Method for the Determination of Dialkyl Phosphate Metabolites in Urine for Studies of Human Exposure to Malathion

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A method for reducing the mono- and dicarboxylic acid urinary metabolites of the organophosphorus pesticide malathion to dialkyl phosphates has been developed as a modification of a standard dialkyl phosphate metabolite procedure. Alkaline hydrolysis of urine samples is conducted prior to extraction and derivatization of dimethyl thiophosphate (DMTP) and dimethyl dithiophosphate (DMDTP). The dialkyl phosphates are derivatized with pentafluorobenzyl bromide (PFBB) and analyzed by GLC/ECD. Recoveries of malathion metabolites were 88%. The procedure was employed in the estimation of exposures in workers mixing and applying malathion in citrus orchards. This approach simplifies chromatographic analysis, increases sensitivity of the overall method, and allows investigators to assess human exposure to malathion within a general procedure for dialkyl phosphate metabolite analysis.

The use of urinary metabolites as a means of biological monitoring for pesticide exposure is gaining wide popularity in both scientific and regulatory circles (van Heemstra-Lequin and van Sittert, 1986; USEPA, 1987). However, animal dosing studies demonstrating substantial (≫50%) urinary excretion of the parent compound or major metabolites are an absolute prerequisite for validation of such a biological monitoring approach. The efficacy of urine excretion monitoring is improved if (1) the parent compound is excreted unchanged or as one or two major metabolites, (2) excretion is relatively rapid (<72 h), and (3) the metabolites are measurable at low levels with relatively straightforward analytical techniques.

Organophosphorus insecticide exposure can be assessed by two basic biological monitoring methods: blood cholinesterase inhibition and urinary metabolite excretion. Routine cholinesterase monitoring of commercial applicators is now required in many states and has been proposed at the federal level in the recently promulgated regulations for farm worker protection (USEPA, 1988). Urine monitoring for worker exposure has been adopted by numerous researchers and was the subject of a recent national symposium (Wang et al, 1989).

The analysis of the dialkyl phosphate metabolites of organophosphorus insecticides has been reported by a variety of investigators (Shafik et al., 1973; Churchill et al., 1978; Daughton et al., 1979; Reid and Watts 1981). This approach has the important advantage of allowing investigators to develop a single laboratory procedure for studying an entire class of insecticides rather than requiring a set of compound by compound procedures.

Malathion [*O,O*-dimethyl *S*-(1,2-dicarbethoxyethyl) phosphorodithioate] presents a special problem in this regard, as it does not proceed through the same mammalian metabolic pathways as most of the organophosphorus compounds. Malathion is metabolized by esterase cleavage at one or both of the carbethoxy esters (Bhagwat and Ramachandran, 1975; Chen et al., 1969), resulting in the excretion of malathiondicarboxylic acid (DCA) and malathion-α-monocarboxylic acid (MCA). These structures are presented in Figure 1.

Table I. Major Mammalian Metabolites on Malathion

name (abbreviation)	% of total metabolities[a]
malathiondicarboxylic acid (DCA)	62.9
malathion-α-monocarboxylic acid (MCA)	19.6
O,O-dimethyl phosphorothionate (DMTP)	8.6
O,O-dimethyl phosphorodithioate (DMDTP)	4.5

[a] From Bradway and Shafik (1977); average percent of total metabolites excreted in rats at three dose levels (0.69–69 mg/day); dimethyl phosphate excretion accounts for remaining 4.4%.

Bradway and Shafik (1977) have identified the major metabolites of malathion in rats and have demonstrated that only small amounts of the dialkyl phosphates are excreted (Table I). The carboxylic acid metabolites constitute more than 80% of the total metabolites excreted. Derivatives of the mono- and diacids are not detected in standard gas chromatographic procedures for alkyl phosphate detection; instead, the rather involved procedures outlined by Bradway and Shafik are required to produce meaningful quantitative results.

The analytical method presented in this paper was developed as part of a comprehensive study of occupational exposure to malathion (Fenske, 1988). The primary purpose of this study was to compare measurements from several dermal exposure monitoring techniques with total (72-h) urinary excretion of malathion metabolites following exposure. Thus, it was deemed more expedient to pool individual metabolites into as few compounds as possible rather than analyze for each separately. This approach has the potential advantages of simplifying the chromatographic analysis and increasing the overall sensitivity of the method. It was therefore decided to hydrolyze the mono- and diacids of malathion (and malaoxon, if present) to their corresponding alkyl phosphates and to analyze all malathion metabolites as dialkyl thiophosphates.

Wolfe et al. (1977) performed an extensive array of hydrolysis experiments on malathion. Although this work was carried out to mimic environmental conditions, it clearly indicated that alkaline hydrolysis is more efficient than acidic hydrolysis for the removal of the succinic acid moiety of the mono- and diacids. Wolfe did not study the acid hydrolysis of the α- and β-monoacids themselves but did examine their alkaline hydrolysis, which cleaves the carbon–sulfur bond, leading to the formation of DMDTP

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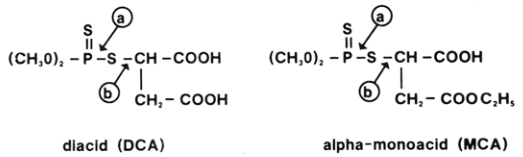

Figure 1. Malathiondi- and -monocarboxylic acid structures and hydrolysis sites for formation of dialkyl thiophosphate metabolites: (a) DMTP, (b) DMDTP.

(see Figure 1). Both acid and basic hydrolyses of the diacid break the phosphorus–sulfur bond, yielding DMTP. These same products also can be anticipated in their own right as metabolites of malathion, as noted previously.

The method of Reid and Watts (1981) was chosen for alkyl phosphate analysis. Bradway et al. (1981) have reported development of a similar method. The use of pentafluorobenzyl bromide (PFBB) as a derivatizing reagent has several advantages over the use of diazoalkanes (Shafik and Enos, 1969; Shafik et al., 1973) and benzylation (Daughton et al., 1979): First, PFBB gives only one product when reacting with DMTP (the $(CH_3O)_2P(O)S$– ester) instead of two (the $(CH_3O)_2P(O)S$– and the $(CH_3O)_2P(S)O$– esters), thus increasing the sensitivity of the method as well as simplifying the chromatography. Second, although PFBB is a strong lacrymator, neither is it as explosive as the diazoalkanes nor is it a suspect carcinogen as are the benzyltriazene and nitrosoguanidine reagents.

EXPERIMENTAL SECTION

Apparatus. A Hewlett-Packard gas chromatograph (Model 5880A) equipped with a flame photometric detector in the phosphorus mode was used throughout the development of this procedure. The column used was a 30-m megabore DB5 (J'W Scientific). Temperatures were as follows: 185 °C, column oven; 205 °C, injector; 193 °C, detector. The following flow rates, as recommended by the manufacturer, were employed: 30 mL/min, carrier gas, helium; 200 mL/min, fuel gas, hydrogen; 50 mL/min, oxidizer gas, Air; 20 mL/min, oxidizer gas, oxygen.

Duplicate 1-μL injections were made by autosampler for all samples and standards. During initial trials, plunger jamming occurred frequently due to buildup of urine residues on the syringe. Thorough rinsing of the syringe immediately following each urine sample injection eliminated this problem.

Sample preparation was carried out entirely in 13 × 100 mm screw-capped culture tubes containing five to seven 3-mm glass beads. The beads were employed to prevent the urine residue from forming a highly viscous mass that reduced the diffusion of the derivatizing agent (PFBB) and the extraction of the dialkyl phosphates. Chemically inert closure was accomplished by the use of Teflon-lined caps. Each tube was tested for its ability to make a positive seal under the conditions used in the method, as recommended by Reid and Watts (1981).

Samples and reagents were dispensed with positive-displacement pipettors (SMI) with the exception of PFBB, which is sold in septum-sealed vials and was dispensed with a 50-μL gas-tight syringe (Hamilton Co.). Acetonitrile for azeotropic drying of the samples was dispensed with a 10-mL syringe.

The hydrolysis reaction was performed in a 90 °C oil bath, while the derivatization reaction was performed in a 40 °C water bath. The azeotrope step was conducted with dry nitrogen on a 12-place Meyer N-Evap (Organomation Industries, Model 111) evaporator set at 40 °C.

Reagents. The derivatization reagent, pentafluorobenzyl bromide, was used neat (Supelco). Hydrolysis of the mono- and diacids was carried out with 1.0 N potassium hydroxide (J. T. Baker Dilutit). Samples of the α-monoacid and diacid were provided by American Cyanamid, as were DMTP and DMDTP standards. Pesticide residue quality acetonitrile was used throughout (J. T. Baker Resi-Analyzed). Tested as catalysts were 1,4,7,10,13,16-hexaoxacyclooctadecane (18-crown-6 ether; Supelco), as suggested by several investigators (Frei and Lawrence, 1981; Durst et al., 1975), and anhydrous potassium carbonate (J. T. Baker AR grade), as recommended by Reid and Watts (1981). Tributyl phosphate (Aldrich Chemical Co.) was used as an internal standard.

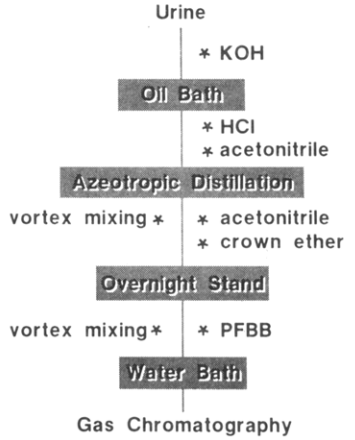

Figure 2. Procedure for determining dialkyl thiophosphates from malathion metabolites.

Table II. Recovery of Dialkyl Phosphates from Urine

	init concn in urine, μmol/mL	% recovery (N = 6)	coeff of var %
total malathion equiv	26.58	88.5	13.4
DMTP	10.56	120.8	7.5
DMDTP	16.02	70.0	24.6

Procedure. The method described here is outlined in Figure 2. Urine (1.00 mL) is pipetted into the sample tube containing the glass beads, followed by 250 μL of 1.0 N potassium hydroxide solution. The samples are placed in the 90 °C oil bath for 4 h and then cooled. Then added are 250 μL of 1.0 N hydrochloric acid solution (J. T. Baker Dilutit) and 7.0 mL of acetonitrile. The sample is evaporated at 40 °C under a stream of dry nitrogen for about 45 min (until water condensation starts to form on the upper portion of the tube); this step is repeated twice more with use of, successively, 5.0 and 3.0 mL of acetonitrile, making certain the sample goes to dryness at the last step.

At this point, 1.00 mL of acetonitrile (containing 18-crown-6 ether at a concentration of 2 mg/mL and the tributyl phosphate internal standard) is added to each sample, together with approximately 100 mg of potassium carbonate, followed by brief vortex mixing. The samples are capped and left to stand overnight. The next day 25 μL of PFBB is added, followed by brief vortex mixing. The sample is tightly capped and placed in a 40 °C water bath for 2 h. Samples are then cooled and analyzed after the supernatant is decanted into a suitable storage or analysis container.

RESULTS AND DISCUSSION

Fortification/recovery studies were conducted by introducing known quantities of DMTP and DMDTP standards into the urine of an unexposed individual. Results of the fortification studies are presented in Table II. Recovery for the total malathion equivalent added to urine was 88.5%. Recoveries for DMTP were consistently higher (121%) than those for DMDTP (70%), indicating that DMDTP is partially converted to DMTP during the extraction/derivatization procedure. Four nonfortified urine sample blanks were taken through all steps of the analytical procedure, with negligible background interference noted.

Samples of the mono- and diacids were not of sufficient purity to allow determination of the efficiency of the alkaline hydrolysis. However, neither repeated nor prolonged hydrolysis increased the alkyl thiophosphate yields significantly. In fact, extending the 90 °C oil bath beyond

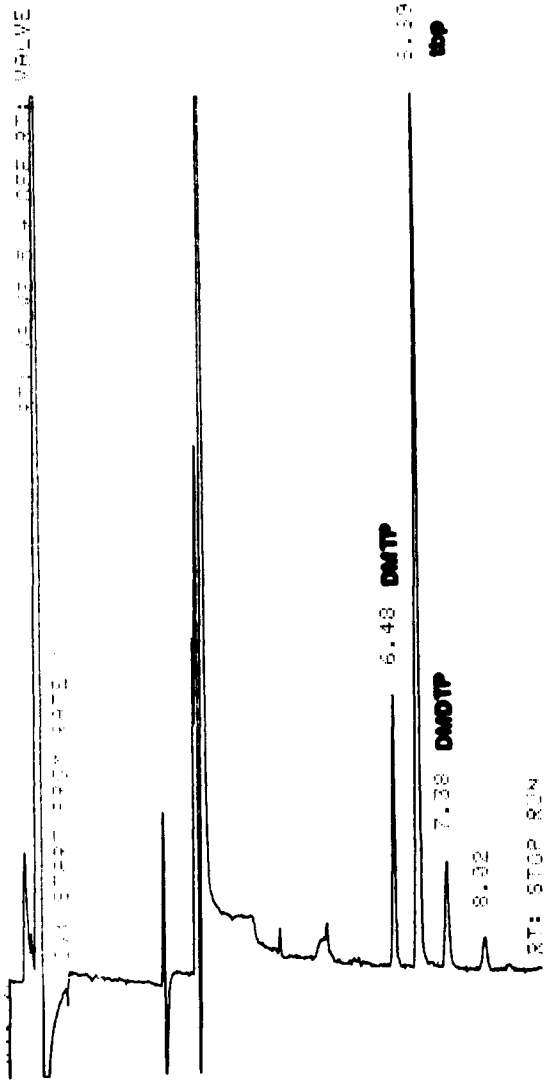

Figure 3. Chromatogram of PFB esters of dialkyl thiophosphate metabolites from urine of a pesticide applicator spraying malathion (DMTP = 0.62 ppm; DMDTP = 0.26 ppm).

4 h led to a reduction in yields. The overnight stand of the sample in acetonitrile allowed penetration of the solvent into the viscous urine material resulting from the azeotropic distillation, improving yields dramatically.

A typical chromatogram of DMTP and DMDTP from a field sample is presented in Figure 3. A 1-mL urine sample was drawn from an 8-h void after a pesticide applicator had sprayed 27 kg of a malathion 25% wettable powder formulation on citrus over 2 h. Retention times were approximately 6.5 and 7.5 min for DMTP and DMDTP, respectively. Tributyl phosphate eluted between these peaks at approximately 7 min. The limit of detection for these compounds with 1-μL injections was 0.014 ppm for DMTP and 0.025 ppm for DMDTP.

The excretion kinetics of DMTP and DMDTP over 48 h for one applicator are presented in Figure 4 to illustrate the pattern seen with nearly all of the 20 workers in the exposure study cited earlier. Excretion of both DMTP and DMDTP was greatest in the 8–16-h period postexposure. Nearly all of the DMTP and DMDTP metabolites were excreted in the first 24 h (85% and 93%, respectively), and neither compound was detectable after 48 h.

DMTP levels were higher than DMDTP levels for each 8-h sampling period. Since the fortification/recovery studies reported earlier demonstrated that some DMDTP is converted to DMTP during the extraction/derivatization procedure, these results do not necessarily reflect the

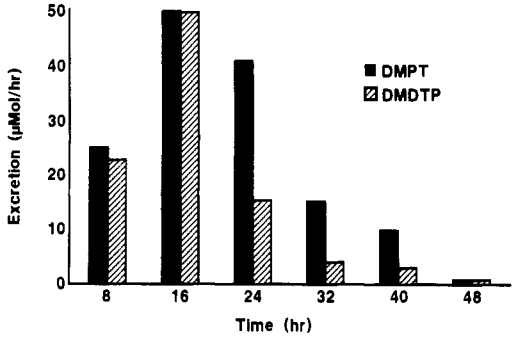

Figure 4. Excretion kinetics of DMTP and DMDTP following airblast application of malathion (25% WP formulation) in citrus orchards.

relative amounts of the two compounds as excreted. However, the amount of DMTP detected over 48 h was nearly twice that of the DMDTP (17.2 and 9.5 μmol/h, respectively), suggesting that hydrolysis occurs primarily at the P–S rather than the S–C bonds. These results are in accord with the findings of Bradway and Shafik (1977) for dialkyl phosphate excretion in rats (see Table I). Since the purpose of metabolite analysis for human exposure assessment is to quantify total malathion equivalent excretion, knowledge of the relative rates of DMTP and DMDTP excretion is not an essential issue.

The alkaline hydrolysis of malathion represents a useful modification of existing urinary alkyl phosphate methodology, simplifying gas chromatographic analysis, increasing sensitivity, and allowing investigators to include malathion with other organophosphorus compounds in a standard analytical procedure.

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Registry No. DMTP, 1112-38-5; DMDTP, 756-80-9; malathion, 121-75-5.

LITERATURE CITED

Bhagwat, V. M.; Ramachandran, B. V. *Biochem. Pharmacol.* **1975**, *24*, 1713–1717.

Bradway, D. E.; Shafik, T. M. Malathion Exposure Studies. Determination of Mono- and Dicarboxylic Acids and Alkyl Phosphates in Urine. *J. Agric. Food Chem.* **1977**, *25*, 1342–1344.

Bradway, D. E.; Moseman, R.; May, R. Analysis of Alkyl Phosphates by Extractive Alkylation. Bull. Environ. Contam. Toxicol. **1981**, *26*, 520–523.

Chen, P. R.; Tucker, W. P.; Dauterman, W. C. Structure of Biologically Produced Malathion Monoacid. *J. Agric. Food Chem.* **1969**, *17*, 86–90.

Churchill, F. C.; Ku, D. N.; Miles, J. W. Gas-Liquid Chromatographic Inlet Block Derivatization of Organophosphorus Pesticides and Related Dialkyl Phosphorothioates. J. Agric. Food Chem. **1978**, *26*, 1108–1112.

Daughton, C. G.; Cook, A. M.; Alexander, M. Gas Chromatographic Determination of Phosphorus-containing Pesticide Metabolites via Benzylation. *Anal. Chem.* **1979**, *51*, 1949–1953.

Durst, H. D.; Milano, M.; Kikta, E. J.; Connelly, S. A.; Grushka, E. Phenacyl Esters of Fatty Acids via Crown Ether Catalysts for Enhanced Ultraviolet Detection in Liquid Chromatography. *Anal. Chem.* **1975**, *47*, 1797–1801.

Fenske, R. A. Correlation of Fluorescent Tracer Measurements of Dermal Exposure and Urinary Metabolite Excretion during

Occupational Exposure to Malathion. *Am. Ind. Hyg. Assoc. J.* **1988**, *49*, 438–444.
Frei, R. W.; Lawrence, J. F. In *Chemical Derivatization in Analytical Chemistry, Volume I: Chromatography*; Plenum Press: New York, 1981; p 175.
Reid, S. J.; Watts, R. R. A Method for the Determination of Dialkyl Phosphate Residues in Urine. *J. Anal. Toxicol.* **1981**, *5*, 126–132.
Shafik, M. T.; Enos, H. F. Determination of Metabolic and Hydrolytic Products of Organophosphorus Pesticides Chemicals in Human Blood and Urine. *J. Agric. Food Chem.* **1969**, *17*, 1186–1189.
Shafik, T. M.; Bradway, D. E.; Enos, H. F.; Yobs, A. R. Human Exposure to Organophosphorus Pesticides. A Modified Procedure for the Gas-Liquid Chromatographic Analysis of the Alkyl Phosphate Metabolites in Urine. *J Agric. Food Chem.* **1973**, *21*, 625–629.
USEPA. *Pesticide Assessment Guidelines, Subdivision U: Applicator Exposure Monitoring*; National Technical Information Service: Arlington, VA, 1987.
USEPA. Environmental Protection Agency: 40 CFR Parts 156 and 170: Worker Protection Standards for Agricultural Pesticides. *Fed. Regist.* **1988**, *53*, 25970–26201.
van Heemstra-Lequin, E. A. H., van Sittert, N. J., Eds. Biological Monitoring of Workers Manufacturing, Formulating and Applying Pesticides *Toxicol. Lett.* **1986**, 33.
Wang, R. A., Franklin, C. A., Honeycutt, R. C., Reinert, J., Eds. *Biological Monitoring for Pesticide Exposure*; ACS Symposium Series 382; American Chemical Society: Washington, DC, 1989.
Wolfe, N. L.; Zepp, R. G.; Gordon, J. A.; Baughman, G. L.; Cline, D. M. Kinetics of Chemical Degradation of Malathion in Water. Environ. Sci. Technol. **1977**, *11*, 88–93.

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Composition of Jimson Weed (*Datura stramonium*) Seeds

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Bulk commercial grain, such as soybeans and wheat, may be contaminated by nongrain impurities, including jimson weed seeds, that coexist with the crop to be harvested. The present study was undertaken to determine the content of the major alkaloids of jimson weed seeds, atropine and scopolamine, as well as protein, carbohydrate, fat, mineral, hemmagglutinin, and tannin. Combined GC–MS analysis of a jimson weed seed extract revealed the presence of atropine and scopolamine plus possibly three additional tropane-like alkaloids. An improved HPLC procedure showed that the alkaloid concentration in samples obtained from different parts of the United States varied by as much as 50%: 1.69–2.71 mg/g for atropine and 0.36–0.69 mg/g for scopolamine. The presence of a strongly fluorescent green compound of unknown structure is also described. Baking experiments with jimson weed seed fortified wheat flour showed that atropine and scopolamine largely survive bread-baking conditions. Jimson weed seeds do not contain protease or amylase inhibitors. These observations provide a rational basis for relating seed composition to biological effects in animals and for assessing the possible significance of low levels of the seeds in food-producing animals and in the human diet.

The plant *Datura stramonium* was grown in England in the 16th century from seeds that came from Constantinopole, Turkey (Claus, 1961). The English presumably imported the plant to the American colonies, as evidenced by the fact that when English soldiers, who were sent to quell Bacon's rebellion at Jamestown in Colonial Virginia, inadvertently ate the plant as part of a salad in 1676, some of them became ill and died. The name jimson weed or Jamestown weed derives from this episode of fatal poisoning (Claus, 1961; Duke, 1984 Feenghaty, 1982; O'Grady et al., 1983). This and related reports of poisonings by jimson weed seeds demonstrate that the plant exerts pharmacological and toxicological effects in animals and humans. In fact, Klein-Schwartz and Oderda (1984) suggest that jimson weed abuse is a potentially serious form of substance abuse in adolescents and young adults. The most common symptoms of jimson weed ingestion are altered perception of the environment, visual hallucinations, mydriasis (dilation of the eye pupils), and tachycardia (increase in heart rate) (O'Grady et al., 1983). High levels may cause depression of the central nervous system, with symptoms ranging from lethargy to coma (Klein-Schwartz and Oderda, 1984). Antidotes include the use of the anticholinesterase drug physostigmine, charcoal to slow down absorption, magnesium citrate to speed passage through the intestinal tract, and ipecac to induce vomiting (Orr, 1975; O'Grady et al., 1983).

The literature on jimson weed covers a variety of aspects including agronomic and botanical (Broekaert et al., 1988; Hagood et al., 1981; Kilpatrick et al., 1984; van De Velde et al., 1988; Weaver, 1986), chemical and pharmaceutical (Cordell, 1981; Duez et al., 1985; List and Spencer, 1976; List et al., 1979), and medical toxicological (Day and Dilworth, 1984; El Dirdiri, 1981; Fangauf and Vogt, 1961; Feenghaty, 1982; Flunker et al., 1987; Gururaja and Khare, 1987; Keeler, 1981; Levy, 1977; Mahler, 1975; Mikolich et al., 1975; Nelson et al., 1982; Shervette et al., 1979; Testa and Fontanelli, 1988; Urich et al., 1982; Weintraub, 1960; Williams and Scott, 1984; Worthington et al., 1981).

The objectives of this study were to (a) develop an improved HPLC procedure for the analysis of atropine and scopolamine in jimson weed seeds, (b) to demonstrate the presence of known and unknown alkaloids in the seeds by GC–MS analysis, (c) to measure the nutrient and antinutrient composition of the seeds, and (d) to assess the

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