Quantitative High-Performance Liquid Chromatography and Mass Spectrometry for the Analysis of the in Vitro Metabolism of the Insecticide Azinphos-methyl (Guthion) by Rat Liver Homogenates

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A method based on high-performance liquid chromatography and mass spectrometry was developed for the quantitative measurement of Gutoxon and benzazimide, two of the metabolic products of Guthion. Analysis of the extracts, without prior purification, was conducted with benzamide as internal standard. The detection limit for Gutoxon was 10 ng and for benzazimide, 5 ng. Extraction efficiencies were determined using samples of heat-inactivated rat liver homogenates containing the appropriate compounds. The advantage of using high-performance liquid chromatography over gas chromatography was that thermal decomposition of Gutoxon was prevented. The metabolites in the incubation mixture were confirmed by mass spectrometry. Metabolic pathways leading to the formation of benzazimide have not been reported previously. The present study verified that Guthion was activated to Gutoxon and was also degraded to benzazimide. Gutoxon was degraded to benzazimide but at a slower rate than that of Guthion.

Although there is considerable information available concerning the metabolism of Guthion (Guthion is the registered trademark name of the Mobay Chemical Co. for the insecticide azinphos-methyl; DuBois et al., 1957; Murphy and DuBois, 1957; Nakatsugana and Dahm, 1962; Johnson and Dahm, 1966; Rao and McKinley, 1969; Adams and Anderson, 1966), the nature and the extent of the formation of its metabolites are not clear mainly due to the lack of quantitative analytical procedures. Both Guthion and Gutoxon, the activated metabolite of Guthion, are thermally labile (Liang and Lichtenstein, 1972) and can not be analyzed by methods which require high temperature. This eliminated the possibility of utilizing the gas chromatographic method for the quantitative determination of the metabolic profile of Guthion. In a preliminary study (Lin et al., 1978), we investigated the GC/MS properties of Guthion and Gutoxon and found that both compounds decomposed in the injector (250 °C) and the separator (250 °C) of the GC/MS system. For this reason, a quantitative method based on high-performance liquid chromatography and mass spectrometry was developed to study the metabolism of Guthion by rat liver homogenates.

In 1972, Motoyama and Dauterman suggested that one of the enzymatic degradative pathways of Guthion in rat liver was via the breakage of the P–S bond of the pesticide, resulting in the formation of mercaptomethylbenzazimide and dimethylphosphorothioic acid. Although the nonphosphorus-containing moiety of the metabolites were not identified, the detection of dimethylphosphorothioic acid with ion-exchange liquid chromatography tended to support this concept. Nevertheless, an alternative metabolic pathway which involved the breakage of the S–C bond of Gutoxon could also result in the same metabolite through a thiono–thiolo tautomerism of thioic acid (Kabachnick et al., 1960). The present study was undertaken to identify and quantitatively determine the nonphosphorus degradation products of Guthion.

EXPERIMENTAL SECTION

Reagents. Guthion [*O,O*-dimethyl *S*-(4-oxobenzotriazino-3-methyl) phosphorothiolothionate] and Gutoxon (oxygen analogue of Guthion) used in this study were of the highest purity available from the manufacturers (>95%). Benzazimide and benzamide were purchased from Plaufz and Bauer, Inc. (Stanford, CT). All solvents were distilled in glass and were obtained from Burdick and Jackson Lab. Inc. (Muskegon, MI).

Incubation Procedures. Male, adult Sprague-Dawley derived rats from Hilltop Co. were used. The animals were housed in air-conditioned rooms and supplied with food and water ad libitum. The rats were killed by decapitation and their livers were immediately removed and homogenized in an ice-cold solution containing 1.15% KCl and 0.25% nicotinamide. All incubations were performed immediately. Incubation mixtures consisted of 40 mg of liver in solutions which were 40 mM in sodium phosphate buffer (pH 7.6), 1.3 mM in nicotinamide adenine dinucleotide phosphate, and 3.3 mM in glucose 6-phosphate in a total volume of 2 mL. Before incubation started, various amounts of Guthion (0, 20, 40, or 80 μL of 0.01 M solution in ethanol) were added. Incubation was carried out in air, with shaking (120/min), at 37 °C for 30 min. In one experiment, it was found that Guthion was decomposed if the incubation mixture was heated in boiling water for 5 min; therefore, termination of enzymatic reaction was conducted by rapidly freezing the incubation mixture with dry ice in 2-propanol.

Extraction. The incubation mixture was extracted twice with 2 mL of ethyl acetate. Internal standard (7 μg of benzamide dissolved in acetonitrile) was added to the tube before the first centrifugation. After centrifuging, the supernatant was transferred to a 4-mL vial equipped with a Teflon-lined screw cap. With the aid of a gentle nitrogen stream, ethyl acetate was evaporated to dryness. To this residue was added 50 μL of acetonitrile and 5 μL of the resulting solution was injected directly into the LC.

High-Performance Liquid Chromatography and Mass Spectrometry. A Waters high-performance liquid chromatograph system was used which included a M-6000 solvent delivery unit, a U-6K universal liquid chromatograph injector, a Waters Model 450 variable wavelength detector, and a 300 × 4 mm i.d. column packed with μ-Porasil. The wavelength of the detector was set at 254 nm. The mobile phase consisted of methylene chloride, acetonitrile, and glacial acetic acid at the ratio of 77.5:22.5:0.02 with the flow rate maintained at 1 mL/min. Quantitative analyses were made based on the measurements of peak height.

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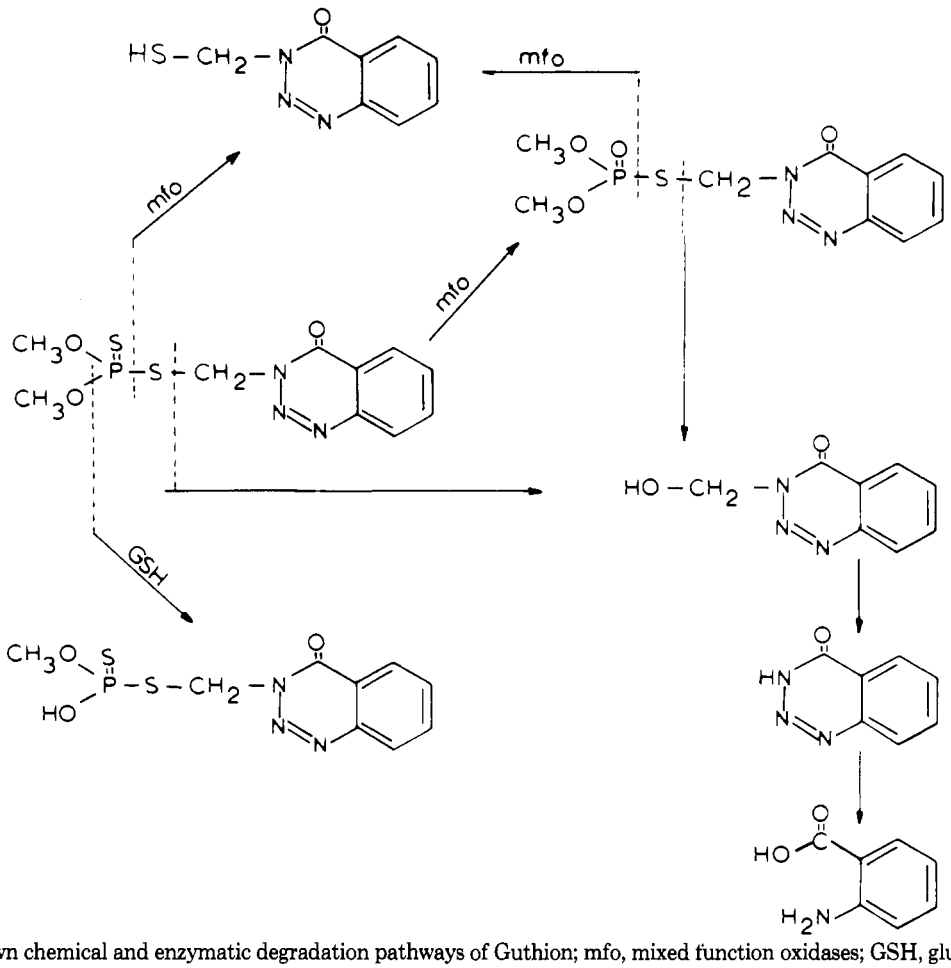

Figure 1. The known chemical and enzymatic degradation pathways of Guthion; mfo, mixed function oxidases; GSH, glutathione-mediated metabolic pathway.

Identification of the metabolites in biological extracts were carried out using a Finnigan 3200 mass spectrometer with a 6000 data system. Electron impact mass spectra were recorded using an ionizing energy of 70 eV at a source temperature of about 100 °C. The direct probe was used to introduce the compounds into the mass spectrometer to avoid thermal decomposition.

RESULTS AND DISCUSSION

One of the prime objectives of this study was to develop a method which could be used to specifically and quantitatively reveal the metabolic fate of the pesticide Guthion, especially when the animal species were pretreated with certain drugs (Murphy et al., 1975). Efforts were made in the past to probe the possibility of utilizing GC/MS methods for this purpose (Stein and Pittman, 1976; Burchfield and Storrs, 1975), but due to the thermal instability of both Guthion and Gutoxon these compounds decomposed during GC/MS analysis. This problem was solved by employing a method based on normal phase high-performance liquid chromatography and mass spectrometry. With the detector wavelength set at 254 nm, the quantitative detection limit of the LC system for Gutoxon was 10 ng and for benzazimide, 5 ng. The linear relationship between the amount of Gutoxon and benzazimide recovered from biological samples with added standards was observed in the range of 10 ng to 3 μg. The measurement was made based on the ratio of their peak heights with respect to that of the internal standard.

Recovery data including 11 separate extractions are shown in Table I. Heat-inactivated rat liver homogenates were mixed with four different combinations of Gutoxon, benzazimide, and benzamide. Recovery of Gutoxon ranged from 86 to 112% with an average of 99%. For benzazimide, it ranged from 75 to 105% with an average of 91%.

In order to eliminate the correction factor which resulted from the differences in the extracting efficiencies among the metabolites and the internal standard, calibration curve bases on known amounts of standards in rat liver homogenates were used for quantitative calculations. Reliability of the analysis was tested by comparing the stated and found level of Gutoxon and benzazimide standards made up in biological samples. Good agreement was observed, and both the precision and accuracy of the analyses were within 5%.

Injections were routinely made every 25 min. However, the polar contaminants in the crude extracts from earlier injections started to elute after ten injections. At this point, the column was first flushed with acetonitrile for 30 min at the flow rate of 1.3 mL/min, followed by flushing with four column volumes of methylene chloride. The clean column was then equilibrated with the mobile phase for 15 min. Changes in the separation efficiency from column to column were observed. Therefore, calibration curves were regenerated each time a new column was installed or when loss of column efficiency was observed. The present study indicated that this method was adequately sensitive and quantitative for studies of Guthion metabolism in vitro.

The schematic diagram presented in Figure 1 shows the known chemical and enzymatic degradation pathways of Guthion. Transformation of Guthion to Gutoxon involved mixed function oxidases (mfo) in the microsomal fraction of rat liver homogenates (Motoyama and Dauterman,

Table I. Recovery of Gutoxon (GO), Benzazimide (BZ), and Benzamide (IS) Added to Rat Liver Homogenates

amount added, μg			amount found, μg			recovery %		
GO	BZ	IS	GO	BZ	IS	GO	BZ	IS
0.05	0.01	0.7	0.04	0.01	0.71	86	91	102
0.20	0.05	0.7	0.20	0.04	0.83	102	90	119
			0.22	0.05	0.76	108	102	109
			0.19	0.05	0.72	97	91	103
			0.22	0.05	0.95	112	98	136
0.39	0.09	0.7	0.41	0.08	0.72	104	83	103
			0.33	0.07	0.59	86	75	84
			0.40	0.08	0.79	103	94	113
			0.36	0.09	0.69	93	105	99
0.78	0.18	0.7	0.75	0.14	0.65	97	78	94
			0.79	0.17	0.73	101	97	104
					mean ± SD	99 ± 8	91 ± 9	106 ± 13

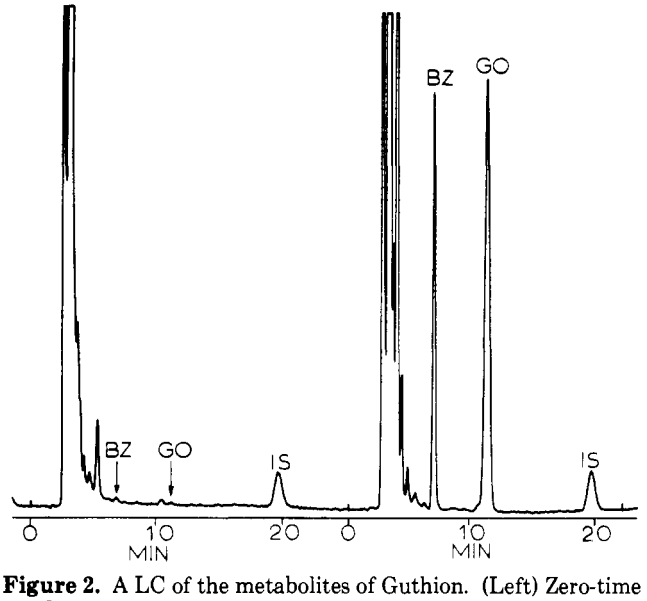

Figure 2. A LC of the metabolites of Guthion. (Left) Zero-time incubation mixture containing the internal standard (IS) and Guthion as substrate. (Right) Incubation at 37 °C for 30 min. GO, Gutoxon; BZ, benzazimide.

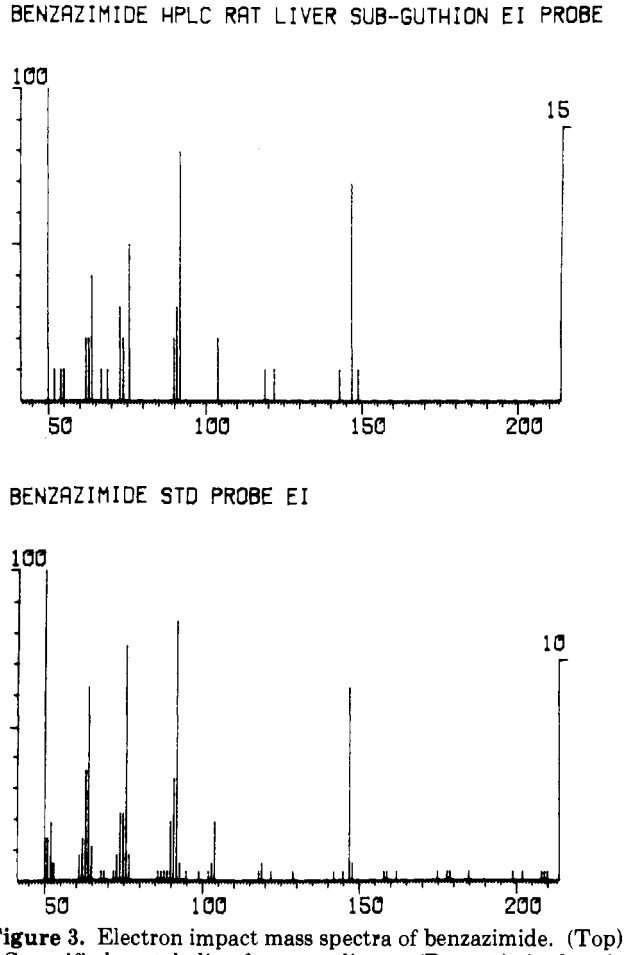

Figure 3. Electron impact mass spectra of benzazimide. (Top) LC purified metabolite from rat liver. (Bottom) Authentic benzazimide.

1972). This pathway could be blocked if the microsomal fraction was gased using carbon monoxide for a short period of time. The same enzyme system also caused the degradation of Guthion as well as Gutoxon to form mercaptomethylbenzazimide and dimethylphosphorothioic acid (Motoyama and Dauterman, 1972).

On addition of glutathione (GSH) to the soluble fraction of the rat liver homogenates, dealkylation of Guthion occurred, producing desmethylguthion as metabolites (Motoyama and Dauterman, 1972). Metabolic pathways involving the breakage of the S–C bond of Guthion and Gutoxon to produce hydroxymethyl benzazimide, followed by the formation of benzazimide and anthranilic acid have not been positively confirmed in the rat (Adam and Anderson, 1966; Motoyama and Dauterman, 1972). However, the determination of Guthion as residue in milk has been accomplished by monitoring its chemical degradation to form anthranilic acid by spectrophotofluorometric methods (Loeffer et al., 1966). It is also interesting to note that metabolic breakage of the S–C bond among dithiophosphates with a thiomethylene bridge between the phosphorus and heterocyclic nitrogen atom has been suggested for phosmet (McBain et al., 1968) and phosalone (Metivier, 1972) in rat.

Figure 2 shows the high-performance liquid chromatogram of two of the metabolic products of Guthion generated from rat liver homogenates. The identities of the metabolites were first confirmed by coinjecting the biological preparation with authentic compounds. Further identifications were made by collecting the LC peaks and analyzing them by mass spectrometry. The peak designated as BZ in Figure 2 was identified to be benzazimide. As indicated in Figure 3, the mass spectrum of this compound was identical with that of the authentic benzazimide (M^+ is m/e 147). The peak designated as GO in Figure 2 was identified to be Gutoxon. Figure 4 shows the mass spectrum of this compound as well as that of authentic Gutoxon. No molecular ion was observed. However, the ion at m/e 109, arising from the phosphorus moiety of the compound, was of diagnostic value (Damico, 1966). The peak designated as IS in Figure 2 corresponded to benzamide which was used as the internal standard. The left panel of Figure 2 demonstrates the chromatogram of a zero-time incubation mixture. It was noted that no ap-

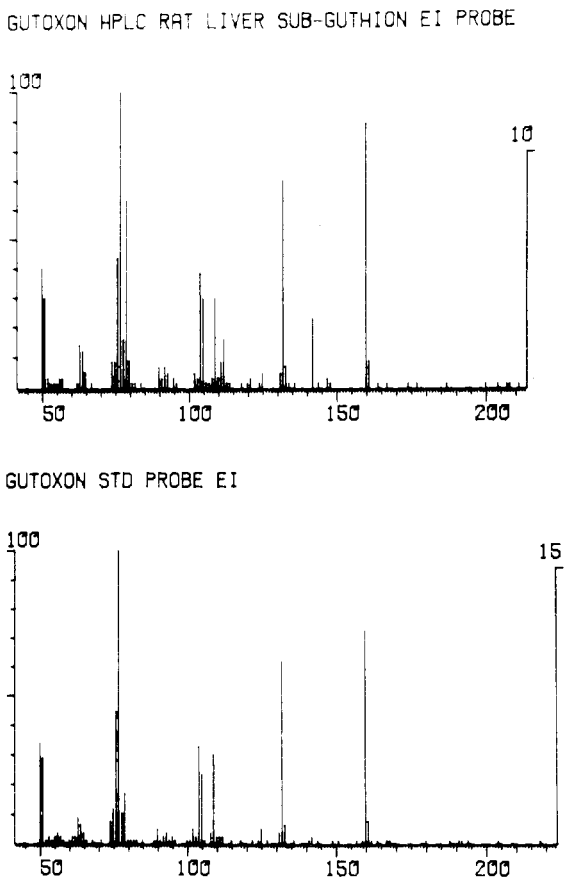

Figure 4. Electron impact mass spectra of Gutoxon. (Top) LC purified metabolite from rat liver. (Bottom) Authentic Gutoxon.

Table II. Degradation of Guthion and Gutoxon by 40 mg of NADP and Glucose 6-Phosphate Fortified Rat Liver Homogenates

substrate, nmol	degradation products, nmol	
	Gutoxon	benzazimide
Guthion		
200	16.9	5.1
400	22.0	9.4
800	26.8	11.4
Gutoxon		
20		0.5
40		0.8
100		1.8
200		2.7
400		4.5
800		7.8

preciable amounts of benzazimide and Gutoxon were observed, suggesting that both components which appeared in the liver homogenate were produced enzymatically. According to the scheme in Figure 1, both Guthion and Gutoxon could be the precursors for benzazimide. Studies on the enzyme kinetics were then carried out with either Guthion or Gutoxon as substrates under the same conditions. Table II shows the formation of benzazimide as a function of the amount of substrate used. It was obvious that both compounds were degraded to benzazimide except that degradation of Guthion proceeded at a faster rate than that of Gutoxon. This experimental result was in agreement with the observations made by Motoyama and Dauterman (1972) in that the overall in vitro degradation of Gutoxon was much slower than that of Guthion. Table II also shows that substantial quantities of Gutoxon were produced when Guthion was substrate.

In conclusion, the present study demonstrates that a combination of high-performance liquid chromatography and mass spectrometry fulfill the requirements for quantitative and qualitative analysis of metabolites of azinphosmethyl. Metabolic pathways were evaluated in that individual metabolites were specifically identified and metabolic kinetic data could also be obtained. In addition, the present study verified that Guthion was activated to Gutoxon and was also degraded to benzazimide. Further, Gutoxon was shown to degrade to benzazimide but at a slower rate.

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