An Improved Method for the Determination of Urinary Dimethyl Phosphate

Derek Blair* and Howell R. Roderick

A new, rapid, procedure is described for the determination of urinary dimethyl phosphate, a metabolite of some important organophosphate insecticides. Selective removal of inorganic phosphate from the urine with calcium hydroxide eliminates a major source of interference. The urine is reacidified with a cation-exchange resin and treated with diazoethane and the ethyl dimethyl phosphate so formed is determined by gas–liquid chromatography (GLC). The derivatization procedure described ensures complete conversion of the dimethyl phosphate, thus eliminating a potential source of error inherent in earlier methods.

Dimethylphosphoric acid (DMPA) is the major metabolite in animals of a number of commercially important organophosphate insecticides including dichlorvos (Hutson and Hoadley, 1972), monocrotophos, and mevinphos (Beynon et al., 1973). The metabolite is eliminated in the urine and it has been proposed (Shafik and Enos, 1969) that the quantity so excreted may be used as an index of the insecticide dose assimilated during experimental or occupational exposure.

Urinary phosphoric acid–ester metabolites have been determined by gas–liquid chromatography (GLC) after treatment with diazoalkanes to convert them into volatile trialkyl phosphates (Shafik and Enos, 1969; Shafik et al., 1973, and references cited therein). A difficulty with urine samples is that they naturally contain large amounts of inorganic phosphate. Unless this is removed, treatment with diazoethane results in triethyl phosphate appearing in the final chromatogram to an extent that can mask peaks corresponding to the derivatized metabolites. Earlier methods have employed solvent extraction to diminish this interference but the partition coefficients are at best unfavorable to the extraction of DMPA and the procedures are not efficient at excluding the inorganic phosphate. This has been overcome by using diazopentane to yield derivatives that are more easily resolved by chromatography (Shafik et al., 1973). However, the higher diazoalkanes are relatively inaccessible and costly.

In the method now described, inorganic phosphate is selectively removed using calcium hydroxide. Ethylation with diazoethane can then be carried out directly, after the sample has been reacidified with a cation-exchange resin. The method is less time consuming and more accurate than hitherto published procedures (Shafik and Enos, 1969; Shafik et al., 1973) and, through the use of ion-exchange resin, avoids the risk of incomplete derivatization of the DMPA which is inherent in the earlier methods.

MATERIALS AND METHODS

Sodium dimethyl phosphate (NaDMPA) was prepared from trimethyl phosphate with sodium iodide (Hutson and Hoadley, 1972). The purity of the sample was established by elemental analysis and by its methylation with diazomethane to yield trimethyl phosphate quantitatively. Ethylation with diazoethane gave ethyl dimethyl phosphate; no triethyl phosphate was detected in the product, demonstrating that the NaDMPA was free from inorganic phosphate.

Diazomethane was prepared from N-methyl-N-nitrosotoluene-4-sulfonamide and diazoethane from N-ethyl-N-nitrosourea according to well-established methods

Shell Research Limited, Tunstall Laboratory, Sittingbourne Research Centre, Sittingbourne, Kent, ME9 8AG, England.

(Fieser and Fieser, 1967,1969). [**Caution:** the diazoalkanes are explosive; both they and the N-ethyl-N-nitrosourea are carcinogenic.]

Calcium hydroxide, laboratory grade (Hopkin and Williams Ltd.), and Amberlite IR-120(H) resin, analytical grade (Rohm and Haas Co.), were used.

The gas–liquid chromatograph was a Pye Model 104 fitted with a flame photometric detector operating in the phosphorus mode. The glass column, 2 m × 3 mm i.d., was packed with 5% Carbowax 6000 on Gas-Chrom Q, 80–100 mesh. The operating temperatures were as follows: inlet port, 145 °C; column, 150 °C; detector, 155 °C. The gas flows were: nitrogen (carrier), 40 ml min^{-1}; air, 215 ml min^{-1}; hydrogen, 195 ml min^{-1}.

A 5-ml sample of the urine was mixed with 0.5 g of calcium hydroxide in a 10-ml centrifuge tube. After centrifugation at 2500 rpm for 3 min, from 2.5 to 3 ml of the treated urine was placed in a vial containing 1 g of Amberlite IR-120(H) resin and the contents were gently swirled. After 1 min an aliquot (0.2 to 1 ml) was transferred to a graduated flask and approximately 4 ml of ethanol was added followed by an excess of ethereal diazoethane. The excess, maintained at ambient temperature for 4 min, was then destroyed by the addition of 1 drop of formic acid and the solution was made up to 10 ml with ethanol. Quantities of up to 10 μl of this solution were injected into the gas chromatograph and the peak heights observed were compared with those obtained from standard solutions of ethyl dimethyl phosphate. These were prepared by treating standard aqueous NaDMPA with ion-exchange resin and then with ethanol and diazoethane as described above.

RESULTS AND DISCUSSION

The use of calcium hydroxide for removing inorganic phosphate from urine samples is a simple but effective method, reducing the concentration from typically 2 mg ml^{-1} by a factor of approximately 10^4. Incomplete removal of the precipitate by centrifugation is the chief source of residual inorganic phosphate. The quantity of reagent does not appear to be critical within ±20% of the prescribed amount and the quantity of resin used for reacidification is sufficient to cope with any excess of calcium hydroxide present.

Recoveries of the order of 95% with satisfactory replication have been obtained consistently in analyses of standard solutions of NaDMPA in both rat and human urine by this method. The lower limit of quantitative detection was approximately 0.05 μg ml^{-1} of NaDMPA in rat urine and the recovery curve was linear at least up to a concentration of 150 μg ml^{-1}. A typical chromatogram, obtained with rat urine, is shown in Figure 1.

The NaDMPA contents of untreated urine samples stored at −20 °C were stable for at least 3 months. After

Table I. Ethylation of NaDMPA with Diazoethane;
NaDMPA (19.8 μg, 0.13 μequiv) in Acidified 75% Ethanol

Acid used	Amount mequiv	% conversion
Hydrochloric	35	32
	69	62
	103	99
Sulfuric	37	50
	56	100

Table II. Methylation of NaDMPA with Diazomethane;
NaDMPA (0.357 mmol l.$^{-1}$) in 1:1
Methanol/Aqueous Acid

Initial hydrogen ion concn, mg ions l.$^{-1}$	% yield of trimethyl phosphate			
	Hydro-chloric acid	Hydro-bromic acid	Sulfuric acid	Nitric acid
2.5	70			
5	86		90	
10	67	81	97	94
25	78		100	100
50	79			
75	90			
100	85	83		100

treatment with calcium hydroxide, the samples could be kept at 0 °C for up to 12 h without significant loss of NaDMPA while after reacidification with resin, samples could be kept at 0 °C for up to 24 h.

In the derivatization of standard solutions of NaDMPA acidified with mineral acids, it was found that substantially quantitative conversion into the triester could be achieved only in the presence of a large excess of acid, although sufficient diazoalkane was always used to give a persistent yellow color with cessation of nitrogen evolution. While these two criteria are usually taken to show that an excess of diazoalkane has been added to a solution to be alkylated the criteria were defective in this case. The results shown in Table I illustrate the effect of using different amounts of hydrochloric and sulfuric acids in the attempted preparation of standard solutions of ethyl dimethyl phosphate from NaDMPA. The presence of unreacted NaDMPA in the incompletely derivatized samples was demonstrated by reacidifying them and adding more diazoethane, when a further yield of the derivative was obtained. On the same basis the derivatization of NaDMPA proceeded quantitatively when a cation-exchange resin was used to acidify the salt solution. In addition, similar results were obtained when diazomethane was used to methylate NaDMPA (Table II), the yields in this case being quantified by comparison of the product with an authentic sample of trimethyl phosphate.

It is accepted (More O'Ferrall, 1967) that the first and an essential step in the esterification of a strong acid with diazoalkane is protonation of the reagent to form a di-azonium ion. This, either immediately or as a carbonium ion following loss of nitrogen, becomes associated with an anion. Collapse of the ion pair leads to the ester. Clearly, when there is an excess of anions over the quantity of protons available to protonate the diazoalkane, competition between the various anions for the protonated reagent can result in incomplete derivatization of an acid, particularly one strongly dissociated. Thus, by using increasing excesses of mineral acid to acidify solutions of NaDMPA before alkylation (and consequently consuming excessive amounts of diazoalkane) quantitative conversion can be approached asymptotically but never be achieved.

In urine analysis, the risk of nonquantitative derivatization arising from the use of mineral acid to acidify the NaDMPA is greatest when concentrated solutions of the
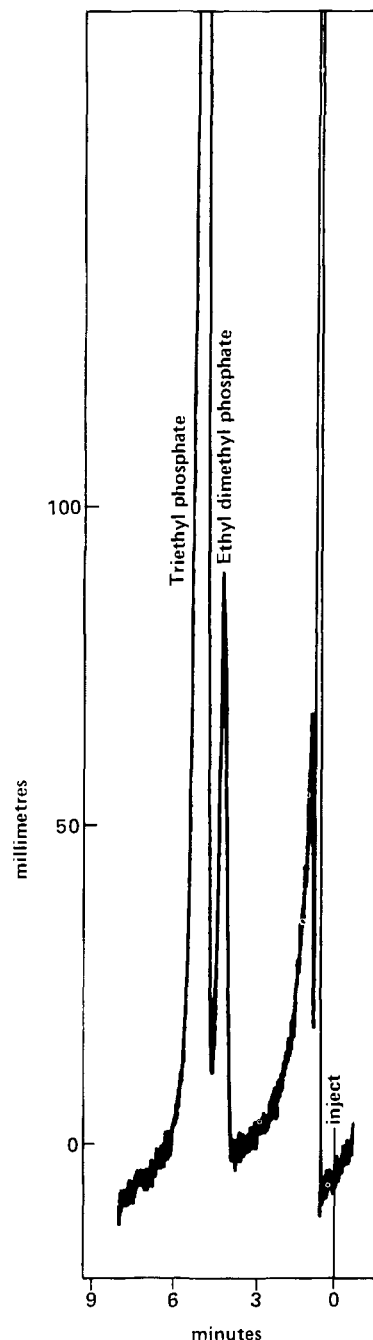
Figure 1. A typical chromatogram obtained with rat urine containing 1 μg ml^{-1} NaDMPA; signal attenuation 2 × 10^3. This injection represented 0.12 μl of urine.

salt are being processed. Standard solutions of the derivatized metabolite are often prepared by alkylating acidified, concentrated, aqueous solutions of NaDMPA, for subsequent dilution. Recovery data based on incompletely derivatized standards would be illusory. When a strong cation-exchange resin is used to acidify either the salt solution or the urine, all of the anions present are converted into the corresponding acid and the problem of nonquantitative derivatization does not arise.

LITERATURE CITED

Beynon, K. I., Hutson, D. H., Wright, A. N. (1973), Residue Rev. 47, 55.
Fieser, L. F., Fieser, M., "Reagents for Organic Synthesis", Vol. 1, Wiley, New York, N.Y., 1967, pp 191–192.
Fieser, L. F., Fieser, M., "Reagents for Organic Synthesis", Vol. 2, Wiley, New York, N.Y., 1969, p 102.

Hutson, D. H., Hoadley, E. C. (1972), *Xenobiotica* **2**, 107.
More O'Ferrall, R. A. (1967), *Adv. Phys. Org. Chem.* **5**, 331.
Shafik, T., Bradway, D. E., Enos, H. F., Yobbs, A. R. (1973), *J. Agric. Food Chem.* **21**, 625.

Shafik, M. T., Enos, H. F. (1969), *J. Agric. Food Chem.* **17**, 1186.

Determination of Benefin and Trifluralin Residues by Quantitative Gas–Liquid Chromatography/Mass Spectrometry

Geoffrey B. Downer, Martin Hall, and David N. B. Mallen*

A quantitative GLC–MS procedure is described for the simultaneous determination of benefin and trifluralin residues in soil. Prior gas–liquid chromatographic separation of the two herbicides is not required due to the selective nature of the detector. Comparable soil residue levels are obtained for both the mass fragmentography (multi-ion detector, MID) procedure and an established electron-capture GLC procedure. The MID method has a similar sensitivity to the electron-capture GLC method but has a shorter analysis time.

Residue levels of trifluralin (2,6-dinitro-N,N-di-n-propyl-α,α,α-trifluoro-p-toluidine) can routinely be determined by gas–liquid chromatography (GLC) (Tepe and Scroggs, 1967). A similar procedure exists for the determination of isomeric benefin [N-(n-butyl)-N-ethyl-2,6-dinitro-α,α,α-trifluoro-p-toluidine]. As the two nitroanilines possess different herbicidal activity, a method is required which can differentiate between them at the residue level.

Simultaneous determination of trifluralin and benefin requires prior GLC separation of the two compounds. The difficult separation of these isomers has been partially achieved on a Durapak-Carbowax 400/Poracil C column (Koons and Day, 1972) and completely on a bonded monomolecular phase (Aue et al., 1973). In their work this latter phase had a very limited lifetime. Recently (Hall and Mallen, 1975) a liquid crystal phase has been used to effect a GLC separation of benefin and trifluralin. However, this phase is not suitable for use in conjunction with an electron-capture detector.

In this approach the specificity advantage of multiple ion detection mass spectrometry (MS) over electron-capture detection GLC is implemented. In consequence the prior separation of the two isomers is not required and conventional GLC columns can be used.

EXPERIMENTAL SECTION

Standard grade benefin and trifluralin were used (Eli Lilly & Co., Indianapolis, Ind.).

Extraction of Residue. Residues of benefin and trifluralin were extracted by the method of Tepe and Scroggs (1967). The extracted residues were dissolved in toluene and analyzed by quantitative GLC–MS and GLC alone.

Gas–Liquid Chromatography/Mass Spectrometry. An LKB 9000S GLC–MS equipped with a four-channel Altema AL5 multi-ion detector (MID) was used. Chromatography was performed on a 5 ft × 4 mm i.d. glass column packed with 1.2% SE30 on Gas-Chrom Q (100–120 mesh). The column temperature and injection port temperature were maintained at 190 and 200 °C, re-

spectively; the carrier gas (helium) flow rate was 30 ml/min. The effluent from the GLC column was passed through a Ryhage jet separator (265 °C) before entering the ion source block (270 °C). The mass spectrometer settings for routine use were as follows: ionizing voltage, 20 eV; accelerating voltage, 3.5 kV; trap current, 60 μA; resolution, 1500. The required masses were brought into focus by switching the accelerating voltage with the Altema MID unit at a fixed magnetic field. Optimum precision was obtained for the method when the GLC–MS system was stabilized overnight. The signal from each ion was recorded on a four-channel Visigraph FR-3017 direct reading oscillograph. Electron-capture GLC was performed on a Pye 104 GLC equipped with a 63 Ni detector.

Calibration Procedure. In samples containing either benefin and no trifluralin or vice versa, the alternative isomer was used as an internal standard (no deuterium-labeled isotopes were available). A series of standards for benefin and trifluralin in the concentration range 0.01–0.06 μg/ml was prepared. Four microliters of each concentration solution was injected into the GLC–MS, and a standard curve prepared by plotting a ratio of selected ion (benefin, m/e 292; trifluralin, m/e 306) peak height against nanograms of the herbicide. When both herbicides were present, an external standard procedure was used. The system was calibrated daily. After the initial calibration, a standard solution was injected between every third sample solution to check that no significant drift occurred from the initial calibration. On occasions unacceptable drift after several hours work necessitated recalibration of the system. All results were corrected for an extraction recovery figure typically of about 80%.

RESULTS AND DISCUSSION

The mass spectra for trifluralin and benefin (m/e >250) are shown (Figure 1). Fragmentation of the molecule to give characteristic imine ions at m/e 292 for benefin and m/e 306 for trifluralin are specific to each component. A relative abundance of 100% also makes these ions very suitable for quantitation. Weaker ions at m/e 276 and 290 can be used to further improve method specificity.

Variation of electron voltage energies did not significantly affect the yield of these ions and hence sensitivity of the method. The detection limit of both herbicides to

Lilly Research Centre Limited, ERL Wood Manor, Windlesham, Surrey GU20 6PH, England.