A New GC On-Column Methylation Procedure for Analysis of DMTP (*O,O*-Dimethyl Phosphorothioate) in Urine of Workers Exposed to Fenitrothion

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A new, improved procedure was developed for determining dimethylphosphorothioate (DMTP) levels in the urine of workers exposed to the organophosphorus pesticide, fenitrothion. The use of ethyl acetate instead of acetonitrile/ether to extract urine significantly increased extraction efficiency (from 57% to 96%). The use of Methelute TM (trimethylanilinium hydroxide) (TMAH) as an on-column derivatizing agent increased sensitivity 10–100-fold, was rapid, gave increased column life, and eliminated the need to work with the more hazardous diazoethane (DAE) compound used in the Shafik ethylation procedure. Total DMTP (μg) excreted by 3 days postexposure is reported for 16 field workers after urine analysis by both TMAH and DAE derivatization.

INTRODUCTION

The assessment of potential health risks associated with pesticide use requires knowledge of the amount of pesticide to which the user was exposed (Franklin et al., 1981). There are numerous problems associated with measuring pesticide exposure, and the traditional method of using absorbent patches to estimate contact exposure may not be totally reliable (Franklin, 1984). Determination of urinary metabolites provides a more satisfactory indicator of exposure. It has been established that, in man, organophosphorus pesticides are hydrolyzed and excreted as urinary alkyl phosphates, which can be analyzed by gas chromatography (GC), as first reported by Shafik and Enos (1969). Although this procedure has been modified in subsequent studies (Shafik et al., 1970, 1971, 1973; Lores and Bradway, 1977; Bradway et al., 1977; Brokopp et al., 1981), the basic methodology remains tedious, lacks reproducible and high extraction efficiency, uses hazardous chemicals, and has a high sample background interference. In addition, isomerization of standards to the S-alkyl derivatives occurs during derivatization (Shafik et al., 1970) and the proportion of the O to S alkyl ester varies, reducing sensitivity (Bradway et al., 1981).

Earlier studies had shown that organophosphorus pesticides could be derivatized on-column with quaternary ammonium hydroxides such as trimethylanilinium hydroxide (TMAH) (Dale et al., 1976; Miles and Dale, 1978). Churchill et al. (1978) also showed that several dialkyl phosphorothioates, including DMTP, could be derivatized by TMAH.

In the present study, an improved procedure for the extraction of DMTP from the urine of workers exposed to the organophosphorus pesticide fenitrothion (*O,O*-dimethyl *O*-(3-methyl-4-nitrophenyl) phosphorothioate) was developed, and the use of TMAH (Methelute TM) as a derivatizing agent for urinary DMTP was established. The data obtained from this new method (TMAH) were compared with those from the old diazoethane (DAE) method of Shafik and Enos, 1969.

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EXPERIMENTAL SECTION

Glassware. Disposable glassware, including round-bottom centrifuge tubes (16 × 100 mm Canlab no. T1290-6), autosampler vials (1.5 mL, Pierce no. 13208), and liquid scintillation vials (20 mL, Fisher no. 3-337-16), was used to minimize contamination.

Solvents and Reagents. Diazoethane (DAE) was prepared with *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine obtained from Aldrich Chemical Co. Trimethylanilinium hydroxide (TMAH) was obtained from Pierce Chemical Co. in the form of Methelute TM (0.02 M in methanol). Solvents were glass distilled, pesticide grade from Caledon Laboratories.

Preparation of Standard Solutions. Analytical grade (98%) *O,O*-dimethyl phosphorothioate phosphorothioate potassium salt (DMTP) was obtained from American Cyanamid Co. A mixed calibration standard of the *O,O*-dimethyl *S*-ethyl phosphorothioate (P=S, 0.14 ng/μL) and *O,O*-dimethyl *O*-ethyl isomer (P=O, 0.48 ng/μL) was synthesized, by standard procedures (Eto, 1974; Churchill et al., 1978), and authenticated by ^{1}H and ^{31}P NMR, and mass spectrometry (Daughton et al., 1976; Greenhalgh et al., 1983).

DMTP stock solutions (1 mg/mL acetone) were kept at −20 °C in the dark. Analytical standard dilutions (0.11 ng/μL, acetone) were prepared daily and kept in foil-wrapped glass volumetric flasks, since preliminary work demonstrated significant reduction (30 ± 3%) in DMTP standard levels after 24 h under laboratory conditions.

Collection of Urine Samples. Total 24-h urine samples were collected from each of 16 workers involved in the aerial application of fenitrothion to control Spruce Budworm in Quebec forests, commencing one day prior to initiation of potential occupational exposure to the chemical, and for up to 3 days thereafter. 100-mL aliquots of each 24-hr sample were frozen and stored at −20 °C in 125-mL Nalgene bottles until analysis.

Gas Chromatographic (GC) Conditions. *Apparatus*: A Varian-Vista 6000 GC equipped with a dual flame photometric detector (FPD) was operated in the phosphorus mode. *Column*: A silanized glass column 2 m × 0.2 cm (id)), packed with 3% Carbowax 20M on Chrom W, H.P., 100/120 mesh was used to minimize tailing of alkyl phosphate peaks (Ives and Guiffrida, 1970). *Flow rates*: Carrier flow was set at 30 mL/min of N_2, and detector flows at 80 mL/min of air (1), 170 mL/min of air (2), and 140 mL/min of H_2. *Operating Temperatures*. Old method (DAE): The injector was held at 160 °C and

0021-8561/85/1433-0464$01.50/0

the detector at 250 °C. The column oven was programmed to hold 2.4 min at 120 °C, then increased to 140 °C at a rate of 50 °C/min, and held for 10 min. *New Method (TMAH)*: The injector was held at 220 °C and the column was isothermal at 160 °C. *Calibration. Old method (DAE)*: The GC was calibrated with intermittent dual injections of 2 μL of the P=S, P=O ethyl DMTP standard (0.62 ng/μL). The average of the sum of the P=S and P=O peak areas in duplicate sample injections (2 μL) was used to calculate the total micrograms of DMTP excreted (Table III) after correcting for total urine volume. Peak area integration was performed with a Varian CDS-401 data acquisition system. The linearity of the detector response for ethyl DMTP (0.03–1.30 ng) was $Y = 0.96X + 0.01$, $r = 0.9997$. The minimum detectable limit (MDL) ranged from 0.01 to 0.10 μg/mL urine (10–100 ppb), depending on sample background interference. *New method (TMAH)*: The total DMTP excreted was calculated by calibration with dual intermittent DMTP standard (0.11 ng/μL of acetone) injections (2 μL) and dual sample injections (2 μL). The linearity response for methyl DMTP (0.01–10 ng) was $Y = 0.99X + 5.18$, $r = 0.9999$. The MDL was 0.001 μg/mL of urine (1 ppb).

Extraction and Derivatization. Urine samples were thawed rapidly under hot running tap water (≤30 min). The samples were then shaken and left for 10–15 min to allow sedimentation.

Extraction. Old method (DAE): Extraction was carried out by the procedure of Shafik et al. (1973) with slight modification to include two extractions as follows. To each 2-mL urine aliquot in a glass centrifuge tube (15 mL) were added ca. 2 g of ether-rinsed NaCl. After vortexing for 1 min, 2 mL of acetonitrile/ether (1/1) and 1 mL of 6 N HCl were added. The tubes were sealed with parafilm, vortexed again for 1 min, and centrifuged (2000 rpm) 1 min, and a 1-mL aliquot of the organic phase was transferred to a second tube, sealed with parafilm, and held in an ice bath. After discarding the residual solvent, the extraction was repeated and the second 1-mL organic extract combined with the first. *New method (TMAH)*: Urine samples were extracted as described above for the old method except that ethyl acetate (2 × 2 mL) was used for extraction. Two aliquots (0.5 mL) of each extract were combined and stored in glass autosampler vials, sealed with screw-cap septa, and stored at −20 °C.

Derivatization. Old method (DAE): An aliquot (ca. 3 mL) of an etherial solution of DAE, prepared as described by Shafik and Enos (1969) was used to derivatize the 2-mL sample extracts. After 30 min at room temperature, the ethylated urine extract was blown to near dryness with N_2, then made up to 0.5 mL with acetone. This solution was vortexed with anhydrous Na_2SO_4 (ca. 0.1 g, acetone-rinsed) and centrifuged for 1 min prior to analysis. *New method (TMAH)*: The extracts were derivatized by dispensing 200 μL of extract into new autosampler vials and then blowing dry under N_2 for 5 min in a Reacti-Therm (Pierce no. 18790) at 56 °C to remove residual water which could have reduced the derivatization efficiency. Drying under N_2 was more efficient in comparison to drying agent (Na_2SO_4 or molecular sieve). Fifteen minutes of drying under these conditions did not significantly (≤5%) reduce DMTP levels. After cooling, 100 μL of acetone was added with swirling followed by 5 μL of TMAH (Methelute TM) directly above the acetone surface with a Hamilton 25-μL syringe. After TMAH addition, the vial was swirled and 2 μL was immediately injected into the GC. Acetone was selected as the injection solvent in order to ensure complete syringe flushing (LeBel and Williams, 1979).

Table I. Comparison of Extraction Efficiencies of the Two Solvents Ethyl Acetate and Acetonitrile/Ether for DMTP-Spiked Urine[a]

vortex time, min	extract no.	% recovery of 0.11 ppm DMTP in urine: ethyl acetate	% recovery of 0.11 ppm DMTP in urine: acetonitrile/ether
5	1	88	48
	2	6	9
	3	<1	<1
	total	94	57
1	1	91	50
	2	5	7
	3	<1	<1
	total	96	57
0.5	1	72	39
	2	11	6
	3	<1	<1
	total	83	45

[a] Recovery corrected for solvent loss of 11.5% for ethyl acetate and 52, 45.5, and 45% for acetonitrile/ether after 5, 1, and 0.5 min vortexting, respectively.

Comparison of Extraction Efficiencies. Control urine samples (preexposure) with no detectable levels of DMTP were spiked at 1.1 ppm (μg/mL) as follows: DMTP (2.2 μg) in 200 μL of acetone was added to a glass centrifuge tube. The acetone was evaporated under N_2 for 1 min and 2 mL of urine was added. Drying of the residue for up to 10 min prior to the addition of the urine did not significantly reduce DMTP levels. The fortified urine was vortexed for 1 min, salt was added, and the sodium acidified and extracted as discussed above. The effect of vortex duration (0.5, 1, and 5 min) on extraction efficiency was tested for three consecutive extractions for both acetonitrile/ether (1/1) and ethyl acetate. The samples were then analyzed following on-column derivatization (TMAH method).

Although the extraction tubes were parafilm sealed, appreciable solvent loss occurred during extraction, especially with acetonitrile/ether (≥45%). Since this concentrated the extract, the amount of solvent loss was accurately measured by triplicate blank extraction trials with distilled water. The extracts were transferred to conical centrifuge tubes and the volumes recorded by drawing into a 1 mL graduated pipet. The ability to discern the miniscus was aided by adding safranin for acetonitrile/ether and methylene blue for ethyl acetate extractions. The reported extraction efficiencies were corrected for solvent loss as follows: corrected % recovery = $X - (X\ 0.01Y)$ where X = % recovery and Y = % solvent loss.

The effect of concentration of DMTP in urine on the extraction efficiency was tested with urine fortified at 11.0, 1.1, 0.1, and 0.011 ppm (μg/mL) of DMTP.

RESULTS AND DISCUSSION

Significantly greater levels of DMTP metabolite were detected following extraction with ethyl acetate compared to acetonitrile/ether (97% vs. 57%). It was also shown that a vortex time of 0.5 min was too short and that there was no difference between a 1 or 5-min vortex time (Table I). Two extractions (2 × 2 mL) were adopted in place of the Shafik 1 × 4 mL extraction, since 4-mL extractions did not demonstrate improved recovery. The recovery of DMTP from fortified urine was only 61% at the lowest concentration of 0.011 μg/mL. This rose to 86% when the urine was spiked at 0.11 and 1.1 μg/mL and to 97% at 11.0 μg/mL (Table II). Daughton et al. (1976) also reported quantitative extraction of DMTP from acidified saline with ethyl acetate. It should be noted that the low recovery (45–57%) obtained here with acetonitrile/ether (Table I)

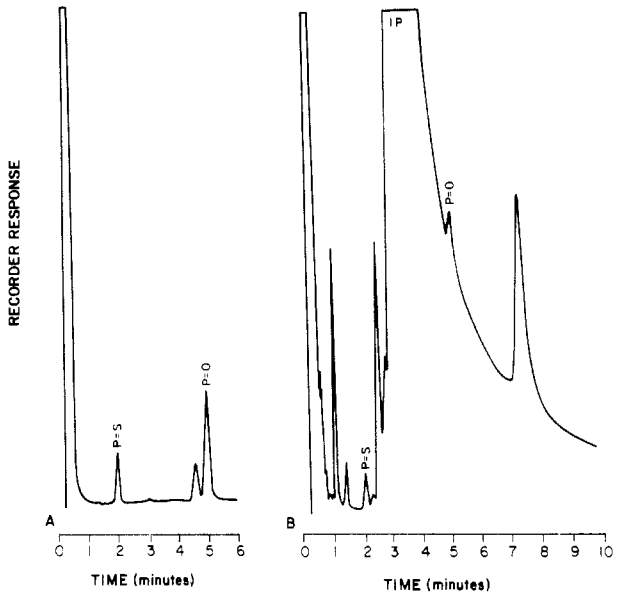

Figure 1. Gas chromatograms showing peaks for the P=S and P=O isomers of ethyl DMTP following the ethylation (DAE) procedure. (A) Ethylated DMTP standard (1.24 ng); (B) ethylated urine extract (subject no. 1, 3 days postexposure), inorganic phosphate (IP).

Table II. Effect of Concentration of DMTP on Extraction Efficiency of Ethyl Acetate

concn of DMTP added to urine, μg/mL (ppm)	% recovery ±SD (n = 3)
11.0	97 ± 3
1.1	86 ± 4
0.11	86 ± 11
0.011	61 ± 7

would have been reported as 95–103% if uncorrected for solvent evaporation, approximating that reported by Shafik et al. (1973) (95.8% at 0.1 ppm).

There were several advantages of using Methelute TM for methylation which include: a single methylated DMTP product (Figure 2 part A), background interference was almost completely eliminated (Figure 2 part B), on-column methylation was rapid, base line current was stable, and column life was ≥3 months. Contrast this with the DAE method in which the variable degree of isomerization obtained during ethylation necessitated quantitation of both the P=S and P=O isomers (Figure 1 part A) (Bradway et al., 1981). One of the main difficulties encountered with the DAE method is the interfering off-scale inorganic phosphate (IP) peak that was present in all samples analyzed, including ethylated ethyl acetate extracts (Figure 1 part B). Although further cleanup procedures for removing IP have been reported (Shafik et al., 1971), they introduce another step and are tedious for routine analysis, leading to possible lowered recoveries. It should be noted that without automated injection and computer data acqusition, the accuracy of the peak integration would have suffered greatly. Further, the ethylated extracts caused substantial base line current increases during runs, necessitating relatively long hold times between runs at high temperature for stabilization. It was also necessary to repack the head of the column packing every few days to maintain sensitivity. In addition, the method was 10 to 100 times less sensitive than the new ethyl acetate/Methelute method.

Diazoethane preparation is tedious and potentially hazardous (Bradway et al., 1981) and batches may vary in composition and purity from one lab to another. In comparison, Methelute TM (TMAH) is commercially available

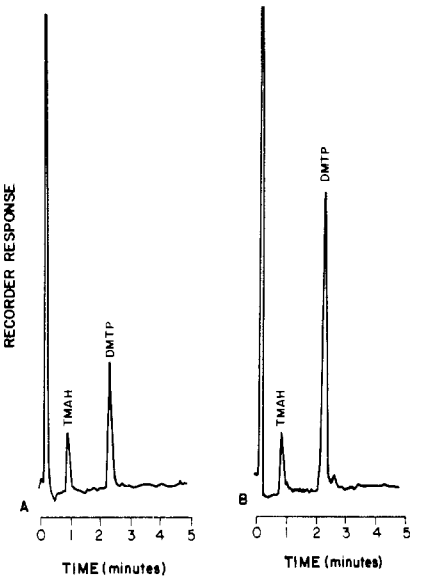

Figure 2. Gas chromatograms showing peaks for TMAH and methyl DMTP following methylation (TMAH) procedure. (A) Methylated DMTP standard (0.22 ng); (B) methylated urine extract (subject no. 1, 3 days postexposure).

Table III. Total Urinary DMTP (μg) Excreted by Workers Exposed to Fenitrothion[a]

	ethyl acetate/methelute method[b]		acetonitrile–ether/diazoethane method[c]	
subject	pre-exposure	total DMTP excreted by day 3, μg	pre-exposure	total DMTP excreted by day 3, μg
1	42	520	27	266
2	ND	66	ND	56
3	ND	97	ND	46
4		273		109
5		257		223
6	65	297	ND	111
7	20	214	ND	220
8		144		74
9		323		280
10	ND	25	ND	ND
11	ND	ND	ND	ND
12		ND	ND	ND
13	ND	ND	ND	ND
14	ND	ND	ND	ND
15	ND	ND	ND	ND
16	ND	ND	ND	ND

[a] DMTP levels were not corrected for extraction efficiency. [b] Limit of detection 0.001 μg/mL of urine. [c] Limit of detection 0.01–0.10 μg/mL of urine.

and was stable under our laboratory conditions. In Figure 2 parts A and B, the peak present at 0.94 min was due to TMAH and was useful for verifying excess TMAH addition. The presence of only one standard peak with TMAH would permit the study of in vivo isomerization of DMTP. In the present study, the samples were commonly analyzed on the day of extraction. However, a comparison of data obtained from analysis of ten samples extracted with ethyl acetate prior to and after one month storage (−20 °C) demonstrated no significant loss (y = $0.99x - 0.50$, r = 0.97) due to storage.

The results of the field sample analysis (Table III) demonstrated higher DMTP levels in a majority of cases for the TMAH procedure. This could be attributed to the greater extraction efficiency obtained with ethyl acetate (new method) as compared to acetonitrile/ether (old method). Loss of the volatile ethyl derivative during concentration under N_2 could also be a factor. Detection of DMTP in some of the preexposure samples (Table III)

could be due to worker contamination through contact with previously used equipment or to exposure to contaminated surfaces in the general work environment.

The developed procedure provides a rapid, reproducible, and sensitive method for monitoring occupational exposure to fenitrothion. The method should be useful for monitoring exposure to organophosphorus pesticides in general and is currently being employed in our laboratory for assessing exposure to azinphos-methyl.

ACKNOWLEDGMENT

The authors are grateful to Anne Danko and Greg Cressman for technical assistance.

Registry No. DMTP, 1112-38-5; TMAH, 1899-02-1; fenitrothion, 122-14-5; ethyl acetate, 141-78-6.

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Received for review September 6, 1984. Accepted January 23, 1985.

Residues of Avermectin B_1a on and in Citrus Fruits and Foliage

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A 3 μg/mL solution of [^{3}H]-avermectin B_1a was applied to citrus fruits and leaves. Both fruits and leaves accepted 0.017 ± 0.004 μg/cm^2 of surface area treated. Postapplication (2~3 h) residues for orange rind, lemon rind, and lemon leaves were 0.066 ± 0.007, 0.038 ± 0.006, and 0.92 ± 0.23 μg/g, respectively, based on total tritiated water obtained by combustion. However, based on reversed isotope dilution analysis of acetone extracts, actual avermectin B_1a residues were 30% for orange rind and 16% for lemon rind of the corresponding combustion value. Residues were <0.001 μg/g in pulp of both treated mature fruits and lemons treated when ~2.5 cm in diameter and analyzed 60 days postapplication. Residues were <0.004 μg/g in new growth leaves sampled from tips of branches whose leaves had been treated 91 days earlier.

The avermectins are a family of new pesticidal agents that are extracted from the mycelia of the actinomycete, *Streptomyces avermitilis*, which was first isolated from a soil sample collected in Japan (Burg et al., 1979; Miller et al., 1979). The chemical structures of the avermectins were elucidated by Albers-Schönberg et al. (1981). Some of the extraordinary biological activities of the avermectins have recently been reviewed (Campbell et al., 1983). MK-936, consisting of at least 80% avermectin B_1a and not more than 20% avermectin B_1b (see Figure 1), has shown good efficacy in laboratory and field tests as an acaricide for the citrus rust mite, *Phyllocoptruta oleivora* (Ashmead), and appears promising for the control of the citrus red mite, *Panonychus citri* (McGregor) (McCoy et al., 1982). The latter is one of the three most serious pests attacking California citrus. It also shows great promise for the control of many other economically important arthropods including imported fire ants (Schuster and Everett, 1983; Putter et al., 1981; Lofgren and Williams, 1982).

To obtain preliminary information on residue levels, dissipation rates, and translocation resulting from a field treatment, tritium-labeled avermectin B_1a was applied at an anticipated use level to orange and lemon fruits and lemon leaves, and samples were collected and analyzed. Field treatment, sample collection, and sample preparation were conducted at the University of California, Riverside (UCR), and sample combustion, liquid scintillation counting, and reversed isotope dilution analyses (RIDA's) were performed at Merck.

EXPERIMENTAL SECTION

Formulation. To 88 mL of 1,2-propanediol in a 500-mL round-bottom flask was added 6.15 mL of a [5-^{3}H]-avermectin B_1a solution (2.88 mg of avermectin B_1a/mL, 3.9 mCi/mL in ethanol). After removing the ethanol under reduced pressure, 174 mg of an avermectin B_1a preparation (L-676,895-00P32), containing 162.3 mg of unlabeled av-

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0021-8561/85/1433-0467$01.50/0