

# 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

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**Abstract**

A method was developed for the separation and quantification of the insecticide chlorpyrifos (*O*,*O*-diethyl-*O*[3,5,6-trichloro-2-pyridinyl] phosphorothioate), its metabolites chlorpyrifos-oxon (*O*,*O*-diethyl-*O*[3,5,6-trichloro-2-pyridinyl] phosphate) and TCP (3,5,6-trichloro-2-pyridinol), the anti-nerve agent drug pyridostigmine bromide (PB; 3-dimethylaminocarbonyloxy-*N*-methyl pyridinium bromide), its metabolite *N*-methyl-3-hydroxypyridinium bromide, the insect repellent DEET (*N*,*N*-diethyl-*m*-toluamide), and its metabolites *m*-toluamide and *m*-toluic acid in rat plasma and urine. The method is based on using solid-phase extraction and high-performance liquid chromatography (HPLC) with reversed-phase $C_{18}$ column, and gradient UV detection ranging between 210 and 280 nm. The compounds were separated using a gradient of 1–85% acetonitrile in water (pH 3.20) at a flow-rate ranging between 1 and 1.7 ml/min over a period of 15 min. The retention times ranged from 5.4 to 13.2 min. The limits of detection ranged between 20 and 150 ng/ml, while the limits of quantitation were between 150 and 200 ng/ml. Average percentage recovery of five spiked plasma samples was 80.2±7.9, 74.9±8.5, 81.7±6.9, 73.1±7.8, 74.3±8.3, 80.8±6.6, 81.6±7.3 and 81.4±6.5, and from urine 79.4±6.9, 77.8±8.4, 83.3±6.6, 72.8±9.0, 76.3±7.7, 83.4±7.9, 81.6±7.9 and 81.8±6.8 for chlorpyrifos, chlorpyrifos-oxon, TCP, pyridostigmine bromide, *N*-methyl-3-hydroxypyridinium bromide, DEET, *m*-toluamide and *m*-toluic acid, respectively. The relationship between peak areas and concentration was linear over a range between 200 and 2000 ng/ml. 

*Keywords:* Chlorpyrifos; Pyridostigmine bromide; *N*,*N*-Diethyl-*m*-toluamide; Combined exposure

## 1. Introduction

Pyridostigmine bromide is used for the treatment of myasthenia gravis patients and was used as a prophylaxis against organophosphate nerve agents [1–3]. Chlorpyrifos is a widely used insecticide in agriculture as well as indoors to control flies and biting insects [2,4]. *N*,*N*-diethyl-*m*-toluamide (DEET) is applied as an insect repellent against mosquitoes and other biting insects [5]. It is estimated that more than 250 000 US veterans were exposed to these chemicals [1–3]. Previous reports implicated combined exposure to these chemicals with Gulf War Illness [2,6–9].

Analytical methods have been developed to analyze these chemicals in plasma and urine when

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applied individually [10–26]. These methods used high-performance liquid chromatography [15,23,24,26–30], high-performance liquid chromatography–mass spectrometry [15], gas chromatography [21,31–35], and gas chromatography–mass spectrometry [36–38]. Recently, we have developed a method for the combined analysis of permethrin, another insecticide used during the Gulf War, DEET and pyridostigmine in rat plasma and urine [39].

We have demonstrated that combined exposure to pyridostigmine bromide, DEET, and chlorpyrifos produced more neurological deficit than that caused by each compound alone [2]. We hypothesized that combined chemical exposure diminished the body's ability to detoxify test compounds since they compete for the same detoxifying enzymes. To test this hypothesis, in vivo and in vitro studies are being carried out to study the metabolism and pharmacokinetics of test compounds alone and in combination. To achieve this goal, our first task was to develop a method for the simultaneous analysis of these compounds and their metabolites. This study reports on the development and validation of a method for simultaneous analysis of the above chemicals and their metabolites in rat plasma and urine using solid-phase extraction coupled with reversed-phase-high-performance liquid chromatography.

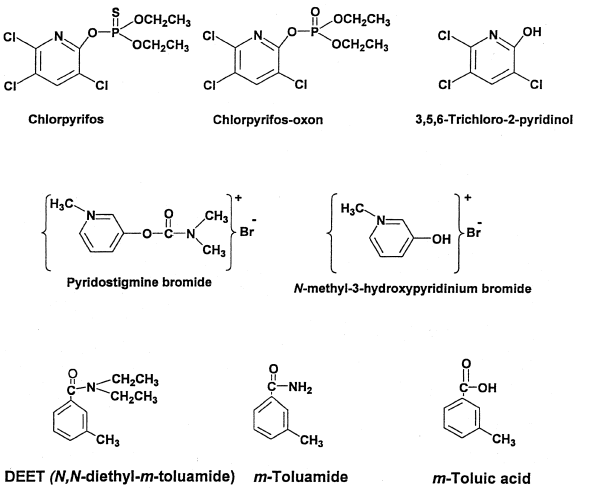


Fig. 1. Structures of chlorpyrifos, chlorpyrifos-oxon, TCP (3,5,6-trichloro-2-pyridinol), pyridostigmine bromide, *N*-methyl-3-hydroxypyridinium bromide, DEET (*N,N*-diethyl-*m*-toluamide), *m*-toluamide and *m*-toluic acid.

## 2. Experimental

### 2.1. Chemicals and materials

Chlorpyrifos (99% *O,O*-diethyl-*O*[3,5,6-trichloro-2-pyridinyl] phosphorothioate) was purchased from Chem Service (West Chester, PA, USA) (Fig. 1). Chlorpyrifos-oxon (98% *O,O*-diethyl-*O*[3,5,6-trichloro-2-pyridinyl] phosphate) was obtained from Dow (Midland, MI, USA). TCP (3,5,6-trichloro-2-pyridinol) was prepared in our laboratory. Briefly, 500 mg of chlorpyrifos was dissolved in 2 ml of ethanol, to this solution 2 ml of 1 N sodium hydroxide solution was added and the reaction mixture was stirred at room temperature for 24 h. The pH of the solution was adjusted to 3.00 using 1 N acetic acid. The solution was allowed to evaporate. The residue was crystallized in ethanol. Then TCP was collected and dried. TCP identity and purity was determined by HPLC (96%) using a pure authentic standard. DEET (>97% *N,N*-diethyl-*m*-toluamide) was obtained from Aldrich (Milwaukee, WI, USA), Pyridostigmine bromide (PB; 3-dimethylaminocarbonyloxy-*N*-methyl pyridinium bromide) was obtained from Sigma (St. Louis, MO, USA). *m*-Toluamide, and *m*-toluic acid were purchased from Fisher Scientific (Pittsburgh, PA, USA) and *N*-methyl-3-hydoxypyridinium bromide was prepared following the method of Somani et al. [20]. Water (HPLC grade) and acetonitrile were obtained from Mallinckrodt Baker (Paris, KY, USA). $C_{18}$ Sep-Pak® cartridges were obtained from Waters (Waters, Milford, MA, USA).

### 2.2. Instrumentation

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

### 2.3. Sample preparation

Rat plasma (0.2 ml) and urine (1.0 ml) samples were spiked with concentrations ranging between 200 and 2000 ng/ml each of chlorpyrifos, chlorpyrifos-oxon, TCP, pyridostigmine bromide, *N*-methyl-3-hydroxypyridinium bromide, DEET, *m*-toluamide, and *m*-toluic acid. Spiked and treated samples were acidified with 1 N acetic acid (pH 5.0). Disposable $C_{18}$ Sep-Pak Vac 3-$cm^3$ (500 mg) cartridges (Waters, Milford, MA) were conditioned with 3 ml of acetonitrile, then equilibrated using 3 ml of water before use. The spiked urine and plasma samples were vortexed for 30 s, centrifuged for 5 min at 1000 *g*, and the supernatant was loaded into the disposable cartridges, then washed with 2 ml of water, and eluted twice with 1 ml of methanol, then twice with 1 ml of acetonitrile, and reduced to 500 μl using a stream of nitrogen, prior to analysis by HPLC.

### 2.4. Chromatographic conditions

A 10-μl solution of plasma or urine residues was injected into HPLC. The mobile phase was water (adjusted to pH 3.20 using 1 N acetic acid). The acetonitrile gradient flow-rate was 1.0 ml/min from zero time to 6 min, then increased to 1.7 ml/min (6–13 min), followed by a return to 1.0 ml/min where it remained for 2 min to re-equilibrate the system. The gradient started at 1% acetonitrile, increased to 75% acetonitrile at 6 min, then increased to 85% acetonitrile by 11 min. Then the system returned to 1% acetonitrile at 15 min where it was kept under this condition for 2 min to re-equilibrate. The eluents were monitored by UV detection of wavelength of 280 nm for chlorpyrifos, chlorpyrifos-oxon, TCP, pyridostigmine bromide and *N*-methyl-3-hydroxypyridinium bromide, and a wavelength of 210 nm for DEET, *m*-toluamide, and *m*-toluic acid. The chromatographic analysis was performed at ambient temperature.

### 2.5. Calibration procedures

Five different calibration standards of a mixture of chlorpyrifos, chlorpyrifos-oxon, TCP, pyridostigmine bromide, *N*-methyl-3-hydroxypyridinium bromide, DEET, *m*-toluamide, and *m*-toluic acid 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 chemicals as a function of the concentration using GraphPad Prism program for windows (GraphPad Software, San Diego, CA, USA). The standard curves were used to determine recovery of the chemicals from plasma and urine samples.

### 2.6. Limits of detection (LOD) and limits of quantitation (LOQ)

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

## 3. Results and discussion

The standard calibration curves of peak area against concentration of chlorpyrifos, chlorpyrifos-oxon, TCP, pyridostigmine bromide, *N*-methyl-3-hydroxypyridinium bromide, DEET, *m*-toluamide and *m*-toluic acid are shown in Fig. 2. Linearity of the calibration curves for the three compounds was

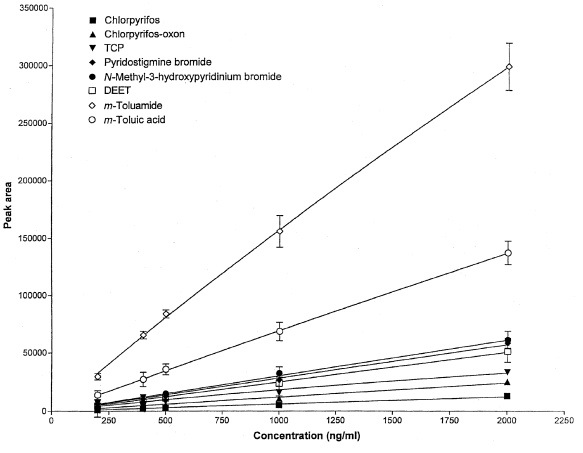


Fig. 2. Standard calibration curves of chlorpyrifos, chlorpyrifos-oxon, TCP (3,5,6-trichloro-2-pyridinol), pyridostigmine bromide, *N*-methyl-3-hydroxypyridinium bromide, DEET (*N,N*-diethyl-*m*-toluamide), *m*-toluamide and *m*-toluic acid.

achieved at concentrations ranging from 200 to 2000 ng/ml. Chromatographic profiles were obtained for rat plasma and urine samples under HPLC conditions, described above (Figs. 3 and 4). Retention times were 9.2, 11.3, 13.2, 6.6, 5.4, 9.9, 7.5 and 8.6 min for TCP, chlorpyrifos-oxon, chlorpyrifos, pyridostigmine bromide, *N*-methyl-3-hydroxypyridinium bromide, DEET, *m*-toluamide and *m*-toluic acid, respectively. The total run time was 15 min. Clean chromatogram shows no interference from endogenous substances in plasma or urine samples.

The average extraction recoveries were determined at concentrations ranging between 200 and 2000 ng/ml (Tables 1 and 2). Spiked plasma and urine samples were extracted and analyzed for each concentration in five replicates. Average percentage recoveries from plasma were 80.2±7.9, 74.9±8.5, 81.7±6.9, 73.1±7.8, 74.3±8.3, 80.8±6.6, 81.6±7.3 and 81.4±6.5, and from urine 79.4±6.9, 77.8±8.4, 83.3±6.6, 72.8±9.0, 76.3±7.7, 83.4±7.9, 81.6±7.9 and 81.8±6.8 for chlorpyrifos, chlorpyrifos-oxon, TCP, pyridostigmine bromide, *N*-methyl-3-hydroxypyridinium bromide, DEET, *m*-toluamide and *m*-toluic acid, respectively. The low recovery for pyridostigmine bromide might have resulted from the use of a solvent system that was not quite suitable for extracting pyridostigmine bromide, but at the same time it was needed for extracting and analyzing the other two chemicals and metabolites under similar conditions. Also hydrolysis of pyridostigmine bromide during extraction is possible [37]. Percentage recovery depends on the matrix, extracting solvent, method of analysis, and the amount to be analyzed. Recoveries of DEET from serum and urine were reported to be 93–95, and 65–70%, respectively using GC–MS as an analytical technique [36], while recovery of DEET from water samples was 45.6% using micellar kinetic chromatography method [40]. Hennis et al. [22] reported a recovery of 50% of *N*-methyl-3-hydroxypyridinium from dog plasma and urine, while Chan et al. [38] reported a recovery of 82% of pyridostigmine bromide from plasma at low concentration of 50 ng/ml, while its recovery was 92% when a concentration of 400 ng/ml was used. In this method, recoveries differed with individual chemicals. Recovery of the analyzed chemicals in this method was between 55 and 83%. This range lies within the reported values in the literature, taking into consideration simultaneous

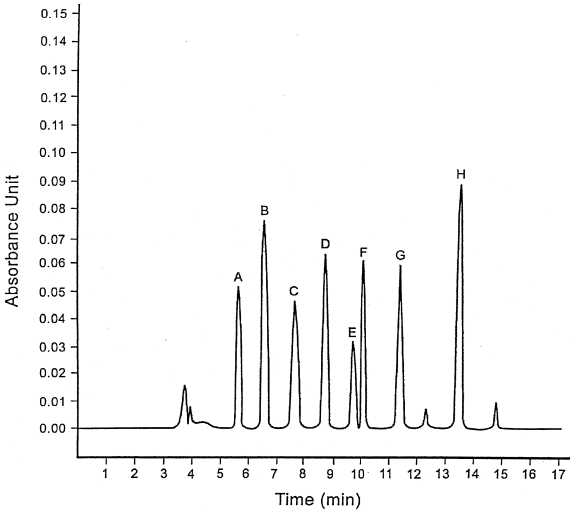


Fig. 3. Chromatogram of spiked plasma sample with concentrations of 500 ng/ml of: (A) *N*-methyl-3-hydoxypyridinium bromide; (B) PB (pyridostigmine bromide); (C) *m*-toluamide; (D) *m*-toluic acid; (E) TCP; (F) DEET; (G) chlorpyrifos-oxon; and (H) chlorpyrifos under established HPLC conditions.

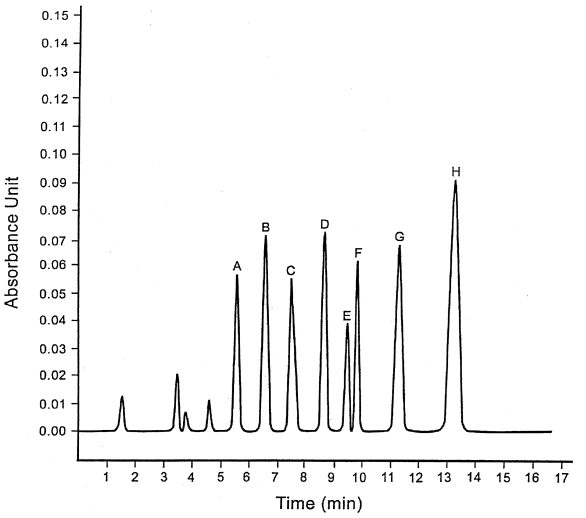


Fig. 4. Chromatogram of spiked urine sample with concentrations of 500 ng/ml of: (A) *N*-methyl-3-hydoxypyridinium bromide; (B) PB (pyridostigmine bromide); (C) *m*-toluamide; (D) *m*-toluic acid; (E) TCP; (F) DEET; (G) chlorpyrifos-oxon; and (H) chlorpyrifos under established HPLC conditions.

Table 1
% Recovery[a] of chlorpyrifos, pyridostigmine bromide, DEET and metabolites from rat plasma

| Concentration (ng/ml) | Chlorpyrifos | Chlorpyrifos-oxon | TCP | Pyridostigmine bromide | *N*-Methyl-3-hydroxy-pyridinium bromide | DEET | *m*-Toluamide | *m*-Toluic acid |
|---|---|---|---|---|---|---|---|---|
| 2000 | 82.0±7.6 | 76.5±6.9 | 87.5±5.9 | 75.2±6.9 | 73.7±9.2 | 83.6±6.2 | 89.1±6.9 | 88.2±6.9 |
| 1000 | 85.4±8.2 | 78.0±9.1 | 83.9±5.8 | 74.8±9.1 | 78.2±6.3 | 80.2±7.2 | 85.6±9.1 | 86.8±7.9 |
| 500 | 79.6±8.3 | 74.8±10.4 | 77.6±9.7 | 74.1±7.6 | 75.2±9.0 | 83.2±5.6 | 82.7±6.3 | 80.6±5.2 |
| 400 | 79.0±7.2 | 71.7±8.0 | 80.4±6.1 | 70.2±8.4 | 74.2±10.2 | 84.2±9.1 | 76.9±5.8 | 76.3±8.4 |
| 200 | 75.2±8.5 | 73.4±8.2 | 79.1±6.9 | 71.2±7.2 | 70.1±6.8 | 72.8±4.9 | 73.8±8.2 | 75.1±4.1 |

[a] Values are expressed as mean±SD of five replicates.

analysis of the parent chemicals and their metabolites.

Limits of detection and quantitation were calculated from a peak signal-to-noise ratio of 3:1 and 10:1, respectively. The resulting detection limits were 150, 150, 100, 100, 100, 50, 20 and 30 ng/ml for chlorpyrifos, chlorpyrifos-oxon, TCP, pyridostigmine bromide, *N*-methyl-3-hydroxypyridine, DEET, *m*-toluamide and *m*-toluic acid, respectively. Limits of quantitation in plasma were determined to be 200 ng/ml for chlorpyrifos, chlorpyrifos-oxon, pyridostigmine bromide and *N*-methyl-3-hydroxypyridine, 150 ng/ml for TCP, and 100 ng/ml for DEET, *m*-toluamide and *m*-toluic acid. In urine limits of quantitation were 200, 200, 150, 150, 150, 100, 100 and 100 ng/ml for chlorpyrifos, chlorpyrifos-oxon, TCP, pyridostigmine bromide, *N*-methyl-3-hydroxypyridine, DEET *m*-toluamide and *m*-toluic acid, respectively. Limits of detection and quantification depended upon the nature of the matrix, rate of application, and method of analysis [16,37]. Limit of detection of chlorpyrifos metabolite TCP in urine was 12 μg/l using GC method [34], while limit of quantitation of chlorpyrifos in blood using GC–MS technique was 0.7 ng/g blood [14]. Pyridostigmine bromide and DEET were also detected in urine samples at 10 h. Hennis et al. [22] reported 50 ng/ml as a limit of detection of *N*-methyl-3-hydroxypyridinium in dog plasma using ion-exchange liquid chromatography. Limit of detection of pyridostigmine bromide in plasma was 10 ng/ml [28], and ranged between 2.7 and 18.6 ng/ml in plasma using GC [38]. The detection limit of DEET was 90 ng/ml and 90 ng/g from urine and serum, respectively, using HPLC–UV method [23], and 15 ng/ml for DEET in human and dog plasma using HPLC [29], while it was 25 ng in cosmetic products using HPTLC method [41]. Limits of detection reported in this method (20–150 ng/ml) are achieved taking into consideration the simultaneous analysis of the combined chemicals and their metabolites.

A rapid and simple HPLC method was developed for simultaneous separation and residual determination of chlorpyrifos, pyridostigmine bromide,

Table 2
% Recovery[a] of chlorpyrifos, pyridostigmine bromide, DEET and metabolites from rat urine

| Concentration (ng/ml) | Chlorpyrifos | Chlorpyrifosoxon | TCP | Pyridostigmine bromide | *N*-Methyl-3-hydroxy-pyridinium bromide | DEET | *m*-Toluamide | *m*-Toluic acid |
|---|---|---|---|---|---|---|---|---|
| 2000 | 84.1±9.1 | 82.8±8.9 | 90.1±6.9 | 72.3±12.8 | 79.2±7.6 | 83.9±7.3 | 86.2±4.5 | 86.1±5.8 |
| 1000 | 83.2±6.4 | 78.1±6.5 | 83.6±7.2 | 71.6±9.4 | 76.5±8.7 | 84.2±9.7 | 83.9±10.3 | 83.9±9.2 |
| 500 | 79.5±6.3 | 74.6±9.4 | 80.9±6.7 | 74.6±7.3 | 77.3±8.10 | 89.1±6.2 | 82.1±5.8 | 81.0±6.2 |
| 400 | 78.0±9.2 | 75.9±6.2 | 81.6±8.3 | 78.6±6.4 | 73.8±6.0 | 81.5±6.7 | 76.6±8.2 | 78.7±6.8 |
| 200 | 72.3±6.5 | 77.6±10.8 | 80.4±3.8 | 67.2±9.2 | 74.5±8.2 | 78.4±9.5 | 79.2±11.1 | 79.2±6.1 |

[a] Values are expressed as mean±SD of five replicates.

DEET and selected metabolites in rat plasma and urine samples. This method could be used in studies to assess pharmacokinetic profiles of these compounds, alone and in combination.

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