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Environmental Health Effects Research Series

THE *IN-VIVO* METABOLISM OF PENTACHLOROANILINE IN RHESUS MONKEYS

Health Effects Research Laboratory
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U.S. Environmental Protection Agency
Research Triangle Park, North Carolina 27711

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THE IN-VIVO METABOLISM OF PENTACHLOROANILINE IN RHESUS MONKEYS

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory develops and revises air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is preparing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

Studies of the metabolic fate of toxic chemicals give the Agency further insight into the significance of these agents in the environment. The metabolism of toxicants generally results in formation of chemicals of unknown toxicological properties. Chemical identification and toxicological evaluation of these chemicals and their metabolites continues to be an integral part of the environmental assessment necessary for continued safe use of chemicals.

John H. Knelson, M.D.
Director
Health Effects Research Laboratory

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INTRODUCTION

Pentachloronitrobenzene (PCNB) was first registered for agricultural use in 1955 and is manufactured under the trade name Terrachlor® by Olin Corporation. The chemical is registered primarily for use as a soil fungicide and as a seed treatment.

Up to 12 percent of the U. S. cotton acreage and 2 to 3 percent of the peanut acreage is treated with PCNB. It is also used as a soil fungicide for nursery plants and vegetables. Maximum tolerance limits on edible food crops is 0.1 ppm, except for peanuts which has a tolerance limit of 1.0 ppm (U. S. EPA, 1976).

PCNB is applied to many types of storage crops, vegetable and grain seeds as a storage fungicide. No tolerance limits have been set since these seeds are not to be used for feed.

PCNB has been shown to rapidly disappear from submerged soil following its application. Ko and Farley (1969) demonstrated that the half-life for PCNB is approximately 7 days, and during that period, a corresponding increase in pentachloroaniline (PCA) occurs. This conversion is favored by anaerobic conditions and is carried out by microorganisms in the soil. The same experiments showed that PCA may be very persistent in soil since no decrease was seen during a two-week period under soil conditions which rapidly lead to the disappearance of PCNB.

Previous work which examined the metabolism of PCNB has been reported. It was learned that PCA is a major metabolite of the pesticide in rabbits (Betts. et al., 1955), cows (St. John, et al., 1965), phytopathogenic fungi (Nakanishi and Oku, 1969), and plant seedlings, rats, and beagle dogs (Kuchar, et al., 1969; Borzelleca, et al., 1971). Methylpentachlorophenyl sulfide has also been shown to be a metabolite of PCNB in plants and animals in several of these studies.

The purpose of this work was to examine the pharmacokinetic properties of PCA in the rhesus monkey and to identify any major metabolites which may be formed *in vivo*.

MATERIALS

Chemicals

Pentachloroaniline (ring-labeled) [^{14}C] was obtained from California Bionuclear Corporation. [illegible] specific activity [illegible] 1.47 mCi/mmole. Unlabeled pentachloroaniline (PCA) was obtained from [illegible] Corporation. All solvents used for organic extractions and thin-layer chromatography were spectral quality (Burdick-Jackson). PCS cocktail (Amersham-Searle) was used as a medium for liquid scintillation counting of samples.

Adult male (6-8 kg) rhesus monkeys were obtained from Primate Imports Company. Animals were quarantined in-house for at least 6 weeks prior to use.

Methods

Five male rhesus monkeys were used in the study. The animals were fasted for 14 hours prior to oral dosing with 100 uCi ^{14}C-PCA at maximal specific activity. Each animal received 17.3 mg PCA equivalent to from 2.2 mg/kg to 2.9 mg/kg. The chemical was administered in 1.0 ml corn oil using a feeding needle. Animals were maintained on water *ad libitum*. Access to monkey chow (Purina) was resumed 2 hours following PCA administration.

Blood samples, urine, and feces were collected at designated times following dosing. To determine total radioactivities, aqueous suspensions of feces and blood samples (0.2 ml) were added to 1 volume of 60 percent per chloric acid, followed by 2 volumes of 30 percent hydrogen peroxide, and digested in sealed scintillation vials for 2 hours at 70 C. Counting cocktail was added to these samples (urine directly) and the total radioactivity in each biological sample was determined by liquid scintillation counting. The data, reported as DPM's by the scintillation counter, were derived from external standard quench [illegible] and [illegible] efficiency curves.

[illegible]

An [illegible] monkey was given an [illegible] dose of ^{14}C-PCA (100 mg; [illegible] mg/kg, 100 [illegible] to [illegible] adequate [illegible] of metabolites [illegible] were [illegible]

The N-hydroxylamine of PCA was chemically synthesized by the method of Berry, et al. (1969) according to the scheme in Figure 1. Hexachlorobenzene (Brothers Chemical Co., Orange, New Jersey), 25 g (0.88 mole); cyclohexylamine (freshly distilled, b.p. 132-134 C), 28.6 ml (24.8 g, 0.25 mole); and sulfolane (tetrahydrothiophene-1, 1-dioxide), 175 ml, were placed in a 250-ml round-bottomed flask equipped with a reflux condenser and heated to 145-150 C with stirring for 14 hours. The solution was cooled and the precipitate collected by suction filtration. The solid was dissolved in ice-cold sulfuric acid, filtered to remove hexachlorobenzene, and poured onto ice. Pentachlorophenyl-cyclohexylamine precipitated and was collected by filtration, air dried, and recrystallized from ethanol to yield 10.9 g (36 percent). The m.p. (64-66 C, lit. 70 C), and the IR spectrum confirmed the identity of this product.

N-pentachlorophenylcyclohexylamine, 5.0 g (14.4 mmole), 19 ml of chloroform, 19 ml of formic acid (90 percent), and 4 ml of hydrogen peroxide (30 Percent) were placed in a flask and stirred magnetically for 2 hours. The precipitate which formed was filtered to give 255 mg (6.3 percent yield) of product. The filtrate was let stand overnight and an additional 393 mg (9.7 percent) of precipitate formed and was collected. A 250-mg portion of the crude product was recrystallized from 25 ml of acetone with only light heating to yield 180 mg of pure N-hydroxypentachloroaniline, m.p. 174-176 C (lit. 161-163 C, impure). The structure of this product was confirmed by IR and mass spectroscopy (Figures 2 and 3).

Three procedures which were initially tried proved to be unsuccessful for the synthesis of N-hydroxypentachloroaniline. They incluced attempts at reduction of pentachloronitrobenzene with (a) zinc metal, (b) Raney nickel-hydrogen, and (c) palladium on charcoal-hydrogen. Procedures (a) and (c) resulted in partial reduction to PCA, while (b) resulted in no appreciable reduction of the starting material.

Mass spectral data were obtained with either a Finnigan Model 1015 electron impact mass spectrometer or a Finnigan Model 3200 gas chromatograph chemical ionization mass spectrometer (GC-CI-MS). A System Industries 250 data system controlled the mass spectrometer scan function and processed the signal output data. A 1.83-m x 2-mm glass column packed with 3 percent OV-1 on 100/120 mesh Gas Chrom Q was used for the GC-CI-MS work. The injection port temperature and the line between the GC and MS were kept at 200 C. The flow rate of the carrier gas (methane) was set at 20 ml/min. The column temperature was programmed from 100 to 280 C at 10 C/min.

The infrared data were obtained with a Digilab FTS-14 Infrared Spectrometer. Fourier transform technique was used in generating the infrared spectrum. The sample was examined as a film on KCl plates.

PCA and its major metabolite were tested for mutagenic activity by the Ames bacterial assay (McCann, et al., 1975). Rat liver microsomes were used as a source of oxidative enzymes in the activating system.

[illegible]

The [illegible] in [illegible] oral administration of [illegible]-PCA [illegible] seen [illegible] Figure [illegible] data [illegible] large variation between individual animals with respect to the time of peak radioactivity, varying from [illegible] hour (Rhesus A and B) to [illegible] hours (Rhesus C and D).

The excreted radioactivity for each animal is given in Table 1. The larger portion of the radioactivity was excreted via the urine (23 to 67 percent) while a lesser amount (6-15 percent) was excreted in the feces.

In an attempt to explain the large variations seen in the blood data (Figure 4), several pertinent data were compiled in Table 2. The higher values for the renal excretion of radioactivity in Monkeys A, B, and E match the days for which peak radioactivities were observed in the blood, as would be expected. The data also suggest a more rapid intestinal removal of radioactivity in these three animals which would also correlate with earlier peak blood levels for these monkeys.

Metabolism of PCA

Organic extraction of radioactivity followed by TLC shows that only unchanged ^{14}C-PCA is excreted in the feces (Figure 5), as the radioactivity cochromatographed with unlabeled PCA (R_f = 0.50).

TLC scans of hexane extracts of urine indicate a more complex pattern of metabolism. Figure [illegible] depicts a typical radiochromatogram of these extracts. Peaks having R_f values [illegible], 0.06, 0.50, and 0.70 were detected. In several extracts [illegible] small [illegible] less than 0.2 percent of the chromatogram [illegible] R_f = 0.[illegible] but quantities were inadequate [illegible]

Th[illegible]) i[illegible] was found [illegible] be PCA [illegible] that of known PCA on silica gel plates [illegible] CH_2Cl_2 and $CHCl_3$. Mass spectral analysis [illegible] standard [illegible] (Figures [illegible] and [illegible]).

[illegible]

believed to be a decomposition product characteristic of organic amines, and further characterization was not pursued.

The substance isolated from the Peak 1 region was ultimately determined to be the primary metabolite of PCA in rhesus monkeys. It was found to be unstable as this region of the TLC plate rapidly became discolored upon standing in the light. Subsequent TLC of this discolored material resulted in the appearance of four peaks (Figure 9) whose R_f values correspond to those seen in the original urine extract radiochromatogram (Figure 6).

Mass spectral analysis was performed on freshly prepared Peak 1 material. Initial analysis of the metabolite by electron impact and isobutane chemical ionization mass spectrometry indicated the molecular weight of the metabolite is 279. The mass spectra are shown in Figures 10 and 11. The cluster of peaks around the molecular ion (Figure 10) and the protonated molecular ion (Figure 11) region exhibit the same isotope ratio of pentachloroaniline in Figures 12 and 13, indicating the metabolite also has five chlorine atom. The proton nuclear magnetic resonance spectrum of the metabolite did not show any peaks in region confirming that none of the chlorine atoms had been displaced by a hydrogen atom. The fragment ion at m/e 262 (Figures 10 and 11) is what one would expect from a hydroxylated metabolite of PCA.

The molecular weight of 279 is odd in number indicating that it contains an odd number of nitrogen atoms. It is also 16 units higher than PCA implying that perhaps an oxygen atom is incorporated into the PCA molecule. In the infrared spectrum, a sharp NH absorption band was observed at 3295 cm^{-1} and a broader NH or OH absorption band centered at 3205 cm^{-1}. The identify of the direct probe mass spectra of the Peak 1 metabolite (Figure 11) and synthetic N-hydroxypentachloroaniline (Figure 3) confirms the structure of the metabolite.

To further characterize the metabolite, trimethylsilylation was performed with bis-trimethylsilyl-trifluoroacetamide. The derivative was analyzed with GC-CI-MS. To enhance the intensity of the peaks at the molecular ion region, ammonia was used as chemical ionization reagent gas. The total ion current (TIC) trace is shown in Figure 14. The mass spectrum of the major peak is shown in Figure 15. The hydroxyl group was silylated giving a protonated molecular ion at m/e 353. This and the fragment ion at m/e 262 confirm that the metabolite has a hydroxyl group and it has a molecular weight of 279. The mass spectrum of trimethylsilylated derivative of synthetic N-hydroxypentachloroaniline is identical to that shown in Figure 16.

Attempts were made to analyze the unsilylated metabolite with GC-MS. The GC trace is shown in Figure 17. Neither of the peaks corresponds to N-hydroxypentachloroaniline. The latter peak gives an identical spectrum to that of PCA, and the earlier peak appears to be pentachloronitrosobenzene (Figure 18). Hydroxylamines are known to undergo disproportionation to form the nitroso and primary amine compounds. This would explain the appearance of PCA and nitrosopentachlorobenzene following exposure of N-hydroxypentachloroaniline to the elevated temperatures of the GC system.

of PCA and N-hydr[illegible]

Mutagenesis Assay

Both PCA and N-hydroxypentachloroaniline were tested for mutagenic activity using two Salmonella tester strains, TA-1535 and TA 98, along with activating systems derived from rat livers. No increase in revertants was seen in the presence of 100 µg of either chemical (Table 3). Both the microsomal activating system and the bacteria were shown to be active through the use of positive chemical controls.

DISCUSSION

Blood levels of radioactivity following ^{14}C-PCA administration to monkeys indicate wide variation between animals. These data would suggest differences between rates of absorption of PCA from the GI tract. All monkeys were fasted for 14 hours prior to treatment which should eliminate food matter as a complicating factor. The use of corn oil as a vehicle for chemical administration to animals has not, to our knowledge, led to irregularities in chemical absorption in the GI tract. However, since the low urinary levels of ^{14}C on Day 1 for Rhesus C and D correlate with low blood levels of radioactivity on that same day, it is apparent that the chemical was not readily absorbed in these two monkeys during the first day. Also, their delayed fecal excretion suggest a decreased GI motility which may play a role in the diminished early absorption of PCA.

TLC scans suggest that only PCA is excreted in the feces. TLC of urinary extracts indicates the presence of four radioactive compounds. Figure 21 is a scheme which summarizes the metabolic information collected in this study with PCA. PCA is metabolized oxidatively to the N-hydroxylamine, as confirmed by mass spectroscopy and chemical synthesis of this compound. Both PCA and N-hydroxypentachloroaniline are conjugated and form water-soluble metabolites which can be deconjugated by aryl sulfatase-glucuronidase.

Formation of nitrosopentachlorobenzene apparently results from a spontaneous disproportionation of N-hydroxypentachloroaniline in the urinary medium prior to extraction procedures. Fresh samples of urine had less of the nitroso compound present upon TLC than did older samples. GC of purified N-hydroxypentachloroaniline demonstrated the formation of the nitroso compound following its exposure to elevated temperatures, and TLC indicates the decomposition of N-hydroxypentachloroaniline to nitrosopentachlorobenzene, as well as to PCA. Both PCA and N-hydroxypentachloroaniline partially decomposed to a brown substance, the structure of which was not pursued.

Although the mutagenesis assays in two bacterial systems indicate that neither PCA or N-hydroxypentachloroaniline is mutagenic, this assay system uses rat liver microsomal enzymes as an activation system. Since the metabolism of PCA in the rat has not been determined, the use of the standard assay activation system leaves unresolved the mutagenic potential of PCA and N-hydroxypentachloroaniline in primates.

	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	6.2	[illegible]
	[illegible]	[illegible]	3.3	[illegible]
	Total	[illegible]	10.2	[illegible]
B	1	20.3	3.6	24.9
	2	8.4	3.5	11.9
	3	4.1	7.1	11.2
	5	1.9	1.0	2.9
	Total	35.7	15.2	50.9
C	1	2.2	.7	2.9
	2	14.5	.8	15.3
	3	10.0	2.7	12.7
	5	6.3	3.9	10.2
	Total	33.0	8.1	41.1
D	1	5.8	.9	6.7
	2	30.6	.7	31.3
	3	10.7	2.6	13.3
	[illegible]	10.9	7.2	18.1
	[illegible]	[illegible]	[illegible]	[illegible]
			[illegible]	
			[illegible]	
	[illegible]		5.7	[illegible]

TABLE 2. RADIOACTIVITY EXCRETION RATIOS FOLLOWING ^{14}C-PCA ADMINISTRATION TO RHESUS MONKEYS.

Rhesus	Day of Peak Blood Radioactivity	Ratio Urinary Radioactivity, Day 1:Day 2	Ratio Fecal Radioactivity, Days 1-3:Days 4-5
A	1	1.24	2.1
B	1	2.54	14.2
C	2	0.15	1.1
D	2	0.19	0.6
E	1	4.69	5.6

TABLE 3. MUTAGENIC ASSAY FOR PCA AND N-HYDROXY-4-CHLOROANILINE

Chemical	Tester Strain	Reverta Colonies/Plate Amount of Chemical µg 20	50	100	50-AS(1)
PCA	TA-1538	23	11.5	17.5	8
	TA-98	37	35.5	47.5	44
N-OH PCA	TA-1538	13.5	14.5	19.5	10
	TA-98	42.5	34	41.5	41
Controls			**+AS**		**-AS**
No chemicals	TA-1583		13		200
	TA-98		29		45
			2NF(2)-AS		**2AA(3)+AS**
Mutagens, 20 µg	TA-1538		2816		2469
	TA-98		2396		3794

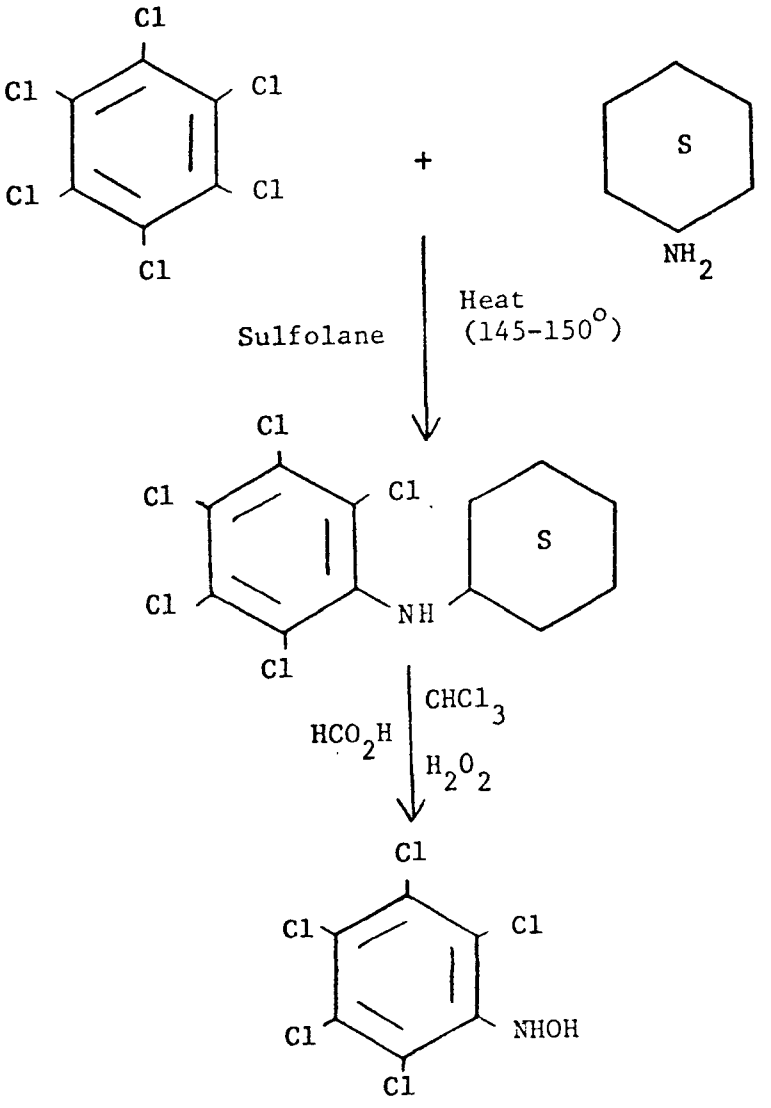
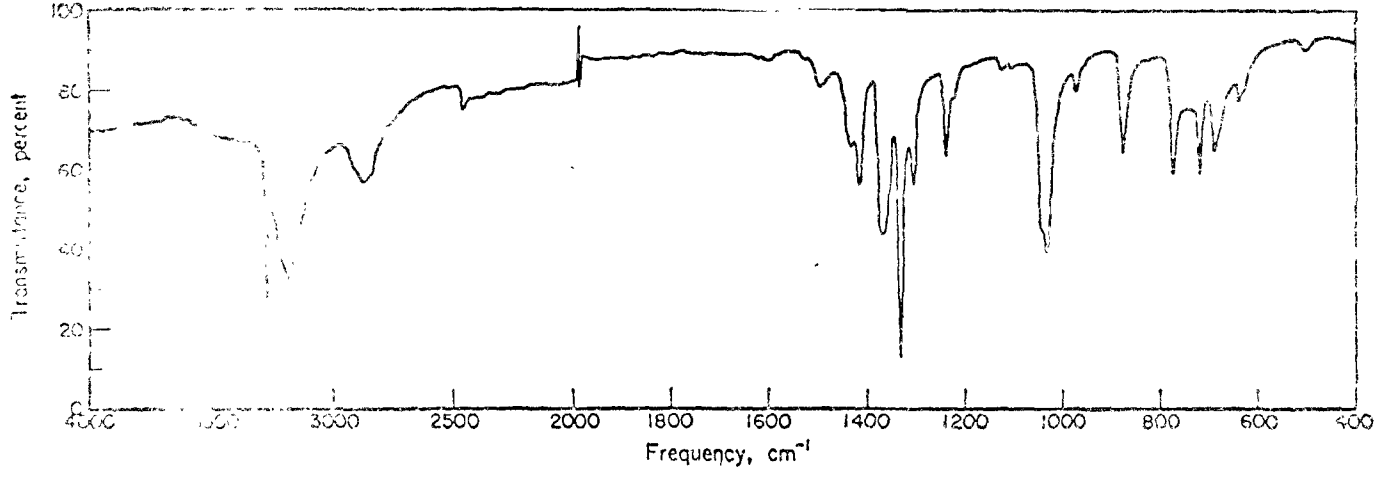


FIGURE 1. OXIDATIVE SYNTHESIS OF N-HYDROXYLPENTACHLOROANILINE



INFRARED SPECTRUM OF SYNTHETIC N-HYDROXYPENTACHLOROANILINE

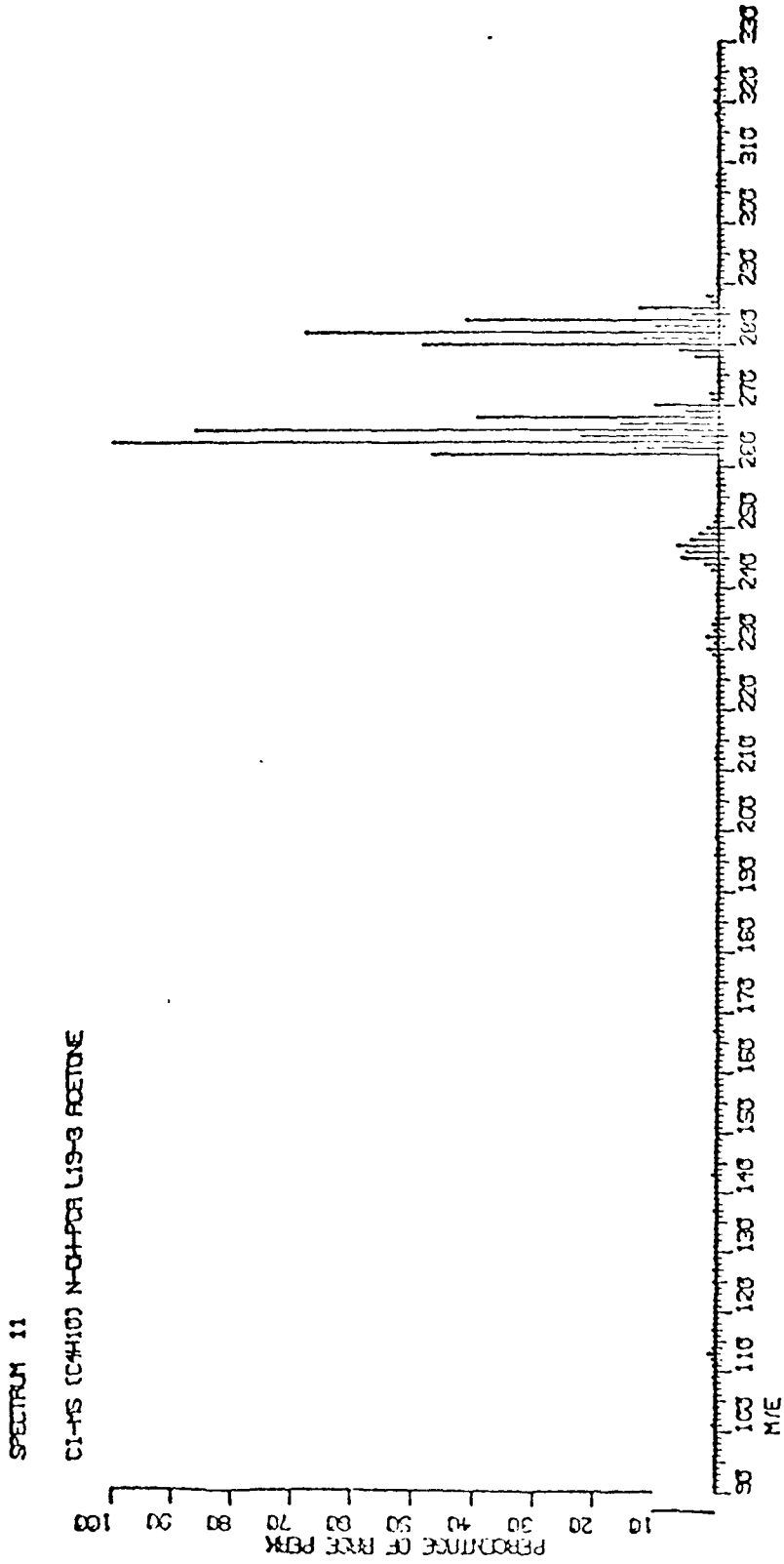


FIGURE 3. MASS SPECTRUM OF SYNTHETIC N-HYDROXYPENTACHLOROANILINE (ISOBUTANE CHEMICAL IONIZATION)

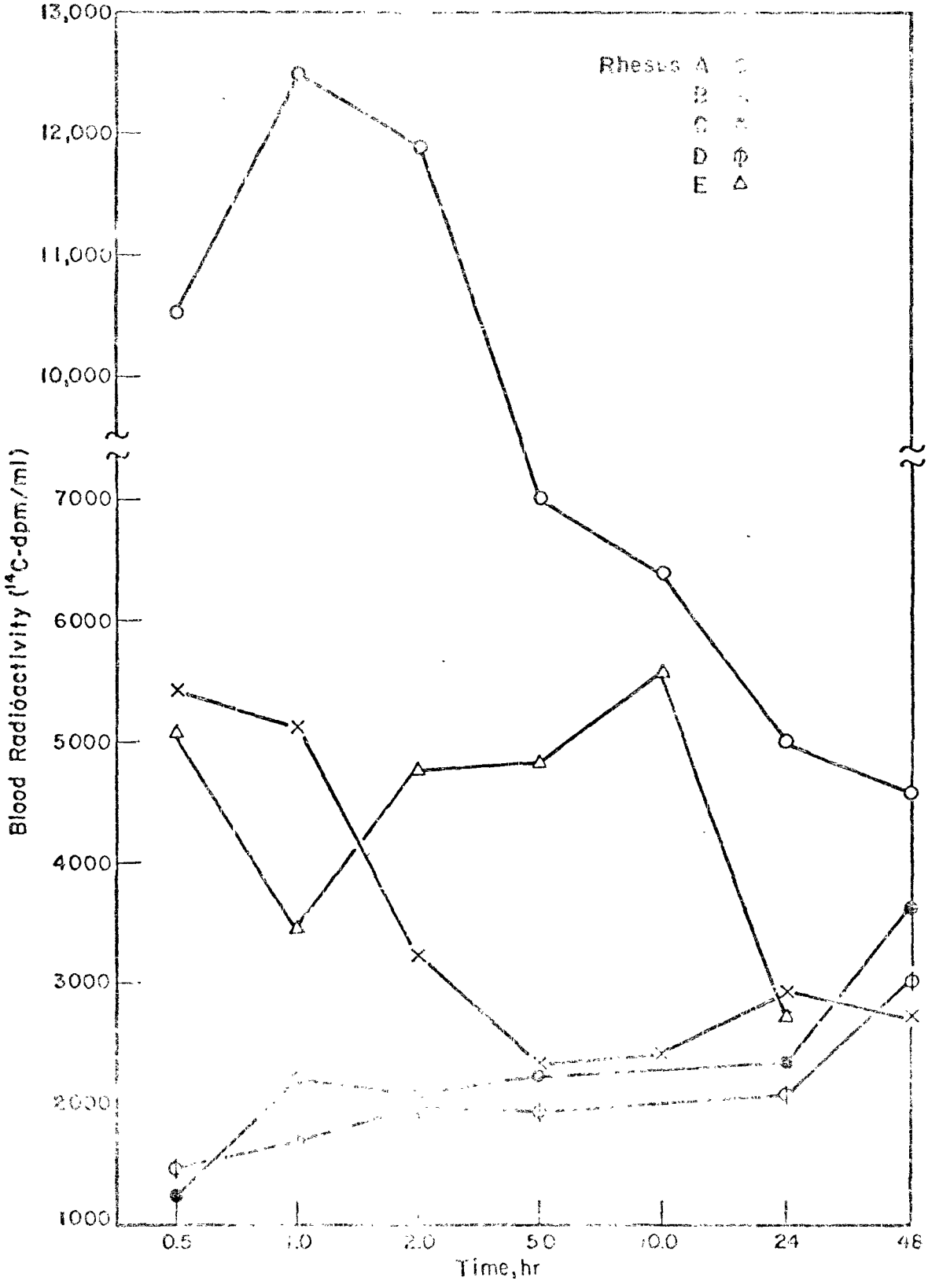


FIGURE 4. BLOOD RADIOACTIVITY FOLLOWING ^{14}C-PCA ADMINISTRATION TO RHESUS MONKEY

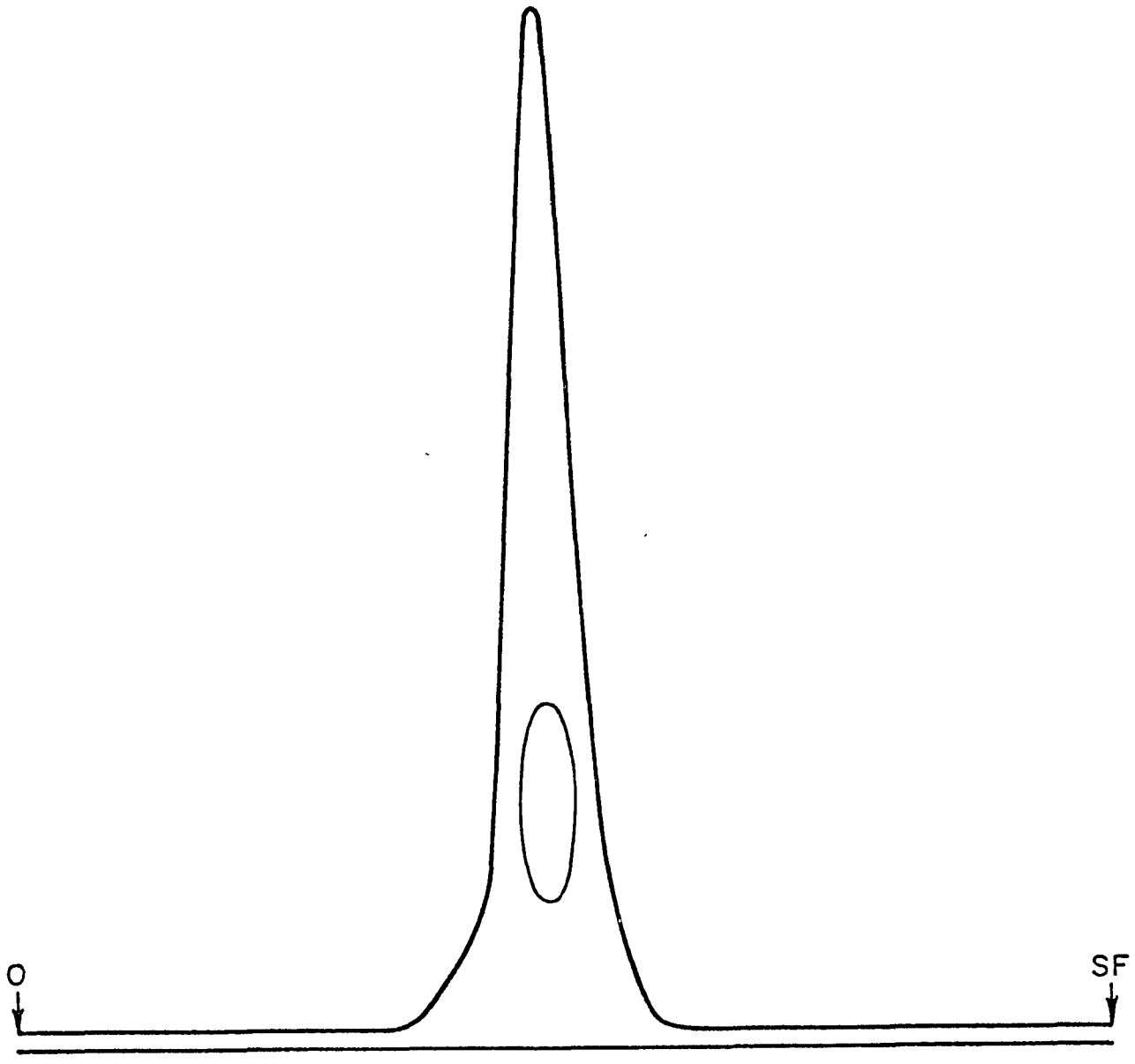


FIGURE 5. THIN-LAYER RADIOCHROMATOGRAM OF HEXANE EXTRACT OF FECES FOLLOWING ^{14}C-PCA ADMINISTRATION TO RHESUS MONKEY

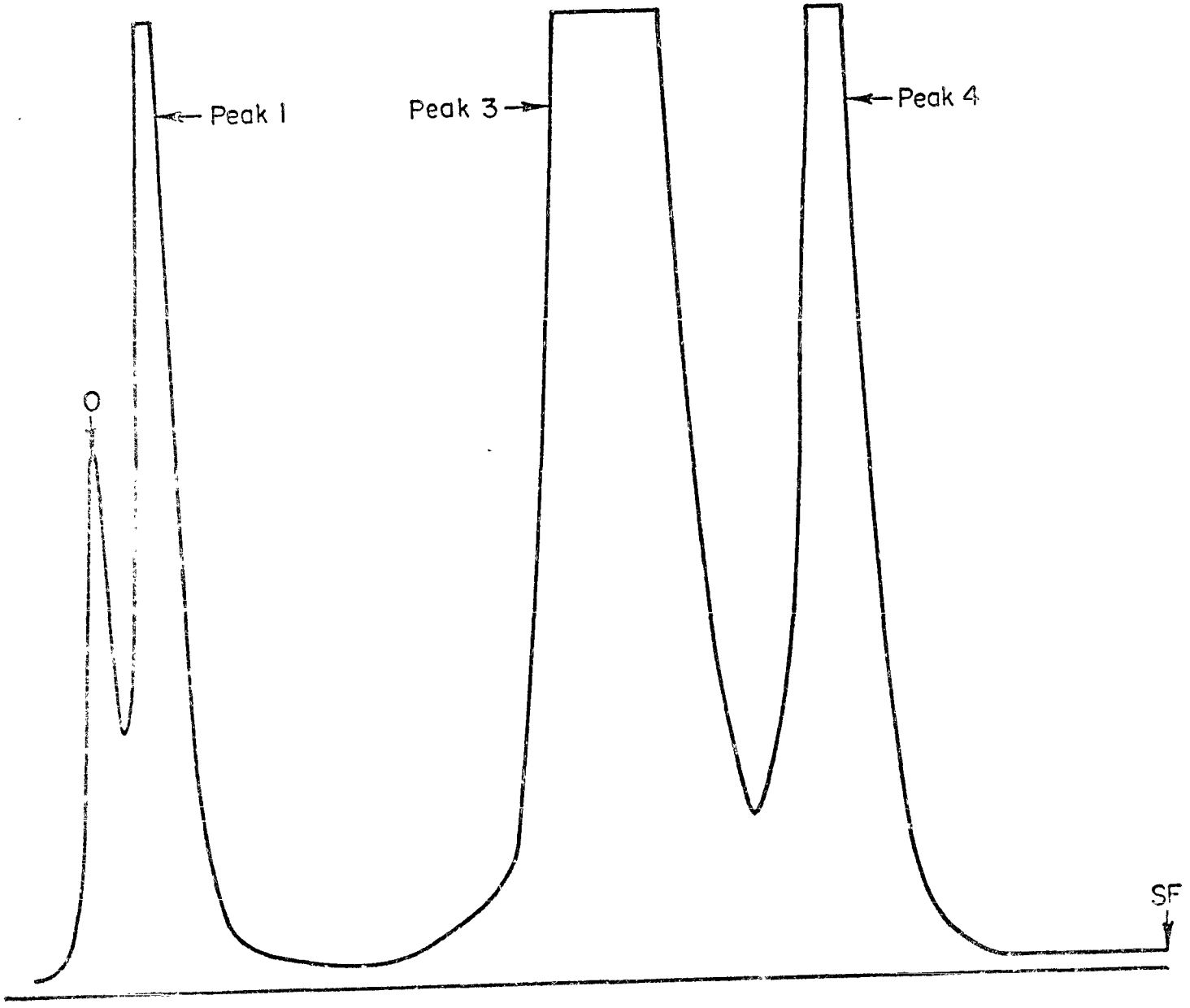
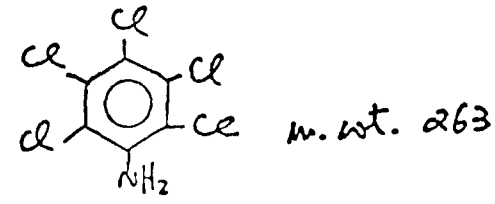


FIGURE 6. THIN-LAYER RADIOCHROMATOGRAM OF HEXANE EXTRACT OF URINE FOLLOWING ^{14}C-PCA ADMINISTRATION TO RHESUS MONKEY



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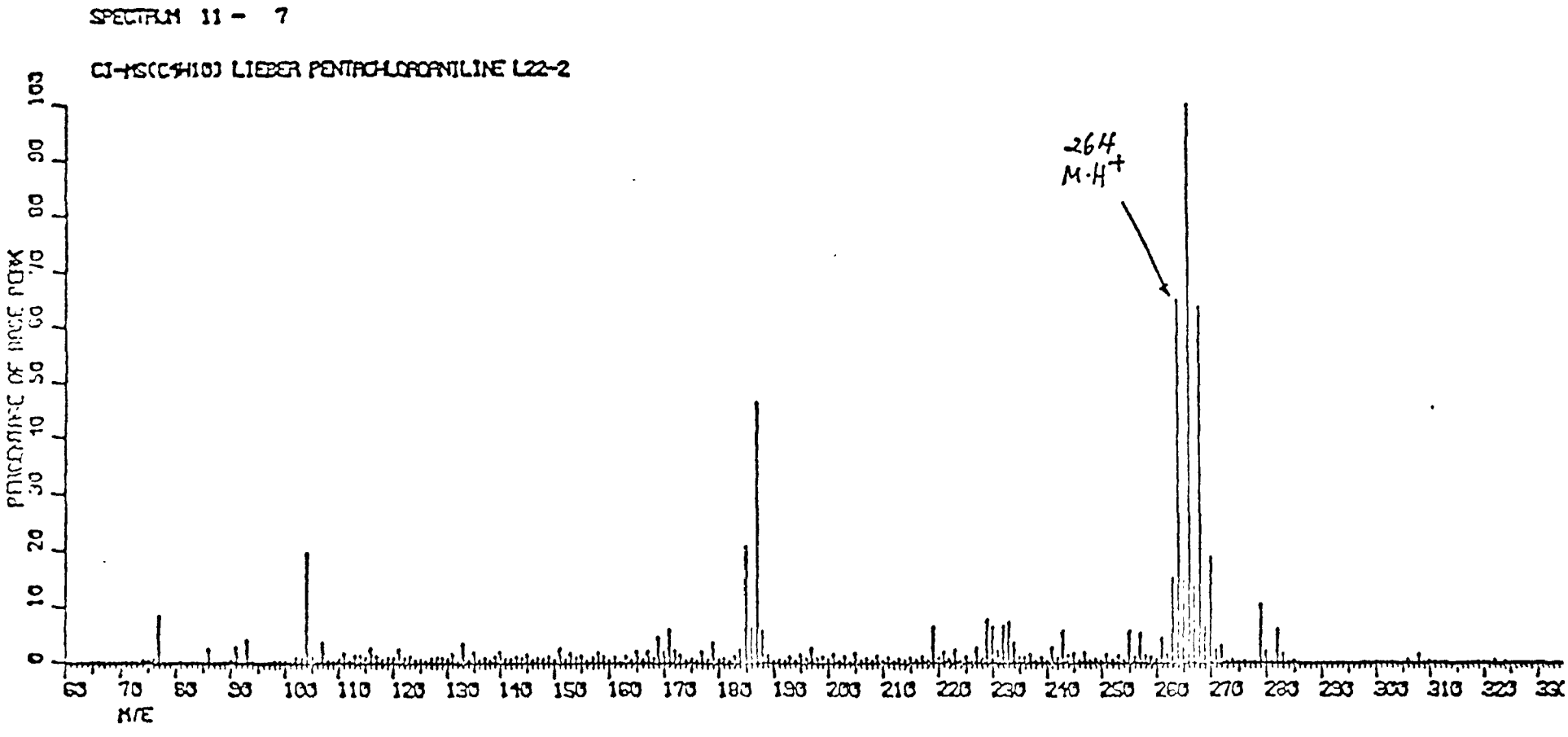


FIGURE 7. MASS SPECTRUM OF PCA STANDARD (ISOBUTANE CHEMICAL IONIZATION)

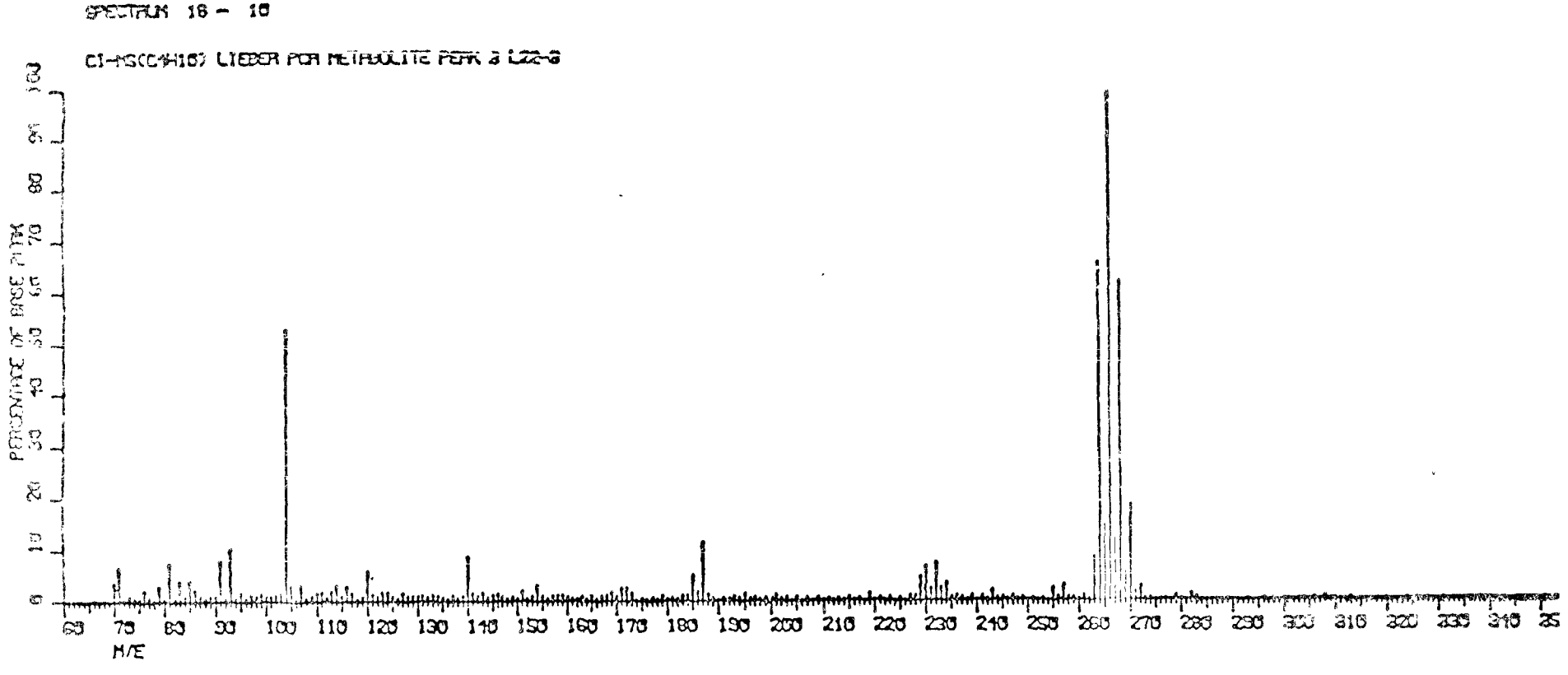


FIGURE 8. MASS SPECTRUM OF PEAK 3 COMPOUND (ISOBUTANE CHEMICAL IONIZATION)

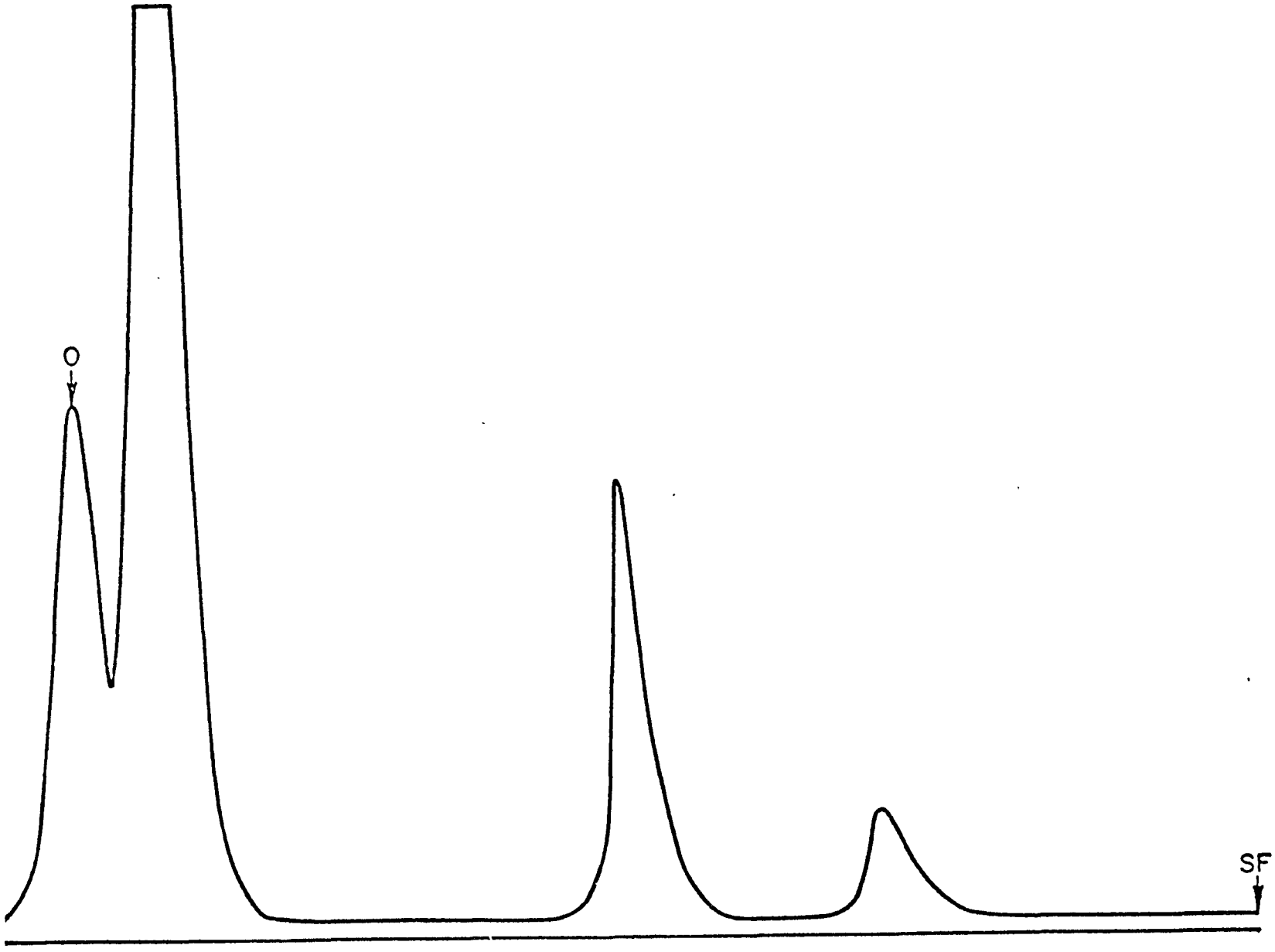


FIGURE 9. THIN-LAYER RADIOCHROMATOGRAM OF PEAK 1 METABOLITE FOLLOWING A 2-HOUR EXPOSURE TO LIGHT

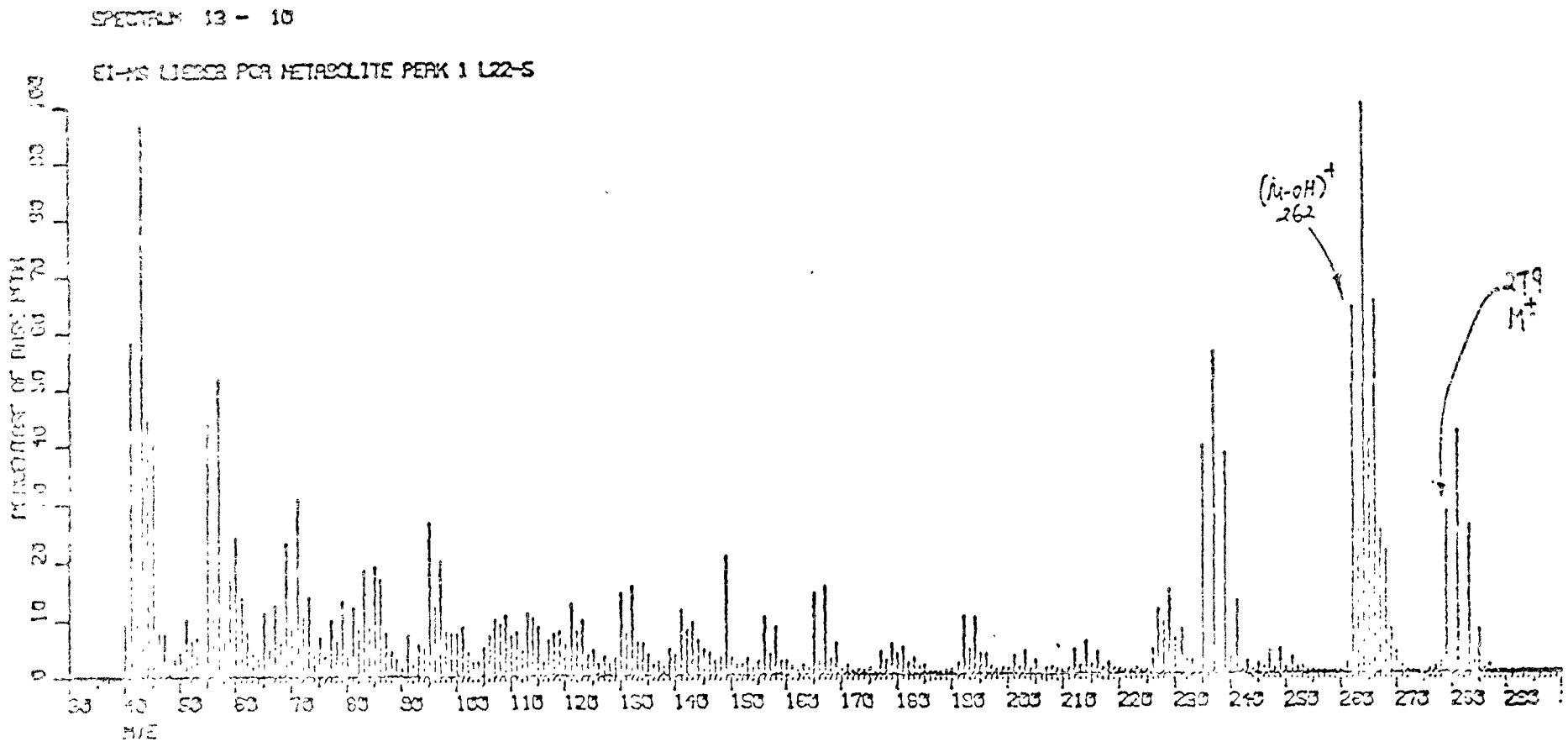


FIGURE 10. MASS SPECTRUM OF PEAK 1 METABOLITE (ELECTRON IMPACT)

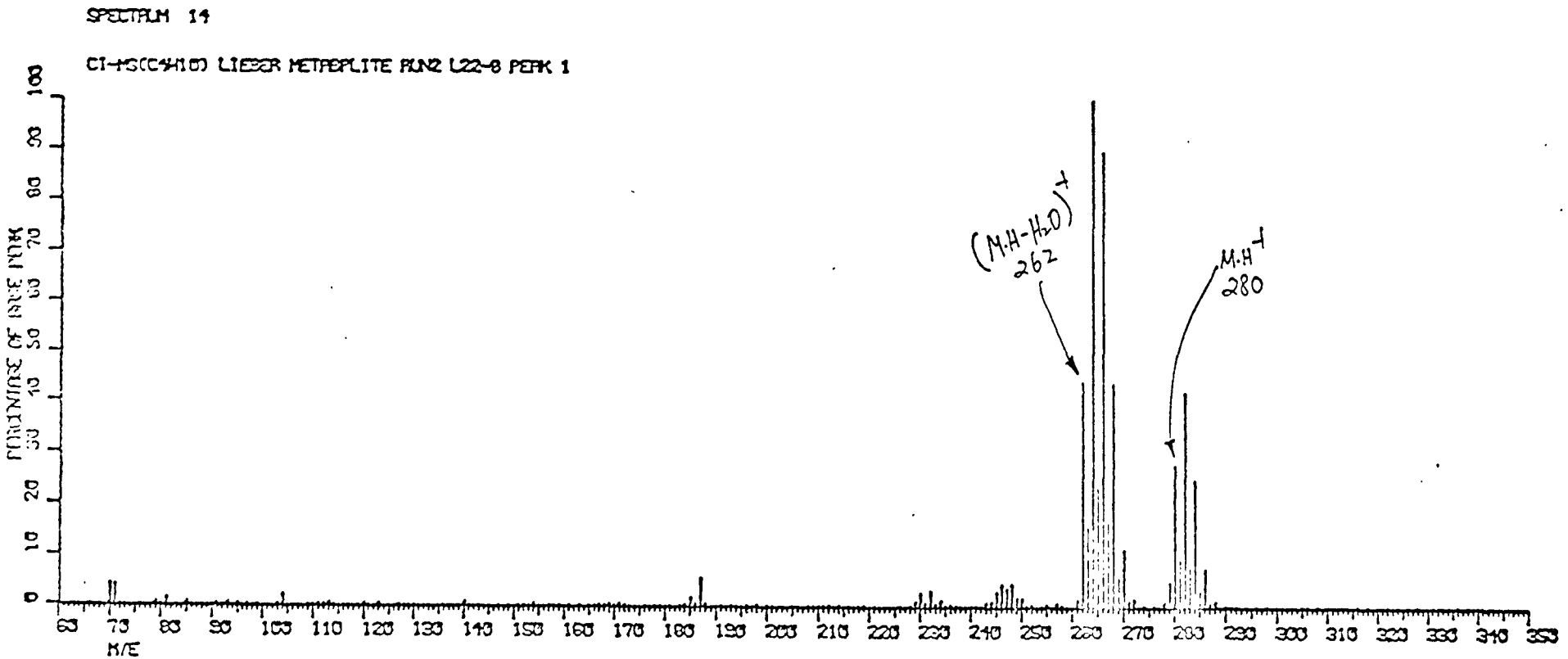
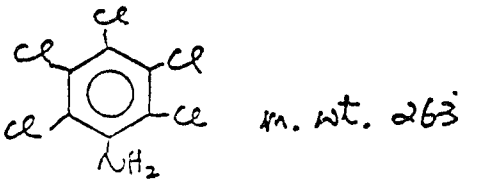


FIGURE 11. MASS SPECTRUM OF PEAK 1 METABOLITE (ISOBUTANE CHEMICAL IONIZATION)



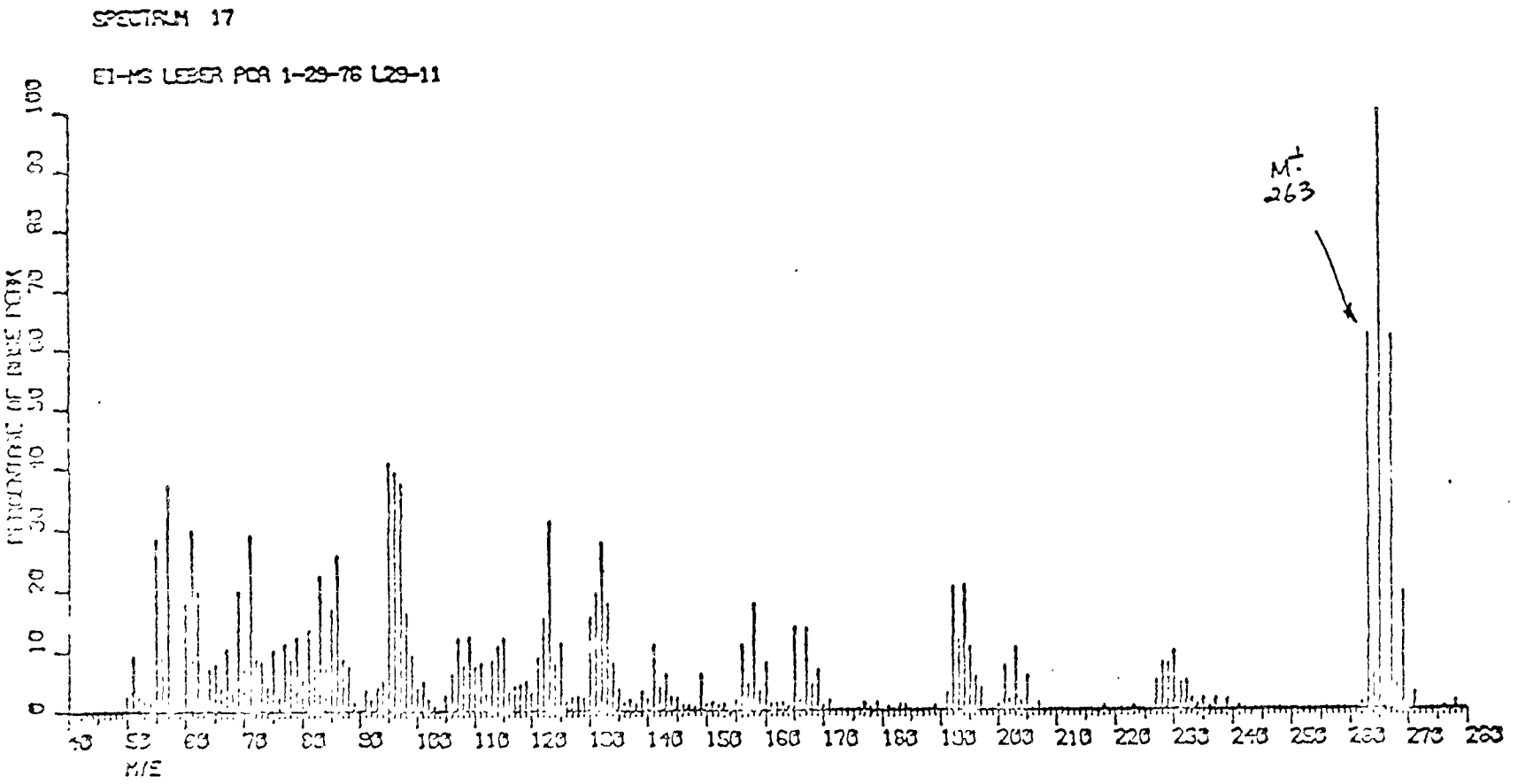
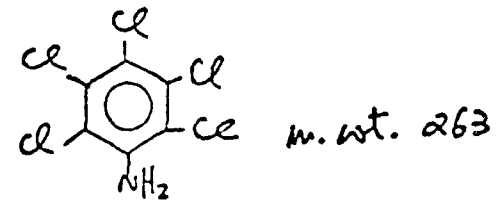


FIGURE 12. MASS SPECTRUM OF PCA STANDARD (ELECTRON IMPACT)



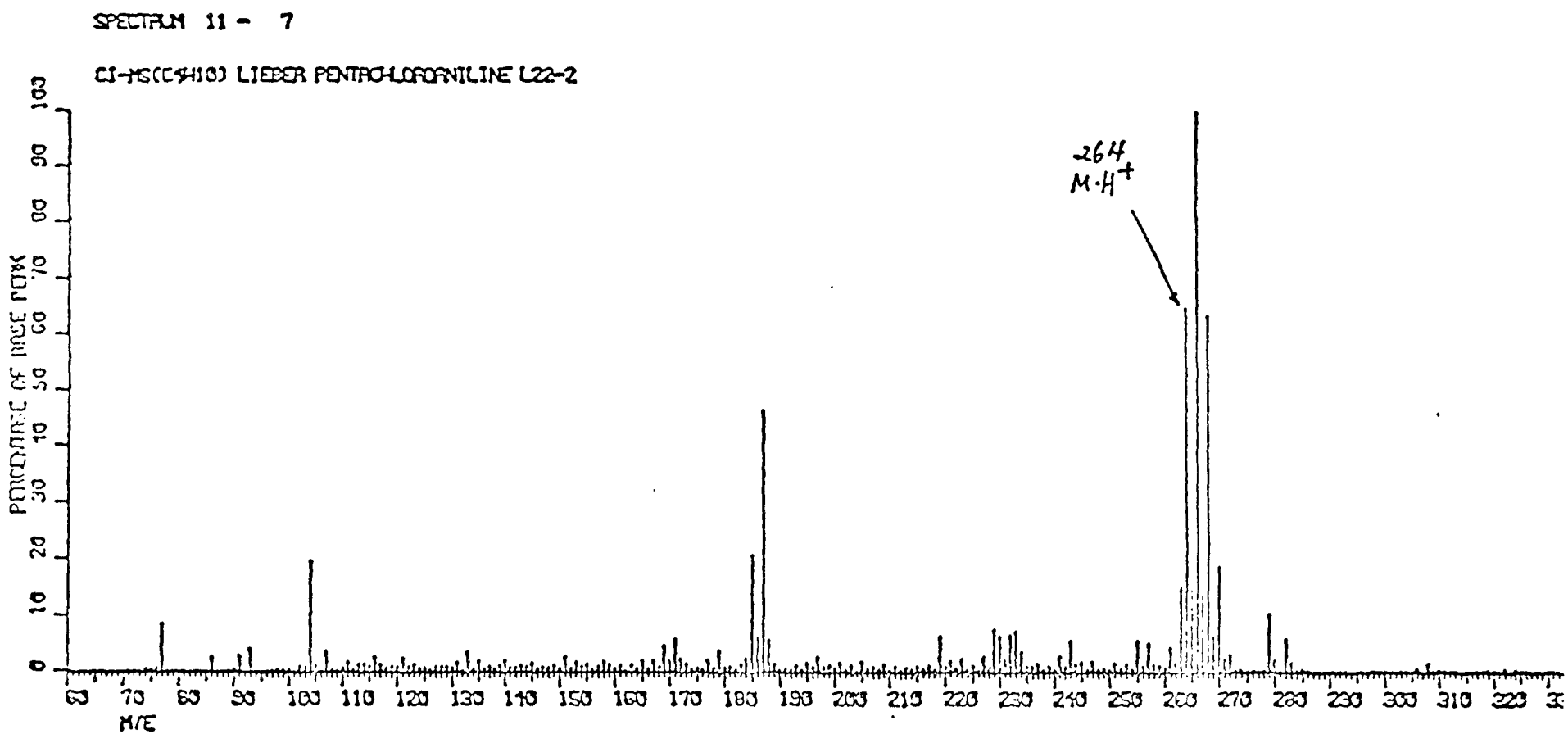


FIGURE 13. MASS SPECTRUM OF PCA STANDARD (ISOBUTANE CHEMICAL IONIZATION)

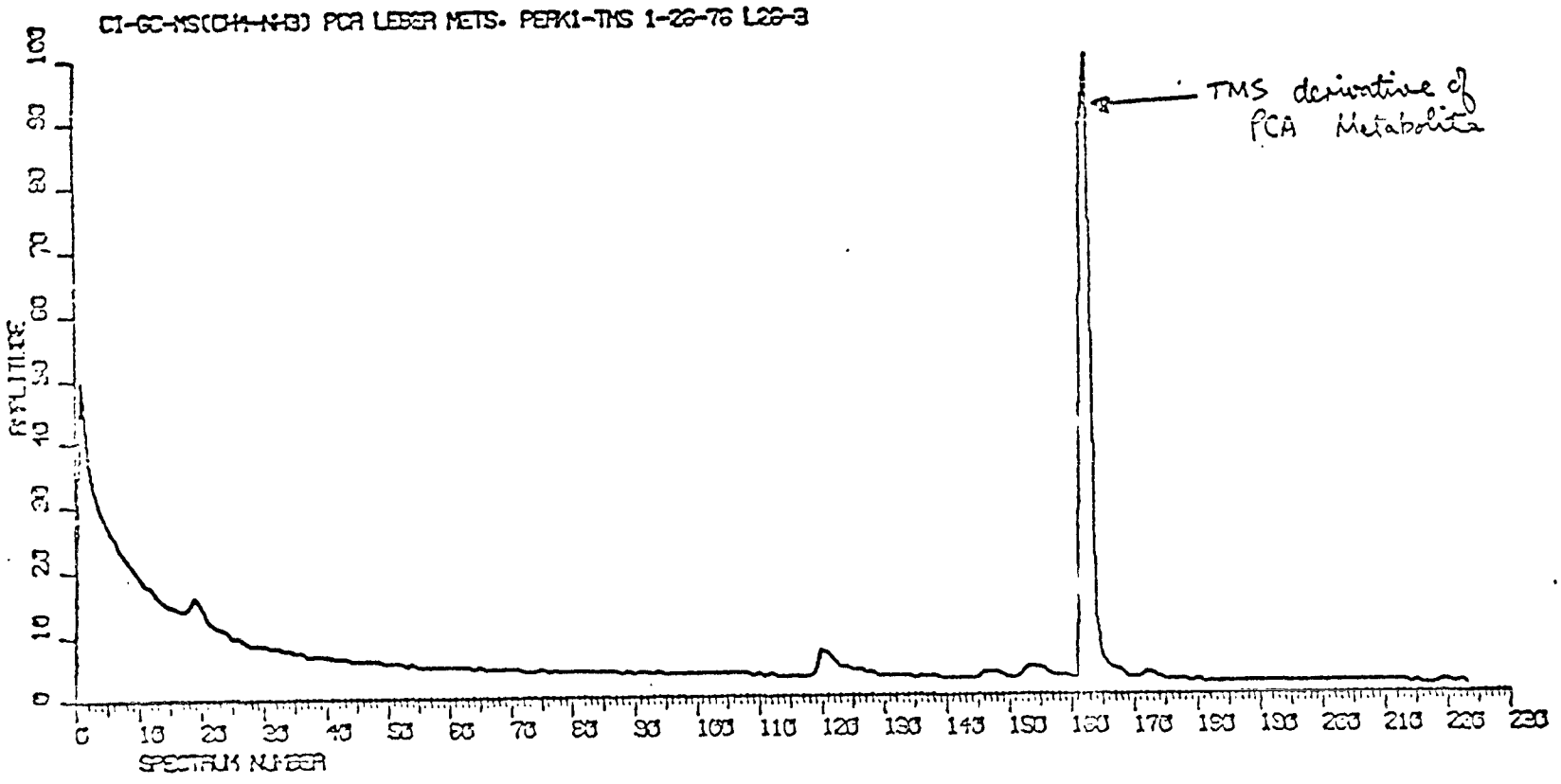


FIGURE 14. GAS CHROMATOGRAM OF THE TRIMETHYLSILYLATED DERIVATIVE OF PEAK 1 METABOLITE

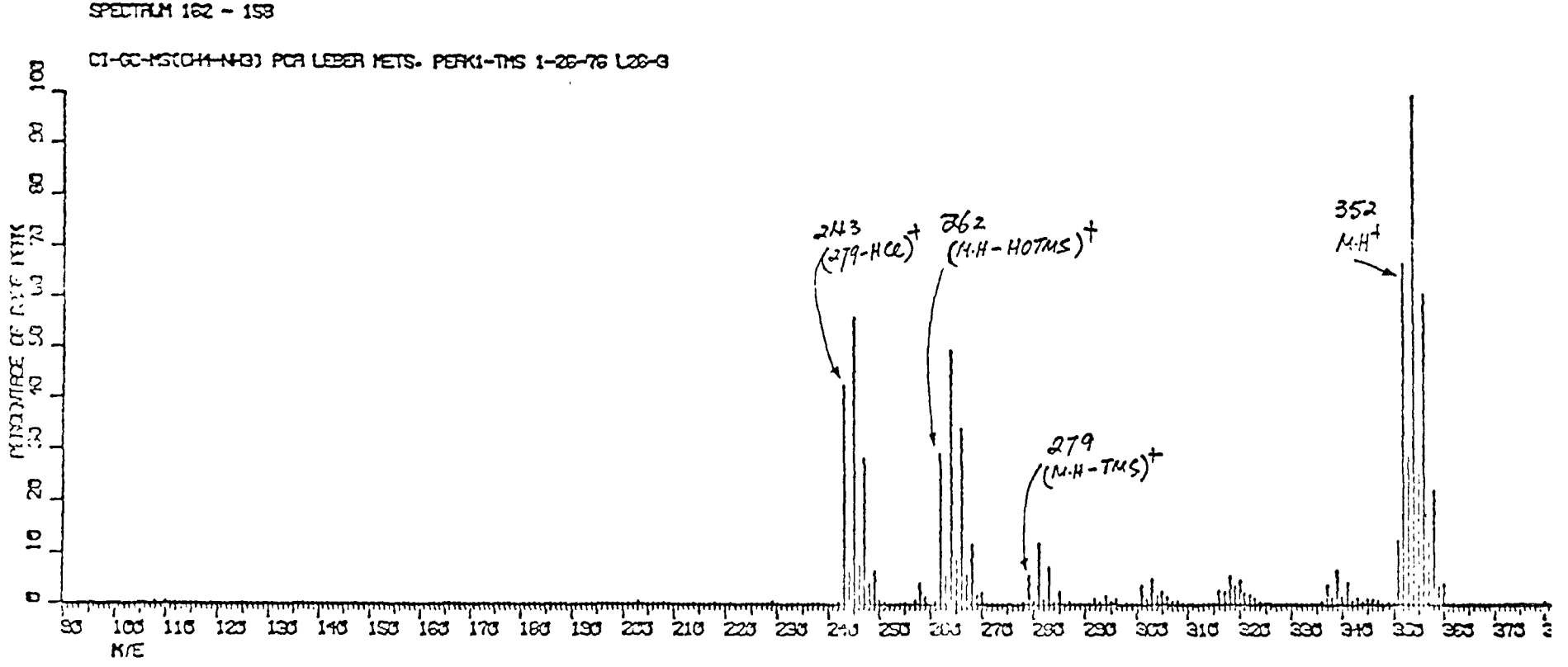


FIGURE 15. MASS SPECTRUM OF THE TRIMETHYLSILYLATED DERIVATIVE OF PEAK 1 COMPOUND (METHANE-AMMONIA CHEMICAL IONIZATION)

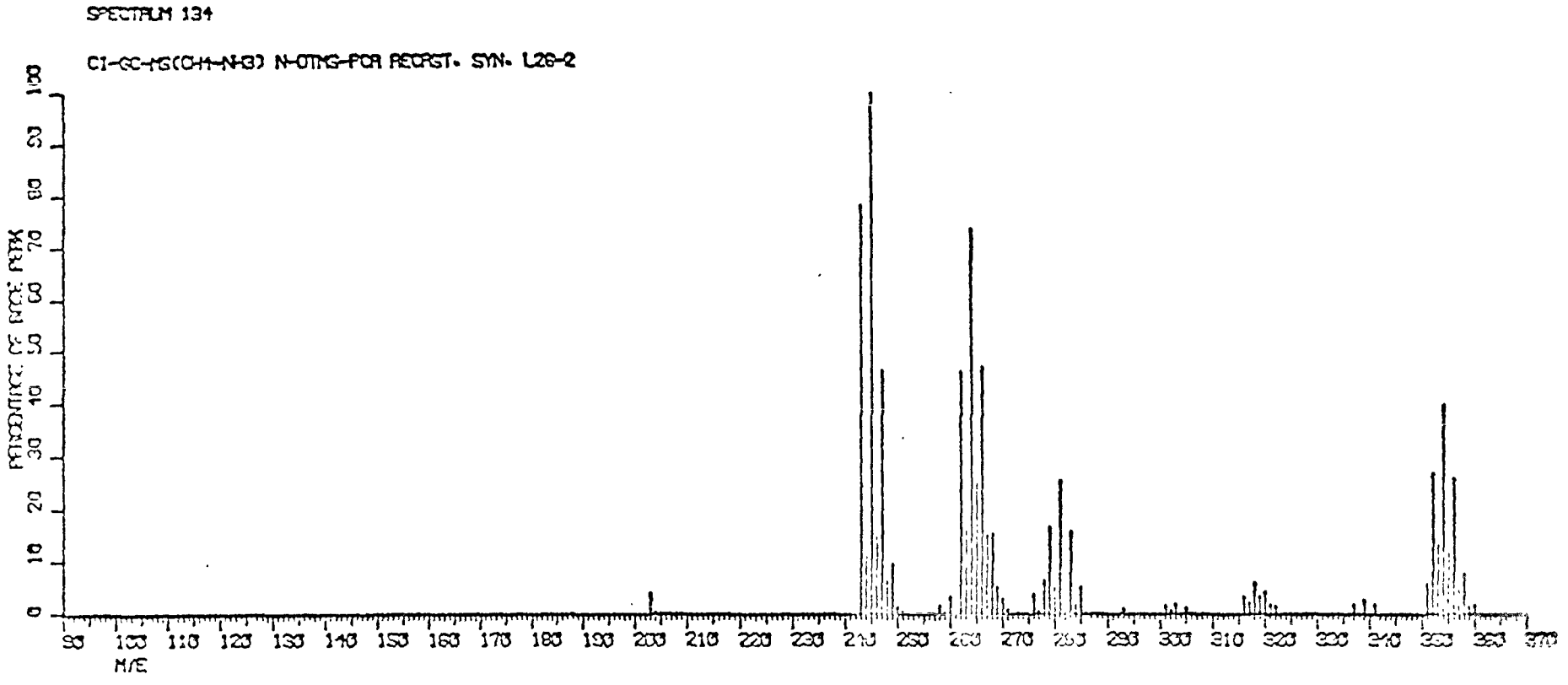


FIGURE 16. MASS SPECTRUM OF THE TRIMETHYLSILYLATED DERIVATIVE OF SYNTHETIC N-HYDROXYPENTACHLOROANILINE (METHANE-AMMONIA CHEMICAL IONIZATION)

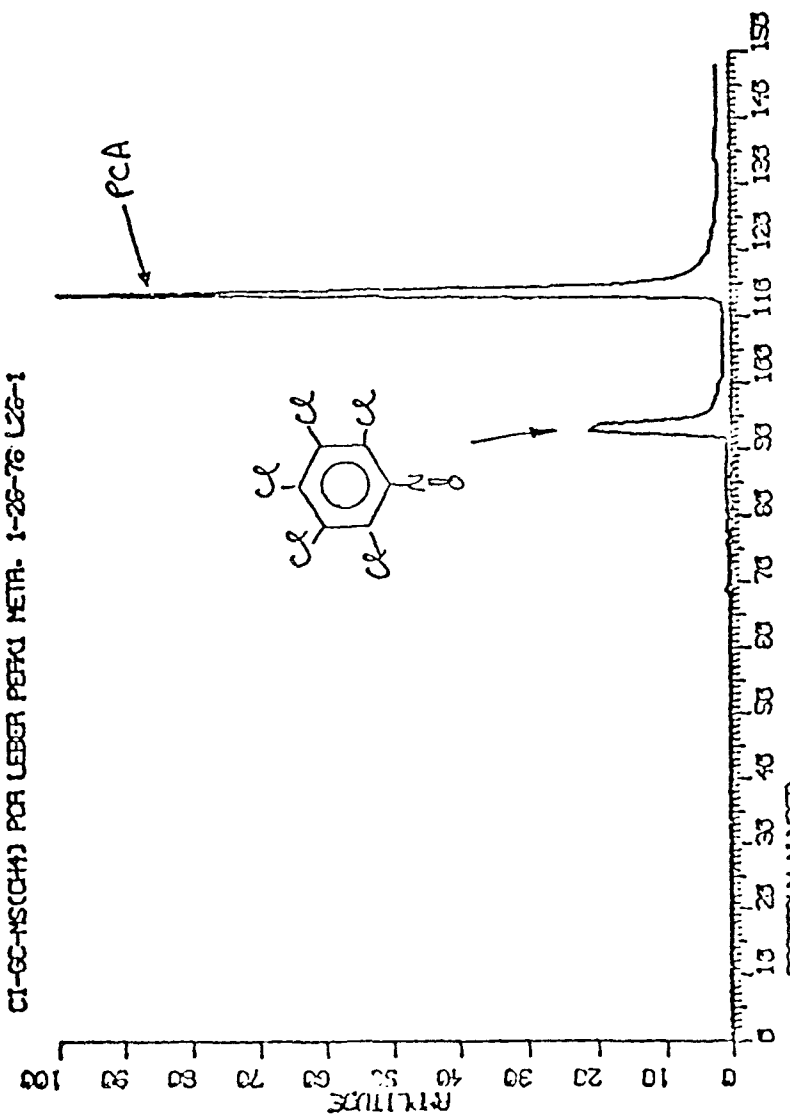
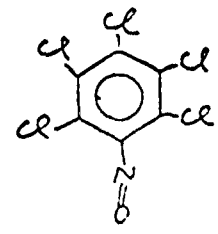


FIGURE 17. GAS CHROMATOGRAPH OF PEAK 1 COMPOUND



m.wt. 277

SPECTRUM 93

CI-GC-MS(CH4) PCB LEEBER PEAK1 METH. 1-26-76 L26-1

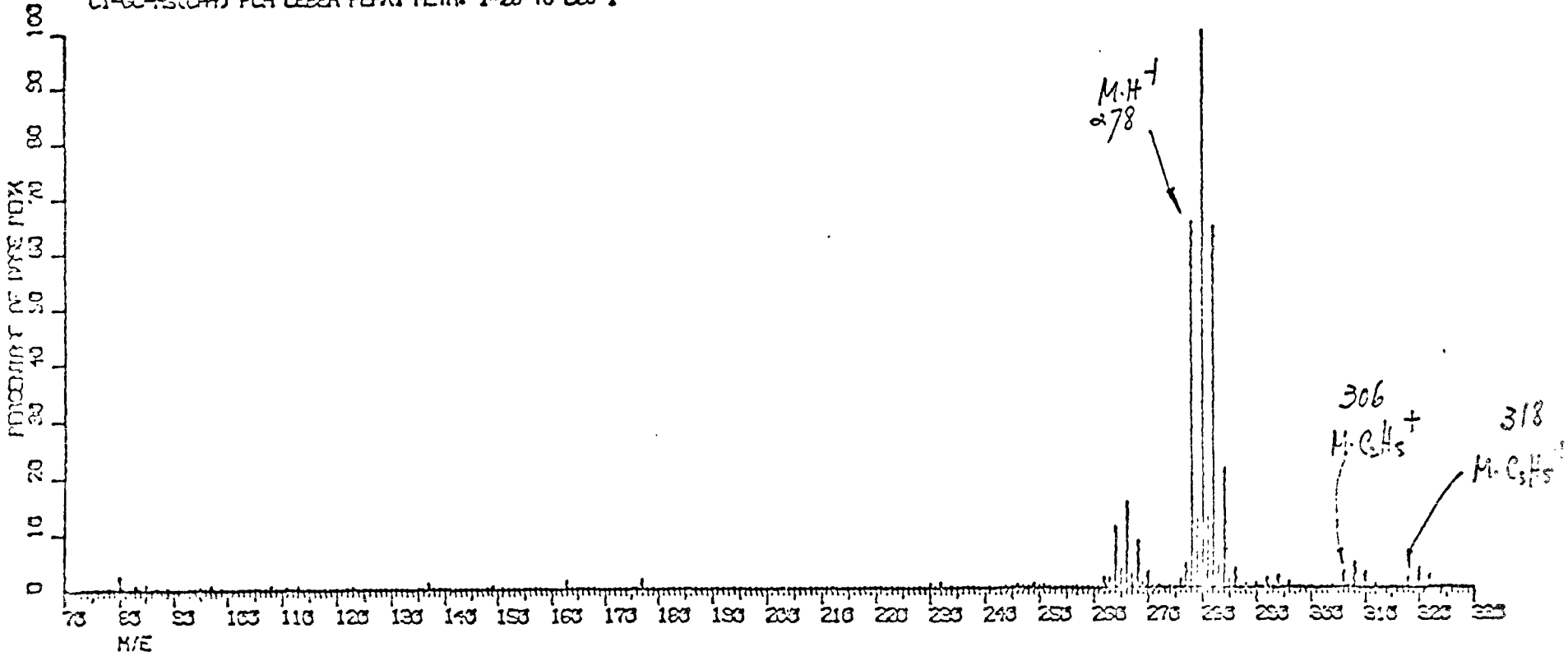


FIGURE 18. MASS SPECTRUM OF THE EARLY GC PEAK IN FIGURE 17
(METHANE CHEMICAL IONIZATION)

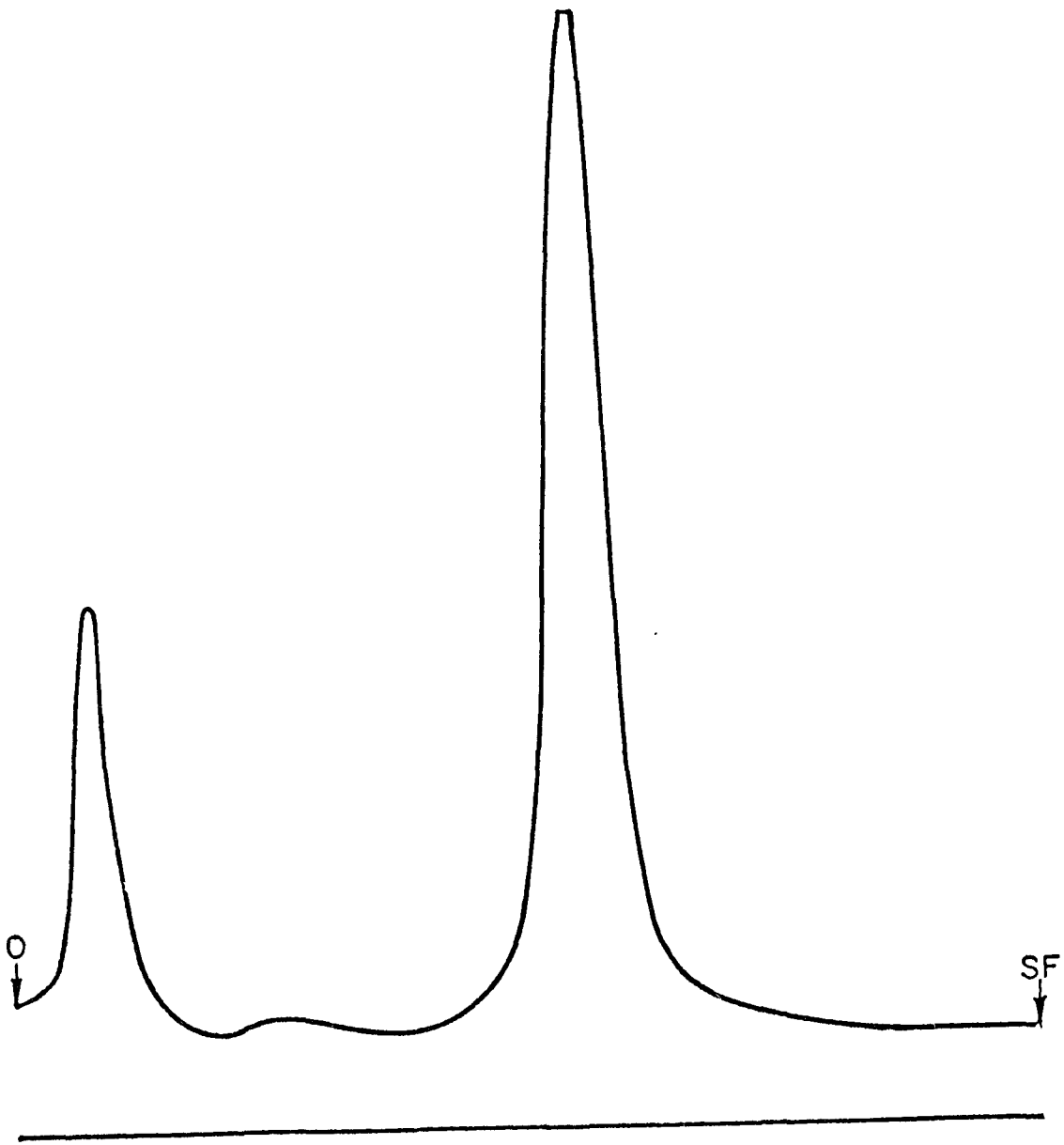


FIGURE 19. THIN-LAYER RADIOCHROMATOGRAM OF HEXANE EXTRACT OF GLUSULASE-TREATED URINE

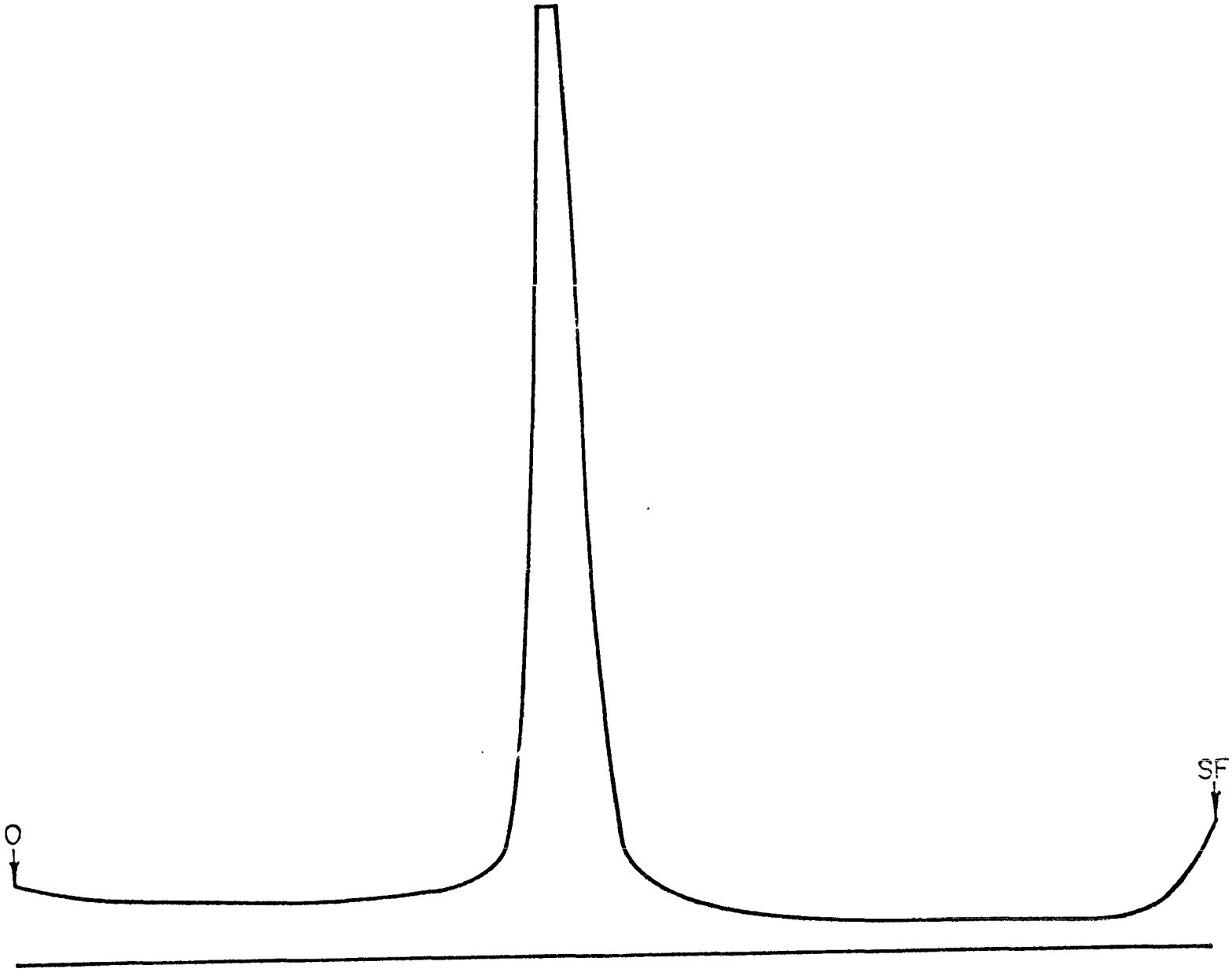


FIGURE 20. THIN-LAYER RADIOCHROMATOGRAM OF HEXANE EXTRACT OF URINE FOLLOWING ^{14}C-PCA ADMINISTRATION TO A MALE RAT

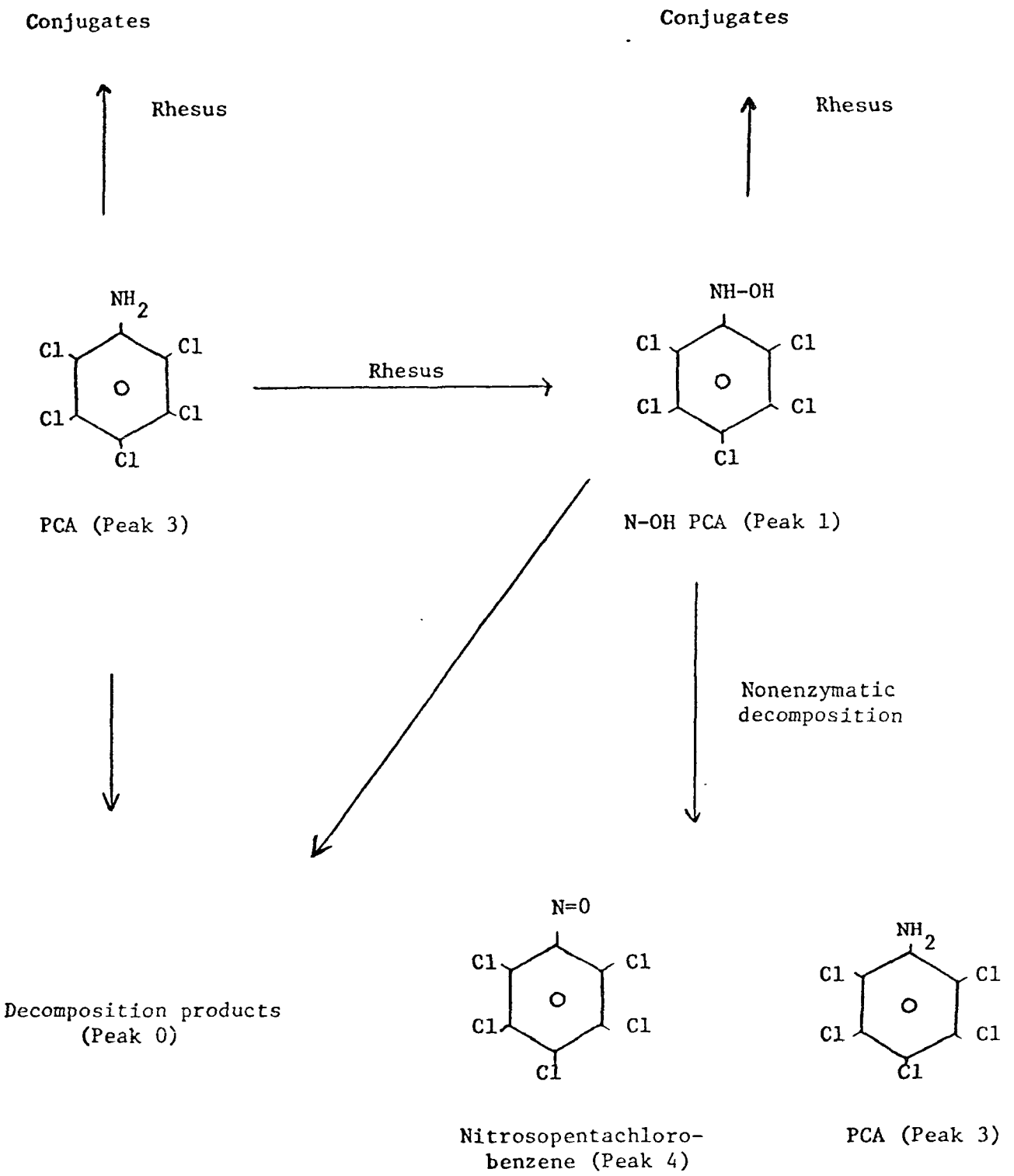


FIGURE 21. METABOLIC SCHEME FOR PCA IN RHESUS MONKEYS

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U. S. EPA, Report on the Initial Scientific Review of PCNB, Office of Pesticide Programs, Washington, D.C., April, 1976.

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15. SUPPLEMENTARY NOTES		

16 ABSTRACT

The metabolism of pentachloroaniline was determined in the rhesus monkey. ^{14}C-pentachloroaniline was orally administered to five rhesus monkeys. Blood, urine and feces were collected at designated times following dosing. The radioactive material in the biological samples was extracted and then separated by chromatographic procedures. The chemical structure of the major metabolite was characterized by mass spectrometry and nuclear magnetic resonance spectrometry, uring a chemically synthesized reference standard.

Radioactivity levels in the blood samples indicate large variation between individual animals with respect to rate of absorption and time of peak plasma radioactivity. Urinary excretion accounts for 33 to 67 percent of the administered dose while from 6 to 15 percent is excreted in the feces.

The major metabolite of pentachloroaniline, N-hydroxypentachloroaniline, is excreted in the urine. Only unchanged pentachloroaniline is found the feces. A small amount of nitrosopentachlorobenzene, found in the urine samples, results from the spontaneous disproportionation of the N-hydroxy metabolite.

Both pentachloroaniline and the N-hydroxy metabolite were tested for mutagenic activity using the two Salmonella tester strains, TA-1358 and TA-98 along with an activating system. Neither pentachloroaniline nor the N-hydroxy metabolite is mutagenic in the Ames assay system.

17 KEY WORDS AND DOCUMENT ANALYSIS		
a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Metabolism In vivo analysis laboratory animals monkeys,	pentachloroaniline	06, T, P
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