Comparison of High-Performance Liquid Chromatography and Anticholinesterase Assay for Measuring Azinphos-methyl Metabolism in Vitro

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The oxon of azinphos-methyl was not thermally stable. Under gas chromatographic conditions, on-column decomposition occurred and tended to produce components which upon electron impact generated the same ions as those generally employed to identify the metabolites of azinphos-methyl by mass spectrometry. Furthermore, approximately 44% of the oxon of azinphos-methyl generated enzymatically by mice liver homogenates was decomposed to benzazimide if the incubation mixture was heated to 100 °C for 3 min. Therefore, it was concluded that analytical methods for studying the metabolism of azinphos-methyl should not involve processes which required high temperature. Investigation of the interchangeability between high-performance liquid chromatography (HPLC) and the anticholinesterase assay (ACHE) was carried out by quantitating the oxon of azinphos-methyl in the same biological samples. Both methods selectively measured the compound to the same degree of accuracy. Whereas the ACHE assay appeared to be faster, HPLC was advantageous in that several metabolites of azinphos-methyl could be simultaneously assayed.

Azinphos-methyl, *O,O*-dimethyl *S*-[[4-oxo-1,2,3-benzotriazin-3(4*H*)-yl]methyl] phosphorodithioate, is a widely used insecticide and acaricide. The compound is frequently referred to as "Guthion", the trademark name of the Mobay Chemical Co. The toxic effect of the compound is attributable to its oxygen analogue (the oxon) formed in vivo which inhibits brain acetylcholinesterase (DuBois et al., 1957; Murphy and DuBois, 1957, 1958). While several analytical procedures have been published to assay azinphosmethyl in biological samples (Cohen et al., 1966; Miles, 1964; Adams and Anderson, 1966; Loeffler et al., 1966; Everett et al., 1966; Szalontai, 1976; Krijgsman and Van De Kamp, 1976), only a few reports have discussed the measurements of the oxon of azinpho-methyl (Murphy and DuBois, 1957; Miles, 1964; Lin et al., 1980; Motoyama and Dauterman, 1972; Hunt and Gilbert, 1970). For example, a colorimetric method (Miles, 1964) was developed to assay both azinphos-methyl and its oxygen analogue in samples of the McIntosh apple, cabbage, and tomotoes. The lengthy procedures included separation on a Florisil column, hydrolysis with strong acid, and formation of a complex between the product and *N*-(1-naphthyl)-ethylenediamine and optical measurements using a colorimeter at a wavelength of 556 μm. The method is quantitative but not sensitive (7-μg dectection limit). The most convenient method for measuring the oxon of azinphos-methyl is by anticholinesterase assay (ACHE) (Murphy and DuBois, 1957). The principle for analyses was based on the observation that the oxygen analogue of azinphos-methyl caused inhibition of the enzyme activities of bovine erythrocyte cholinesterase, whereas the highly purified cholinergic azinphos-methyl did not. The method is fast and sensitive. However, to be sure that the method is specific, it is necessary to ascertain that other metabolites besides the oxygen analogue of azinphos-methyl do not inhibit the enzyme activity as well.

Recently, a high-performance liquid chromatographic (HPLC) method has been developed to investigate in vitro metabolism of azinphos-methyl by mice liver homogenates (Lin et al., 1980). The method is both sensitive and specific, with the lower limit of detection being 5 ng of the oxon. Although mass spectrometric method has been used to identify some of the metabolites of azinphos-methyl during the course of developing this methodology by gas chromatography–mass spectrometry (GC–MS), it was found that on-column decomposition of the oxon of azinphos-methyl did occur. The products so produced tended to confuse the identification of metabolites of azinphos-methyl. In this study, we will discuss the GC properties of the oxon of azinphos-methyl and the interchangeability of the methodologies of ACHE and HPLC for measuring in vitro metabolism of azinphos-methyl by mice liver homogenates.

EXPERIMENTAL SECTION

Chemicals. Azinphos-methyl, 98.7% purity, and its oxygen analogue, the highest purity available, were obtained from Chemagro Division, Mobay Chemical Corp., Kansas City, MO. Glucose 6-phosphate, and NADP, bovine erythrocyte cholinesterase, DTNB [5,5-dithiobis(2-nitrobenzoic acid)], and acetylthiocholine iodide were purchased from Sigma Chemical Co., St. Louis, MO. Benzazimide and benzamide were purchased from Plaufz and Bauer, Inc., Stanford, CT. All organic solvents were distilled in glass obtained from Burdick and Jackson Laboratories, Inc. (Muskegon, MI).

Liver Incubation Procedures. Adult male Charles mice weighing between 25 and 35 g were used. The animals were housed in an air-conditioned room and were provided with food and water ad libitum. Mice were sacrified by cervical dislocation. Their livers were quickly removed and blotted and homogenized at 0 °C in 4 volumes of solution containing 1.15% KCl and 0.25% nicotinamide. To the incubation mixture, coonsisting of 40 mg of liver, was added 40 mM sodium phosphate, 3.8 mM glucose 6-phosphate, and 1.4 mM NADP, giving a total volume of 2 mL. After a 5-min preincubation period, 400 nmol of a 0.1 M solution of azinphos-methyl in absolute ethanol was added. Incubation was carried out at 37 °C for 30 min in a reciprocal water bath shaker. The resulting solution was divided into two tubes. To determine the effect of elevated temperature on the degradation of the oxon of azinphos-methyl, one tube was heated to 100 °C for 3 min by a boiled water bath. After centrifugation of the heated sample two consecutive 10 μL of the supernatant were transferred for use in the ACHE assay. The remaining mixture of 0.98 mL was stored in a freezer at

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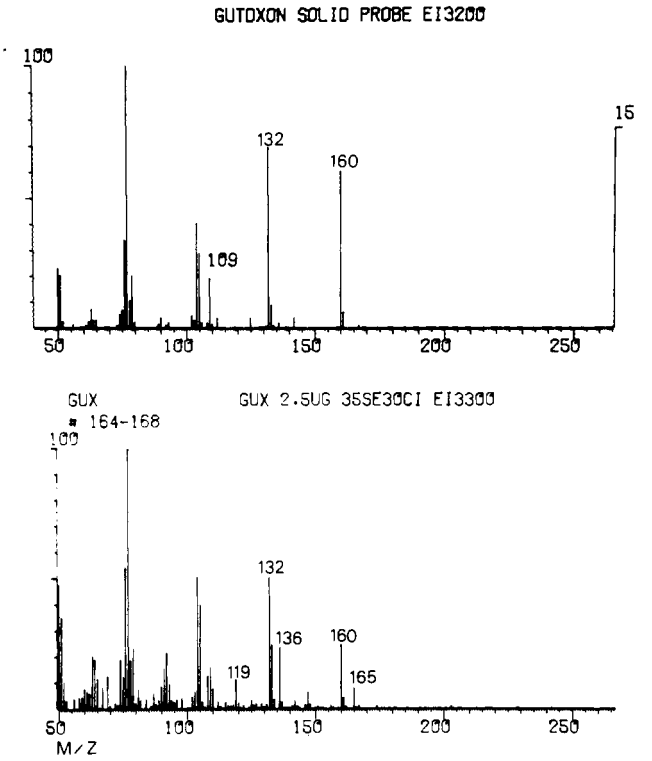

Figure 1. (Upper) Mass spectrum of the oxon of azinphos-methyl by using direct probe sampling techniques. (Lower) Mass spectrum obtained after GC separation.

−40 °C to be analyzed by HPLC. The incubation mixture in the other tube, after removal of 20 μL for the ACHE assay, was immediately frozen by dry ice in 2-propanol and stored for the HPLC assay.

Gas Chromatography–Mass Spectrometry. A 3300 Finnigan GC–MS equipped with a 6000 data acquisition system was used. A standard solution was prepared by dissolving 1 mg of insecticide in 1 mL of acetonitrile. A 1.5 m × 3 mm i.d. U-shaped glass column packed with 3% SE-30 was used. The oven temperature was programmed from 100 to 250 °C at 10 °C/min. Both the GC injector and the GC–MS separator were maintained at a constant temperature of 250 °C. Electron impact mass spectra were recorded at an electron energy of 70 eV.

High-Performance Liquid Chromatography. Procedures of HPLC analysis of metabolites of azinphos-methyl in liver homogenate were previously described by Lin et al. (1980). After 7 μg of benzamide in 30 μL of acetonitrile was added to the incubation mixture, extractions were carried out twice with 2.5-mL portions of ethyl acetate. The pooled organic extracts were evaporated to dryness by a N_2 stream. Acetonitrile, 50 μL, was added to the residue and 5 μL used for analysis.

Anticholinesterase Assay. The method as described by Benke et al. (1974) was used. To 10 μL of sample, derived from biological tissues as described above was added 5 μg of bovine erythrocyte cholinesterase in 0.1 M phosphate buffer (pH 8.0) to yield a total volume of 5 mL. The tube was allowed to stand at room temperature for 60 min in the absence of substrate. The Ellman reagent, 0.54 μmol of DTNB, was added in the cold, followed by 5 μmol of acetylthiocholine iodide. Enzyme activity was measured as the change in absorbance at 412 nm during a 30-min incubation at 27 °C. The amount of active cholinesterase inhibitor (presumably the oxon of azinphos-methyl) present in the incubation mixture was measured by comparison with standard inhibition curves using the authentic oxygen analogue of azinphos-methyl.

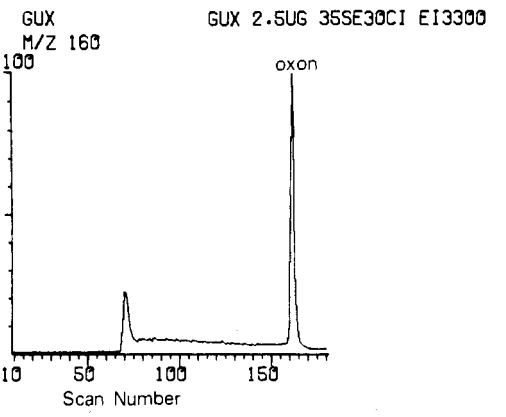

Figure 2. Reconstructed mass chromatogram of the oxon of azinphos-methyl based on the ion intensity of m/z 160. The observations of an early GC peak and the disruption of the base line of the chromatogram idicate that on-column decomposition of the component occurs.

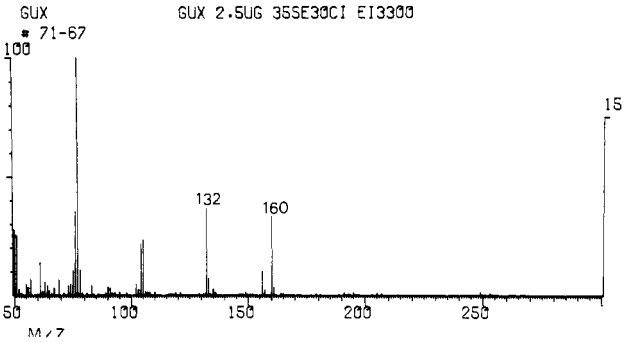

Figure 3. Mass spectrum of the early GC peak shown in Figure 2. Tentative structural evaluation of the mass spectrum indicated that the compound was methyl(mercaptomethyl)benzazimide, possibly as a result of on-column thermal transalkylation of the oxon of azinphos-methyl.

Preliminary experiments indicated that varioous amounts of the oxon of azinphos-methyl (2.0, 3.0, 4.0, 5.0, 10.0, 20.0, 30.0, 40.0, and 50.0 nmol as a 10^{-5} M solution) added to the cholinesterase test system would give between 15 and 90% inhibition of bovine erythrocyte cholinesterase.

RESULTS AND DISCUSSION

In order to reduce thermal decomposition of the oxon of azinphos-methyl, the direct probe sampling technique for the mass spectrometer was used. The resulting spectrum is given in the top panel of Figure 1 and is in agreement with the spectrum reported by Damico (1966). The molecular ion is not observed. However, m/z 160 corresponds to an ion produced by α-cleavage of the S–C bond and further elimination of CO to form m/z 132. The ion at m/z 109 has an ionic structure of $[(CH_3–O)_2P{=}O]^+$ (Damico, 1966; Casida, 1974). The mass spectrum of the oxon of azinphos-methyl after GC separation is shown on the lower panel of Figure 1. The enhancements in the intensities of ions m/z 165, 147, 136, 133, and 119 indicate that the mass spectrum is generated by a composite of compounds. In Figure 2 , the reconstructed mass chromatogram based on the ion intensity of m/z 160 is given. In addition to the peak corresponding to the insecticide, there is an early GC peak at scan no. 71. The mass spectrum of this compound is shown in Figure 3. It is found to be similar to that observed for the oxon of azinphos-methyl with the exception that there is no m/z 109. The identification of this compound was verified by matching its mass spectrum with that of authentic methyl(mercaptomethyl)benzazimide. Further purification of the oxon of azinphos-methyl by the HPLC method did not

Table I. HPLC Measurements of the Oxon of Azinphos-methyl (GO)[a] and Benzazimide (BZ)[a] Produced in Vitro by Mice Liver Homogenates: The Resulting Incubation Mixtures Were either Panel A, Frozen by Dry Ice in 2-Propanol, or Panel B, Heated to 100 °C for 3 min

	panel A		panel B		
samples	GO	BZ	GO	BZ	adjusted,[b] GO
C-1[c]	0	0	0	0.83	
C-2[d]	0	0	0	0.57	
1	4.90	0.49	1.95	2.52	4.41
2	5.37	0.54	1.76	2.64	4.36
3	4.95	0.46			
4	3.97	0.39	2.05	2.65	4.98
5	4.53	0.41	1.86	2.64	4.73
6	4.34	0.39	1.79	2.45	4.31
7	4.42	0.45	1.76	2.45	4.16
8	3.97	0.37	1.97	2.81	5.27
9	3.76	0.40	1.88	2.36	4.22
10	4.76	0.43	1.67	2.46	4.13
av ± SD	4.50 ± 0.48	0.43 ± 0.05	1.85 ± 0.11	2.55 ± 0.13	4.51 ± 0.38
RSD,[e] %	±10.7	±11.6	±5.9	±5.1	±8.4

[a] All units in micrograms per total incubate. [b] The adjusted GO values were obtained by substracting the amounts of BZ in panel A and 0.83 μg of BZ in row C-1 from the amount of BZ in panel B. The resulting values were added to the values of GO in panel B on an equimolar basis. [c] Mixture of azinphos-methyl and liver homogenates was heated to 100 °C for 3 min without incubation. [d] Azinphos-methyl in a solution containing all the cofactors except liver homogenate was heated to 100 °C for 3 min. [e] Relative standard deviation.

eliminate the early GC peak. The chemical event leading to the formation of this component is not known. However, it was reported (Eto, 1974) that when the compound was merely heated, the methyl group of dimethyl isoxazolyl phosphorothioate could migrate to the nitrogen atom of isoxazolyl ring. Likewise, a thermal effect also caused the migration of the alkyl group of *O,O*-dialkyl *S*-aryl phosphorothioate to form alkyl aryl sulfide in high yield (Hilgetag and Teichmann, 1965). Therefore, it is logical to speculate that on-column transalkylation of the oxon of azinphos-methyl may also occur. The striking aspect of Figure 2 is that the base line of the chromatogram is abruptly disrupted right after the first GC peak, forming a plateau until the parent compound elutes from the column. The explanation of this observation is that thermal decomposition of the oxon of azinphos-methyl occurs in the GC injector because of high temperature (250 °C) and also on the column as the temperature is programmed. The intensity of the early GC peak can be partially reduced if the GC column is repeatedly conditioned by injecting 4–5 times 2 μg of the oxon of azinphos-methyl. In their study, Hunt and Gilbert (1970) also indicated that the gas chromatographic column required periodic priming with oxygen analogue, approximately 200 ng at 2-h intervals, in order to maintain maximum sensitivity.

Recently, a comparison between ACHE and GC methods for measureing parathion metabolism in vitro indicated that the ACHE method was as precise as the GC method to measure paraoxon in biological samples (Mirer et al., 1975). The same kind of experiments to evaluate the accuracy of the ACHE method for measureing the oxon of azinphos-methyl in biological samples have not been reported. In this study, the interchangeability between the ACHE and HPLC methods for measuring the metabolism of azinophos-methyl by mouse liver homogenates was tested by performing analyses by both methods on the same biological samples.

Table I shows the HPLC measurements of the oxon of azinphos-methyl and benzazimide produced in vitro, with tests for temperature effects. A total of 10 mice were used in the experiments. Panel A of Table I shows the data of those incubation mixtures in which the enzyme activities are terminated by freezing with dry ice in 2-propanol, whereas panel B, those by heating to 100 °C for 3 min. It

Table II. ACHE Measurements of the Oxon of Azinphos-methyl (GO) Produced in Vitro by Mice Liver Homogenates[a]

	GO[b]	
samples	panel A	panel B
1	5.51	2.32
2	6.02	1.96
3	5.14	2.32
4	5.14	3.07
5	5.14	3.07
6	5.14	2.78
7	4.57	2.19
8	4.57	2.72
9	4.45	2.64
10	4.89	2.26
av ± SD	5.05 ± 0.47	2.54 ± 0.37
RSD,[c] %	±9.3	±14.6

[a] Biological samples were derived from the same incubation mixtures of A and B in Table I. [b] The amount of the oxon in the incubation mixture were expressed in a unit equivalent to micrograms of the oxon of azinphos-methyl present in the standard which could cause the same percent inhibition of the bovine erythrocyte cholinesterase. [c] Relative standard deviation.

is interesting to note that the amount of the oxon of azinphos-methyl measured in the B system is 44% less than that in the A system. On the other hand, the amount of benzazimide is 2.12 μg greater in the B panel (2.55 ± 0.13 μg) than that of A panel (0.43 ± 0.05 μg). Since thermal decomposition of 400 nmol of azinphos-methyl in a zero-time incubation mixture (row 1) produces only 0.83 μg of benzazimide, the extra amount of 1.29 μg (2.12–0.83) must result from chemical decomposition of metabolites in the mixture. Furthermore, if this amount of 1.29 μg of BZ is added to the GO panel B on an equal molar basis, the resulting adjusted value of the oxon (4.51 μg) is comparable to the measured amount of 4.50 μg in panel A. This result suggests that benzazimide is the sole product of thermal decomposition of the oxon of azinphos-methyl produced in the incubation mixture. Thus, thermal deactivation of liver enzyme activity should be avoided. According to Table I, the relative standard deviations for the measurements of the oxon of azinphos-methyl in 10 liver homogenates are within 11% and that of benzazimide, 12%.

Table II shows the results by ACHE assay on the same samples as those shown in Table I. In agreement to Table I, the effect of temperature on the decomposition of the oxon of azinphos-methyl is indicated by a 44% less in the measured amount of GO in panel B than that in panel A. The relative standard derivation of measurements of the oxon of azinphos-methyl by the ACHE method is within 15%, comparable to that of HPLC method, although the rate of formation of the oxon of azinphos-methyl of the A system measured by the ACHE method appears to be 13% greater than those measured by the HPLC method. However, the Student's t test indicates that this difference is statistically insignificant (>0.05). To further test whether there are any acetylcholinesterase inhibitors remaining in the liver homogenates after extraction with ethyl acetate, various volumes of the aliquot portion of A samples (10, 20, 30, 100, and 500 μL) were subjected to the ACHE assay. No response could be detected.

In conclusion, a comparsion between the two techniques indicates that both the ACHE and HPLC methods measure the oxon of azinphos-methyl to the same degree of precision. Since there are no significant difference in the measured amounts of the oxon of azinphos-methyl by both methods, it is likely that the compound may be the sole active metabolite produced in the liver which inhibits acetylacholinesterase. The limit of detection of the oxon of azinphos-methyl by the ACHE method, using the workup procedure described here, is 6 ng while the described procedure for the HPLC method was reliable to 5 ng (Lin et al., 1980). For some purposes of routine assays of the oxon of azinphos-methyl, the ACHE method would be more labor efficient (extraction and cleanup are not required) and more rapid (several metabolic samples can be assayed simultaneously, instead of serially as with HPLC). However, the HPLC method holds the advantage that several metabolites can be measured simultaneously.

Registry No. Azinphos-methyl oxon, 961-22-8; methyl(mercaptomethyl)benzazimide, 85850-18-6; azinphos-methyl, 86-50-0.

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Gas–Liquid Chromatographic Determination of Residue Dissipation of 3,6-Dichloropicolinic Acid in Sugar Beets

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Cyronal emulsifiable concentrate was applied at rates of 120, 150, 180, and 360 g of 3,6-dichloropicolinic acid/ha as postemergence herbicide on a sugar beet crop to follow the dissipation of residues in roots, tops, and leaves from the treatment to the harvesting. The method used was a gas–liquid chromatography after derivatization of the acid to form the methyl ester. Results show a light persistence of the pesticide respectively at a rate of 0.11, 0.13, 0.17, and 0.27 ppm in the beets (root plus top).

3,6-Dichloropicolinic acid (3,6-DCP) is a relatively specific, postemergence, growth-regulator herbicide manufactured by Dow Chemical Co. It is intended for use in the control of important phenoxy-tolerant weeds such as thistles which infest Graminaceae, sugar beet, and flax. It is absorbed by roots and leaves and translocated throughout the plant. In susceptible plants, it induces characteristic auxin-type response (Brown et al., 1976; Martin and Worthing, 1977; Jones, 1977).

However, 3,6-DCP is chemically related to another herbicide, picloram (4-amino-3,5,6-trichloropicolinic acid), which is very persistent in crops and soils. Because many problems due to that picloram persistence have been observed, it was interesting to study the dissipation of 3,6-

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