Gas Chromatographic Analysis of Phorate and Five Metabolites in Monkey Liver Homogenates

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During recent investigations on the interaction of organophosphorus pesticides and enzymes in different crude liver homogenates it was necessary to develop a quantitative method for phorate and its metabolites. A number of procedures can determine the total quantity of phorate and its metabolites in plant samples (1,2,3, 4,5). However, these methods are not suitable for separating and determining phorate, phoratoxon, phorate sulfone, phorate sulfoxide, phoratoxon sulfone or phoratoxon sulfoxide in nanogram quantities.

This paper describes a rapid and quantitative procedure using gas-liquid chromatography with a Melpar

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photometric emission detector (phosphorus model 526 mu) to separate and determine phorate and 5 metabolites. Two extraction solvents, acetone and ethanol were evaluated.

Apparatus and Reagents

1. Gas chromatograph, MicroTek MT-220 fitted with Melpar photometric emission detector and phosphorus 526 mu filter; Column, glass, U-shaped, 20" x 1/4" o.d., packed with 5% DEGS on 80/100 mesh HMDS treated Chromosorb W, prepared by the method of Mendoza et al. (6); Nitrogen flow, 120 ml/min.; Hydrogen flow, 150 ml/min.; Oxygen flow, 15 ml/min.; Air flow, 15 ml/min.; Detector temperature, 145°C; Inlet block temperature, 210°C; Oven temperatures, 150°C or 195°C; Electrometer setting, 32 x 10^3; Recorder, Westronics, 1 mv. full scale.

2. Standards were obtained from the American Cyanamid Co.; phorate, analytical standard, phorate sulfoxide, phorate sulfone, phoratoxon, phoratoxon sulfoxide and phoratoxon sulfone as chromatographic standards and stated to be slightly contaminated with other members of the phorate family.

Standard stock solutions were prepared at the following concentrations in ethanol; phorate 6.0 mg/ml and each of the metabolites at 5.0 mg/ml. No attempt was made to correct the final concentrations for impurities.

A dilute stock solution was prepared to contain in ug/ml: phorate 500; phoratoxon, phorate sulfone, phorate sulfoxide, 357 each; phoratoxon sulfoxide and phoratoxon sulfone 1785 each. A GLC reference solution was prepared to contain in ng/ul; phorate 6.0; phoratoxon, phorate sulfoxide, phorate sulfone 4.7 each; phoratoxon sulfoxide and phoratoxon sulfone 23.8 each.

3. Solution A - A water solution 0.15 M in NaCl, 0.015 M in $MgCl_2$ and 0.008 M in nicotinamide was prepared.

4. Liver Homogenate - At death the liver* was excised, immediately frozen and all subsequent operations were carried out at 4-5°C. Five grams were cut into small portions and ground in an all glass homogenizer with Solution A (final volume 20 ml). The particulate matter was removed by centrifuging the mixture at 6000 x g and discarded. The supernatant was transferred to 10 ml glass tubes, covered with Saran® wrap and the contents frozen until used.

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*Monkey livers were obtained from the Virus Research Laboratory, Department of National Health and Welfare. These animals had been killed by exsanguination after dosing with barbiturate.

PROCEDURE

Recovery of Phorate and Metabolites from fortified monkey liver homogenate incubation mixture.

Aliquots of the following mixtures were transferred to a 10 ml glass stoppered centrifuge tube;

2.0 ml liver homogenate supernatant

2.0 ml solution A

0.5 ml phosphate buffer pH 8.0

The contents were thoroughly mixed and heated to 90°-100°C for several minutes to denature the enzyme systems. After cooling the mixture to room temperature 0.2 ml of the standard dilute stock solution mixture was added, then 5 ml of acetone or 95% ethanol and the contents shaken vigorously for 1 minute. Tube contents were centrifuged at 6000 x g and the supernatant decanted into a 15 ml centrifuge tube. The residue was extracted with a second 5 ml portion of acetone or 95% ethanol, centrifuged and the supernatant combined with the first extract. The volume was adjusted to 15 ml with solvent and mixed thoroughly.

GLC Analysis

An oven temperature of 150°C was used for the phorate and phoratoxon separation. A 0.5 ul aliquot of the 15 ml supernatant mixture was injected. The phorate response peak occurred at 1.8 minutes followed by

phoratoxon at 2.4 minutes. Five minutes of the detector response was recorded. Similarly a 0.5 ul aliquot of the GLC standard solution was analyzed.

Phorate sulfoxide and sulfone, phoratoxon sulfoxide and sulfone were analyzed by the above procedure but with the GLC oven temperature equilibrated at 195°C. Under these conditions the phorate sulfoxide response peak occurred at 2.8 minutes, phoratoxon sulfoxide at 3.4 minutes, phorate sulfone at 4.2 minutes and phoratoxon sulfone at 4.7 minutes. The recovery values were calculated by comparing the response peak height of extracted compounds with those for a 0.5 ml aliquot of the GLC standard reference solution.

RESULTS AND CONCLUSIONS

Figure 1 illustrates the GLC separation and response of 3.0 ng phorate (A) and 2.3 ng phoratoxon (B) recovered from the liver homogenate incubation mixture. Figure 2 illustrates the response of 2.3 ng phorate sulfoxide (C) 11.7 ng of phoratoxon sulfoxide (D) 2.3 ng of phorate sulfone (E) and 11.7 ng phoratoxon sulfone (F) from the same incubation mixture. Response peaks overlap but are adequately separated one from another for identification and peak height measurements when working with known amounts.

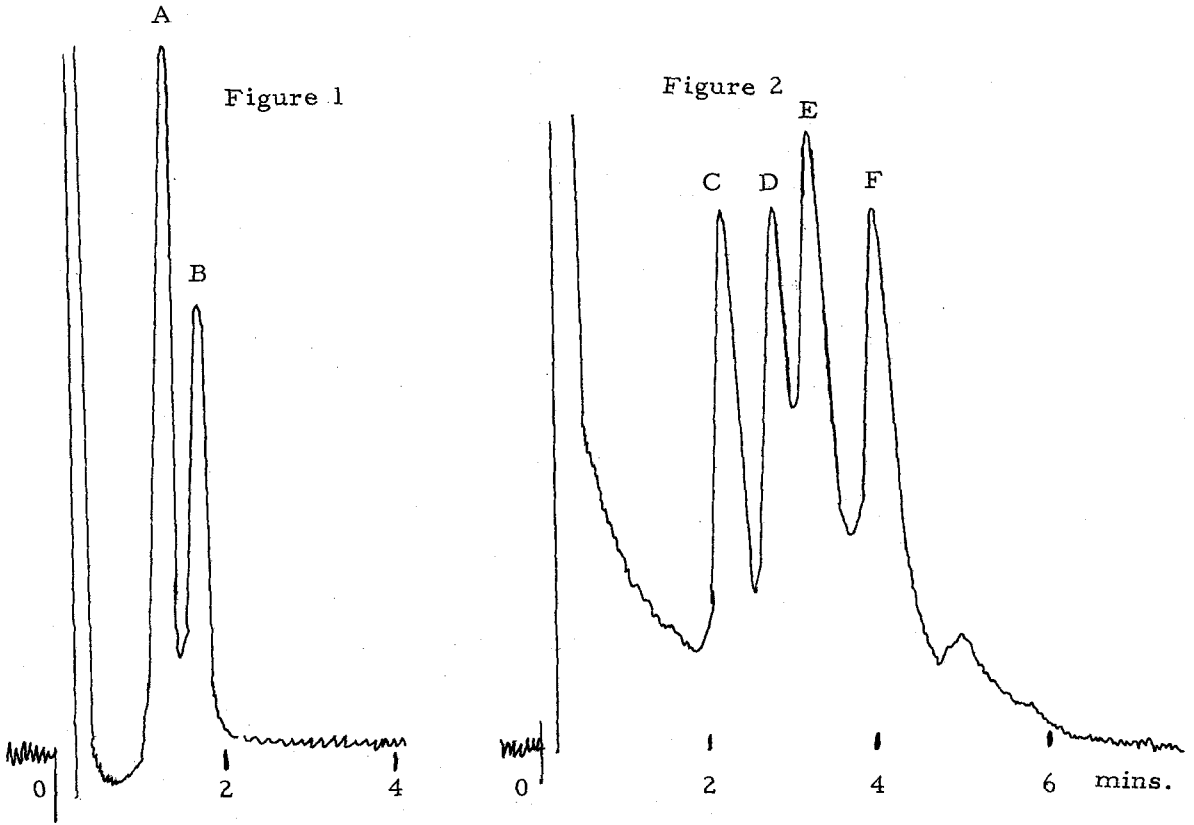

Figure 1

Figure 2

Typical GLC chromatograms of phorate and metabolites in monkey liver extracts. Figure 1, phorate (A) and phoratoxon (B); column temperature 150 C. Figure 2, phorate sulfoxide (C), phoratoxon sulfoxide (D), phorate sulfone (E) and phoratoxon sulfone (F); column temperature 195 C.

Recovery values could be in error where the ratio of metabolite concentrations are different from the standard mixture. This can be overcome by preparing a standard reference solution that approximates the response of the sample or by establishing mathematical correction factors for each compound under various concentration ratios.

Recovery values in duplicate are given in Table 1 for all compounds extracted with acetone or ethanol. These recoveries are good for most compounds (80-106%).

Table 1

Percent Recoveries (duplicate) of Phorate and Five Metabolites Added to Monkey Liver Homogenates

Pesticide	Ug Per 500 mg Liver	Solvent	
		Acetone	Ethanol
Phorate	100	106;106	93;99
Phorate Sulfoxide	71	95;92	98;106
Phorate Sulfone	71	97;93	84;96
Phoratoxon	71	94;106	98;104
Phoratoxon Sulfoxide	357	69;80	94;98
Phoratoxon Sulfone	357	85;73	76;92

Three low values were obtained; phoratoxon sulfoxide 69%, and phoratoxon sulfone 73 and 76%. These latter values are not excessively low and for this type of analysis were acceptable. Acetone and ethanol were considered to be equally efficient for extracting phorate and metabolites.

An advantage of this procedure is the ability to carry out a quantitative determination by GLC without an involved cleanup. In addition, residue losses are kept to a minimum by directly injecting an aliquot of the clear supernatant from the incubation mixture.

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