

Vinyl chloride

VINYL CHLORIDE URINARY METABOLITES:
ISOLATION AND IDENTIFICATION

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Abbreviated Title: Vinyl Chloride Metabolites

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Vinyl Chloride Urinary Metabolites: Isolation and Identification. McGowan, G. R., Watanabe, P. G. and Gehring, P. J. (1975). Toxicol. Appl. Pharmacol. ____, ____-____. Three major urinary metabolites have been isolated by high pressure liquid chromatography (HPLC) from rats dosed orally with 0.05 to 100 mg/kg of ^{14}C -vinyl chloride. The primary metabolites, accounting for about 70% of the total ^{14}C activity in the urine have been identified as S-(2-hydroxyethyl)-cysteine and N-acetyl-S-(2-hydroxyethyl)-cysteine by gas chromatography-mass spectrometry (GC-MS), high resolution GC-MS, and co-elution on HPLC. The third major metabolite, accounting for 20-30% of the urinary ^{14}C activity has been identified as thiodiglycolic acid using similar techniques. The identification of cysteine conjugates as the major urinary metabolites in rats suggests that the primary detoxification of VC proceeds by initial conjugation with glutathione.

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INTRODUCTION

The fate of inhaled vinyl chloride (VC) has been studied in rats (Hefner et al., 1975). Consideration of this data led to the hypothesis that the carcinogenicity of VC was due to alkylating metabolites formed in vivo. While the metabolism of many alkylating agents has been studied, VC has received little interest until recently. Studies by Rannug et al. (1974), Bartsch et al. (1975), and Malaveille et al. (1975) have shown that the mutagenicity of VC towards Salmonella typhimurium is greatly increased by the presence of fortified liver homogenates. This is consistent with the hypothesis that a metabolic product of VC rather than VC per se may be responsible for the mutagenicity and by analogy the carcinogenic effects.

Elucidation of the urinary metabolites of VC would lend insight into the metabolism of VC in vivo. Earlier work in this laboratory with rats exposed to 7900 ppm of ¹⁴C-VC for one hour had indicated the presence of 3 major and several minor urinary metabolites which could be separated by thin layer chromatography and column chromatography. One of the major metabolites reacted as a mercapturic acid in several chemical derivitizations but could not be identified as such by instrumental techniques.

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The objective of the present study was to isolate and identify the major urinary metabolites of VC from rats.

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METHODS

Animals and Dosing. Male albino Sprague-Dawley (Spartan substrain) rats weighing from 180-225g purchased from Spartan Research were used throughout the studies. All animals were fasted overnight and the VC administered between 8 and 10 AM the following morning. 1,2-¹⁴C-vinyl chloride was synthesized directly from 1,2-dichloroethane (1,2-¹⁴C, New England Nuclear Corp.) by the method of Wagner and Muelder, (1975). The ¹⁴C-VC and non-labeled VC (Matheson Gas Products, >99% purity) were bubbled directly in 15g of USP corn oil in a sealed septum vial to the desired specific activity. Typically all animals received from 2-5 μ Ci at doses of 0.05, 1, 20 or 100 mg/kg VC. The VC-corn oil solution was administered by gastric intubation using a stainless steel dosing needle connected to a glass syringe. The rats were housed in Roth-type metabolism cages and the urine collected for 24 hours. For additional details of the administration procedure refer to Watanabe et al. (1975).

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High Pressure Liquid Chromatography (HPLC). Urine (5 to 10 ml) containing 0.05 to 1.0 μC of ^{14}C activity (ranging from 9-2000 μg VC equivalents) was lyophilized to dryness and the solid residue extracted once with 5 ml of methanol, once with 0.5 ml of water and twice more with 5 ml of methanol. The solution was centrifuged after each extraction and the clear supernatants removed and combined. The extraction of ^{14}C activity into the combined supernatants was 100 ± 2 (SD)%. The combined supernatants were evaporated to dryness under a stream of nitrogen at room temperature and the residue reconstituted in 1 ml of methanol.

The liquid chromatography column was prepared by packing a 2 mm ID x 50 cm glass column with Corasil II, 37-50 μ (Waters Associates, Framington, Ma.). The pumping system used was a Waters Model 660 Solvent Programmer with two Waters Model 6000 Pumps. The flow rate was maintained at 2.0 ml/min while the solvent was programmed to form non-linear gradient #7 from hexane:dioxane (7:1) to 2-propanol:methanol (3:2) over 20 min (Figure 1). The column temperature was ambient, about 23°C. The

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sample was injected in 25 μ l aliquots and washed onto the column with hexane:dioxane (7:1) for about 10 sec between injections. Normally from 10-75 μ l of methanol solution containing from 1000-150,000 dpm of ^{14}C activity were injected for each run.

The eluant was monitored at 254 or 280 nm using a Chromatronix 220 UV monitor and collected in 2 ml fractions. For analytical work 10 ml of Aquasol (New England Nuclear, Boston, Mass.) was added to each fraction and the samples counted in a Searle MK II liquid scintillation spectrometer using external standardization and channels ratio for determining counting efficiency.

Urine used for the identification of the major metabolites was obtained from rats administered 20 mg/kg VC. The urine samples contained about 800 μ g VC equivalents and 1 μCi ^{14}C -activity per ml. The radioactivity in these samples were separated by HPLC as previously described. Aliquots (25-250 μ l) of each fraction from the liquid chromatographic columns were counted and the fractions making up individual peaks of ^{14}C activity were combined, evaporated to dryness under N_2 and picked up in a small volume of methanol. A one meter Porasil B(250) column (Waters

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Associates) was also used for preparative work with a linear gradient from hexane:chloroform (1:1) to methanol. The results were similar to those obtained from the Corasil II column except for a higher column capacity and poorer peak resolution. With both columns the recovery of ^{14}C activity was quantitative and the columns could be reused numerous times before peak resolution decreased significantly.

For further purification of Metabolite A additional HPLC was done using alumina. The column was prepared by packing a 2 mm ID x 1 meter glass column with acidic alumina AG-4 (40 μ) (Bio-Rad Laboratories, Richmond, Ca.). The column was repacked for each run. The packed column was washed with methanol until a stable baseline was indicated by the UV monitor. About 50 μg VC equivalents of Metabolite A from the initial Porasil separation were injected on the column and eluted with 6% concentrated aqueous NH_4OH in methanol at a flow of 1 ml/min. The eluant was collected in fractions of 1-2.5 ml and an aliquot counted as before. The fractions making up the peak of ^{14}C activity were combined and evaporated to dryness under N_2 .

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Gas Chromatography (GC). The collected eluant fractions from the initial HPLC separation on Corasil II or Porasil B(250) were combined to give 3 fractions containing the 3 peaks of ^{14}C activity. The first two major peaks, designated Metabolites A and B, were evaporated to dryness under N_2 , dissolved in methanol:diethylether (1:1) and methylated using diazomethane. They were then evaporated to dryness and dissolved in methanol to give a concentration of 0.5 to 1 μg VC equivalents and 500-1200 dpm of ^{14}C activity/ μl .

The third peak, designated Metabolite C was dissolved in methanol, methylated using diazomethane, and the tri-fluoroacetyl derivative formed (Darbe and Blau, 1965). The methylated N-trifluoroacetylated material was dissolved in a minimum volume of diethyl ether for injection into the gas chromatograph.

The derivatized fractions were chromatographed on one of two columns, (A); 6' x 2 mm ID glass packed with 10% UCW-98 on 80/100 Gas Chrom Q or (B); 6' x 2 mm ID glass packed with 3% OV-210 on 80/100 Chromsorb 750. Column A was programmed from 100° to 250°C at 10°/min and column B was run at 125°C isothermally.

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The outlet of the column was routed into an effluent splitter using a 5:1 split ratio. One part was fed into the flame ionization detector while five parts exited through a 1/8" OD stainless steel heated exit line to a fraction trapping apparatus. A Hewlett-Packard 5750B gas chromatograph was used with the injection port and flame ionization detector maintained at 250 and 275°, respectively. The helium carrier gas flow rate was 35 ml/min.

When fractions were trapped from the GC for counting of ^{14}C labeled metabolites, the glass capillary containing the condensed metabolite was washed into a scintillation vial using 2 ml of Aquasol. Eight additional ml of Aquasol were added to the vial. When fractions were trapped for infrared spectrophotometric analysis, multiple GC runs were made using the same capillary and the metabolite removed with 5 μl of solvent and placed in a cavity cell.

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Mass Spectroscopy (MS). Low resolution mass spectra were run on a Finnigan Model 3000D GC-MS operating at 70 KeV using both probe and GC inlets interfaced with the Model 6000 MS data system. When using the GC inlet the columns and GC conditions were identical to those previously given. Probe samples were run by placing about 1 μg of metabolite in a quartz cup in the probe and slowly raising the temperature from ambient to 250°C.

High resolution GC-MS were run on an AEI MS-30/DS-50 double beam mass spectrometer at Dow Corning Analytical Services. The resolution was 4300 and the mass measuring accuracy was generally within ± 0.005 mass units over the range of interest. The GC conditions were as previously described.

Synthetic Metabolite Standards. Samples of N-acetyl-S-(2-hydroxyethyl)-cysteine and thiodiglycolic acid were synthesized by Dr. N. Peet of Dow Lepetit, Pharmaceutical R&D. Samples of ^{14}C -labeled N-acetyl-S-(2-hydroxyethyl)-cysteine and thiociglycolic acid were synthesized by D. Gransden of Dow Environmental Sciences Research.

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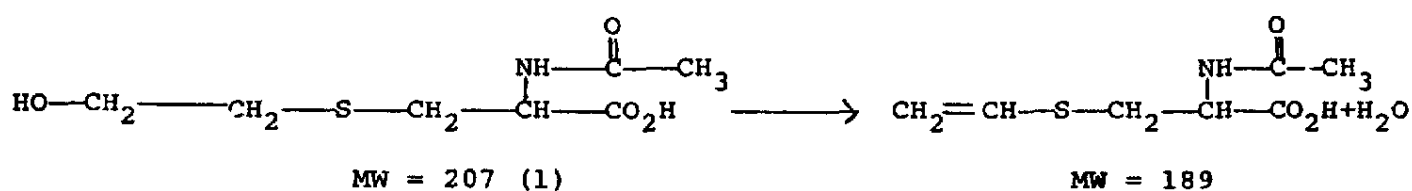
RESULTS

Using HPLC on a Corasil II column, methanol extracts of urine from rats given 0.05 to 100 mg/kg ^{14}C -VC orally were separated into three major peaks containing about 95% of the applied ^{14}C activity and several minor peaks. Figure 1 shows a chromatogram of a typical sample. The metabolites were designated by their elution order from the column as A, B, and C, comprising 38, 28, and 30% respectively of the total radioactivity in the urine.

Additional purification of the metabolite A fraction from the initial Corasil II or Porasil separation was carried out on an acidic alumina HPLC column with a methanol: aqueous NH_4OH eluant. The ^{14}C activity eluted as a single peak containing over 90% of the applied radioactivity. A probe mass spectrum was run on the combined fractions from the alumina column containing the bulk of the ^{14}C activity. The sample was shown to be a mixture of several components including one which had an apparent weak molecular ion of $m/e = 189$ and an important peak at $m/e = 130$. Earlier work with $\underline{\text{S}}$ -(2-hydroxyethyl)-cysteine (1) had shown that the highest mass peak found corresponded to the dehydrated molecular ion (M-18).

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If a similar dehydration had occurred in the metabolite the mass spectrum obtained corresponded to that expected for N-acetyl-S-(2-hydroxyethyl)-cysteine (1).

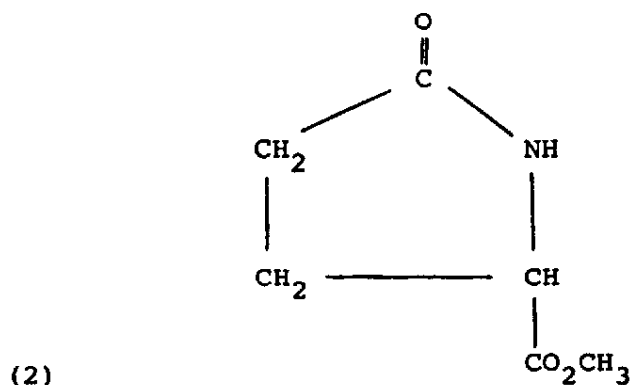


The mass spectrum of a synthesized sample of N-acetyl-S-(2-hydroxyethyl)-cysteine was found to be virtually identical to that of the material found in the Metabolite A fraction (Figure 2).

Using a 10% UCW-98 column programmed from 120° to 250°C, the methylated Metabolite A fraction was shown to contain a peak which had the identical retention time and mass spectrum as that of the methyl ester of the previously synthesized N-acetyl-S-(2-hydroxyethyl)-cysteine standard (Figure 3). It was found that only fresh samples of the Metabolite A fraction contained this peak. When the methylated sample was allowed to sit for several days at room temperature the peak would disappear, probably due to decomposition of the metabolite. It was found that the

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chromatogram of both the Metabolite A fraction and the synthesized N-acetyl-S-(2-hydroxyethyl)-cysteine contained a significant peak with a molecular ion at $m/e = 143$. The mass spectrum corresponded to that expected for:



Structure (2) was theorized to arise from the elimination of mercaptoethanol from N-acetyl-S-(2-hydroxyethyl)-cysteine. Additional confirmation of the identity of Metabolite A was obtained by co-injection of a synthetic ^{14}C -labeled N-acetyl-S-(2-hydroxyethyl)-cysteine with the urinary metabolites using HPLC. It was found that the added ^{14}C activity co-eluted quantitatively with metabolite A on both the Corasil II and acidic Alumina columns described previously.

When the methylated metabolite B fraction from the initial Porasil separation was run on the gas chromatograph it

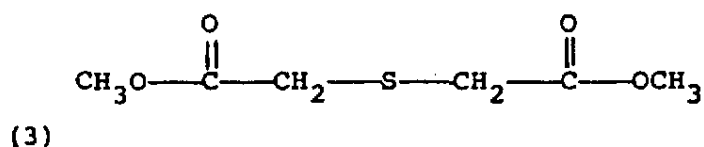
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was possible to associate a single peak in the chromatogram with the ^{14}C activity in the sample by trapping the material at the column outlet. The best separation was obtained using an OV-210 column at 125° isothermally, but Metabolite B was also present at low levels in the Metabolite A fraction run previously on the UCW-98 column (Figure 3). The methylated Metabolite B fraction used in the GC trapping was also run under identical conditions in the Finnigan GC-MS to obtain the mass spectrum of the peak associated with the trapped ^{14}C activity (Figure 4).

By high resolution GC-MS Metabolite B was shown to be a carboxylic acid (methyl ester) with a nominal molecular weight 178 and molecular formula $\text{C}_6\text{H}_{10}\text{O}_4\text{S}$. A listing of the important peaks is shown in Table 1.

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A sample of thiodiglycolic acid (dimethyl ester) (3) was run on the Finnigan GC-MS. The mass spectrum was found to be identical to that of Metabolite B (Figure 4).



Sufficient material was trapped from the GC column to obtain an IR spectrum of Metabolite B. About 12 μg of the metabolite dissolved in 5 μl of CCl_4 and run in a Beckman IR-9 infrared spectrophotometer gave bands at 1435 cm^{-1} and 1745 cm^{-1} .

The band at 1435 cm^{-1} would indicate a $-\text{S}-\text{CH}_2-\overset{\text{O}}{\underset{\text{||}}{\text{C}}}-$ structure and that at 1745 cm^{-1} an ester. An authentic sample of thiodiglycolic acid (dimethyl ester) was run for comparison and was found to contain major bands identical to those obtained in Metabolite B.

Finally, co-injection of a sample of synthesized ^{14}C -labeled thiodiglycolic acid with the urinary metabolites on the Corasil II HPLC column showed co-elution of the added ^{14}C activity with Metabolite B.

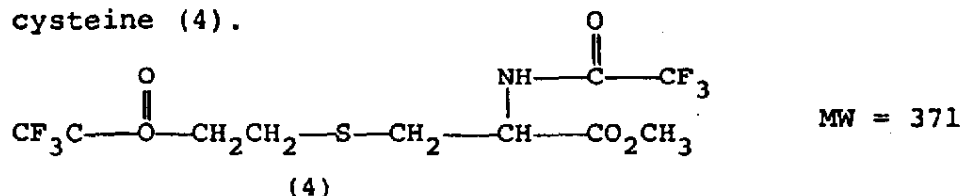
Because of the identification of Metabolite A it was suspected that a major metabolite might be S-(2-hydroxyethyl)-cysteine. When the methyl ester, trifluoroacetyl derivative of a standard sample of S-(2-hydroxyethyl)-cysteine was run on the UCW-98 GC column (Column A) at 170°C , peaks

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cysteine (4).

$$\begin{array}{c}
 \text{O} \\
 \parallel \\
 \text{CF}_3\text{C}-\text{O}-\text{CH}_2\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}-\text{CO}_2\text{CH}_3 \\
 | \\
 \text{NH}-\text{C}(=\text{O})-\text{CF}_3
 \end{array}$$

(4) MW = 371



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When the Metabolite C fraction from the initial Corasil II HPLC separation was derivatized and gas chromatographed in a similar manner, two peaks corresponding in retention time and mass spectrum to those from the derivatized standard S-(2-hydroxyethyl)-cysteine were observed. The mass spectra for the major peak of the Metabolite C fraction and the standard S-(2-hydroxyethyl)-cysteine are shown in Figure 5. High resolution GC-MS were run on both the standard and metabolite C derivatized samples which confirmed the proposed structure and the identity of Metabolite C as S-(2-hydroxyethyl)-cysteine (Table 2).

DISCUSSION

The identification of two of the major urinary vinyl chloride metabolites as cysteine conjugates, N-acetyl-S-(2-hydroxyethyl)-cysteine and S-(2-hydroxyethyl)-cysteine lends support to earlier indications that glutathione conjugation may be the principle route of vinyl chloride detoxification (Hefner et al., 1975). The third major metabolite identified, thiodiglycolic acid could arise from the transamination of a cysteine conjugate to the corresponding mercaptopyruvate followed by an oxidative decarboxylation. The identification of hydroxyethyl cysteine conjugates by mass spectroscopy is complicated by the lack of a molecular ion; the hydroxyethyl group dehydrates readily and the highest mass peak observed is the M-18. The reactivity of the hydroxyethyl group must also be considered during derivatization as O-acetylation and O-trifluoroacetylation will occur during attempts to acetylate or trifluoroacetylate the amino function of cysteine.

HPLC on Corasil II provides a convenient method for the quantitation of the 3 major urinary VC metabolites. Compared to TLC and classical ion exchange chromatography the HPLC

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procedure is rapid, provides good resolution and does not subject the sample to extremes of pH. In addition, by varying the shape and range of the gradient, metabolites of widely varying polarity can be separated by a single procedure.

In a study reported recently, Green and Hathway (1975) indicated that after multiple dosing of ^{14}C -VC in rats (50 mg/kg orally, 3 times at 3 hr intervals), thiodiglycolic acid was the major metabolite of vinyl chloride (about 47% of the total urinary ^{14}C activity). Our work confirms that thiodiglycolic acid is one of the major metabolites of vinyl chloride but in our study it accounted for only 20-30% of the ^{14}C activity in the urine in all samples analyzed.

Two other major metabolites identified by Green and Hathway (1975) were S-(2-chloroethyl)cysteine and its acetylated analog, N-acetyl-S-(2-chloroethyl)-cysteine. Since we have identified two major metabolites S-(2-hydroxyethyl) cysteine and its acetylated analog, the question is raised whether the vinyl chloride metabolites exist as hydroxyethyl or chloroethyl conjugates.

S-(2-chloroethyl)-cysteine is a monofunctional sulfur mustard and has been shown to be mutagenic (Fahmy and Fahmy, 1970). It seems doubtful, however, that it could be detected intact in the urine even if formed because of its susceptibility to hydrolysis to S-(2-hydroxyethyl)-cysteine. Jones (1973) reviewing the metabolism of 1,2-dibromoethane reported that the initially formed S-(2-bromoethyl)-glutathione is unstable and spontaneously hydrolyzed to S-(2-hydroxyethyl)-glutathione producing S-(2-hydroxyethyl)-cysteine as the primary metabolite. A similar hydrolysis would be expected for the chloroethyl conjugate. More direct evidence for the instability of S-(2-chloroethyl)-cysteine is its reported half-life of 7 minutes in aqueous solution at 37°C and pH 7 (Ross, 1962).

Green and Hathway (1975) identified the vinyl chloride metabolites by preparing the N-trifluoroacetyl n-butyl esters by the method of Gehrke and Stalling (1967). This involves the formation of the methyl ester using 1.25 M HCl (gas) in methanol at room temperature followed by the transesterification to the n-butyl ester using 1.25 M HCl (gas) in n-butanol at 100°C. It has been

reported by Connors and Ross (1958) and Carson and Wong (1964) that S-(2-chloroethyl)-cysteine can be prepared by heating S-(2-hydroxyethyl)-cysteine with concentrated HCl. It seems likely, therefore, that if the urinary vinyl chloride metabolites were present as S-(2-hydroxyethyl)-cysteine and its acetylated analog they may be converted into the corresponding chloroethyl compounds by the derivatization procedure used by Green and Hathway.

We feel therefore that the vinyl chloride metabolites identified by Green and Hathway (1975) correspond to those identified in this study but that the chloroethyl conjugates were in fact artifacts of their derivitization procedure. This does not, however, rule out the initial formation of the chloroethyl conjugate in the animal followed by hydrolysis to the corresponding hydroxyethyl compounds before excretion.

ACKNOWLEDGEMENTS

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LEGENDS

- Figure 1 Separation of urinary metabolites by high pressure liquid chromatography (HPLC) on a Corasil II column. The profile formed by the solid line shows the separating of radioactivity representing metabolites A, B, and C, by HPLC versus min (flow rate = 2 ml/min). The dashed line depicts the non-linear gradient profile used to effect the separation expressed as % solvent b (Solvent a, hexane:dioxane (7:1); solvent b, 2-propanol:methanol(3:2)).
- Figure 2 Probe mass spectra of N-acetyl-S-(2-hydroxyethyl)-cysteine (top) and urinary metabolite A (bottom). All peaks m/e greater than 140 were expanded by a factor of 10.
- Figure 3 Total ion chromatogram of metabolite A subjected to gas chromatography-mass spectroscopy (GC-MS). Relative detector response versus scan number (2 sec/scan) run on a 10% UCW-98 column.

Figure 4 Mass spectra of the methyl ester of ..
thiodiglycolic acid (top) and the methyl
ester of metabolite B.

Figure 5 Mass spectra of the methyl ester, trifluoroacetyl
derivative of S-(2-hydroxyethyl)-cysteine and
the similarly derivatized metabolite C.
All peaks m/e greater than 162 were expanded .
by a factor of 5.

TABLE 1

High Resolution GC-MS of Metabolite B

<u>m/e</u>	<u>Molecular Formula</u>	<u>Structure</u>
178.0302	$C_6H_{10}O_4S$	$[CH_3O-\overset{O}{\parallel}C-CH_2-S-CH_2\overset{O}{\parallel}C-OCH_3]^+$
146.0064	$C_5H_6O_3S$	$[M^a \text{ minus } -CH_3OH]^+$
119.0165	$C_4H_7O_2S$	$[M^a \text{ minus } -\overset{O}{\parallel}C-OCH_3]^+$
118.0127	$C_4H_6O_2S$	
91.0247	C_3H_7OS	$[-CH_2-S-CH_2-O-CH_3]^+$
74.0393	$C_3H_6O_2$	$[CH_3\overset{O}{\parallel}C-OCH_3]^+$
61.0142	C_2H_5S	
59.0118	$C_2H_3O_2$	$[-\overset{O}{\parallel}C-OCH_3]^+$
45.9905	CH_2S	
45.0379	C_2H_5O	$[CH_2CH_2OH]^+$

^aMolecular ion

TABLE 2

High Resolution GC-MS of N,O-trifluoroacetyl-methyl ester of
S-(2-hydroxyethyl cysteine) and Corasil II metabolite C fraction

Hydroxyethyl Cysteine	Metabolite C	Molecular Formula	Structure
<u>m/e</u>	<u>m/e</u>		
258.0221	(258.0370) ^a	C ₈ H ₉ O ₄ F ₃ S	$[M^b \text{ minus } -\text{NH}_2\overset{\text{O}}{\parallel}\text{C}-\text{CF}_3]^+$
230.0126	(--) ^a	C ₆ H ₇ O ₃ NF ₃ S	$[M^b \text{ minus } -\text{CH}_2\text{CH}_2\overset{\text{O}}{\parallel}\text{C}-\text{CF}_3]^+$
198.0267	198.0274		
187.0042	187.0081	C ₅ H ₆ O ₂ F ₃ S	$[\text{CH}_2-\text{S}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{CF}_3]^+$
169.9939	169.9986	C ₄ H ₃ ONF ₃ S	$[\text{S}-\text{CH}=\overset{\text{NH}}{\text{CH}}-\overset{\text{O}}{\parallel}\text{C}-\text{CF}_3]^+$
144.0240	144.0215	C ₆ H ₈ O ₂ S	$[\text{CH}_2=\text{CH}-\text{S}-\text{CH}=\text{CH}-\text{CO}_2\text{CH}_3]^+$
141.0178	141.0153	C ₄ H ₄ O ₂ F ₃	$[\text{CF}_3\overset{\text{O}}{\parallel}\text{C}-\text{O}-\text{CH}_2\text{CH}_2]^+$

^aTo weak to detect or measure accurately

^bMolecular ion

FIGURE 1

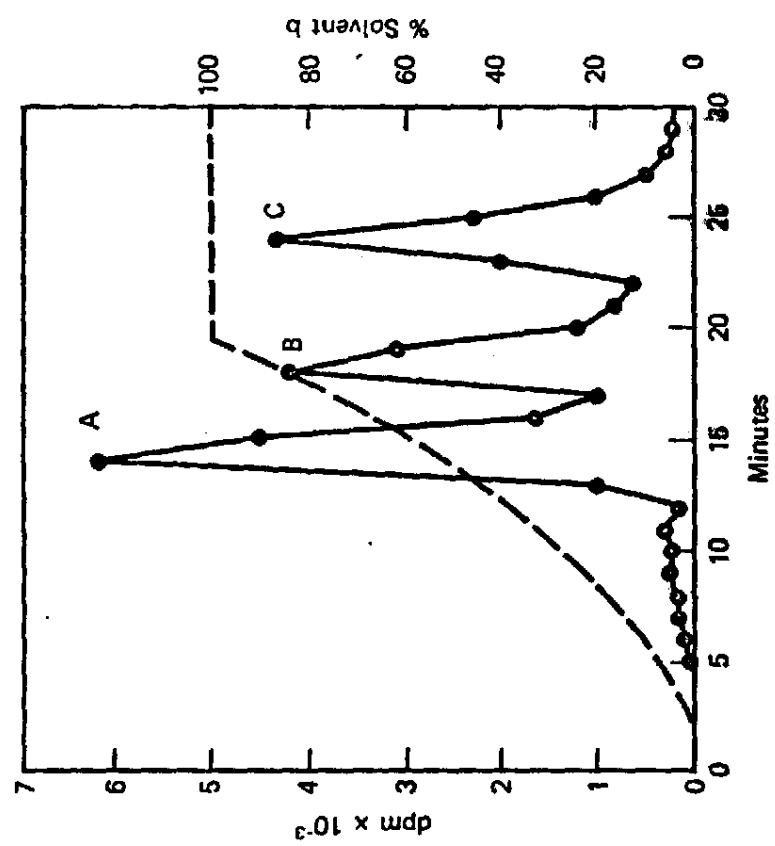


FIGURE 2

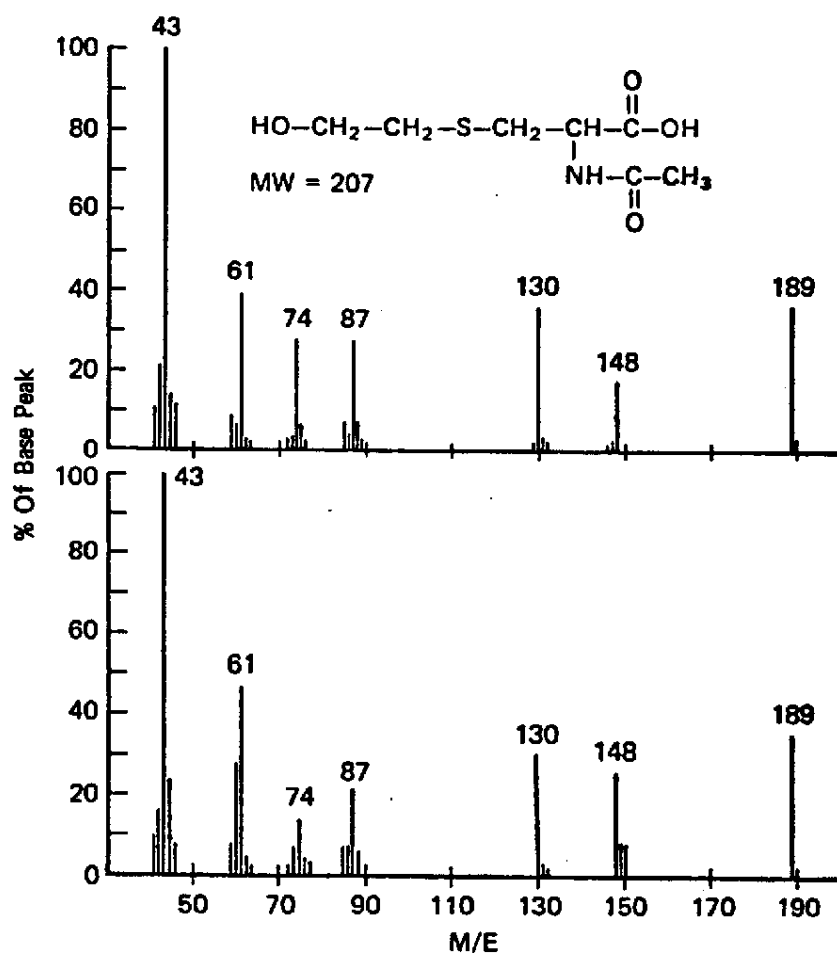
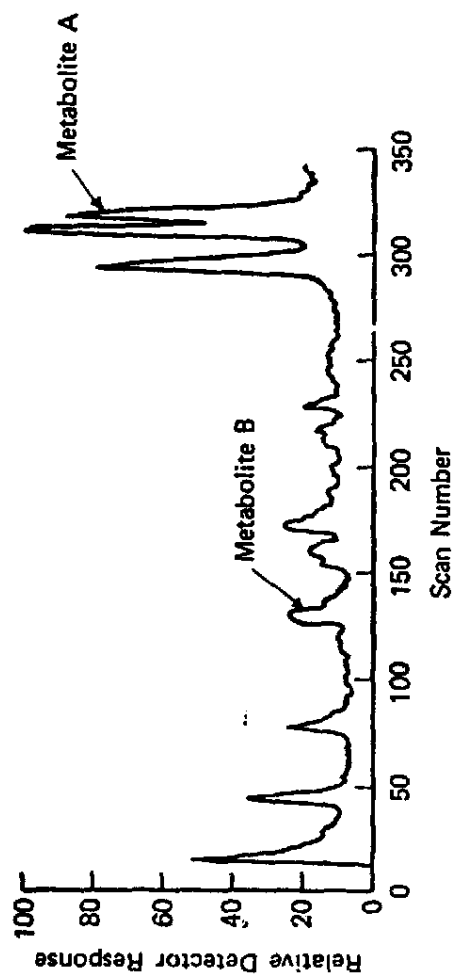


FIGURE 3



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FIGURE 4

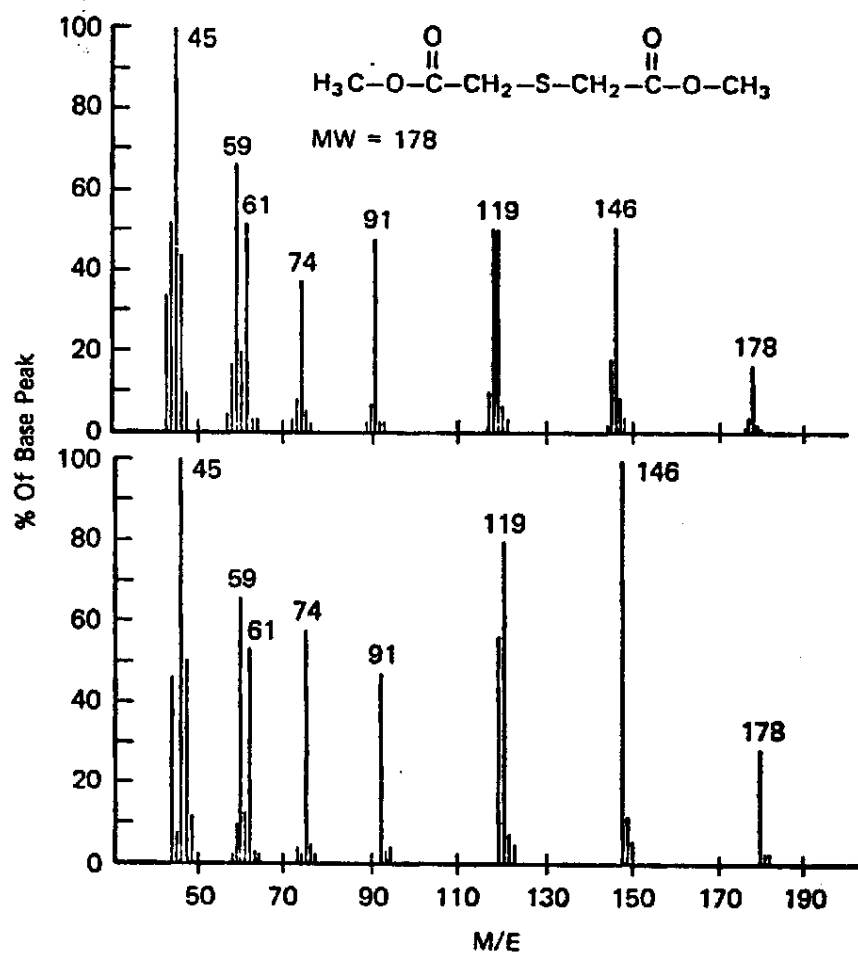
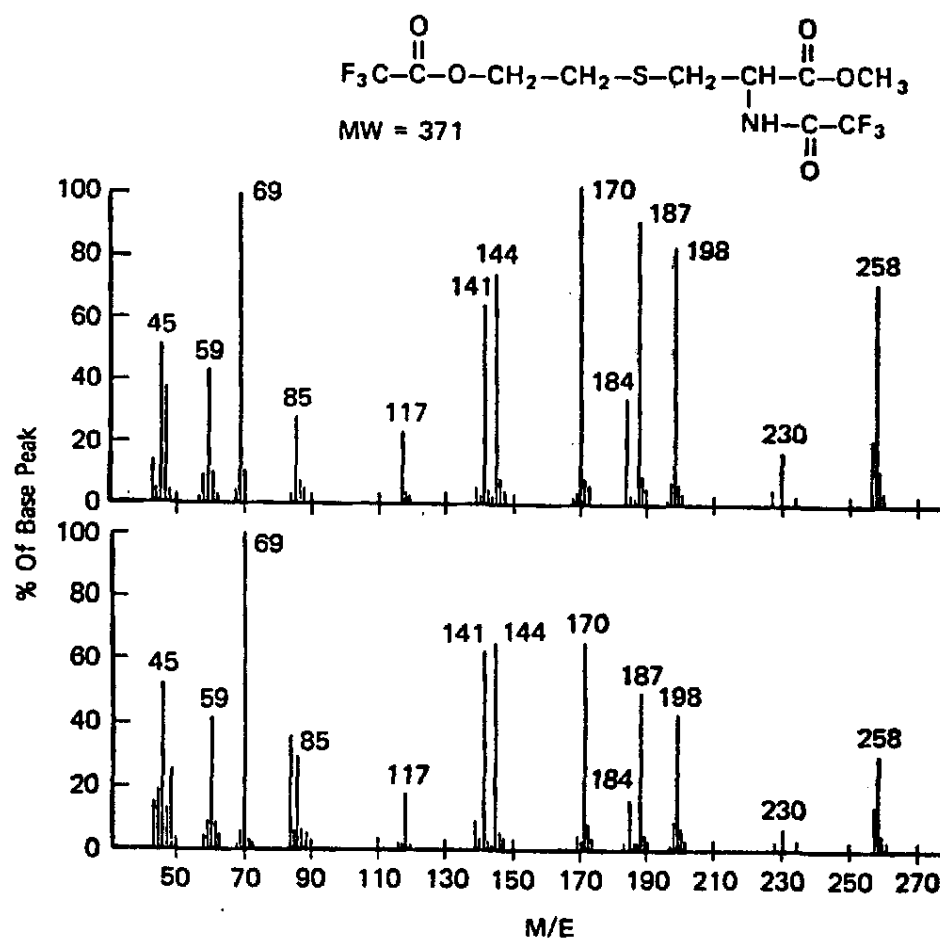


FIGURE 5



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