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Biological Monitoring of Exposure to Fenitrothion by High Performance Liquid Chromatography

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Fenitrothion [FNT, O,O-dimethyl O-(3-methyl-4-nitrophenyl) phosphorothioate], the active ingredient in the formulated product Sumithion, is extensively used in Taiwan, not only for agricultural purposes, but also for household pest control. The consumption for the latter purpose was registered as 11,900 kilograms for 1991. FNT is used in North American for the control of spruce budworm (Volpe and Mallet 1981) and in east Asia and south east Asia for the control of malaria (Gandahusada et al. 1984) and Japanese encephalitis (Self et al. 1973).

FNT inhibits cholinesterase both in rats and humans (Anand et al. 1977; Ecobichon et al. 1977). Acute toxicity in humans including nausea, vomiting, diarrhea, dyspnea and sometimes delayed neurotoxicity in salivation and fasciculation (Kojima et al. 1989; Yoshida et al. 1987; Sakamoto et al. 1984). It has been shown that FNT interferes with the normal behavioral development in newborn rats and induces aggressive behaviour in adult rats (Lehotzky et al. 1989; Anand et al. 1989). A postmortem tissue analysis of a male suicide victim found that the highest concentration of fenitrothion was in fat, followed in decreasing order by the pancreas, muscle, lung, and brain (Yoshida et al. 1987).

Due to its high lipid solubility, dermal absorption of FNT can be an important exposure route (Sax and Lewis 1989). Thus, an investigation of the feasiblity of biological monitoring of urinary metabolites for the management of occupational exposure to FNT seemed appropriate. The in vivo metabolism of FNT involves (i) oxidation of P=S to P=O, (ii) hydrolysis of the P-O-aryl linkage, (iii) demethylation, and (iv) reduction of the nitro- group to an

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amino-group (Miyamoto et al. 1976; Kojima et al. 1989). The hydrolysis of the P-O-aryl linkage results in the production of 3-methyl-4-nitrophenol (3MpNP). In rats, 3MpNP is mainly conjugated with sulfate and excreted in urine (Miyamoto et al. 1976).

The analysis of 3MpNP in a simple aqueous matrix can be done by spectrophotometry (HSDB 1990). When 3MpNP exists in a complex matrix such as blood and urine, chromatographic separation becomes necessary. Previously, 3MpNP was derivatized and analyzed by GC/MS, or via a derivation and extraction with organic solvent and then analyzed by GC/FID (Kojima et al. 1989). An HPLC method, reported for the analysis of 3MpNP in lake water by Volpe and Mallet (1981), was modified in this laboratory to analyze urinary 3MpNP of rats that were orally dosed with FNT. The method was further verified for its feasibility in the analysis of human urines collected from a small group of professional pest-control sprayers.

MATERIALS AND METHODS

Male Wistar rats, four per dose group, were given an oral dose of 5 mL/kg of Mazola corn oil in which was dissolved FNT (98%, Sumimoto Chemical Co, Japan) at 0, 25, 50, and 100 mg/kg. Prior to the intubation of FNT, 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 for the day before exposure 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 (Chang et al. 1993) until analyzed. The volume of each urine sample was recorded.

Urinary creatinine was determined by the Jaffe reaction. The sample preparation 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 1 hr to release 3MpNP from its sulfate and glucuronide conjugates, and (iv) addition of the internal standard (IS), p-nitrophenol, prior to HPLC analysis.

An Eldex 9600 HPLC coupled with an Alcott 738 autosampler and a

Linear UVIS 205 at 315 nm was used. The column (15 cm x 4.6 mm i.d.) was packed with a reversed-phase packing, 5μm Chemcosorb ODS. The mobile phase consisted of a mixture of acetonitrile and water (44:56 by volume) containing 1% acetic acid. The flow rate was 0.5 mL/min. Injection volume was 20 μL. A working standard curve, which consisted of 5 spiked urine samples, ranging from 0.03 to 0.48 μg 3-MpNP per mL (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 both series. The recovery was calculated as the ratio of the slope.

The feasibility of the method for the biological monitoring of an occupational exposure to fenitrothion was verified with an analysis of urine samples collected from several professional pest-control workers. Two minor modifications were implemented into the final protocol. They were (i) 2-hour acid hydrolysis instead of 1-hour, and (ii) o-nitrophenol to replace p-nitrophenol, the IS, since the latter is a non-specific metabolite that may come from other pesticide exposure such as parathion (ACGIH 1993).

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 $< \pm 10\%$), and good reproducibility (the interday variation i.e., CV < 10% at all levels). The percent recovery was 93.3 ± 1.3 %. Based on the analysis of the lowest standard prepared in mobile phase at 0.015 μg/mL in 5 replicates, a detection limit of 0.068 ng per 20 μL was obtained. It was calculated as 3 standard deviations of the mean response of the 5 replicates. This detection limit was about 75 times more sensitive than the one reported by Volpe and Mallet (1981).

The relationship between administered dose versus urinary 3MpNP in Wistar rats is summarized in Table 2. The data showed a linear dose response relationship at the dose levels studied, both for the non-conjugated i.e., free 3MpNP determined without acid hydrolysis and the acid hydrolysis-released 3MpNP. With an acid hydrolysis,

Table 1. Recovery and linearity studies of the 3-methyl-4-nitrophenol spiked control rat urine

	3MpNP [µg/mL]	mobile phase std. $\overline{\text{pk ht ratio}}^a \pm$ SD	CV, % n = 5	% rel. error
A.	0.015	0.0939 ± 0.0071	7.5	-7.4
	0.03	0.1579 ± 0.0078	4.9	-2.2
	0.06	0.2905 ± 0.0104	3.6	+2.2
	0.12	0.5358 ± 0.0079	1.5	+0.4
	0.24	1.0338 ± 0.0293	2.8	+0.2
	0.48	2.0254 ± 0.0305	1.5	-0.1
	3MpNP [µg/mL]	spiked urine std. $\overline{\text{pk ht ratio}}^a \pm$ SD	CV, % n = 5	% rel. error
B.	0.03	0.1460 ± 0.0094	6.4	-5.3
	0.06	0.2677 ± 0.0141	5.3	-0.2
	0.12	0.4960 ± 0.0150	3.0	-0.9
	0.24	0.9833 ± 0.0335	3.4	+2.0
	0.48	1.8847 ± 0.0232	1.2	-0.4

a. $\overline{\text{Pk ht ratio}}$ stands for the mean peak height ratio, the IS was p-nitrophenol at 0.14 µg/mL. The linear regression equation for A. the mobile phase standards was $y = 0.249\ x \pm 0.036$; B. spiked urine standards, $y = 0.232\ x \pm 0.036$. r^2 of both > 0.99.

0.5 N perchloric acid at 85°C for 1-hour, a significant increase in urinary 3MpNP was observed. The hydrolysis was used to deconjugate the phenolic metabolite, resulting in the formation of about 22 to 27 times more of the free 3MpNP. According to Miyamoto et al. (1976) the major conjugation of 3MpNP in rat was to sulfate and minor to ß-glucuronic acid. A storage-stability study for total acid hydrolyzable 3MpNP was conducted on 12 rat urine samples stored at 4°C for 0, 8, 12, and 17 days. The acid hydrolyzable 3MpNP was found to be stable in all samples for the time span studied. The concentration range of the acid hydrolyzable 3MpNP evaluated was from 10 to 110 µg/mL.

For the analysis of urine samples collected from 5 professional pest-control sprayers, a calibration curve ranging from 0.05 to 2.00 µg 3MpNP per mL was prepared with control human urine. A linearity

Table 2. Dose-response of the free (non-conjugated) and acid (hydrolysis)-released urinary 3MpNP in rats

	mean (μg 3MpNP/mg creatinine) ± SD, n = 4	
Dose (mg/kg)	free 3MpNP	acid-released 3MpNP
25	4.2 ± 1.5	98.4 ± 38.2
50	11.4 ± 3.6	294.2 ± 32.3
100	16.2 ± 1.5	454.4 ± 44.1
r^2	0.91	0.94

Wistar rats, 4 per dose group, were gavaged with a single dose of FNT in corn oil. From each rat, 24-hour urine samples were collected at room temperature and protected from light.

of $r^2 > 0.99$ was obtained with all of the percent relative errors of $< \pm 10\%$.

Summarized in Table 3 are data of some biological monitoring of potential human exposure to FNT following a pest-control spraying section on 2 different dates. Urine samples were collected at 2 to 3 hours right after spraying (post-shift) as well as the next day before shift and analyzed for total 3MpNP. The results showed that (i) on the first sampling date, no 3MpNP was found in worker #3 who did not work with FNT on that particular day, (ii) on the second sampling date, all workers had worked with more FNT, a significantly higher concentration of urinary 3MpNP was measured, for both of the post-shift and pre-shift of the next day, and (iii) except the first 2 low dose samples, all others of the next day sample still had significant quantities of 3MpNP. This last observation suggested that the in vivo clearance of FNT was not a fast process (not within 24-hour). FNT or its intermediate metabolites would accumulate in the body to reach a steady state should some consecutive daily exposure to FNT occur.

In conclusion, a sensitive isocratic HPLC/UV method with o-nitrophenol as an internal standard was developed for the quantitative analysis of 3MpNP, which is the chemical specific urinary metabolite of FNT. The straightforward sample preparation included an ice-cold acid denaturation of the urinary macromolecules, a low speed centrifugation to remove the denatured

Table 3. 3MpNP measured in urines of 5 pest-control workers, expressed as (A) μg/mL urine (B) μg/mg creatinine

worker ID #	FNT (kg) used[a]	post-shift (A)/(B)	next day pre-shift (A)/(B)
1	0.4	0.37/0.22	b
2	0.4	0.84/0.36	b
3	0	0	0
4	0.4	0.16/0.52	0.10/0.07
5	0.4	c	0.57/0.27
1	1.0	2.24/0.74	0.78/0.44
2	1.0	2.52/0.87	1.10/0.59
3	1.0	1.54/0.12	1.41/0.68
4	1.0	0.64/0.57	1.56/0.87
5	1.0	1.48/0.53	0.77/0.35

a. Within 24-hour of urine collection.
b. Below the lowest standard, 0.05 μg/mL.
c. No sample collected.

precipitate, and an acid hydrolysis of the 3MpNP conjugates. The feasibility of the method to be used as a non-invasive chemical specific biological monitoring of an occupational exposure to FNT was adequately demonstrated with urine samples collected from professional pest-control applicators.

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