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Quantification of nicotine, chlorpyrifos and their metabolites in rat plasma and urine using high-performance liquid chromatography

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Abstract

This study describes a high-performance liquid chromatographic method for the separation and quantification of nicotine, its metabolites nornicotine and cotinine, 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. The compounds were separated using gradient mobile phase of methanol, acetonitrile and water (pH 3.20) at a flow-rate of 0.8 ml/min in a period of 17 min, and gradient UV detection ranging between 260 and 280 nm. The retention times ranged from 3.4 to 16.7 min. The limits of detection were ranged between 20 and 150 ng/ml, while limits of quantitation were 50–200 ng/ml. Average percentage recovery of five spiked plasma samples were 84.7±8.3, 78.2±7.6, 80.1±7.6, 79.0±6.4, 74.0±7.4, 87.6±7.5, and from urine 85.1±5.2, 75.9±7.0, 82.1±6.1, 79.5±6.1, 71.3±7.4 and 81.3±6.9 for nicotine, nornicotine, cotinine chlorpyrifos, chlorpyrifos-oxon and TCP, respectively. Intra-day accuracy and precision for this method were ranged between 2.2–3.6 and 2.1–2.8%, respectively. The relationship between peak areas and concentration was linear over range between 200 and 2000 ng/ml. This method was applied to analyze the above chemicals and metabolites following combined oral administration in rats.

Keywords: Nicotine; Chlorpyrifos; Nornicotine; Cotinine; Chlorpyrifos-oxon; 3,5,6-Trichloro-2-pyridinol

1. Introduction

Nicotine is a natural alkaloid obtained from the leaves of the tobacco plant, *Nicotiana tabacum* [1]. People are exposed to nicotine during smoking or inhalation of environmental tobacco smoke [2]. Chlorpyrifos is a widely used organophoshorous insecticide in agriculture and indoors [3]. Exposure to chlorpyrifos resulted in toxic signs attributed to inhibition of acetycholinesterase enzymes [4]. Cigarette smoking increases the risk of lung and heart diseases [5], and induces oxidative stress and DNA damage [6,7]. Nicotine is primarily metabolized to cotinine in human liver [8], even other metabolites such as nornicotine, norcotinine and hydroxycotinine have been identified in human urine [9]. Analytical methods have been developed for identification and quantification of the above chemicals and their metabolites, when applied alone in plasma and urine samples [10–17]. These methods used high-performance liquid chromatography [12,18–20], high-performance liquid chromatog-

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raphy–mass spectrometry [9,21,22], gas chromatography [23], gas chromatography–mass spectrometry [10,11,16,17,24,25], and thin-layer chromatography with densitometry [10]. Both nicotine and chlorpyrifos act on nicotinic acetylcholine receptor (AChR) and cause adverse effects on the reproductive and developmental system [26]. Nicotine is a direct agonist for AChR while chlorpyrifos acts indirectly on AChR via inhibiting acetylcholinesterase and then accumulation of acetylcholine at the receptor. As a result, both chemicals cause overstimulation of nicotinic AChR. We hypothesized that combined exposure to the two chemicals could generate toxic interactions. To examine this possibility, we plan to study the pharmacokinetic interactions between nicotine and chlorpyrifos in rats. The first task is to develop a method for simultaneous analysis of these chemicals and their metabolites following combined exposure in rats. This study reports on a method for simultaneous analysis of nicotine, chlorpyrifos and their metabolites in rat plasma and urine using solid-phase extraction coupled with reversed-phase high-performance liquid chromatography.

2. Experimental

2.1. Chemicals and materials

Nicotine (98%), nornicotine (98%) and cotinine (99%) (Fig. 1) were obtained from Sigma (St. Louis, MO, USA). Chlorpyrifos (99% *O*,*O*-diethyl-*O*[3,5,6-trichloro-2-pyridinyl]phosphorothioate) was purchased from Chem Service (West Chester, PA, USA). Chlorpyrifos-oxon (*O*,*O*-diethyl-*O*[3,5,6-trichloro-2-pyridinyl]phosphate) was obtained from Dow Chemical (Midland, MI, USA). TCP (96% 3,5,6-trichloro-2-pyridinol) was prepared in our laboratory. Water (HPLC grade) and acetonitrile were obtained from Mallinckrodt Baker (Paris, KY, USA). C_{18} Sep-Pak^{R} cartridges were obtained from Waters (Waters, Milford, MA, USA).

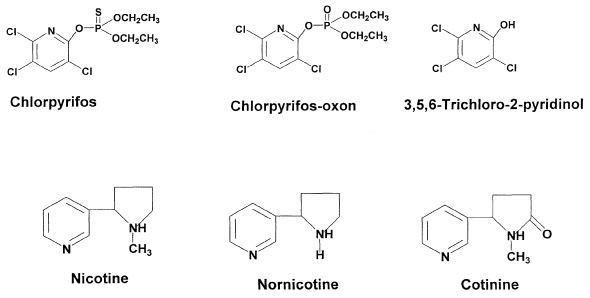

Fig. 1. Structures of nicotine, nornicotine, cotinine, chlorpyrifos, chlorpyrifos-oxon and TCP.

2.2. Animals

Rats (Sprague Dawley) were purchased from Zivic Miller (Zelienople, PA, USA). Five rats were treated with a combined single oral dose of 10 mg/kg of nicotine and a single dose of 10 mg/kg of chlorpyrifos. Five untreated control rats were treated with oral dose of ethanol. Urine samples were collected from treated and control rats, 12 h after dosing. Then the animals were anesthetized with halothane and sacrificed by heart exsanguinations. Blood was collected via heart puncture with a heparinized syringe and centrifuged at 2400 rpm for 15 min at 5°C to separate plasma.

2.3. Instrumentation

The liquid chromatographic system (Waters 2690 Separation Module), consisted of a Waters 600E Multisolvent delivery system pumps, a Waters Ultra WISP 715 autoinjector, and a Waters 2487 Dual λ absorbance detector (Waters). 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} 125A° 10 μm, 3.9×300 mm were used (Waters).

2.4. Sample preparation

Plasma (0.5 ml) and urine (1.0 ml) samples from untreated rats were spiked with concentrations ranging between 200 and 2000 ng/ml of each of nicotine, nornicotine, cotinine, chlorpyrifos, chlorpyrifos-oxon and TCP. Spiked and treated samples were acidified with 1 N acetic acid (pH 4.0). Disposable C_{18} Sep-Pak Vac 3cc (500 mg) cartridges (Waters) 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, centrifuged for 5 min at 1000 *g*, and the supernatant was loaded into the disposable cartridges, then washed with 3 ml of water, and eluted 2 times by 2 ml of methanol and reduced to 500 μl using stream of nitrogen, prior to analysis by HPLC.

Table 1
HPLC conditions

Time (min)	Flow rate (ml/min)	Wavelength (nm)	%*A* (Water pH 3.2)	%*B* (Acetonitrile)	%*C* (Methanol)
0	0.8	260	90	0	10
7	0.8	260	75	25	0
8	0.8	280	75	25	0
10	0.8	280	60	40	0
12.5	0.8	280	50	50	0
14	0.8	260	90	0	10

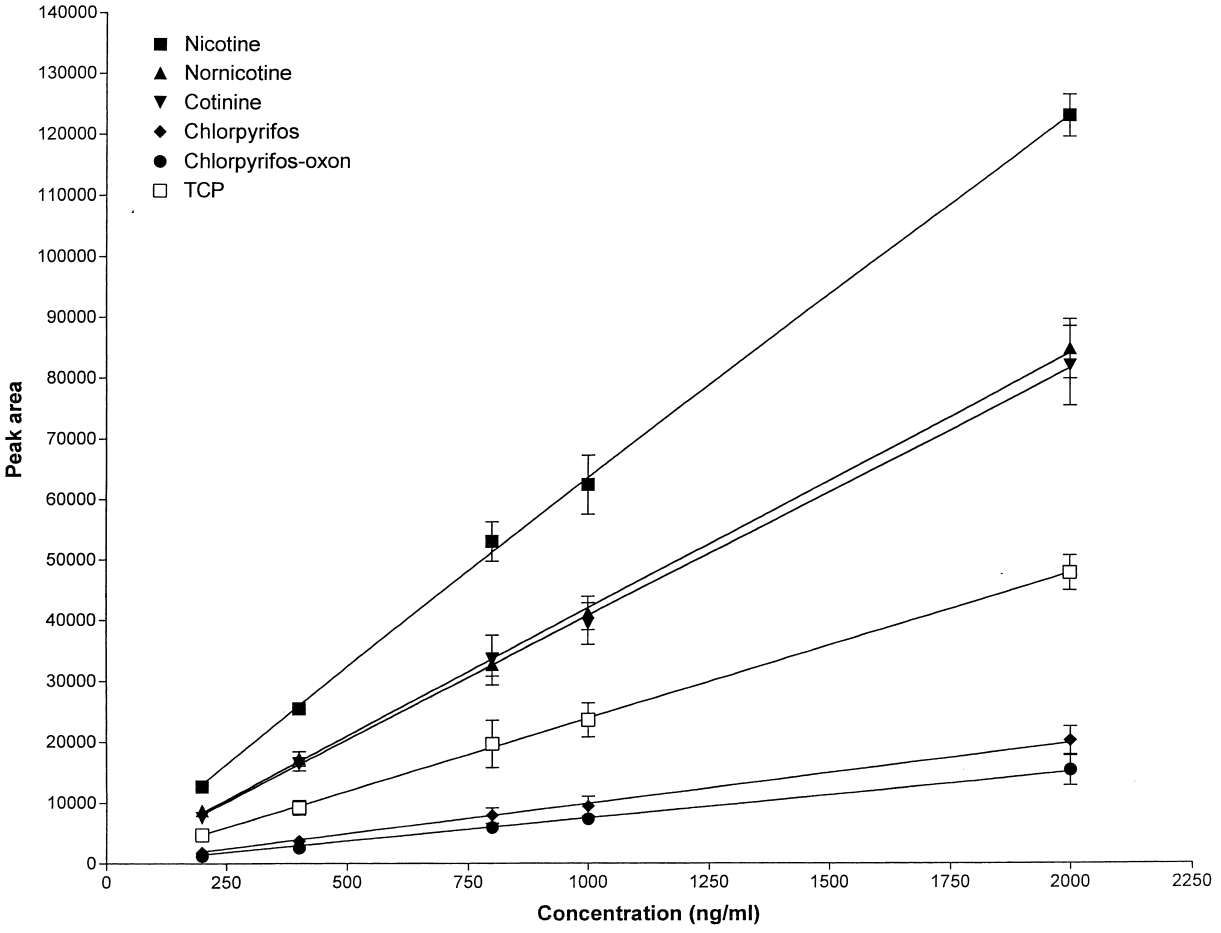

Fig. 2. Standard calibration curves of nicotine, nornicotine, cotinine, chlorpyrifos, chlorpyrifos oxon and TCP.

2.5. Chromatographic conditions

A 10 μl solution of plasma or urine residues was injected into HPLC. HPLC conditions are shown in Table 1. The chromatographic analysis was performed at ambient temperature.

2.6. Calibration procedures

Five different calibration standards of a mixture of nicotine, nornicotine, cotinine, chlorpyrifos, chlorpyrifos-oxon and TCP were prepared in acetonitrile. Their concentrations ranged from 200 to 2000 ng/ml. The standard curves were used to determine recovery of the chemicals from plasma and urine samples.

2.7. Accuracy and precision

Intra-day precision and accuracy of the method were determined in plasma, and urine samples spiked with the compounds. Plasma and urine samples ($n=5$) were spiked with concentrations of 100, 200, 400, 500, and 1000 ng/ml. The samples were analyzed on the same day. The relative error percentage accuracy was determined as mean of detected concentration/added concentration$\times$100. For determination of precision, the coefficient of variation (C.V.) was calculated.

2.8. 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 nicotine, nornicotine, cotinine, chlorpyrifos, chlorpyrifos-oxon and TCP are shown in Fig. 2. Linearity of the calibration curves for the three compounds was achieved at concentrations ranging from 200 to 2000 ng/ml. This range was selected taking into consideration the lowest level of limits of quantitation (200 ng/ml) of the above chemicals. In previous study, linearity of nicotine was achieved over 10–10000 ng/ml [21]. Chromatographic profiles were obtained for rat plasma and urine samples after solid-phase extraction using C_{18} Sep Pak^R cartridges under HPLC conditions as described above (Figs. 3 and 4). The chromatogram shows no interference from plasma and urine endogenous substances, indicating an efficient cleanup method using solid-phase extraction and UV detection.

The average extraction recoveries of nicotine, nornicotine, cotinine, chlorpyrifos, chlorpyrifos-oxon and TCP were determined at concentrations ranged between 200 and 2000 ng/ml, taking into consideration the highest LOQ of analyte (200 ng/ml) (Tables 2 and 3). Average percentage recoveries were: from plasma, 84.7±8.3, 78.2±7.6, 80.1±7.6, 79.0±6.4, 74.0±7.4 and 87.6±7.5; and from urine, 85.1±5.2, 75.9±7.0, 82.1±6.1, 79.5±6.1, 71.3±7.4 and 81.3±6.9 for nicotine, nornicotine, cotinine, chlorpyrifos, chlorpyrifos-oxon and TCP, respectively. The low recovery of chlorpyrifos-oxon might due to its rapid degradation. The reported range of recoveries in this method lies within the reported values in the literature [20], taking into consideration

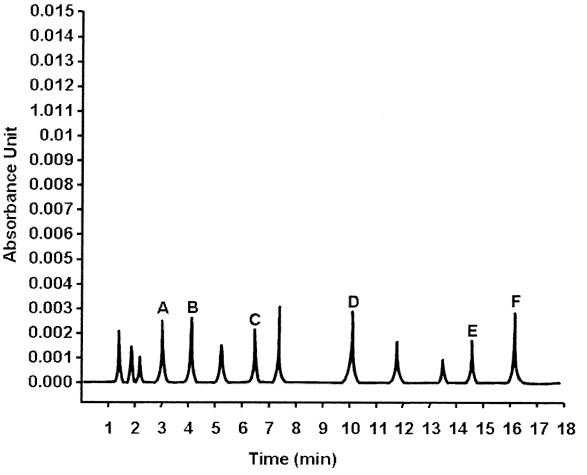

Fig. 3. Chromatogram of spiked plasma sample with concentrations represent limits of detection (LOD): (A) nornicotine (30 ng/ml), (B) nicotine (20 ng/ml), (C) cotinine (20 ng/ml), (D) TCP (100 ng/ml), (E) chlorpyrifos-oxon (150 ng/ml), and (F) chlorpyrifos (150 ng/ml) under established HPLC conditions.

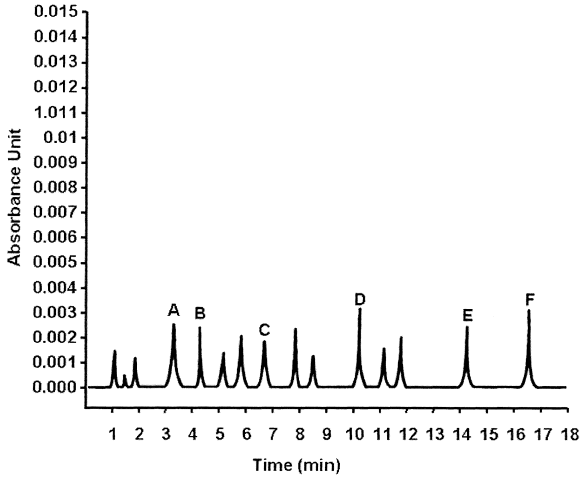

Fig. 4. Chromatogram of spiked urine sample with concentrations represent limits of detection (LOD): (A) nornicotine (30 ng/ml), (B) nicotine (20 ng/ml), (C) cotinine (20 ng/ml), (D) TCP (100 ng/ml), (E) chlorpyrifos-oxon (150 ng/ml), and (F) chlorpyrifos (150 ng/ml) under established HPLC conditions.

simultaneous analysis of the six chemicals in this method.

Average percentage relative error of accuracy for all added concentrations to plasma samples was 3.6±0.5, 3.0±0.7, 2.4±0.9, 2.8±0.6, 3.5±1.2 and 2.8±1.0%, and in urine samples was 2.6±0.8, 2.2±0.3, 3.2±0.7, 3.0±0.6, 3.1±1.4 and 2.8±0.7% for nicotine, nornicotine, cotinine, chlorpyrifos, chlorpyrifos oxon, and TCP, respectively. Intra-day precision was determined as percent coefficient of variation (%C.V.) for plasma and urine samples ranged between 2.1±0.8 and 2.8±1.1%.

The resulting detection limits range were 20, 30, 20, 150, 150 and 100 ng/ml for nicotine, nornicotine, cotinine, chlorpyrifos, chlorpyrifos-oxon and TCP, respectively. Limits of quantitation in plasma were determined to be 50, 70, 50, 200, 200, and 150 ng/ml for nicotine, nornicotine, cotinine, chlorpyrifos, chlorpyrifos-oxon, and TCP, respectively. In urine limits of quantitation were 50, 100, 50, 200, 200, and 150 ng/ml for nicotine, nornicotine, cotinine, chlorpyrifos, chlorpyrifos-oxon and TCP, respectively. Limits of detection and quantification depends upon nature of the matrix, and method of analysis [27]. In previous study, limit of quantitation of chlorpyrifos using GC–MS was 0.7 ng/ml in blood [17]. While limit of detection of cotinine was 10 ng/ml in smokers plasma using HPLC–UV [18]. The reported LODs and LOQs in this method are bound to the simultaneous analysis of the six analytes in one run and to the use of HPLC for determination of the polar metabolites.

The method was applied for analysis of the

Table 2
Percent recovery[a] of nicotine, chlorpyrifos and their metabolites from rat plasma

Concentration (ng/ml)	Nicotine	Nornicotine	Cotinine	Chlorpyrifos	Chlorpyrifos -oxon	TCP
200	81.2±6.1	71.6±8.2	75.3±8.3	78.4±4.0	69.9±6.0	80.9±8.1
300	83.9±9.6	74.2±6.5	74.2±7.5	76.9±5.3	71.9±8.3	85.4±9.2
400	85.3±8.2	83.2±10.5	83.4±5.9	75.1±9.3	72.6±7.2	88.2±7.9
500	88.2±7.6	81.9±6.9	83.2±6.9	81.9±5.7	79.2±5.4	90.5±6.8
1000	84.9±10.2	80.2±5.7	85.8±9.2	82.1±6.9	77.1±8.4	92.2±5.6

[a] Values are expressed as mean±S.D of three replicates.

Table 3
Percent recovery[a] of nicotine, chlorpyrifos and their metabolites from rat urine

Concentration (ng/ml)	Nicotine	Nornicotine	Cotinine	Chlorpyrifos	Chlorpyrifos -oxon	TCP
200	78.8±3.9	76.2±5.3	80.1±6.3	76.0±3.8	66.4±7.6	81.3±7.1
300	83.2±6.8	74.7±7.1	81.6±7.5	77.1±6.3	70.7±5.3	80.2±6.2
400	85.2±5.8	74.9±6.1	83.2±6.2	80.1±6.2	70.1±8.2	82.3±8.6
500	88.1±4.6	77.1±6.8	81.9±5.8	82.9±8.1	73.2±9.4	80.6±4.8
1000	90.1±4.8	76.8±9.7	83.5±4.8	81.6±6.0	76.2±6.4	82.1±7.6

[a] Values are expressed as mean±S.D of three replicates.

chemicals and their metabolites in treated rats. TCP was determined in its free (non-conjugated form). Following combined oral dose of chlorpyrifos and nicotine, the animals developed, tremor, seizure, and several neurotoxic symptoms. In plasma, their levels were 519±173, 198±43 and 608±195 ng/ml for nicotine, cotinine, and chlorpyrifos, respectively. Nornicotine, chlorpyrifos-oxon, and TCP have not been detected in plasma 12 h after dosing. This is, either due to their low levels, or to instability of chlorpyrifos oxon, or rapid excretion or conjugation of TCP. TCP was excreted in urine in the free or conjugated forms following administration of chlorpyrifos animals [27–29]. In urine, cotinine and TCP have been identified. Their levels 12 h after dosing were 1073±217 and 823±178 ng/ml for cotinine and TCP, respectively. The results were corrected based on the percentage recoveries of the above chemicals from untreated plasma and urine samples. The described method is being used for studying the pharmacokinetic profile of nicotine and chlorpyrifos, alone and in combination in rats.

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