Determination of Metabolic and Hydrolytic Products of Organophosphorus Pesticide Chemicals in Human Blood and Urine

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The procedure of St. John and Lisk for the determination of the hydrolytic metabolites of organophosphorus insecticides in cow urine has been modified for application to human blood and urine. The extracting solvent, a 1 to 1 mixture of acetonitrile and diethyl ether, resulted in an average recovery of 90, 89, and 87%, of the six dialkyl phosphates from water, urine, and blood, respectively. The interference from inorganic phosphates was eliminated by making the methyl and ethyl derivatives of the dialkyl phosphates. The use of a column packed with 20% Versamid on Gas Chrom Q allowed for the simultaneous determination of the six major metabolites of organophosphorus and thiophosphorus insecticides. The level of alkyl phosphates excreted in the urine of exposed individuals was considerably higher than in the nonexposed group.

The metabolism and urinary hydrolysis of organophosphorus pesticide chemicals in mammals results in the excretion of a variety of alkyl phosphates. These include the salts of dimethyl or diethyl phosphate, thiophosphate, or dithiophosphate. The separation and quantitation of such products in blood or urine is of value in estimating the extent of exposure to the parent pesticide chemical.

The methylation and ethylation of mono- and dimethyl phosphates and the mono- and diethyl phosphates produced the esters dimethyl monoethyl phosphate, diethyl monomethyl phosphate, trimethyl phosphate, and triethyl phosphate. The preparation of both the methyl and ethyl esters can be used for the definite identification of the various mono- and dialkyl phosphates by gas chromatography (Stanley, 1966).

Gas chromatographic analysis of the methylated metabolites, utilizing a thermionic detector, has been described (Gutenmann *et al.*, 1968; St. John and Lisk, 1968). An investigation of the applicability of this procedure to human urine and blood samples has resulted in changes in the extraction, alkylation, and chromatographic steps which make the method more sensitive, eliminate interferences from inorganic phosphates, and allow for the simultaneous determination of six major metabolites or hydrolysis products of organophosphorus insecticides.

EXPERIMENTAL

Solvents and Reagents. Extracting solvent, mixture of acetonitrile and diethyl ether (1 to 1).

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wis.).

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Dimethyl phosphate (DMP) 10 μg./5 ml.

Dimethyl thiophosphate (DMTP) 10 μg./5 ml.

Dimethyl dithiophosphate (DMDTP) 25 μg./5 ml.

Diethyl phosphate (DEP) 5 μg./5 ml.

Diethyl thiophosphate (DETP) 10 μg./5 ml.

Diethyl dithiophosphate (DEDTP) 10 μg./5 ml.

Trimethyl phosphate (TMP), in hexane, 1 μg./ml.

Triethyl phosphate (TEP), in hexane, 1 μg./ml.

Potassium dihydrogen phosphate, in water, 100 μg./ml. Prepare the solutions of the potassium salts of the dialkyl phosphates in water for use as standard solutions and store them in a freezer. Frozen solutions are stable for about three months.

Diazoalkane reagent (Stanley, 1966), prepared as follows: Dissolve 2.3 grams of potassium hydroxide in 2.3 ml. of distilled water in a 125-ml. Erlenmeyer flask, cool, and add 25 ml. of ethyl ether. Cool the flask in a freezer. Carry out the following preparation in a highly efficient hood. Add either 1.5 grams of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine for diazomethane, or 1.6 grams of *N*-ethyl-*N'*-nitro-*N*-nitroso guanidine for diazoethane in small portions over a period of a few minutes to the flask, and shake the flask vigorously after each addition. Decant the ether layer into a bottle capped with a "poly seal" cap (A. H. Thomas Catalog No. 2849-E) and store in a freezer. (Caution: Diazoalkanes are skin irritants and carcinogens.) Do not use a ground-glass-stoppered bottle. (If it is kept in a tightly capped bottle, the diazoalkane solution may be stored at −20 °C. for a week.) This procedure gives about 16 ml. of ether solution. The diazoalkane can be prepared in a larger quantity by increasing the quantities of the reagents used but the proportions must not be changed because a higher diazoalkane concentration might result in an explosion. Do not use etched or scratched glassware and avoid exposure to strong light.

Apparatus. Flame Photometric Detector. (Melpar Corp., Falls Church, Va., available from MicroTek Instrument Co., Baton Rouge, La.) equipped with the 394-mμ and 526-mμ filters (to determine sulfur and phosphorus compounds, respectively) attached to a MicroTek MT 220 gas chromatograph.

Modifications of Gas Chromatograph. (a) Equip bucking control on electrometer Type E-2 with a 10-megohm Victoreen resistor.

(b) Install Carle switching valve No. 2011 with automatic actuator No. 2050 interfaced between gas chromatographic column and flame photometric detector. (The valve, which is heated with a 432-watt, 2 1/2- × 24-inch insulated heating tape, permits interchange of column effluent and nitrogen purge). Adjust the nitrogen purge flow rate to equal the flow from the gas chromatographic column so that when an interchange of flows is made for the purpose of venting solvent, no change is observed in the recorder base line. This arrangement avoids extinguishing the flame when sample injections are made.

Gas Chromatographic Column. Pack an aluminum column (1/4-inch o.d. × 12 feet) with 60- to 80-mesh Gas

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Chrom Q coated with 20% Versamid 900. (On-column injection is used for all analyses.)

COLUMN PACKING. Dissolve Versamid 900 in hot 1 to 1 (v./v.) mixture of chloroform and *n*-butanol. (The volume of the mixed solvent should be just enough to produce a slurry when added to the Gas Chrom Q.) Place the weighed Gas Chrom Q in an evaporating dish, then add the dissolved Versamid and mix gently. Leave the evaporating dish in a hood overnight or until all the solvent has evaporated. Place in an oven overnight at 150 °C. Do not make any attempt to sieve the preparation. (The presence of agglomerates does not affect the efficiency of the column packing.) Condition the prepared column at 200 °C. for four days.

DISPOSABLE CAPILLARY PASTEUR PIPETS were also used.

Methods. PREPARATION OF STANDARD CURVES. Dialkyl Phosphates. Pipet an aliquot of each of the dialkyl phosphate standard solutions into a 100-ml. centrifuge tube. Dilute the combined aliquots of 5 ml. with distilled water. Add 15 grams of sodium chloride and exactly 10 ml. of the extracting solvent (1 to 1 v./v. mixture of acetonitrile and diethyl ether) to the centrifuge tube. Add 1 ml. of 5*N* HCl, stopper the tube, and proceed with immediate, vigorous mixing for 1 minute with the aid of a Vortex-Genie mixer (Scientific Industries, Inc.). Centrifuge for 1 minute to obtain complete separation of the two layers. Immediately pipet an aliquot (1 to 9 ml.) of the organic solvent layer into a 15-ml. glass-stoppered graduated centrifuge tube. Avoid prolonged contact of the sample with the 5*N* HCl solution to prevent the decomposition of the dialkyl phosphate. Concentrate aliquots larger than 2 ml. to 1 to 2 ml. with a gentle stream of nitrogen. Alkylate by adding either 10 drops of methanol and 2 ml. of diazomethane solution or 10 drops of ethanol and 2 ml. of diazoethane solution to the ether concentrate. Allow the mixture to stand for 20 minutes in a well-ventilated hood. Bubble dry nitrogen through the solution using a disposable pipet connected to a nitrogen cylinder. When the yellow color of the diazoalkane disappears, cease bubbling nitrogen and dilute the standard solution to 2 to 10 ml. with either methanol or ethanol, depending on the alkyl derivative being made. Inject 5 to 50 μl. into the gas chromatograph.

Inorganic Phosphates. Pipet an aliquot of KH_2PO_4 standard solution (0.01–0.1 ml.) into a 10-ml. graduated test tube. Add one drop of concentrated HCl and 10 drops of either methanol or ethanol followed by 2 ml. of the corresponding diazoalkane solution. Proceed as described above for dialkyl phosphates.

Trimethyl Phosphate and Triethyl Phosphate. Inject appropriate amounts of the standard solutions directly into the gas chromatograph.

Extraction of Blood and Urine. Analyze each of two 5-ml. aliquots of a blood or urine sample in the following manner: Pre-extract a 5-ml. aliquot of blood or urine contained in a 100-ml. centrifuge tube, with two 5-ml. portions of diethyl ether and discard the extracts. Pre-extraction is necessary only when the urine has been contaminated with feces as would be the case in using this procedure on urine collected from experimental animals housed in cages. The pre-extraction does not remove any of the metabolites described in this paper. Add sodium chloride and extracting solvent, acidify, and proceed as described under preparation of standard curves.

Alternatively, if alkyl phosphate levels permit, extract one 5-ml. portion of sample, and pipet two equal aliquots of the organic solvent extract into two 15-ml. graduated centrifuge tubes. (It is necessary to methylate and ethylate each sample; see Results and Discussion.) After complete removal of the diazoalkane, inject 10 μl. of the solution into the gas chromatograph as a trial sample to determine whether the sample must be diluted or concentrated before proceeding to the gas chromatographic step. As in the case of the standards, dilute the methylated derivative with methanol and the ethylated derivative with ethanol. Use injections of 5 to 50 μl. from a final volume of 2 to 50 ml. for satisfactory results.

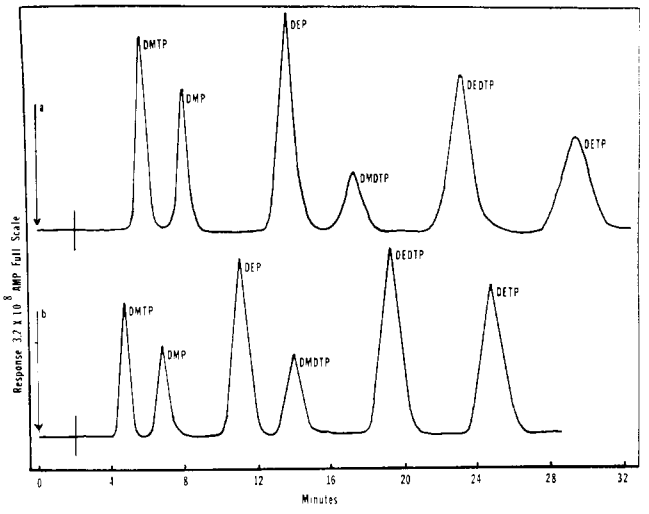

Figure 1. Chromatograms of trialkyl phosphate esters using the phosphorus filter, 526 mμ

a. Ethylated: 6.5 ng. DMTO; 8 ng. DMP; 4 ng. DEP; 45 ng. DMDTP; 10.5 ng. DEDTP; 8.5 ng. DETP.
b. Methylated: 11 ng. DMTP; 5.7 ng. DMP; 3.2 ng. DEP; 35 ng. DMDTP; 10 ng. DEDTP; 8.4 ng. DETP

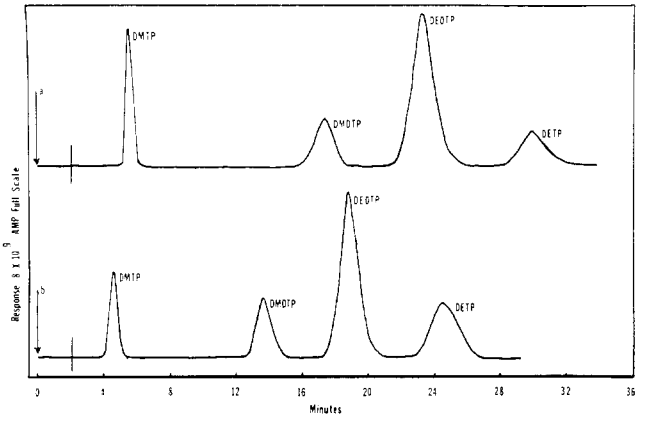

Figure 2. Chromatograms of trialkyl phosphate esters using the sulfur filter, 394 mμ

a. Ethylated: 25 ng. DMTP; 120 ng. DMDTP; 20 ng. DEDTP; 20 ng. DETP
b. Methylated: 50 ng. DMTP; 120 ng. DMDTP; 20 ng. DEDTP; 20 ng. DETP

Gas Chromatography. Inject appropriate aliquots of the sample and standard extracts into the gas chromatograph. Set the operating temperatures of the column, injection block, detector, transfer lines, and switching valve to 142, 143, 130, 145, and 145 °C., respectively. Adjust the gas flow rates for nitrogen (carrier), nitrogen (purge), hydrogen, air, and oxygen to 90, 90, 180, 80, and 10 ml. per minute, respectively.

RESULTS AND DISCUSSION

Chromatograms in Figures 1 and 2, of the ethylated and methylated mixtures of dialkyl phosphates, were obtained with the use of the phosphorus filter and sulfur filter, respectively.

Table I. Isothermal Gas Chromatography of Trialkyl Phosphates with Phosphorus Flame Photometric Detection System

Trialkyl Phosphate	Relative Retention Time	Detector Sensitivity (4/1 Signal-to-Noise Ratio), Nanograms	Limit of Detectability, P.P.M.
TMTP	0.70		
TMP	1.00		
DEMMP	1.72	0.4	0.01
TMDTP	2.09	15.0	0.20
DEMMDTP	2.98	1.0	0.02
DEMMTP	4.12	1.0	0.02
DMMETP	0.71	1.5	0.02
DMMEP	1.00	1.5	0.02
TEP	1.75		
DMMEDTP	2.18		
TEDTP	3.08		
TETP	3.96		

TMTP = Trimethyl thiophosphate; TMP = Trimethyl phosphate; DEMMP = Diethyl monomethyl phosphate; TMTP = Trimethyl dithiophosphate; DEMMDTP = Diethyl monomethyl dithiophosphate; DEMMTP = Diethyl monomethyl thiophosphate; DMMETP = Dimethyl monoethyl thiophosphate; DMMEP = Dimethyl monoethyl phosphate; TEP = Triethyl phosphate; DMMEDTP = Dimethyl monoethyl dithiophosphate; TEDTP = Triethyl dithiophosphate; TETP = Triethyl thiophosphate.

Table II. Recovery of Dialkyl Phosphates from Water, Urine, and Blood

Dialkyl Phosphate	Added, μg.	Recovered from 5 ml, μg[a]		
		Water	Urine	Blood serum
DMTP	10	9.9	9.9	9.0
DMP	5	4.2	4.2	4.3
DEP	5	4.7	4.7	4.6
DMDTP	50	42.0	38.0	37.0
DEDTP	10	8.3	8.3	8.2
DETP	10	10.0	10.0	9.5

[a] Average of three determination.

The following dialkyl phosphate derivatives were found to give a linear response in the range of 0 to 10 ng.: Ethylated DMTP and DMP; methylated DEP, DEDTP, DETP. On the other hand, the methylated DMDTP was found to give a linear response in the range of 0 to 100 ng. Quantitation was made using the phosphorus filter.

A comparison of the standard curves reveals that the ethylated DMP and DMTP produce a greater detector response than the corresponding methylated compounds. Conversely, the methylated derivatives of DEP, DMDTP, and DETP produce a greater detector response than the corresponding ethylated derivatives. For this reason, quantitation is based on the methylated DEP, DMDTP, DEDTP, DETP, and the ethylated DMTP and DMP. However, it should be pointed out that this difference in sensitivity is not the only reason for methylating and ethylating each sample. The extraction step removes inorganic phosphates from the sample. The phosphate, in turn, converts to either TMP or TEP, depending on the diazoalkane used for derivatization. In one case, the TMP interferes with methylated DMP; in the other, the TEP interferes with the ethylated DEP. This problem can be circumvented by splitting the extract and making both derivatives. The TMP produced during methylation is vented in much the same manner as the solvent peak; likewise, in the case of ethylation, the TEP peak is vented.

Table I lists the relative retention times, detector sensitivity, and limits of detectability for the methylated and ethylated dialkyl phosphates using the phosphorus filter.

In order to determine the efficiency of the alkylation step, a quantitative comparison was made between commercially available TMP and TEP and the corresponding derivatives produced by methylating DMP or ethylating DEP. In both cases, the standard curves were superimposable, indicating a 100% conversion. Furthermore, the methylation or ethylation of acidified KH_2PO_4 produced TMP and TEP which were quantitatively indistinguishable from the standard curves discussed above.

Primary standards for the other trialkyl phosphates discussed were not available and, therefore, they were not tested. However, it was assumed that the efficiency of derivatization is the same for these compounds as for those actually used.

As shown in Table II, the average recovery of six dialkyl phosphates from water, urine, and blood was 90, 89, and 87%, respectively.

The ability to interchange the sulfur and phosphorus filters in the single detector or the use of the base assembly for dual phototube operation with both filters (Bowman and Beroza, 1968) greatly enhances the specificity of this method. Suspected thiophosphate can be confirmed with the sulfur filter by simply increasing the concentration of the compound injected into the gas chromatograph by a factor of 5 to 10. With the sulfur filter in the detector, up to 200 ng. of TMP or TEP can be injected without producing a significant detector response.

The monoalkyl phosphates undergo derivatization by this technique to produce dimethyl monoalkyl phosphates on methylation or diethyl monoalkyl phosphates on ethylation—*i.e.*, methylated DEP is used as a standard for ethylated monomethyl phosphate metabolite. A mixture of monomethyl and dimethyl phosphate after ethylation would yield DEMMP and DMMEP compounds which are readily separated on the Versamid gas chromatographic column.

The results for any particular compound can be confirmed by forming the *n*-propyl derivative as in the method described for methylation and ethylation, except that *N*-*n*-propyl-*N'*-nitro-*N*-nitrosoguanidine is used as the diazoalkane precursor. The *n*-propyl derivative can also be used to circumvent the interference by inorganic phosphates except that the analysis time would be unduly lengthened.

Urine and blood serum samples from different individuals were analyzed for their alkyl phosphate content with the results grouped into three categories, shown in Table III. The classification is somewhat arbitrary and based on information supplied by Health Officers. The first group of subjects was exposed to dichlorvos, malathion, and carbophenothion, and the samples were taken 14 days after the last exposure. Members of the second group were operators of a nursery and landscaping organization and the third were nonexposed individuals. The results of the analysis of these samples are presented as a first indication of the potential of this method for determining the extent of exposure to organophosphorus compounds. Additional experiments with humans and animals are in progress in order to establish this relationship more precisely.

Figures 3 and 4 illustrate chromatograms of methylated and ethylated urine extract of an exposed individual.

Equipping the gas chromatograph with an automated switching valve greatly simplifies the operation of the flame photometric detector. The flame and, hence, the base line are not disturbed during the analysis because excess solvents

Table III. Urine and Blood Serum Alkyl Phosphate Content of Exposed and Nonexposed Individuals Concentration, P.P.M.

Degree of Exposure[a]	MMP[b]	DMTP	DMP	DEP	DMDTP	DEDTP	DETP	Serum[c] DEP	Serum[c] DMDTP
(1) Heavy	*N.D.*[d]	*N.D.*	0.13	0.20	1.52	*N.D.*	0.21	0.20	3.50
	0.02	0.11	0.12	1.72	1.20	*N.D.*	0.18	0.15	3.40
	N.D.	*N.D.*	*N.D.*	0.12	1.86	*N.D.*	*N.D.*	0.20	3.00
(2) Moderate	*N.D.*	*N.D.*	0.05	0.03	0.69	*N.D.*	0.04	*N.D.*	0.11
	0.03	*N.D.*	*N.D.*	*N.D.*	0.73	*N.D.*	0.04	*N.D.*	...
	N.D.	*N.D.*	*N.D.*	0.02	1.76	*N.D.*	*N.D.*	*N.D.*	0.15
	N.D.	*N.D.*	*N.D.*	*N.D.*	0.56	*N.D.*	*N.D.*	...	...
	N.D.	*N.D.*	*N.D.*	0.05	0.96	*N.D.*	0.04	...	0.16
	0.02	*N.D.*	*N.D.*	0.07	1.40	*N.D.*	*N.D.*	*N.D.*	0.12
	0.03	0.04	*N.D.*	0.04	1.47	*N.D.*	0.04	*N.D.*	0.16
	0.03	*N.D.*	*N.D.*	*N.D.*	1.22	*N.D.*	0.04	...	...
(3) Light or None	*N.D.*	*N.D.*	*N.D.*	0.02	0.40	*N.D.*	*N.D.*	*N.D.*	*N.D.*
	N.D.	*N.D.*	*N.D.*	0.04	0.38	*N.D.*	*N.D.*	*N.D.*	*N.D.*
	N.D.	*N.D.*	*N.D.*	*N.D.*	0.22	*N.D.*	*N.D.*	*N.D.*	*N.D.*
	N.D.	*N.D.*	*N.D.*	*N.D.*	<0.20	*N.D.*	*N.D.*	*N.D.*	*N.D.*

[a] Selection category is arbitrary and based on information supplied by Health Officers.
[b] Ethylated monomethyl phosphate, calculated using a methylated diethyl phosphate standard.
[c] Other alkyl phosphates were not found in the serum samples.
[d] Not determined.

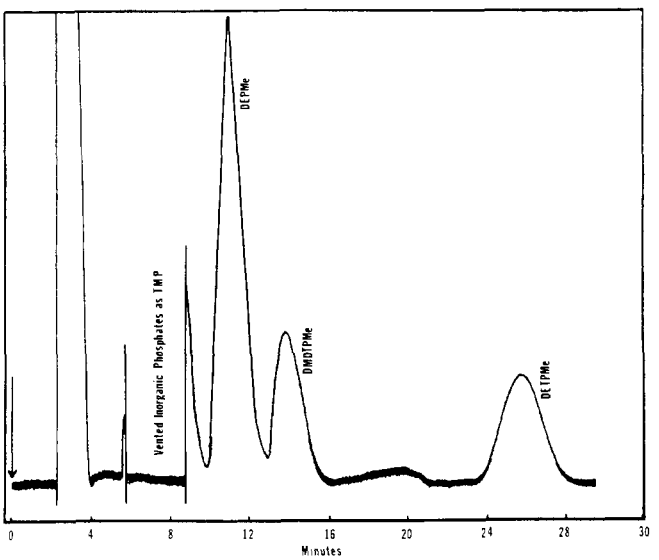

Figure 3. Chromatogram of methylated urine extract

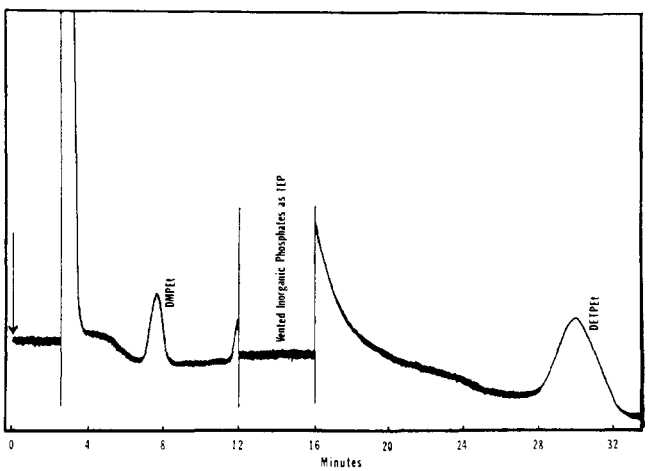

Figure 4. Chromatogram of ethylated urine extract

and other compounds are vented to the atmosphere with essentially no interruption of any of the gases flowing to the detector.

Aside from the specific application cited in this paper, the method described probably can be used in the following applications:

Determine the rate of decomposition of organophosphorus pesticide formulations through analysis of the resulting alkyl phosphates.

In both animal and plant tissue extracts, determine the presence of organothiophosphates and their corresponding oxons through alkaline hydrolysis and subsequent derivatization.

Determine the presence of gas chromatographically unstable organophosphorus insecticides (vinyl phosphates, TEPP, etc.) in a variety of substrates *via* a preliminary alkaline hydrolysis, followed by derivatization and quantitation using a phosphorus detector.

Study metabolism reactions such as dealkylation, isomerization, and oxidation of organophosphorus pesticides without recourse to radioactive labelled compounds.

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