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1-(4-Nitrobenzyl)-3-(4-tolyl)triazene as a Derivatizing Reagent for the Analysis of Urinary Dialkyl Phosphate Metabolites of Organophosphorus Pesticides by Gas Chromatography

Dennis Y. Takade,[1] John M. Reynolds,* and James H. Nelson

Human exposure to organophosphorus compounds may be monitored by the determination of urinary levels of dialkyl phosphate metabolites. A sensitive, reproducible method for the determination of the *O,O*-dimethyl and *O,O*-diethyl derivatives of phosphoric, phosphorothioic, and phosphorodithioic acids is reported. The methodology consists of lyophilization of the urine, derivatization of dialkyl phosphates with a triazene alkylating agent [1-(4-nitrobenzyl)-3-(4-tolyl)triazene], cleanup by adsorptive removal of certain contaminants with anhydrous nickel sulfate, separation of P=O and P=S compounds by silica gel chromatography into two fractions, and quantitation of individual dialkyl phosphates in each fraction by gas chromatography. The method is sufficiently sensitive such that each of six urinary dialkyl phosphate metabolites studied may be determined at levels as low as 0.01 ppm. The method was evaluated by the analysis of urine samples collected from pesticide formulators and from unexposed control workers.

The determination of dialkyl phosphates is becoming increasingly important in connection with the evaluation of both environmental and occupational hazards associated with the use of organophosphorus (OP) chemicals and pesticides. In human beings, dialkyl phosphates occur as metabolic products of OP compounds, and these metabolic derivatives are excreted in the urine of individuals exposed to OP pesticides. Accordingly, their determination in urine provides a convenient means for estimating exposure to such compounds. These urinary alkyl phosphate metabolites (UAPM) include the *O,O*-dialkylphosphoric, *O,O*-dialkylphosphorothioic, and *O,O*-dialkylphosphorodithioic acids (see Table I). Previously published methods for determining these metabolites involve alkylation to increase their volatility, thereby permitting quantitation by gas chromatographic (GC) analysis (St. John and Lisk, 1968; Shafik and Enos, 1969; Shafik et al., 1973; Blair and Roderick, 1976; Daughton et al., 1976; Lores and Bradway, 1977). In each of the published methods alkylation was achieved using a diazoalkane.

Although diazoalkanes are effective alkylating reagents, they have undesirable characteristics. These include the necessity for generation of the diazoalkane immediately prior to use as well as problems related to toxicity, mutagenicity, carcinogenicity, volatility, high reactivity, and explosiveness (deBoer and Backer, 1963; Osterman-Golkar, 1974). Other difficulties are also associated with the nature of the derivatives which are generated by the use of diazoalkanes. For example, the methyl and ethyl derivatives of alkyl phosphates are not as stable to hydrolysis and do not have as favorable GC characteristics as higher molecular weight derivatives (Shafik et al., 1973). Their gas chromatographic peaks are also easily obscured by alkylated inorganic phosphate unless special procedures are employed (Shafik and Enos, 1969; Daughton et al., 1976; Blair and Roderick, 1976). Each of these difficulties is avoided through the formation of the higher molecular weight pentyl or hexyl derivatives (Shafik et al., 1973). However, repeated experimental trials in our laboratory with these diazoalkanes resulted in the generation of several different phosphorus-containing products, presumably arising from rearrangement of the diazoalkane carbon backbone before or during derivatization. Such rearrangement is possible and has been reported previously (Zollinger, 1961). These problems indicate a need for a more suitable derivatizing reagent.

Aryl–alkyl triazenes have been recommended as substitutes for diazoalkanes as alkylating reagents (White et al., 1968). This document describes a method by which suitable esters of UAPM can be prepared for GC analysis

Table I. Organophosphate Compounds and Designated Abbreviations

compound	abbreviation
O,O-dimethylphosphoric acid	H-DMP
O,O-diethylphosphoric acid	H-DEP
potassium *O,O*-dimethyl phosphorothioate	K-DMPT
potassium *O,O*-diethyl phosphorothioate	K-DEPT
potassium *O,O*-dimethyl phosphorodithioate	K-DMPDT
potassium *O,O*-diethyl phosphorodithioate	K-DEPDT
O,O-dimethyl *O*-(4-nitrobenzyl) phosphate	*O*N-DMP
O,O-diethyl *O*-(4-nitrobenzyl) phosphate	*O*N-DEP
O,O-dimethyl *O*-(4-nitrobenzyl) phosphorothionate	*O*N-DMPT
O,O-diethyl *O*-(4-nitrobenzyl) phosphorothionate	*O*N-DEPT
O,O-dimethyl *S*-(4-nitrobenzyl) phosphorothiolate	*S*N-DMPT
O,O-diethyl *S*-(4-nitrobenzyl) phosphorothiolate	*S*N-DEPT
O,O-dimethyl *S*-(4-nitrobenzyl) phosphorodithioate	*S*N-DMPDT
O,O-diethyl *S*-(4-nitrobenzyl) phosphorodithioate	*S*N-DEPDT

University of Utah Research Institute, Salt Lake City, Utah 84108.

[1]Present address: Velsicol Chemical Corporation, Chicago Illinois 60611.

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using 1-(4-nitrobenzyl)-3-(4-tolyl)triazene (*p*-nitrobenzyltolyltriazene, PNBTT). This reagent, unlike the diazoalkanes, is a crystalline solid, stable in solution, ready to use without prior generation, and is nonvolatile and nonexplosive. The method permits detection of at least 0.01 ppm of each of several UAPM compounds in urine without apparent interference from inorganic phosphate.

EXPERIMENTAL SECTION

Apparatus. A Tracor Model MT 222 gas chromatograph equipped with a dual flame photometric detector (FPD) was used in the GC analysis. A Valco four-port high-temperature column switching valve was supplied with the instrument for solvent venting and was heated by the outlet cover block. A glass column, 27 in. × $^1/_4$ in. o.d. × 4 mm i.d., was silanized (Leibrand and Dunham, 1973) and packed with 5% OV-101 on 100/120 mesh Chromosorb G-HP for gas chromatographic determinations. Operating temperatures were as follows: injector port, 225 °C; oven, 200 °C; and detector, 235 °C. The carrier gas was nitrogen; flow rate was 210 cm^3/min.

In use, the analytical column was protected with a precolumn from potential adverse effects of thermal decomposition products derived from nonvolatile extractives of urine. Precolumns provided better protection and better peak efficiencies than glass wool alone. Each precolumn was prepared by inserting a silanized glass tube (3 in. × $^1/_4$ in. o.d. × 4 mm i.d.) into the inlet chamber. The outlet opening of the glass tube was allowed to constrict during fire polishing to an opening of approximately 2 mm in order to retain the packing. The packing consisted of $^1/_4$ in. silanized glass wool, 1 in. of 5% OV-101 on Chromosorb G-HP, and 1 in. of loosely packed silanized glass wool. In order to prevent carrier gas from bypassing the precolumn, a positive seal between the outside of the precolumn and the bottom of the inlet port was achieved by placing a $^1/_4$ in. diameter Teflon ferrule on the outside of the precolumn and between the bottom of the inlet chamber and the metal sleeve. The bottom of the metal sleeve was beveled on the inside in order to fit the ferrule. The retainer, which is normally not tightened, was screwed down tightly onto the metal sleeve on top of the ferrule, forcing the ferrule to form a tight seal between the precolumn and the bottom of the port. The metal sleeve and the retainer are stock items with the Tracor Model MT 222 gas chromatograph. The analytical column was cut $^1/_4$ in. shorter than normal so that the precolumn would extend past the ferrule into the narrower part of the inlet chamber. This precolumn was cleaned and repacked daily during sample analysis.

A Dupont Model 291B mass spectrometer equipped with a Hewlett-Packard Model 2100 computer was used for mass spectral analysis. Spectra were obtained at 20 eV with a probe temperature between 50 and 80 °C. Mass range was scanned from 0 to 400.

Proton magnetic resonance (^{1}H NMR) spectra of the derivatives were recorded using a Varian EM 390 NMR spectrometer at 90 MHz. Solvent was deuteriochloroform ($CDCl_3$) with 1% Me_4Si as internal standard.

A rotary evapo-mix (Buchler Instruments, Fort Lee, NJ) was used for incubating the reactions under vortex type stirring. A Roto-Rack (Fisher Scientific, Pittsburgh, PA) was used for mixing samples at room temperature. Lyophilizations were achieved with the use of a Thermovac Portable lyophilizer (Thermovac Industries, Copiaque, NY) equipped with a Welch Duo Seal Model 1400 vacuum pump.

Reagents. All solvents were distilled in glass (Nanograde; Mallinkrodt, St. Louis, MO). Diethyl ether (anhydrous) was reagent grade (supplied with 2% ethanol preservative) (Mallinkrodt). 1-(4-Nitrobenzyl)-3-(4-tolyl)triazene was purchased from Regis Chemical Co. (Morton Grove, IL). Caution: Triazenes are potential carcinogens (Preussman et al., 1969).

O,O-Dimethylphosphoric acid, *O,O*-diethylphosphoric acid, potassium *O,O*-dimethyl phosphorothioate, and potassium *O,O*-diethyl phosphorothioate were supplied by American Cyanamid (Princeton, NJ). Potassium *O,O*-dimethyl phosphorodithioate and potassium *O,O*-diethyl phosphorodithioate were graciously supplied by D. A. Wustner (University of California, Riverside, CA). Primary stock solutions were prepared in methanol to correspond to 1000 ppm free acid as follows: H-DMP, 10.0 mg/10 mL; H-DEP, 10.0 mg/10 mL; K-DMPT, 12.7 mg/10 mL; K-DEPT, 12.2 mg/10 mL; K-DMPDT, 12.4 mg/10 mL; K-DEPDT, 12.0 mg/10 mL. (See Table I for abbreviations used throughout this paper.) Working standard solutions were prepared in methanol to correspond to 10 ppm of the free acid.

Reagent grade nickel sulfate (granulated, Mallinckrodt) was dehydrated at approximately 200 °C overnight, ground in a porcelain mortar, and stored in an air-tight bottle for at least 1 week prior to use. Freshly prepared nickel sulfate is ineffective in the adsorptive removal of contaminants, while nickel sulfate stored for at least 1 week is effective. We do not know the reason for this observed phenomenon. Caution: Various forms of nickel have been associated with adverse biological reactions (National Research Council, 1975).

Silica gel (Mallinckrodt SilicAR CC-7, 200–325 mesh) for column chromatography was dried overnight at 190 °C and stored in an air-tight bottle. Columns were prepared in 6.5 in. glass Pasteur pipets by adding in sequence the following materials: a $^1/_4$ in. silanized glass wool plug, $^1/_8$ in. of anhydrous sodium sulfate, approximately 0.5 g of anhydrous silica gel, and $^1/_2$ in. of anhydrous sodium sulfate. Columns were prepared on same day of use.

Analytical Procedures. *Derivatization.* A 2-mL aliquot of urine, frozen at –75 °C for storage, was transferred to a 15 × 25 mm screw-cap tube. Samples were processed in duplicate. Each urine sample was saturated with approximately 500 mg of sodium bitartrate monohydrate and lyophilized. To each lyophilate was added 1 mL of 0.002 M PNBTT in chloroform, and the tubes were capped and incubated with gentle agitation at 40 °C for 2 h. Screw-cap tubes with Teflon-faced liners were used for this purpose. Alternatively, incubation can be accomplished overnight at room temperature in a Roto-Rack set at 50 rpm. At the end of incubation, 1 mL of chloroform was added to each tube followed by 5 mL of 0.1 N HCl to destroy excess PNBTT, to dissolve solid residues, and to facilitate the extraction of reaction byproducts. The organic layer was washed with 10 mL of distilled water and the aqueous layer was discarded by careful aspiration in a manner to prevent loss of chloroform. The chloroform was dried by decanting through 5 g of anhydrous sodium sulfate supported by a glass wool plug in a $^1/_2$ × 3 in. filter funnel. The tube and filter funnel were rinsed three times with approximately 1 mL of chloroform each time. Each rinse was blown from the funnel with a rubber squeeze bulb. The combined volume of the chloroform fractions was 4–5 mL collected in a clean 15-mL tube. Two blank urine samples and a set of fortified urine samples (employed as standards) were routinely processed with each set of analytical samples. The blank urine, obtained from laboratory personnel, consisted of a pooled urine sample collected from at least three different individuals. Appropriate aliquots of the pooled sample were fortified with

Table II. Mass Spectrometry (MS) and Proton Magnetic Resonance (^{1}H NMR) Data for Dimethyl Phosphate Derivatives and for *N*-(4-Nitrobenzyl)toluidine

compound	structure	^{1}H NMR data (solvent: $CDCl_3$) δ (Me_4Si)	MS data *m/e* (rel intensity)
*O*N-DMP	$(CH_3O)_2P(O)OCH_2C_6H_4NO_2$	3.78 (d, 6 H, *J* = 12 Hz, OCH_3), 5.18 (d, 2 H, *J* = 10 Hz, $OCH_2C_6H_4$), 7.57 (d, 2 H, *J* = 9 Hz, *H2,6*-$ArNO_2$), 8.25 (d, 2 H, *J* = 9 Hz, *H3,5*-$ArNO_2$)	261 (19) (M), 244 (14), 137 (9), 121 (10), 119 (19), 117 (17), 110 (100), 109 (9), 95 (13), 89 (14), 80 (16), 79 (11)
*O*N-DMPT	$(CH_3O)_2P(S)OCH_2C_6H_4NO_2$	3.76 (d, 6 H, *J* = 13 Hz, OCH_3), 5.18 (d, 2 H, *J* = 10 Hz, $OCH_2C_6H_4$), 7.53 (d, 2 H, *J* = 9 Hz, *H2,6*-$ArNO_2$), 8.22 (d, 2 H, *J* = 9 Hz, *H3,5*-$ArNO_2$)	279 (14) (M + 2), 278 (95) (M + 1), 277 (100) (M), 261 (20), 260 (25), 151 (10), 136 (29), 110 (74), 93 (66), 90 (10), 78 (39)
*S*N-DMPT	$(CH_3O)_2P(O)SCH_2C_6H_4NO_2$	3.72 (d, 6 H, *J* = 13 Hz, OCH_3), 4.10 (d, 2 H, *J* = 15 Hz, $SCH_2C_6H_4$), 7.55 (d, 2 H, *J* = 9 Hz, *H2,6*-$ArNO_2$), 8.20 (d, 2 H, *J* = 9 Hz, *H3,5*-$ArNO_2$)	277 (20) (M), 260 (10), 156 (7), 151 (7), 136 (6), 117 (7), 110 (100), 109 (11), 93 (7), 80 (14), 79 (15), 78 (7)
*S*N-DMPDT	$(CH_3O)_2P(S)SCH_2C_6H_4NO_2$	3.64 (d, 6 H, *J* = 23 Hz, OCH_3), 4.10 (d, 2 H, *J* = 26 Hz, $SCH_2C_6H_4$), 7.52 (d, 2 H, *J* = 13.5 Hz, *H2,6*-$ArNO_2$), 8.16 (d, 2 H, *J* = 13.5 Hz, *H3,5*-$ArNO_2$)	293 (21) (M), 157 (22), 151 (9), 136 (6), 126 (24), 125 (12), 93 (100), 90 (8), 89 (9), 79 (9), 78 (8)
*N*N-T[a]	$CH_3C_6H_4NHCH_2C_6H_4NO_2$	2.24 (s, 3 H, $C_6H_4CH_3$), 4.44 (s, 2 H, $NHCH_2C_6H_4$), 6.48 (d, 2 H, *J* = 9 Hz, *H2,6*-ArNH), 6.96 (d, 2 H, *J* = 9 Hz, *H3,5*-ArNH), 7.51 (d, 2 H, *J* = 9 Hz, *H2,6*-$ArNO_2$), 8.17 (d, 2 H, *J* = 9 Hz, *H3,5*-$ArNO_2$)	243 (24) (M + 1), 242 (100) (M), 241 (20), 196 (12), 195 (11), 120 (40), 107 (14), 106 (57), 90 (11), 89 (20), 79 (18)

[a] *N*-(4-Nitrobenzyl)toluidine.

the dialkyl phosphates to obtain a set of standards. Each fortified sample contained all six metabolites, each at the same concentration (with the exception of DMP). A set of standards for each analytical run was comprised of duplicate samples prepared at each of the following concentration levels for each dialkyl phosphate except DMP: 0.01, 0.05, 0.10, and 1.00 ppm. Corresponding DMP levels were twice those listed above. Low recovery of DMP dictates use of standards of higher concentration levels for this metabolite. If required, other standards of varying concentrations were processed to match the metabolite levels observed in the analytical samples. A competent analyst can process an average of approximately ten analytical samples per day utilizing this procedure.

Nickel Sulfate Cleanup. To the 4–5 mL of dried chloroform was added 1.5 g of powdered anhydrous nickel sulfate. The tube was capped and mixed for 15 min on a Roto-Rack at 50 rpm at room temperature. The nickel sulfate was removed by *suction* filtration through a 15-mL medium porosity fritted disk Büchner funnel. The test tube, nickel sulfate, and funnel were rinsed three times with 1 mL of chloroform and the filtrates were collected in a 15-mL graduated tube. The combined filtrates were evaporated under a gentle stream of nitrogen in a 40 °C water bath and taken up in 0.5 mL of benzene.

Silica Gel Chromatography. A column of silica gel (5 × 0.5 cm i.d.) was washed with approximately 0.2 mL of dichloromethane and 5 mL of hexane. After the hexane entered the upper sodium sulfate layer, the 0.5 mL of benzene concentrate was applied and the collection of the effluent begun. The concentration tube was rinsed three times with approximately 0.2 mL of benzene and each rinse was applied to the column immediately after the previous rinse had entered the sodium sulfate layer. After the last rinse entered the column, 4 mL of dichloromethane was applied. The effluent (fraction I) contained the P=S derivatives. The column was transferred to a new collection tube and eluted with 2 mL of acetone. The effluent (fraction II) contained the P=O derivatives. The fractions were taken to dryness under nitrogen in a water bath at 40 °C. Each residue was dissolved in 200 μL of ethyl acetate. An appropriate aliquot of the resulting solution (up to 6 μL) was injected for gas chromatographic analysis.

Structural Confirmation. Ten milliliters of chloroform containing 2 mmol of PNBTT was carefully added to 1 mmol of each dialkyl phosphate. These preparations were allowed to stand loosely capped overnight at room temperature. Salts were converted to the acid form by the addition of 1 mmol of hydrochloric acid (0.2 mL of 5 N HCl) prior to the addition of chloroform. The reaction mixtures were concentrated and the derivatives purified by preparative thin-layer chromatography (TLC). The P=S derivatives were developed in benzene and P=O derivatives were developed in diethyl ether. Bands were observed under ultraviolet light and product bands localized on a test plate using 4-nitrobenzylpyridine (Watts, 1965). Corresponding bands on the preparative plates were scraped off and the respective derivatives were eluted from the scrapings with acetonitrile. The acetonitrile was evaporated to dryness in a rotary evaporator at 35 °C. The residue of each derivative was dissolved in benzene, reapplied, and redeveloped on new TLC plates. A third TLC development was effected by repeating this process. *O*N-DMP required a fourth development in ethyl acetate to achieve purity. Thin-layer chromatography of each derivative resulted in a single spot in each of seven different solvent systems. The detection of P=O and P=S groups in the derivatives was achieved using TLC–bromine vapor oxidation (Gardner, 1971) and by the use of 2,6-dibromo-*N*-chloro-*p*-quinonimine spray reagent (Menn et al., 1957) on analytical TLC plates. Structural characteristics of each TLC-purified derivative were assessed by mass spectrometry and nuclear magnetic resonance spectroscopy with determination of the structures illustrated in Tables II and III.

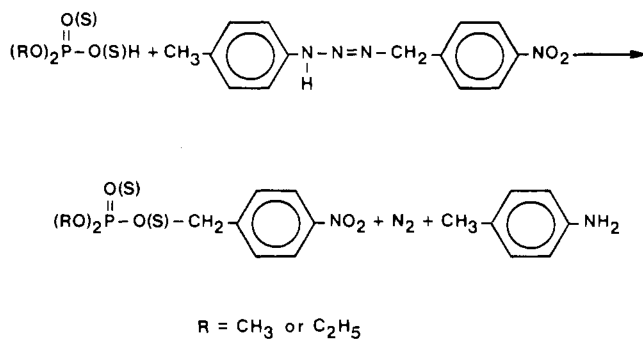
Analysis of Urine Samples. The analytical method was evaluated by the analysis of urine samples collected from 12 different pesticide formulators during normal work exposure to a variety of OP compounds; samples were also obtained from ten petroleum refinery workers (controls) expected to have minimal exposure. All samples (morning void) were collected in clean polyethylene containers

Table III. Mass Spectrometry (MS) and Proton Magnetic Resonance (^{1}H NMR) Data for Diethyl Phosphate Derivatives

compound	structure	^{1}H NMR data (solvent: $CDCl_3$) δ (Me_4Si)	MS data m/e (rel intensity)
ON-DEP	C_2H_5O, O, P, C_2H_5O, OCH_2, NO_2	1.31 (t, 6 H, J = 7 Hz, OCH_2CH_3), 4.11 (m, 4 H, J = 7,7 Hz,[a] OCH_2CH_3), 5.14 (d, 2 H, J = 8 Hz, $OCH_2C_6H_4$), 7.54 (d, 2 H, J = 9 Hz, H2,6-$ArNO_2$), 8.22 (d, 2 H, J = 9 Hz, H3,5-$ArNO_2$)	289 (87) (M), 261 (54), 233 (77), 216 (86), 153 (72), 152 (100), 136 (74), 125 (92), 89 (48), 82 (75)
ON-DEPT	C_2H_5O, S, P, C_2H_5O, OCH_2, NO_2	1.32 (t, 6 H, J = 7 Hz, OCH_2CH_3), 4.13 (m, 4 H, J = 7,10 Hz,[a] OCH_2CH_3), 5.18 (d, 2 H, J = 10 Hz, $OCH_2C_6H_4$), 7.56 (d, 2 H, J = 9 Hz, H2,6-$ArNO_2$), 8.24 (d, 2 H, J = 9 Hz, H3,5 $ArNO_2$)	307 (23) (M + 2), 306 (100) (M + 1), 305 (79) (M), 153 (20), 138 (22), 136 (43), 121 (29), 97 (22), 90 (37), 89 (46), 78 (29)
SN-DEPT	C_2H_5O, O, P, C_2H_5O, SCH_2, NO_2	1.28 (t, 6 H, J = 7 Hz, OCH_2CH_3), 4.06 (m,[b] 4 H, OCH_2CH_3), 4.12 (d, 2 H, J = 16 Hz, $SCH_2C_6H_4$), 7.57 (d, 2 H, J = 9 Hz, H2,6-$ArNO_2$), 8.20 (d, 2 H, J = 9 Hz, H3,5-$ArNO_2$)	307 (20) (M + 2), 306 (100) (M + 1), 305 (80) (M), 169 (22), 153 (15), 138 (22), 136 (16), 122 (15), 121 (30), 111 (22), 89 (31)
SN-DEPDT	C_2H_5O, S, P, C_2H_5O, SCH_2, NO_2	1.27 (t, 6 H, J = 11 Hz, OCH_2CH_3), 4.09 (m,[b] 4 H, OCH_2CH_3), 4.15 (d, 2 H, J = 26 Hz, $SCH_2C_6H_4$), 7.53 (d, 2 H, J = 13 Hz, H2,6-$ArNO_2$), 8.18 (d, 2 H, J = 13 Hz, H3,5-$ArNO_2$)	321 (20) (M), 185 (17), 154 (20), 136 (11), 129 (16), 121 (100), 97 (20), 93 (21), 89 (10), 65 (20)

[a] The first coupling constant listed pertains to methylene-methyl proton coupling. The second pertains to methylene proton-phosphorus coupling. [b] Due to the complexity of the methylene multiplet pattern and also the spectral overlap of the methylene and benzylic proton signals, coupling constants are not assigned.

Scheme I



without preservative. They were frozen immediately upon collection, maintained in the frozen state during shipment to the laboratory, and stored at -75 °C until analysis.

Because recovery of the dialkyl phosphates is somewhat variable from one compound to another and from one day to the next, standards for these analyses were prepared by fortifying base line urine samples (having no detectable OP compounds) with appropriate levels of each dialkyl phosphate and processing these standard preparations along with the samples. Standard curves were constructed from the results obtained with fortified samples and the concentration range of standards was matched to those of the samples. This technique automatically compensates for recovery factors for each dialkyl phosphate.

The specific gravity of each urine sample was determined and urinary alkyl metabolite concentrations were adjusted by multiplication of each observed concentration by the ratio: 1.024/sp gr. Morgan et al. (1977) prefer correction in relationship to a standard urinary solute, e.g., creatinine.

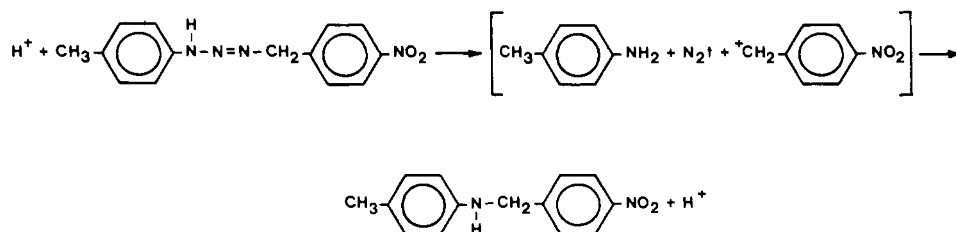
RESULTS AND DISCUSSION

The reaction of PNBTT with the dialkyl phosphates proceeds according to Scheme I. The reaction of aryl-alkyl triazenes with acids has been investigated by White et al. (1969). Protonation of the triazene appears to be the first step. This is followed by the expulsion of nitrogen and the formation of a reactive carbonium ion. Alkylation of the aryl amine is a major side reaction reported by White et al. (1969). This reaction was also observed in this study with PNBTT (Scheme II). The resulting N-alkylated toluidine is one of the major interferences of the assay. Its identity was confirmed by ^{1}H NMR and mass spectral data (see Table II).

Alkylation. Aryl-alkyl triazenes react more rapidly with strong acids than with weak acids (White and Scherrer, 1961). This characteristic permits the dialkyl phosphates, which are strong acids (pK_a = 1-2) (Charton, 1969), to be derivatized in the presence of a large excess of weak acid. Reaction of PNBTT with pure dialkyl phosphoric acids is essentially instantaneous. However, derivatization of the metabolites in the presence of urinary constituents requires approximately 2 h at 40 °C or overnight incubation at 25 °C to complete the reaction.

The concentration of PNBTT specified in this paper (0.002 M) is evidently not critical. It represents a compromise between increased yields of ON-DMP and ON-DEP achieved with higher PNBTT concentrations (~0.01 M) and reduced levels of undesirable side-reaction con-

Scheme II



taminants observed with the use of lower PNBTT concentrations (~0.0005 M).

Highest yields were obtained with the use of chloroform as the solvent for PNBTT. Other solvents evaluated were benzene, diethyl ether, ethyl acetate, acetone, acetonitrile, and methanol. Beginning with acetone, increased solvent polarity resulted in increased extraneous phosphorus peaks by flame photometric GC and decreased yields of the desired derivatives.

Structural Confirmation. Each of the eight derivatives listed in Table I yielded a single TLC spot in seven different solvent systems.

Both ^{1}H NMR and mass spectral data were obtained for each TLC-purified derivative. These data are concisely summarized for dimethyl compounds and for diethyl compounds in Tables II and III, respectively. Also presented in Table II are spectral data pertaining to the major undesirable byproduct [*N*-(4-nitrobenzyl)toluidine] of the derivatization reaction.

The mass spectral data are comprised of fragmentation patterns consistent with characteristic structural features for each class of dialkyl phosphate compound (dimethyl or diethyl). Ion fragments expected from the 4-nitrobenzyl group were also observed in each case. The ^{1}H NMR spectrum of each derivative verified the presence of protons belonging to an aromatic nitro moiety. Dimethyl phosphate ^{1}H NMR spectra are characterized by methoxy protons coupled to phosphorus, whereas the corresponding data for diethyl phosphates shows coupled methylene and methyl protons of an ethoxy group. The methylene proton signals are split due to phosphorus coupling. The observed chemical shifts of benzylic protons are appropriately either oxyester (δ 5.1–5.2) or thioester (δ ~4.1). The benzylic proton signals are observed as doublets due to coupling with phosphorus.

Silica Gel Chromatography. Incomplete resolution of *O*N-DEP from *O*N-DMPT, *S*N-DMPT from *O*N-DEPT, and *S*N-DEPT from *S*N-DMPDT was experienced using the OV-101 gas chromatographic column. Accordingly, silica gel chromatography was employed for preliminary separation of P=O derivatives from P=S derivatives prior to gas chromatographic analysis. Chloroform cannot be substituted for dichloromethane in the elution of P=S derivatives since in chloroform the P=O and P=S derivatives are not completely resolved on the silica gel column. The separation of P=S compounds from P=O compounds by silica gel chromatography is illustrated in Figure 1.

Gas Chromatography. Figure 1 also illustrates typical gas chromatograms of P=S and of P=O derivatives from urine fortified at 0.1 ppm with each of the test metabolite compounds (DMP at 0.2 ppm); note that DMPT and DEPT each yield both the P=S and the P=O compound during derivatization. The relative retention times of each derivative on OV-101, the recommended gas chromatographic column, are presented in Table IV. In addition, the presence of each of these derivatives was confirmed by chromatography on 5% OV-210 on 100/120 mesh Gas-Chrom Q. Relative retention times on OV-210 are also summarized in Table IV.

Preparation of Samples Prior to Derivatization. Previous studies by Shafik et al. (1973) involved solvent partitioning of dialkyl phosphates from acidified urine prior to derivatization with diazoalkanes. These workers employed 1:1 acetonitrile/diethyl ether for extraction. In our laboratory, chloroform yielded much less extraneous material than did acetonitrile/diethyl ether. Therefore, chloroform was used for the preparation of urine in our

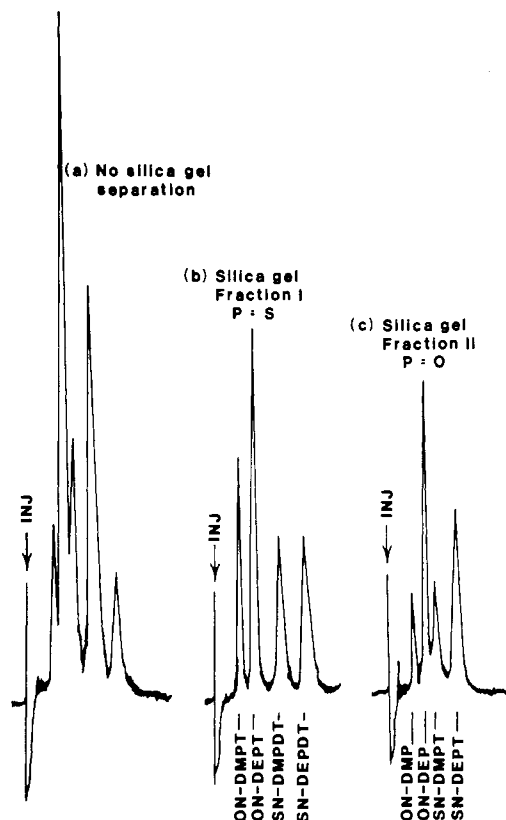


Figure 1. Gas chromatographic output (FPD) obtained for a mixture of urinary dialkyl phosphate derivatives with: (a) no silica gel separation; (b) fraction I from preliminary silica gel chromatography; and (c) fraction II from preliminary silica gel chromatography.

Table IV. Retention Time of Nitrobenzyl Derivatives Relative to Parathion

compound	RRT (OV-101)[a]	RRT (OV-210)[b]
Compounds from Silica Gel Fraction I (P=S)		
*O*N-DMPT	1.11	1.15
*O*N-DEPT	1.49	1.44
*S*N-DMPDT	2.19	2.15
*S*N-DEPDT	2.82	2.54
Compounds from Silica Gel Fraction II (P=O)		
*O*N-DMP	0.94	1.67
*O*N-DEP	1.23	2.13
*S*N-DMPT	1.57	2.29
*S*N-DEPT	2.05	2.77

[a] 5% OV-101 on Chromosorb G-HP, 100/120 mesh, 27 in. × 1/4 in. o.d. glass column; oven temperature 200 °C. [b] 5% OV-210 on Gas-Chrom Q, 100/120 mesh, 27 in. × 1/4 in. o.d. glass column; oven temperature 200 °C.

initial derivatization experiments. We determined, however, that better results are obtained when the preparative chloroform extraction procedure is replaced by lyophilization of the urine sample, particularly if addition of sodium bitartrate to the sample precedes the lyophilization step. When recovery experiments were conducted with urine fortified at dialkyl phosphate levels less than 0.1 ppm, direct treatment of lyophilized urine with the derivatizing reagent yielded cleaner results than did solvent partitioning with chloroform. The level of interferences in the solvent partition method prevented quantitation of organophosphates below 0.1 ppm. The cause of these interferences is likely the result of products created in acidifying the urine. The addition of 500 mg of sodium bitartrate to a 2-mL sample of urine prior to

Table V. Urinary Alkyl Phosphate Levels (ppm) in Samples from Nonexposed Individuals

identification no.	DMP	DMPT	DMPDT	DEP	DEPT	DEPDT
1	0.07	<0.01	<0.01	<0.01	<0.01	<0.01
2	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
3	0.03	0.01	<0.01	<0.01	<0.01	<0.01
4	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
5	0.02	<0.01	<0.01	<0.01	<0.01	<0.01
6	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
7	0.01	<0.01	<0.01	<0.01	<0.01	<0.01
8	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
9	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
10	0.06	0.09	<0.01	<0.01	<0.01	<0.01

Table VI. Urinary Alkyl Phosphate Levels (ppm) in Samples from Pesticide Formulators Exposed to Organophosphorus Pesticides

identification no.	DMP	DMPT	DMPDT	DEP	DEPT	DEPDT
11	2.53	1.01	0.59	0.27	0.82	<0.01
12	2.72	0.56	0.70	0.23	0.11	<0.01
13	0.06	0.03	0.05	0.04	0.03	0.04
14	0.04	0.04	0.08	0.02	0.14	0.02
15	2.20	0.08	<0.01	0.15	0.04	<0.01
16	4.74	0.04	<0.01	0.36	0.09	<0.01
17	0.48	0.02	0.01	0.06	0.02	<0.01
18	0.98	0.01	0.01	0.09	0.01	<0.01
19	0.45	0.03	<0.01	0.07	0.01	<0.01
20	0.82	0.01	<0.01	0.14	0.02	<0.01
21	0.37	0.03	<0.01	0.05	0.02	<0.01
22	0.72	0.03	<0.01	0.18	0.03	<0.01

lyophilization resulted in optimum analytical performance in various comparative experiments we conducted to investigate procedures for preparation of the urine for derivatization. Use of the bitartrate resulted in a lyophilate which was porous and powdery. The lyophilate obtained with either the addition of sodium bisulfate or with no additive was glutinous and somewhat impervious (in the presence of chloroform) to the PNBTT reagent. The exact quantity of bitartrate was not critical. A large change (e.g., twofold increase) in the amount of added bitartrate resulted in slightly different (e.g., 5–10%) yields for some of the derivatives.

Other acids such as oxalic, boric, tartaric, succinic, and acetic acids and salts such as sodium chloride, sodium acetate, disodium tartrate, and sodium borate were tried as additives prior to lyophilization. In general, strong acids ($\mathrm{p}K_a < 2$) resulted in discoloration of the urinary residue during lyophilization and reduced yields of derivatives. Salts with an alkaline reaction in water also gave reduced or no yields of derivatives. Neutral salts had little effect. Slightly acidic salts or weak acids were beneficial in improving yields.

The bitartrate/lyophilization method is preferred for assessing levels of urinary alkyl phosphate metabolites below 0.1 ppm. The detection limit of this method is approximately 0.01 ppm in urine for each of the six dialkyl phosphates investigated in this study. The detection limit is defined as twice the base line noise which is observed in the chromatograms of fortified urine samples. This detection limit is somewhat variable, depending on the condition of the gas chromatographic column and on the characteristics of the sample. The detector sensitivity for parathion was 0.1 ng at the minimum detection limit. The detector sensitivity for *O*N-DEPT, an analogue of parathion, was also 0.1 ng.

Results of Analysis of Urine Samples. Urine samples from ten different petroleum refinery workers, expected to have minimal exposure to OP compounds, were analyzed for six different OP metabolites. Results are shown in Table V. These data demonstrate the practical sensitivity of the method as well as the fact that these nonexposed individuals have, in general, only very small urinary levels of each of the UAPM compounds. It is interesting to note, however, that two of the workers (1 and 10) had relatively high concentrations of urinary DMP. These results were unexpected. Subsequent observations in our laboratory have verified that unexpectedly high levels of DMP are occasionally found in the urine of individuals whose known exposure to OP pesticides is minimal. Similar high background DMP values have been reported by Morgan et al. (1977). We speculate that the high DMP concentrations observed in our work are the result of an interfering constituent having chromatographic properties similar to those of DMP. However, it is possible that unexpectedly high DMP levels are actually realized. Resolution of this problem requires additional research and such studies are currently being pursued in our laboratory. Summarized in Table VI are similar data for pesticide formulators. During sample collection, each of these workers was exposed to a variety of OP compounds at varying levels. The results demonstrate that our method is effective in detecting dialkyl phosphates in the urine of exposed workers. With the exception of the DEPDT data, exposed individuals have, in general, UAPM levels which are at least an order of magnitude higher than such levels in nonexposed subjects. These results were obtained with urine samples comprised of only the morning void. Morgan et al. (1977) have shown that of the total DMP eliminated (within 24 h) in the urine of human subjects who had previously ingested methyl parathion, 67% was excreted within 8 h of dosing. Similar ingestion of ethyl parathion resulted in the excretion of 41% of the total (24 h) recovered DEP and 99.5% of recovered DEPT within 8 h. Although the excretion of urinary metabolites might be expected to be more protracted under conditions of occupational exposure (in comparison to ingestion), these results indicate that alkyl phosphate levels might possibly be higher in complete 24-h urine samples than in morning void collections as reported here. Therefore, monitoring of worker exposure to OP compounds with this procedure

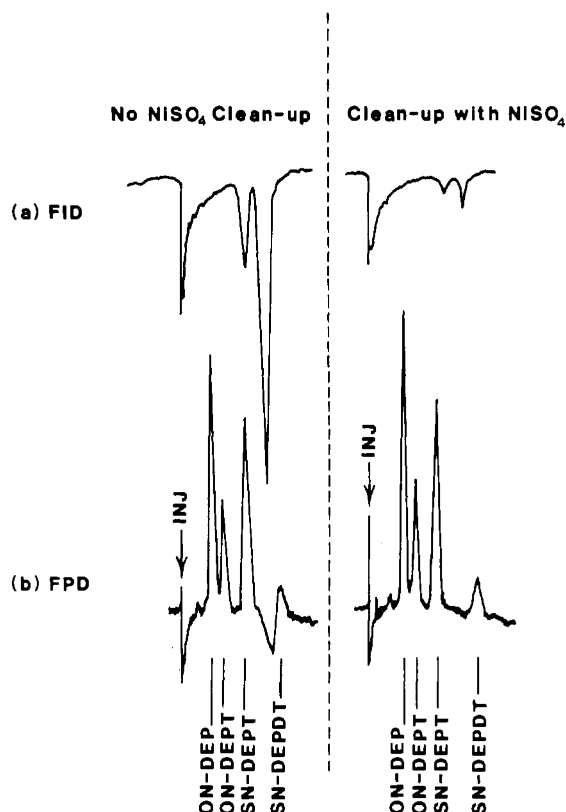


Figure 2. Effect of nickel sulfate clean-up procedure on the (a) flame ionization detector (FID) output and (b) flame photometric detector (FPD) output in the gas chromatography of derivatives of DEP, DEPT, and DEPDT from urine fortified with 0.1 ppm of each compound. The FPD peak for *S*N-DEPDT is partially obscured by interferences in the absence of nickel sulfate cleanup.

appears very promising, particularly if 24-h urine samples can be obtained. Additional advantages of the method are that it reflects actual absorption of the OP compounds and, in addition, it is much more sensitive than determination of either plasma or RBC cholinesterase. For example, it is estimated that depression of either plasma or RBC cholinesterase requires absorption of more than 10 mg/day of methyl parathion (Rider et al., 1969). Considering the data of Morgan et al. (1977), this would correspond to a daily excretion of approximately 0.6 mg of DMP. Our method is capable of detecting 0.01 μg of DMP/mL of urine. Assuming a daily excretion of 1500 mL of urine, a minimum of 0.015 mg of DMP/day may be detected. Therefore, exposures which could be overlooked by cholinesterase determinations would be detected by appropriate UAPM monitoring.

Interfering Compounds. Byproducts resulting from internal side-reactions of the triazene reagent gave the most serious interferences observed in the GC chromatograms. The N-alkylated toluidine byproduct was found to be one of several components of a large GC peak which was observed with the flame ionization detector. These interfering compounds eluted almost simultaneously with *S*N-DEPDT on the OV-101 column. When their concentrations were sufficiently high, they caused a depression in the output of the flame photometric detector (FPD), thus preventing accurate quantitation of *S*N-DEPDT. The presence of these interferences was observed on the FPD as a negative deflection in the base line just as *S*N-DEPDT began to elute (see Figure 2). Levels of *S*N-DEPDT higher than 0.1 ppm are observable in the presence of this interference, but extension of sensitivity to 0.01 ppm requires removal of these side-reaction contaminants. (With reference to Figure 2, it is interesting to note the diminished response of *O*N-DEPT and *S*N-DEPDT in comparison with the corresponding peaks in Figure 1. This phenomenon has also been observed with dimethyl derivatives. Such diminished response is attributed to the accumulation of urine degradation products on the precolumn. Frequent repacking of the precolumn restores expected sensitivity.) A rapid and convenient clean-up procedure for removal of undesirable contaminants involves the adsorbance of byproducts on anhydrous nickel sulfate. The rationale for our investigating this clean-up procedure was, in part, based on the observation that metal cations have been used successfully in the ligand chromatographic separation of aromatic amines (Chow and Grushka, 1977). None of the other metal salts which we studied (zinc sulfate, cobalt sulfate, and copper sulfate in both hydrated and anhydrous states) proved to be as effective as nickel sulfate. The binding capacity of nickel sulfate for the interfering compounds increased with increasing state of dehydration. Only suction filtration was used to remove spent nickel sulfate since interfering compounds were occasionally observed in the filtrate when pressure filtration was used.

Extraneous peaks representing phosphorus-containing compounds occasionally appeared in the FPD gas chromatograms. It was found that the origin of such peaks could be attributed variously to the use of glass wool, contaminated anhydrous sodium sulfate, or the direct exposure of polyethylene bottles to organic solvents. Accordingly, Teflon or glass containers were used for procedures involving direct interface with organic solvents. Reagents, packing materials, and other items were carefully preextracted, when necessary, to circumvent problems with interferences.

CONCLUSIONS

The assay for dialkyl phosphate metabolites in urine using PNBTT is sensitive, specific, and reproducible. In addition to the excellent sensitivity of the method, the use of an aryl–alkyl triazene for derivatization is an effective alternative to diazoalkanes because it eliminates many of the attendant problems intrinsically associated with the latter. This has also been demonstrated in recent experiments reported by Shafik and Peoples (1976) in which a different triazene, 1-benzyl-3-(4-tolyl)triazene was employed as the derivatizing agent.

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Determination of Arprinocid in Chicken Tissues by Gas Chromatography–Mass Spectrometry

Patricia Cala Tway,* James S. Wood, Jr., and George V. Downing

A procedure has been developed for the isolation and determination of the coccidiostat arprinocid and its metabolites in tissue. Analysis can be performed with a sensitivity of 0.04 ppm using a combined gas chromatographic–mass spectroscopic detection system. Recoveries of arprinocid spikes average 76% for liver, kidney, and muscle and 70% for skin/fat.

Arprinocid [6-amino-9-(2-chloro-6-fluorobenzyl)purine] (Figure 1) is a newly developed coccidiostat which is used prophylactically in broiler chickens. The drug is effective at the 70 ppm level against all important economic strains of coccidia and has been tested against more than 50 strains of *Eimeria tenella* and *Eimeria acervulina* (Miller et al., 1977; Tamas et al., 1978; Olson et al., 1978).

To study the tissue distribution of the arprinocid residue in chickens and to satisfy the requirements of governmental regulatory agencies, a sensitive, specific, and reliable chemical assay for the parent drug and its major drug-related metabolites was needed. Metabolism studies using radioactive tracer have shown that the largest and most persistent drug residues are found in the liver. While on-drug liver residue is primarily the parent compound, the residue in livers from chickens that are withdrawn from the drug for 5 days is only approximately 5% arprinocid. Most of the drug residue at this withdrawal time is covalently bonded to macromolecular constitutents of the tissue and is not easily solvent extractable from the tissue. It was found that acid hydrolysis of the tissue released approximately 70% of the radioactive residue; the acid hydrolyzes the residue, including any arprinocid, to form 2-choro-6-fluorobenzylamine. On the basis of this metabolism work (Carlin et al., 1976), liver was chosen as the target tissue and 2-chloro-6-fluorobenzylamine formed by acid hydrolysis as the marker substance. Both the use of liver as the target tissue and fluorochlorobenzylamine as the marker substance fulfill the requirements of the Food and Drug Administration for an acceptable animal drug residue assay (Federal Register, Feb 22, 1977).

Many different analytical techniques have been used to determine amines including fluorescence measurement after derivatization (Rinde and Troll, 1976; Sturgeon and Schulman, 1975), liquid chromatography using UV detection (Majors, 1973), and gas chromatography using electron-capture detection (Cala et al., 1972). Recently workers have used gas chromatography (GC) coupled with mass spectrometry (MS) as the detection system to quantitate low-level drug residues in biological extracts, primarily in plasma and urine (Summons et al., 1974; Narasimhachari et al., 1978; Carrington and Frigerio, 1977).

During the early work it was found that dansylation of the 2-chloro-6-fluorobenzylamine was quantitative (Carlin et al., 1976), and the product (Figure 1) could be manipulated without the losses due to volatility that were likely with the underivatized amine. It could also be determined by gas chromatography using electron-capture detection. However, initial experiments on arprinocid showed that gas chromatography with electron-capture detection did not have the necessary specificity for an assay with the desired sensitivity. A material was found to extract from the tissue which interfered with the dansyl derivative upon chromatography, and no combination of chromatographic conditions or extraction/purification steps were found to eliminate this interference. However, GC/MS using specific ion monitoring was found to provide the necessary sensitivity and specificity.

The procedure developed utilizes gas chromatography–mass spectrometry in the chemical ionization mode as the method of separation and detection. The combined drug residue is hydrolyzed to yield 2-chloro-6-fluorobenzylamine which is extracted from the tissue, dansylated, and cleaned up further through a series of extractions, and finally quantitated by GC/MS. The assay has a sensitivity of 0.04 ppm and a limit of detection of approximately 0.01 ppm. Recoveries of arprinocid spikes averaged 76% for liver, kidney, and muscle and 70% for skin/fat.

EXPERIMENTAL SECTION

Reagents. All organic solvents were nanograde quality. All other reagents were of analytical grade purity. The 5-dimethylamino-1-naphthalenesulfonyl chloride (dansyl chloride) was reagent grade from Matheson, Coleman, & Bell. The dansyl derivative of 2-chloro-6-fluorobenzylamine was obtained from Merck Sharp & Dohme Research Laboratories, Rahway, NJ. All aqueous solutions were prepared with water which had been doubly distilled.

A standard solution of 2-chloro-6-fluorobenzylamine dansyl derivative was prepared by dissolving 1 mg of the

Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065.

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