A Cleanup Procedure for the Determination of Low Levels of Alkyl Phosphates, Thiophosphates, and Dithiophosphates in Rat and Human Urine

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A method has been developed for the cleanup of rat and human urine extracts which permits quantitative analysis of alkyl phosphates at a level considerably lower than heretofore possible. The method has been used for analysis of urine from rats exposed to parathion. Results indicate that the analysis of urine for alkyl phosphates constitutes an excellent tool for monitoring both acute and chronic exposure to parathion in the general population.

An investigation has been initiated to determine whether a correlation exists between the extent of exposure to an organophosphorus pesticide and the urinary secretion of the corresponding alkyl phosphate. As part of the study, Shafik and Enos (1969) developed a method for the determination of alkyl phosphates in the urine of humans occupationally exposed to organophosphorus pesticides. However, application of this method to the determination of these compounds in rat urine required a preliminary cleanup. Although several authors (Askew *et al.*, 1969; Gutenmann *et al.*, 1968) have described analytical methods for determining alkyl phosphates in a variety of substrates, no cleanup procedure has been suggested. This study was therefore undertaken to develop a suitable sample cleanup.

The preparation of standard curves, extraction of urine, and gas chromatographic determination of the methylated and ethylated alkyl phosphates have been described in a previous publication (Shafik and Enos, 1969). *O,O*-Diethyl phosphorothionate is quantitated after methylation as *O,O*-diethyl *S*-methylphosphorothiolate (Shafik *et al.*, 1970).

The initial phase of this investigation involved the separation of mixtures of trialkyl phosphate standards by solvent partitioning and column chromatography.

For the convenience of the reader, the following list includes the abbreviations used when referring to alkyl phosphates: DMP—*O,O*-dimethyl phosphate; TMP—*O,O,O*-trimethyl phosphate; DMEP—*O,O*-dimethyl *O*-ethyl phosphate; DEP—*O,O*-diethyl phosphate; DEMP—*O,O*-diethyl *O*-methyl phosphate; TEP—*O,O,O*-triethyl phosphate; DMTP—*O,O*-dimethyl phosphorothionate; TMTP—*O,O,O*-trimethyl phosphorothionate; DMETP—*O,O*-dimethyl *O*-ethyl phosphorothionate; DETP—*O,O*-diethyl phosphorothionate; DEMTP—*O,O*-diethyl *O*-methyl phosphorothionate; TETP—*O,O,O*-triethyl phosphorothionate; DEPTh—*O,O*-diethyl phosphorothiolate; DEMPTh—*O,O*-diethyl *S*-methyl phosphorothiolate; TEPTh—*O,O*-diethyl *S*-ethyl phosphorothiolate; DMDTP—*O,O*-dimethyl phosphorodithioate; DMEDTP—*O,O*-dimethyl *S*-ethyl phosphorodithioate; TMDTP—*O,O*-dimethyl *S*-methyl phosphorodithioate; DEDTP—*O,O*-diethyl phosphorodithioate; DEMDTP—*O,O*-diethyl *S*-methyl phosphorodithioate; TEDTP—*O,O*-diethyl *S*-ethyl phosphorodithioate.

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MATERIALS AND METHODS

Solvents and Reagents. Silica gel, Woelm, Activity Grade I (Waters Associates, Inc.), was activated at 135° C for 48 hr and stored in a desiccator. Standard solution for determining silica gel elution pattern was prepared as a mixture of the trialkyl phosphates in hexane at the following concentrations: TMTP, 5 μg/ml; TETP, 5 μg/ml; TMP, 20 μg/ml; DMEP, 19 μg/ml; DEMP, 12 μg/ml; TEP, 12 μg/ml; TMDTP, 25 μg/ml; TEDTP, 10 μg/ml; and TEPTh, 10 μg/ml.

Animal Treatments. Pairs of male Sprague-Dawley rats (250–270 g body weight) were dosed by gavage with peanut oil solutions of parathion at 160, 16, 3.2, and 1.6 μg/ml, representing, respectively, $^1/_{10}$, $^1/_{100}$, $^1/_{500}$, and $^1/_{1000}$ of the LD_{50} of parathion (LD_{50}, 4 mg/kg), and an aqueous solution of the potassium salt of diethyl phosphorothioate at a level equivalent to the DETPK content of $^1/_{10}$ of the LD_{50} of parathion (114 μg/ml). The doses were administered daily for 12 days. The animals were maintained in stainless steel metabolism cages (Acme Metal Products Inc.) which were cleaned twice daily to minimize fecal contamination. Urine samples were collected in glass bottles, with no preservative added, at 24-hr intervals before, during, and for 12 days after exposure. Twenty-four-hour urine samples from rats fed parathion were analyzed for alkyl phosphates at day 0, 1, 6, 12, and 14. For the DETPK feeding, an extra 24-hr sample was analyzed at day 2.

Solvent Partitioning of Trialkyl Phosphates. Evaporate the methylated or ethylated dialkyl phosphate standard solution to 0.5 ml using a gentle stream of filtered nitrogen and a water

Table I. Typical Elution Pattern of Trialkyl Phosphates from Silica Gel. Recovery % of Indicated Amount[a] of Test Compound

Solvent	TMTP, 5 μg	TETP, 5 μg	TMP, 20 μg	DMEP, 19 μg	DEMP, 12 μg	TEP, 12 μg	TMDTP, 25 μg	TEDTP, 10 μg	TEPTh, 10 μg
Hexane	...	...	...	...	...	...	...	...	...
20% (v/v) benzene in hexane	...	...	...	...	...	...	...	...	...
40% (v/v) benzene in hexane	...	...	...	...	...	...	13	51	...
60% (v/v) benzene in hexane	100	100	...	...	...	...	87	49	...
80% (v/v) benzene in hexane	...	...	...	...	...	...	...	...	...
Benzene	...	...	...	...	...	...	...	...	...
20% (v/v) ethylacetate in benzene	...	...	...	...	...	...	...	...	84
40% (v/v) ethylacetate in benzene	...	...	...	2	17	46	...	...	16
60% (v/v) ethylacetate in benzene	...	...	19	57	71	51	...	...	...
80% (v/v) ethylacetate in benzene	...	...	66	40	12	2	...	...	...
Ethylacetate	...	...	15	...	...	...	...	...	...

[a] The original amount of trialkyl phosphates in 1 ml of mixed standard.

Table II. Recovery of Known Amounts of Trialkyl Phosphates Added to Human and Rat Urine Extracts Using the Cleanup Procedure. Concentration[a] of Indicated Compound, ppm

Trialkyl phosphate	Human urine		Rat urine	
	Added	Recovered	Added	Recovered
TMTP	0.10	0.092	0.50	0.42
	0.010	0.009	0.050	0.046
TETP	0.10	0.093	0.50	0.44
	0.010	0.009	0.050	0.044
TMDTP	0.50	0.49	2.50	2.40
	0.050	0.046	0.25	0.24
TEDTP	0.20	0.19	1.0	0.91
	0.020	0.016	0.10	0.080
TEPTh	0.20	0.18	1.0	0.94
	0.020	0.016	0.10	0.10
DMEP	0.40	0.22	2.0	1.57
	0.040	0.027	0.20	0.17
DEMP	0.20	0.17	1.0	0.79
	0.020	0.019	0.10	0.10

[a] All results calculated as the potassium salt of the corresponding dialkyl phosphate.

bath maintained at 40° C. Add 9.5 ml of water and 10 ml of *n*-hexane, mix on a Vortex mixer for 1 min, and allow the layers to separate. The hexane will extract the sulfur-containing trialkyl phosphates: methylated and ethylated DMTP, DMDTP, DETP, DEPTh, and DEDTP. The nonsulfur trialkyl phosphates will remain in the aqueous layer. Add 5 g of NaCl and 10 ml of benzene to the aqueous layer. Mix on a Vortex mixer for 1 min and allow the layers to separate. The benzene extract contains DMEP, DEMP, TEP, and a portion of the TMP.

Elution of Trialkyl Phosphates from Silica Gel. Partially deactivate the silica gel by shaking with 3% (w/w) distilled water. Allow to stand for about 4 hr. To a size 22 chromatographic column (Kontes #420100) plugged with glass wool, add 1 g of silica gel, followed by 2 g of anhydrous sodium sulfate. Prewash the column with 10 ml of *n*-hexane and discard. Introduce 1 ml of the solution of trialkyl phosphates to the silica gel column. Elute successively with 8 ml of each of the solvents listed in Table I, collecting each eluate separately.

Cleanup Procedure for Trialkyl Phosphates in Rat and Human Urine. A combination of the benzene partitioning step and the silica gel column chromatography was applied to the cleanup of both rat and human urine using the following procedure. Concentrate the methylated or ethylated ether–acetonitrile extract of urine (Shafik and Enos, 1969) to 0.5 ml in a water bath maintained at 40° C, using a gentle stream of nitrogen. Add 9.5 ml of water, 5 ml of benzene, and 5 g of NaCl. Mix on a Vortex mixer for 1 min and allow the layers to separate. With the aid of a disposable pipet, transfer the benzene extract quantitatively to a silica gel column prepared as previously described. Immediately begin collection of the eluate. Extract the aqueous layer with an additional 3 ml of benzene, transferring the benzene extract to the column just as the previous extract reaches the sodium sulfate layer. Rinse the sides of the column with 2 ml of benzene. The benzene eluate collected up to this point will contain the methylated and ethylated thionates and dithioates. Change receivers and continue elution of the column with 8 ml of 40% ethyl acetate in benzene. This fraction contains methylated and ethylated diethyl phosphorothiolate. A third fraction, eluted with 8 ml of 80% ethyl acetate in benzene, contains methylated and ethylated DEP and ethylated DMP. Most of the TMP is retained on the silica gel column. Eluates are quantitated by gas chromatography (MicroTek MT 220), using a dual flame photometric detector operating in the phosphorus and sulfur modes.

Rat and human urine extracts to which known quantities of trialkyl phosphates had been added are analyzed by this method to obtain recovery data.

RESULTS AND DISCUSSION

Chromatograms representing standard mixtures of trialkyl phosphates were previously presented (Shafik and Enos, 1969; Shafik *et al.*, 1970). It has been established that quantitation should be based on ethylated DMP and DMTP and methylated DEP, DMDTP, and DEDTP; quantitation of DETP should be based on the methylated thiolate isomer.

Table I shows the order of elution of a mixture of trialkyl phosphate standards from silica gel. Although both the methyl and ethyl derivatives of each sulfur-containing dialkyl phosphate were available, only one derivative of each compound was included in the standard mixture. Preliminary studies showed that the methyl and ethyl derivative elute in the same fraction.

The recoveries of known amounts of trialkyl phosphates from rat and human urine extracts are shown in Table II.

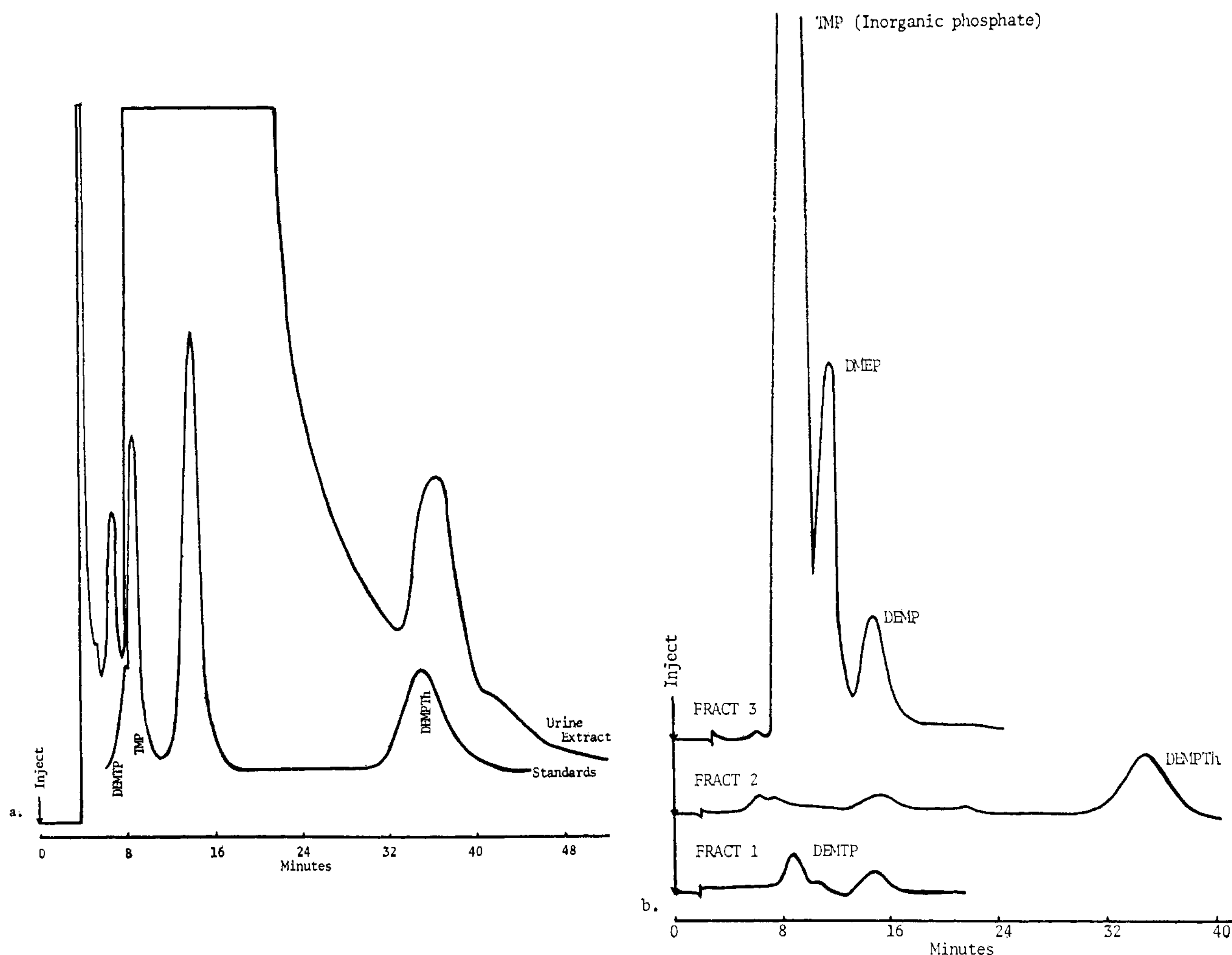

Figure 1. a. Chromatograms of standard alkyl phosphates and noncleaned-up methylated urine extract from rats exposed to $^1/_{100}$ LD_{50} parathion. Extract equivalent to 0.1 ml rat urine injected. Phosphorus mode, 526 mμ. b. Chromatograms of the three fractions from cleanup of methylated rat urine extract following exposure to $^1/_{100}$ LD_{50} parathion. Each injection represents 0.1 ml rat urine

Because of the instability of aqueous standard solutions of the potassium salts of dialkyl phosphates, these salts were not added directly to urine. The trialkyl phosphates were added to the alkylated urine extracts. Previous work (Shafik and Enos, 1969) has established that dialkyl phosphates are quantitatively extracted from acidified urine with acetonitrile–ether. Two spiking levels were used for each compound. As in the study of the elution pattern, only one of the two derivatives of each dialkyl phosphate was used. Trimethyl phosphate and triethyl phosphate were not included, since these compounds could also result from the alkylation of inorganic phosphate. Recoveries from human urine extracts spiked at levels of 0.01 to 0.50 ppm are generally greater than 80%. Recoveries from rat urine extracts spiked at levels of 0.05 to 2.5 ppm are in the range of 80 to 100%.

Table III shows limits of detectability of trialkyl phosphates in rat and human urine with and without the cleanup. Data are given only for the derivatives used for actual quantitation of alkyl phosphates in urine, *i.e.*, methylated DEP, DMDTP, DEDTP, and DEPTh and ethylated DMP and DMTP. These data clearly indicate the value of cleanup of urine extracts for gas chromatographic analysis. It is possible to realize at least a fivefold increase in sensitivity for most trialkyl phosphates. Before cleanup injections of urine extracts could not exceed the equivalent of 0.02 ml of rat urine and 0.1 ml of human urine and still permit quantitation of all six trialkyl phosphates. The addition of a cleanup step permits the injection of urine extracts equivalent to 0.1 and 0.5 ml of rat and human urine, respectively, as shown in Figures 1a and 1b.

Application of the cleanup procedure to urine of rats exposed to parathion gave satisfactory quantitative results at the two higher levels of exposure, $^1/_{10}$ of the LD_{50} and $^1/_{100}$ of the LD_{50}. However, cleanup was not sufficient to permit quantitation of alkyl phosphates at the two lower levels. The results of the exposure experiment are presented in

Table III. The Limits of Detectability of Trialkyl Phosphates in Rat and Human Urine Before and After Cleanup. Lower Limits[a] of Detection, ppm

Trialkyl phosphate	Human urine		Rat urine	
	Cleanup	No cleanup	Cleanup	No cleanup
DMETP	0.001	0.01	0.007	0.1
TMDTP	0.1	0.7	0.1	2
DEMDTP	0.002	0.01	0.01	0.1
DEMTP[b]	0.003	0.01	0.01	0.2
DMEP	0.002	0.01	0.01	0.1
DEMP	0.001	0.01	0.006	0.5

[a] All results calculated as the potassium salt of the corresponding dialkyl phosphate. [b] Calculated as DEMPTh.

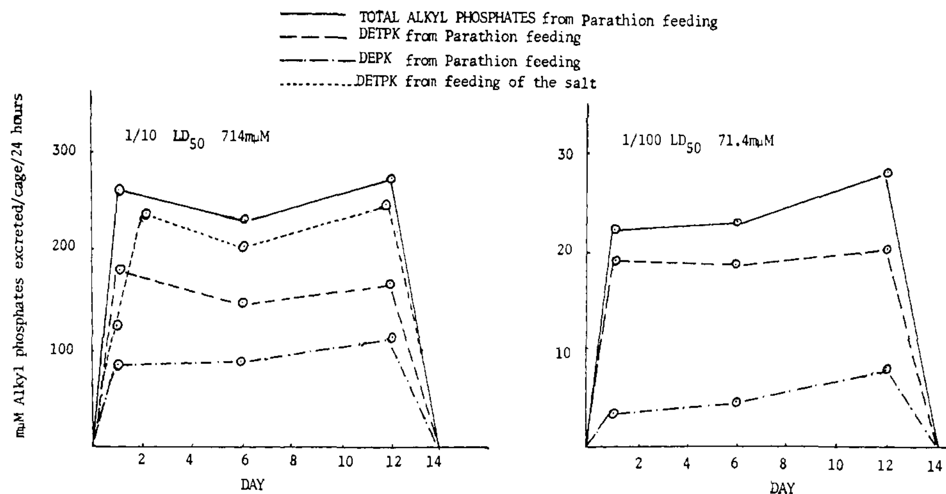

Figure 2. Alkyl phosphate excretion resulting from exposure of rats to parathion at the $^1/_{10}$ and $^1/_{100}$ LD_{50} levels, and DETPK at a level equivalent to $^1/_{10}$ LD_{50} parathion

Figure 2. In each case two metabolites, DEP and DETP, result from exposure of rats to parathion and account for 30 to 40% of the parathion initially fed. The total amount of these two compounds appeared to remain relatively constant from the first 24 hr through the remaining days of exposure and dropped rapidly to 0 upon cessation of feeding. There appears to be a small but steady increase in the amount of DEP formed, which is especially evident at $^1/_{100}$ of the LD_{50} exposure.

A linear relationship appears to exist between level of exposure and total alkyl phosphate excretion. In some cases there may be an advantage in using the sum total of DETP and DEP excreted in the urine as an index of exposure to parathion. However, from a practical point of view, it may be preferable to determine exposure from DETP alone since coextracted inorganic phosphate interferes to some extent with the quantitation of low levels (0.01 ppm) of DEP.

The results of the experiment in which DETPK was fed to rats are also included in Figure 2. The levels and excretion pattern of DETP are very similar to those of total alkyl phosphates (DEP + DETP) following exposure to an equivalent amount of parathion. It is interesting to note that no oxidation of the potassium salt of DETP occurs in the rat, as evidenced by the absence of DEP in the urine. This suggests that the DEP which is found following exposure to parathion arises from the action of hydrolytic enzyme(s) on paraoxon, a metabolite of parathion.

Table IV. Cholinesterase Levels in Rats Following Exposure to Parathion and DETPK

Exposure level	Cholinesterase, μm/ml/min Plasma	Red blood cell
$^1/_{10}$ LD_{50} parathion	1.5	1.6
	2.5	2.5
$^1/_{100}$ LD_{50} parathion	4.4	3.3
	4.4	2.9
$^1/_{500}$ LD_{50} parathion	4.0	2.7
	4.4	2.6
$^1/_{1000}$ LD_5 parathion	3.6	3.4
	. . .	2.3
68 μg DETPK	2.3	2.5
	2.9	3.3
Control	4.6	3.6
	4.4	3.4

In this work the cleanup procedure has been applied specifically to the alkyl phosphate metabolites resulting from parathion exposure. However, it is an extremely versatile method and has intentionally been presented in detail to allow its use in determining exposure to virtually all organophosphorus compounds. Depending upon the compound of exposure and the resulting alkyl phosphate metabolites, or the level of exposure, an analyst is free to select those steps of the procedure which satisfy his particular needs. If the dialkyl thioates or dithioates are of specific interest, hexane partitioning may provide sufficient cleanup. If further cleanup is required, silica gel column chromatography may be employed using the solvent which elutes the compound(s) of interest.

Similarly, when interest centers on nonsulfur-containing alkyl phosphates, the hexane preextract may be discarded and the compounds of interest isolated with benzene partitioning. For further cleanup the benzene extract may be introduced into a silica gel column and eluted with a suitable prior solvent to remove less polar interfering compounds, followed by elution with 80% ethyl acetate in benzene to recover the alkyl phosphate. An analyst may use his experience to decide whether any steps may be added or eliminated and which fractions may be combined or discarded.

An additional advantage inherent in the method is its ability to separate the alkyl phosphate esters by group and thus provide additional confirmation of identity. Solvent partitioning separates sulfur-containing compounds from those which do not contain sulfur. The elution pattern from silica gel provides even more characteristic separation and confirmation.

In addition to cleanup of urine extracts and confirmation of the identity of trialkyl phosphates, the procedure may be used for the preparation of pure standards, the separation of thionates from thiolates, and the isolation of sufficient quantities of the compounds for mass spectroscopy or other confirmatory procedures.

Since depression of blood cholinesterase has long been considered an index of exposure to organophosphorus insecticides, these values were obtained for comparison to urinary alkyl phosphate excretion. Blood taken from each rat approximately 2 hr following administration of the final doses of parathion and DETPK and from control rats was analyzed for plasma and red blood cell cholinesterase activity (Cranmer and Peoples, 1970). Table IV lists the plasma and red blood cell cholinesterase levels for each rat. Cholines-

terase inhibition is evident following exposure to $^1/_{10}$ of the LD_{50} of parathion but at lower levels of exposure no inhibition was observed. In comparison alkyl phosphates are easily detectable at a level of exposure of $^1/_{100}$ of the LD_{50}, which is equivalent to 40 μg of parathion per kilogram of body weight.

An unexpected result of this experiment was the apparent inhibition of cholinesterase by the potassium salt of DETP. This same inhibition was observed in the subsequent exposure of two additional pairs of rats to DETPK. Analysis of the potassium salt revealed no gross impurities which might account for these observations. Further studies are being conducted on this problem.

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