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DETERMINATION OF HYDRALAZINE AND ITS METABOLITES BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Metabolism of the vasodilator hydralazine was investigated by *in vivo* and *in vitro* studies. Standards to identify metabolic products were synthesized. Determination and quantification of hydralazine and its metabolites were accomplished by gas chromatography–mass spectrometry. A deuterium-labeled internal standard was used for quantification. ^{14}C-labeled internal standards were synthesized and used to demonstrate recoveries from the biological samples.

INTRODUCTION

Hydralazine (1-hydrazinophthalazine) is a widely used vasodilator antihypertensive drug[1–17] its administration in combination with propranolol[2] represents one of the most efficacious antihypertensive regimens currently in use.

Despite the importance of hydralazine as a drug, many aspects of its metabolic fate remain unsolved. The instability of hydralazine at physiologic pH[3] has been one of the main adversities of its analysis. So far, lack of both specificity and sensitivity characterized the methods employed to: (1) measure hydralazine levels in blood or urine and (2) distinguish definitely between the parent drug and its metabolites[4–7]. Recently, the first unambiguous analytical method to measure the parent drug concentration in plasma was reported[8].

N-Acetylation was found to be one of the major metabolic pathways in rat, rabbit, and man[6,9–11,14]. This so called N-acetyl-hydralazine[12] was subsequently found to be the hydrate of 3-methyl-*s*-triazolo[3,4-*a*]phthalazine[13–15]. The time course of plasma levels and urinary and fecal excretion was determined[4,6,11,16]. It was found that more than 70% of an administered dose was excreted within 24 h in the urine (as measured with ^{14}C-hydralazine)[11]. Feces accounted for up to 10% of administered dose excreted within several days[11]. The same authors also showed that hydralazine and some of its metabolites are highly bound to human albumin and plasma.

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Our objective was to develop a gas chromatographic–mass spectrometric (GC–MS) method to unequivocally determine hydralazine and its metabolites in biological samples. In order to do so, we synthesized most of the metabolites as well as a deuterium-labeled internal standard to quantify these compounds. ^{14}C-Labeled metabolites were synthesized as well and their recovery from biological samples was determined.

EXPERIMENTAL

Reagents

All solvents were obtained from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.). The hydralazine administered to rats was Apresoline. The silylating reagent Regisil TMCS (trimethylchlorosilane), was obtained from Regis (Chicago, Ill., U.S.A.).

Other derivatizing agents were purchased from Fisher Scientific (Houston, Texas, U.S.A.), except for d_6-acetic anhydride, which was obtained from Merck (St. Louis, Mo., U.S.A.). [^{14}C]Hydralazine was obtained from California Bionuclear (Sun Valley, Calif., U.S.A.).

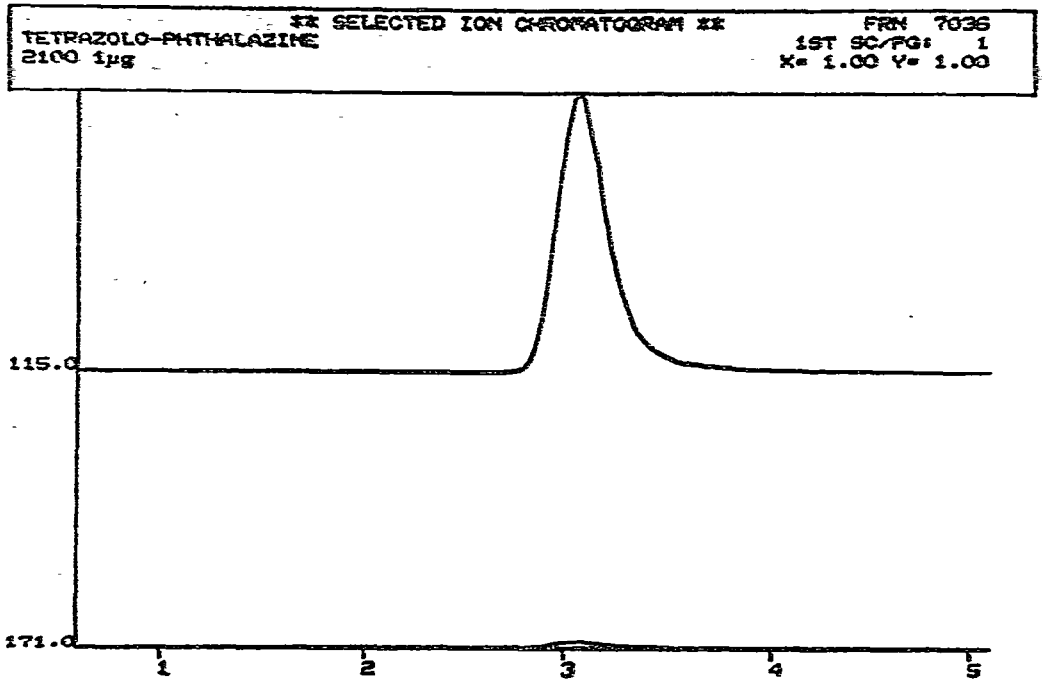
Synthesis

(1) Tetrazolo[1,5-a]phthalazine (tetrazolo-P)[18]. 1-Hydrazinophthalazine-HCl (495 nm; 2.4 mmoles) was dissolved in 2 ml of 4 *N* acetic acid. Upon addition of an excess of a concentrated solution of sodium nitrite in water, the reaction mixture was allowed to stand for 1 h at room temperature. The crystalline product was collected and dried. Yield: 390 mg or 90% of theory. Its mass spectrum showed a molecular ion at *m/e* 171. The base peak is observed at *m/e* 115. Since the molecular ion was of low abundance under the instrumental parameters used, the ion at *m/e* 115 was chosen for single ion monitoring (SIM)

(2) 3-Methyl-s-triazolo[3,4-a]phthalazine (MTP)[18]. 1-Hydrazinophthalazine-HCl (395 nm; 2 mmoles) was heated to reflux with 2 ml of acetic anhydride for 2 h. The solvent was evaporated to dryness and the residue dissolved in hot water. Upon cooling, MTP crystallized. The crystals were collected and dried over P_2O_5. Yield: 280 mg or 75% of theory. The mass spectrum gave the molecular ion at *m/e* 184 as the base peak; a prominent fragment ion was observed at *m/e* 115. Both ions were chosen for SIM (Fig. 2a).

(3) s-Triazolo[3,4-a]phthalazine (TP)[18]. 1-Hydrazinophthalazine-HCl (395 mg; 2 mmoles) was refluxed with 2 ml of 85% formic acid. After evaporation of the solvent, the residue was dried over P_2O_5. Yield: 335 mg or 98% of theory. The mass spectrum gave the molecular ion at *m/e* 170 as the base peak; the second most abundant ion was observed at *m/e* 115. Both ions were chosen for SIM (Fig. 3).

(4) 1-Hydrazinophthalazine acetone hydrazone (acetonide). 1-Hydrazinophthalazine-HCl (395 mg; 2 mmoles) was dissolved in 2.5 ml of acetone and allowed to react for 1 h with one drop of acetic acid added to the mixture. The solvent was evaporated and the slightly yellow material was dried *in vacuo*. Yield: 411 mg or 99% of theory. The pass spectrum showed the moleculiar ion at *m/e* 200, with the base peak being observed at *m/e* 185. A prominent ion was alse observed at *m/e* 115. Both *m/e* 185 and *m/e* 115 were chosen for SIM (Fig. 4).



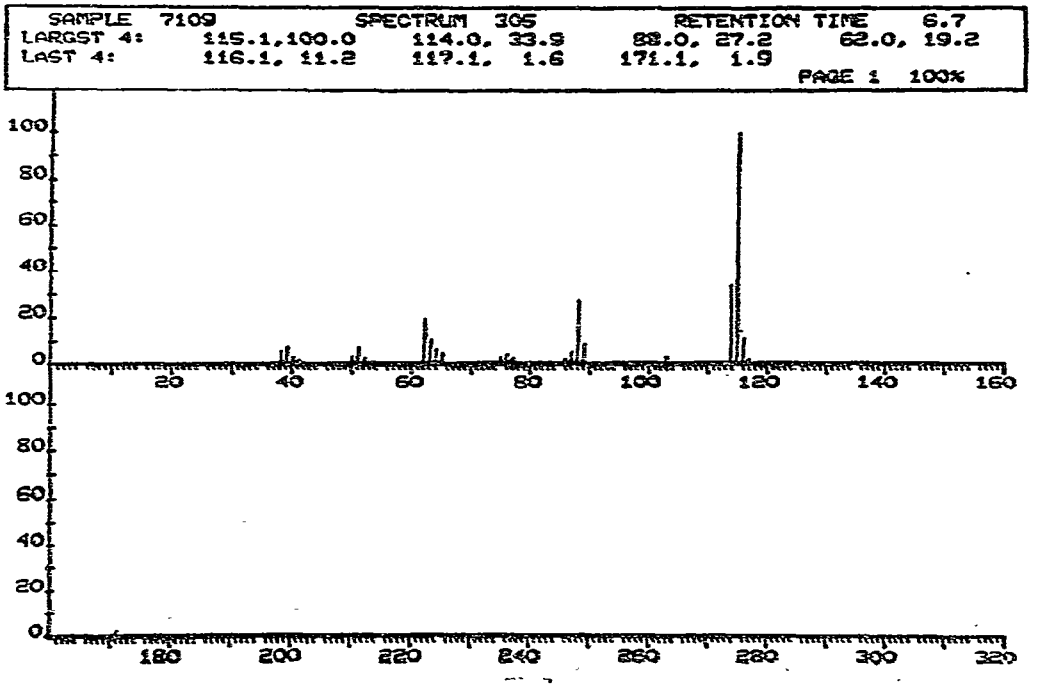


Fig. 1. Mass spectrum of tetrazolo-P.

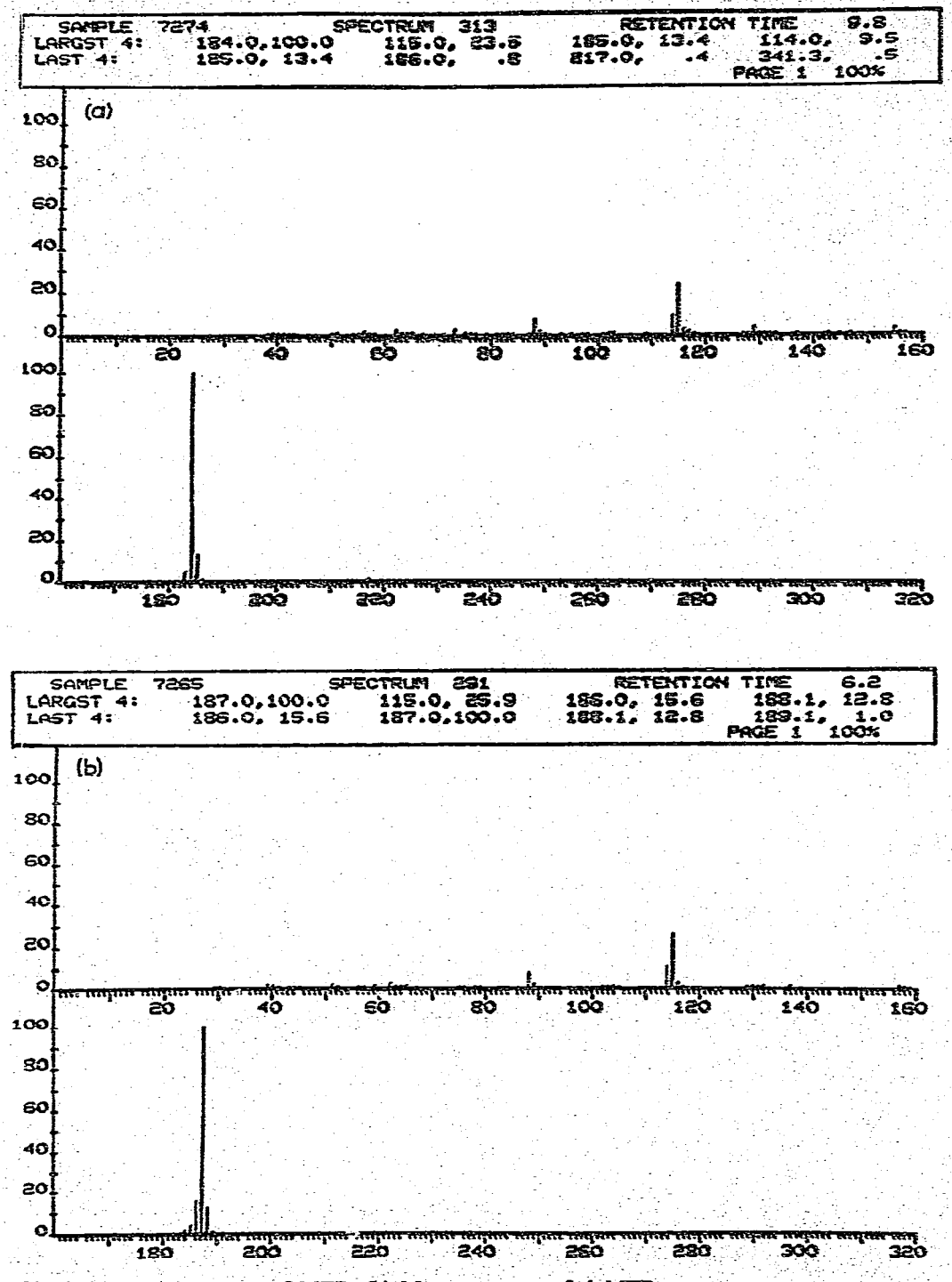
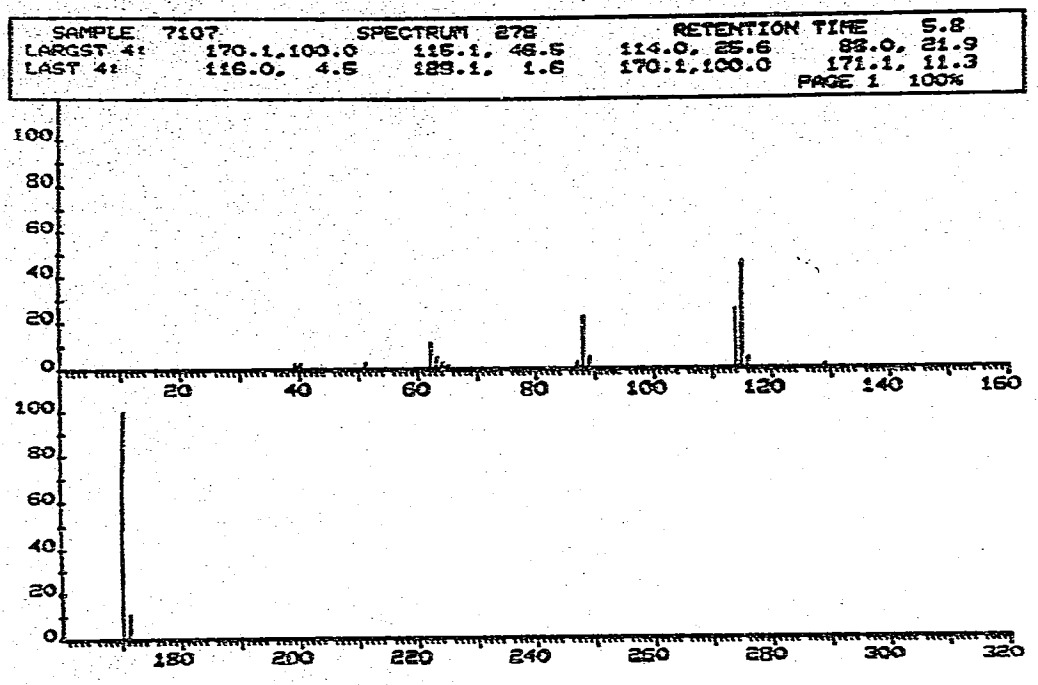


Fig. 2. (a) Mass spectrum of MTP; (b) Mass spectrum of d_5-MTP.



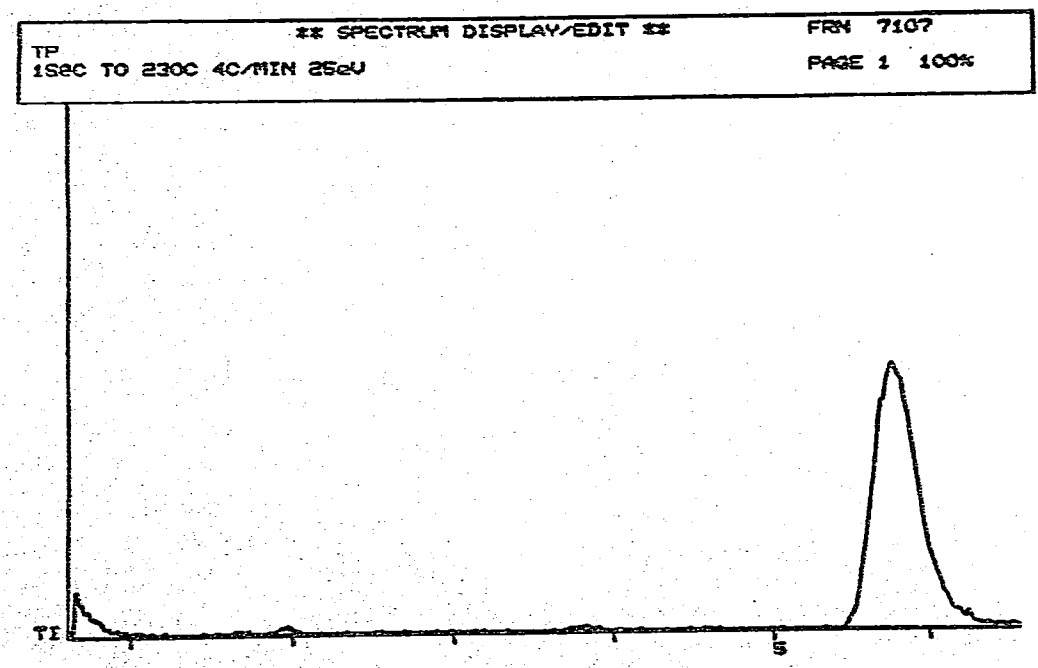


Fig. 3. Mass spectrum of TP.

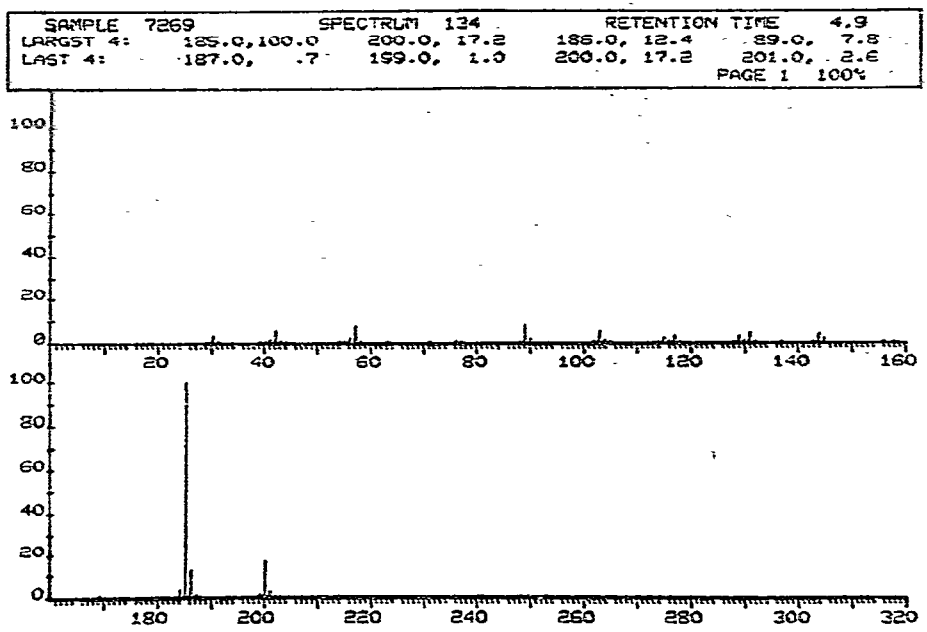


Fig. 4. Mass spectrum of acetonide.

(5) 1-Hydrazinophthalazine pyruvic acid hydrazone (pyruvate)[18]. 1-Hydrazinophthalazine-HCl (395 mg; 2 mmoles) and 1 ml pyruvic acid were refluxed in 5 ml of methanol. The methanol was evaporated. After addition of 1 ml of water, the sample was extracted with isopropanol–chloroform (1:3). The extracts were first dried over sodium sulfate and the solvent was subsequently evaporated. Yield: 70 mg or 15% of theory. The compound was esterified with trimethylsilylating reagent and injected onto the gas chromatograph for identification. No molecular ion was observed. The base peak of the spectrum was represented by the ion at *m/e* 212 (M — TMS-OH). Other ions of significance were *m/e* 184 (212 — CO) and *m/e* 115. Methylating the compound with Methelute (Pierce, Rockford, Ill., U.S.A.) yielded the N,O-dimethylated derivative with the molecular ion at *m/e* 258. Loss of $\cdot COOCH_3$ from the molecular ion forms *m/e* 199, the base peak of the spectrum. These mass spectra therefore showed the compound to be indeed pyruvate. The ions at *m/e* 212 and *m/e* 115 were selected for SIM (Figs. 5 and 6).

(6) Phthalazine (P). Treatment of 1-hydrazinophthalazine-HCl (395 mg; 2 mmoles) with 5 ml of 1 *N* sodium hydroxide and extraction with chloroform–isopropanol (3:1) yielded 215 mg of P (82% of theory). The mass spectrum of P gave the molecular ion at *m/e* 130 as the base peak. Elimination of HCN froms *m/e* 103, loss of another HCN moiety forms *m/e* 76. The ions at *m/e* 130 and *m/e* 103 were chosen for SIM (Fig. 7).

(7) d_3-3-Methyl-s-triazolo[3,4-a]phthalazine (d_3-MTP). Synthesis of d_3-labeled MTP was accomplished analogously as described in Section 2 but with d_6-acetic anhydride. Yield after recrystallization from ethanol–water: 72 mg or 19% of theory. MS yielded the molecular ion at *m/e* 187 as the base peak, *m/e* 115 is the most abundant fragment ion found. These two ions were utilized for SIM of the compound (Fig. 2b).

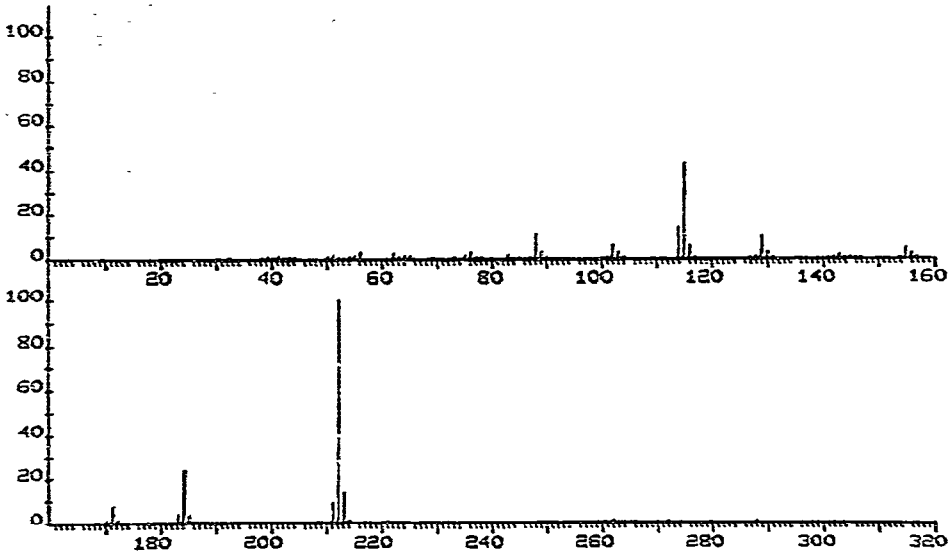


Fig. 5. Mass spectrum of pyruvate, TMS ester.

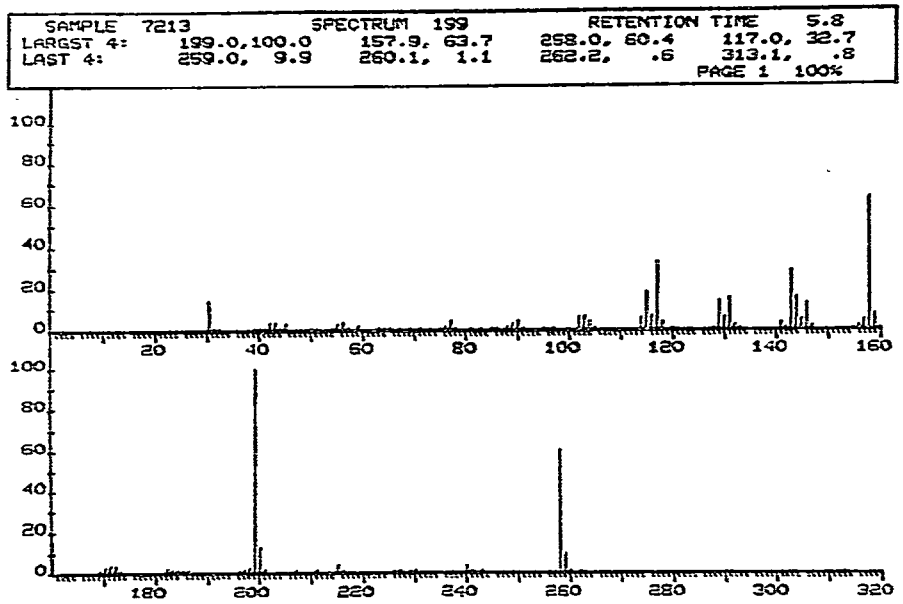


Fig. 6. Mass spectrum of pyruvate, N,O-dimethylated.

Gas chromatography–mass spectrometry

MS analysis was carried out on a combined gas chromatograph–mass spectrometer–computer system (Hewlett-Packard Models 5110A, 5980A, and 5933A). The column was a 3 ft. × 4 mm I.D. glass coil packed with 3% OV-17 on Chromosorb W (80–100 mesh). Helium carrier gas was kept at a flow-rate of 40 ml/min. The column

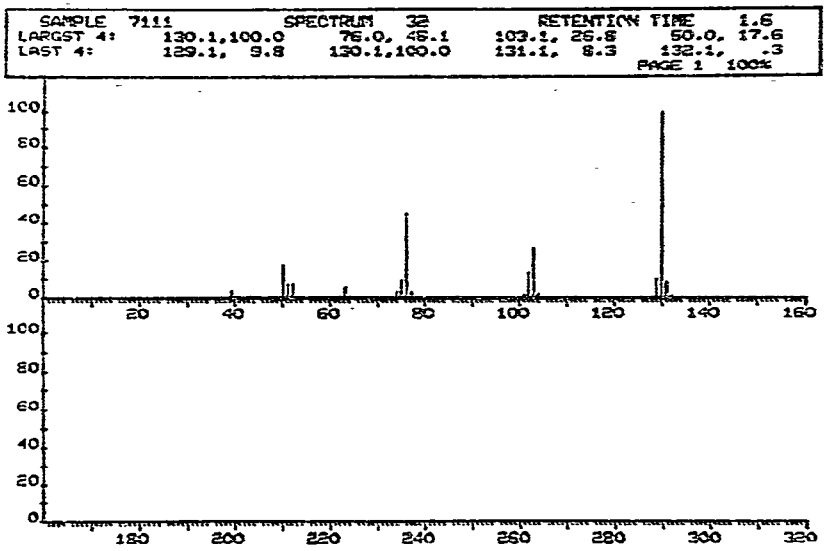


Fig. 7. Mass spectrum of P.

temperature was programmed from 170–240° at a rate of 4°/min. The injector temperature was 250°. The gas chromatograph was interfaced via a membrane separator (Hewlett-Packard) to the mass spectrometer. The ion source temperature was 210°, ionization energy 25 eV (electron impact). The mass spectrometer was interfaced to a Hewlett-Packard Model 5933A computer system which controlled the scan of the mass spectrometer, and processed and displayed mass spectral data.

Metabolic studies

(1) In vitro —Hydralazine metabolism as studied by rat liver homogenate. Rats weighing 450–600 g were fasted for a minimum of 12 h prior to decapitation. The liver was immediately removed and placed into 0° buffer (0.05 *M* potassium phosphate, pH 7.4). The liver was minced and then homogenized in 40 ml of buffer using a standard glass and PTFE tissue grinder (A. H. Thomas, Philadelphia, Pa., U.S.A.). The homogenate was centrifuged at 3000 × *g* (max.) and the supernatant decanted. For the incubations, 4.0 ml of this supernatant was added to 1.0 ml of buffer containing hydralazine (and cofactors when used). This mixture was incubated with shaking for 90 min at 37° in room atmosphere.

Immediately prior to extraction, the samples were adjusted to pH 2 and derivatized by reacting with 50% aqueous $NaNO_2$ to a final concentration of 3% (w/v) for 15 min at room temperature. This converts unmetabolized hydralazine to tetrazolo-P, which, unlike hydralazine, can be extracted from aqueous solution with organic solvents[8].

After derivatization with HONO, the samples were extracted first at the acidic pH and then again at pH 10–11. The extraction procedure involved transferring the aqueous sample to a conical centrifuge tube with 25 ml of chloroform–isopropanol (3:1). The contents were shaken for a minimum of 1 min on a vortex mixer and centrifuged. The organic (bottom) phase was removed and filtered into a round-

bottom flask. The aqueous phase was extracted two more times with 10 ml of chloroform–isopropanol and the organic phases combined and evaporated to dryness under vacuum at a maximum temperature of 35°. The aqueous phase was then adjusted to pH 10–11 and extracted again as described. Further processing of the sample for GC–MS was carried out as described for the *in vivo* experiments.

(2) In vivo —Hydralazine metabolites in rat urine. Male Sprague-Dawley rats weighing 450–600 g were anesthetized with 40 mg/kg pentobarbital sodium intraperitoneally (i.p.). Supplemental doses were administered i.p. as needed. The bladder was exposed via a small midline abdominal incision. A short segment of PE 50 tubing was inserted into the bladder and urine collected into an ice-cooled graduated cylinder. As soon as urine flow had been established, 1 mg/kg hydralazine (Apresoline) was administered i.p. in a volume of 0.5 ml. Care was taken that there was no effluent from the area of incision. The hydralazine solution contained a total of 8×10^5 cpm of ^{14}C-labeled hydralazine (specific activity 6.77 mCi/mmole). Urine was collected for 4–5 h. Upon termination of the procedure, the urine was either analyzed directly or frozen immediately for subsequent analysis within 24 h. Before acidification of the urine (2–3 ml), 1 mg of EDTA was added. This was followed by 1 ml of 2 *N* HCl and 0.2 ml of a 50% $NaNO_2$ solution. The reaction mixture was allowed to stand for 15 min. Estraction was carried out with three 10-ml portions of chloroform–isopropanol (3:1). The urine was then adjusted to pH 10 with 4 *N* NaOH solution and re-extracted with three portions chloroform–isopropanol. The extracts were combined and dried over Na_2SO_4.

Aliquots of this solution were transferred to Reactivials (Pierce, Rockford, Ill., U.S.A.) and evaporated to dryness. The sample was redissolved in 50 μl acetonitrile. Then 50 μl of Regisil-TMCS were added and the sample heated at 100° for 15 min. After addition of the deuterium-labeled internal standard (d_3-MTP), aliquots were injected onto the GC column for analysis by GC–MS.

RESULTS AND DISCUSSION

Mass spectrometric fragmentation pathway

It is interesting to note that, except for P itself, all the tricyclic derivatives of hydralazine as well as acetonide and pyruvate yield an abundant fragment ion at *m/e* 115. Little or no contribution from background impurities of the biological sample was observed for this ion. Therefore *m/e* 115 served as a characteristic feature to recognize hydralazine metabolites in the sample by MS even when no standard was available. The formation of this ion is rationalized in the fragmentation pathway for the TMS ester of pyruvate given in Fig. 8.

Elimination of TMS-OH from the unobserved molecular ion leads to *m/e* 212. Expulsion of a CO moiety from *m/e* 212 yields MTP at *m/e* 184. Further losses of CH_3CN and nitrogen lead to *m/e* 115. This explains why *m/e* 115 occurs in MTP as well as in d_3-MTP, TP, tetrazolo-P, and in the hydrazone derivatives. Expulsion of HCN from this ion leads further to *m/e* 88, a fragment ion also found in most of the hydralazine derivatives, but due to background contribution, this ion is less suitable for SIM.

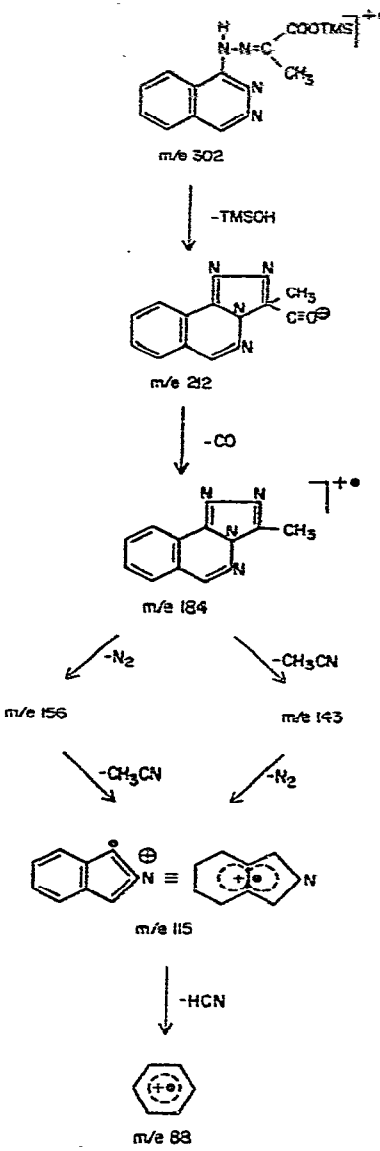


Fig. 8. Fragmentation pathway of pyruvate, TMS ester.

Recovery studies

^{14}C-Labeled internal standards were synthesized analogously to the syntheses for corresponding nonradioactive standards. ^{14}C-Hydralazine was directly added to urine and converted to tetrazolo-P as already described. Extraction was carried out in the same manner as described for the nonradioactive compounds. Radioactivity was measured with a Packard 3375 liquid scintillation spectrometer. Table I shows the recoveries obtained.

When ^{14}C-hydralazine was administered to rats and their urine collected for

TABLE I

RECOVERIES OF STANDARDS FROM BIOLOGICAL SAMPLES

Compound	*Recovery (%)*
MTP	98
TP	99
Tetrazolo-P	95
Pyruvate	76

5 h following a single i.p. dose, recovery was 79.4%. When this urine was incubated with Glusulase (Endo Labs., Garden City, N.J., U.S.A.) for 24 h at 37°, recovery increased as follows: recovery of radioactivity previously unrecovered, 45.6%; total recovery, 88.7%. The additional recovery of 9.3% radioactivity indicates that this amount was originally present in the urine as glucuronides or sulfates.

GC–MS analysis

Table II list the ions chosen for SIM.

TABLE II

GC–MS ANALYSIS OF STANDARDS

Compound	*Retention time (min)*	*Ions for SIM (m/e)*	*Base peak of mass spectrum (25 eV)*
P	1.7	103; 130	130
Phthalazinone	2.3	117; 146	146
Acetonide	4.9	115; 185; 200	185
TP	8.0	115; 170	170
Tetrazolo-P	8.9	115	115
MTP	9.6	115; 184	184
d_3-MTP	9.6	115; 187	187
Pyruvate, TMS ester	17.0	115; 184; 212	212

Figs. 9 and 10 show the SIM curves for the standards: P, acetonide, TP, tetrazolo-P, MTP, d_3-MTP, and pyruvate, TMS ester.

Up to four ions can be monitored at once with the Hewlett-Packard GC–MS–computer system, but up to four groups of ions can be followed during a GC run. Fig. 11 shows a SIM chromatogram of a rat urinary extract. The group chosen monitors, MTP, d_3-MTP, TP, and tetrazolo-P, represented by the ions at *m/e* 184, 187, 170, and 115. The first three *m/e* values represent the molecular weights and base peaks in the mass spectra of these compounds, respectively; *m/e* 115 is the characteristic fragment ion common to all four of these compounds.

All of the compounds listed in Table III were found to be present in the sample. The occurence of P[11,18], phthalazinone[19], MTP[13–15], TP[19,20], and pyruvate[9] has been reported in the literature previously. The findings that acetonide is also a metabolite of hydralazine is therefore not surprising. It was suggested[11,20] that TP was formed by oxidative demethylation of MTP and 3-hydroxymethyl-TP was indeed identified in human urine[20]. Since no standard was available for this compound,

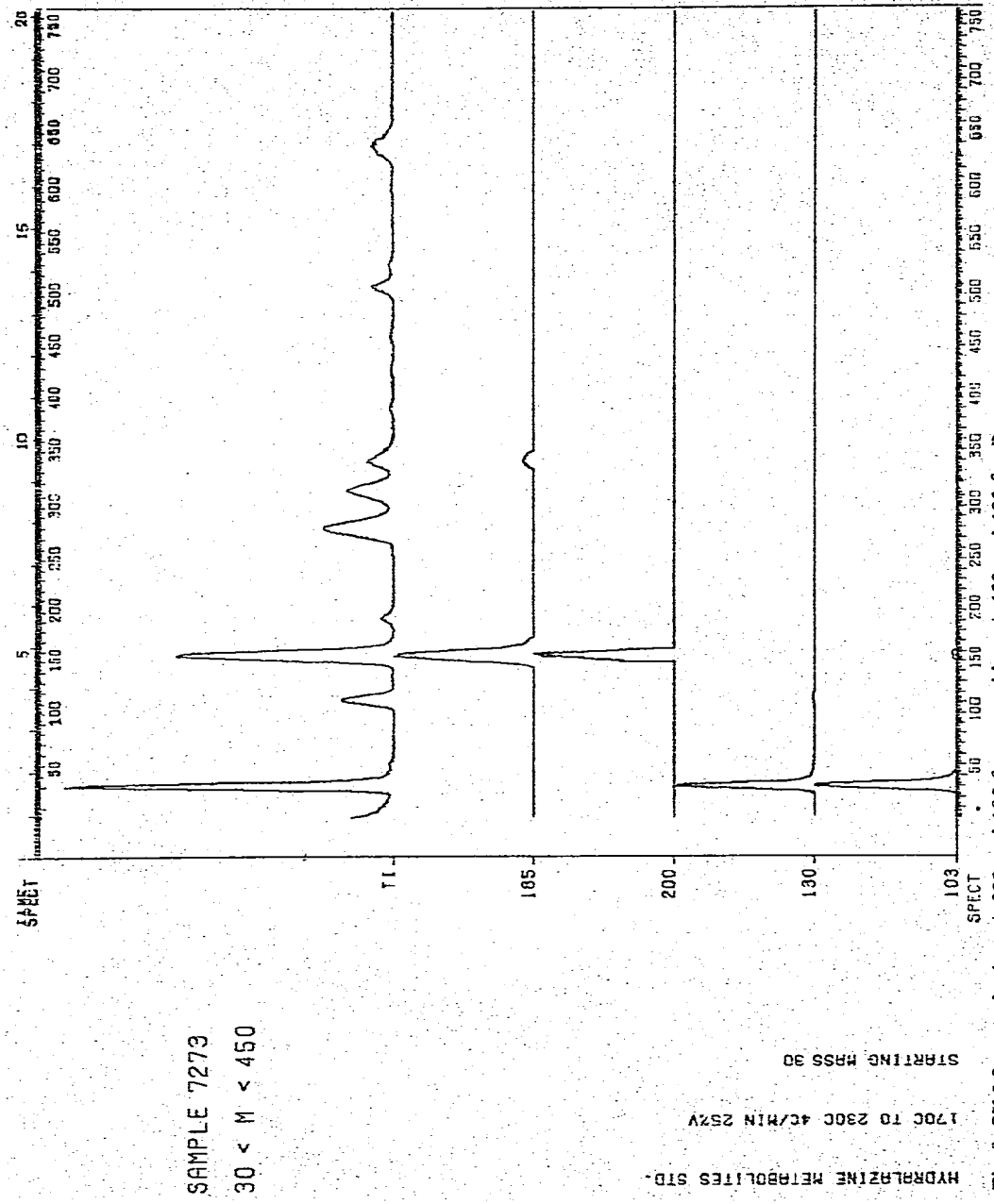


Fig. 9. SIM for standards: *m/e* 200 and 185 for acetonide; *m/e* 130 and 103 for P.

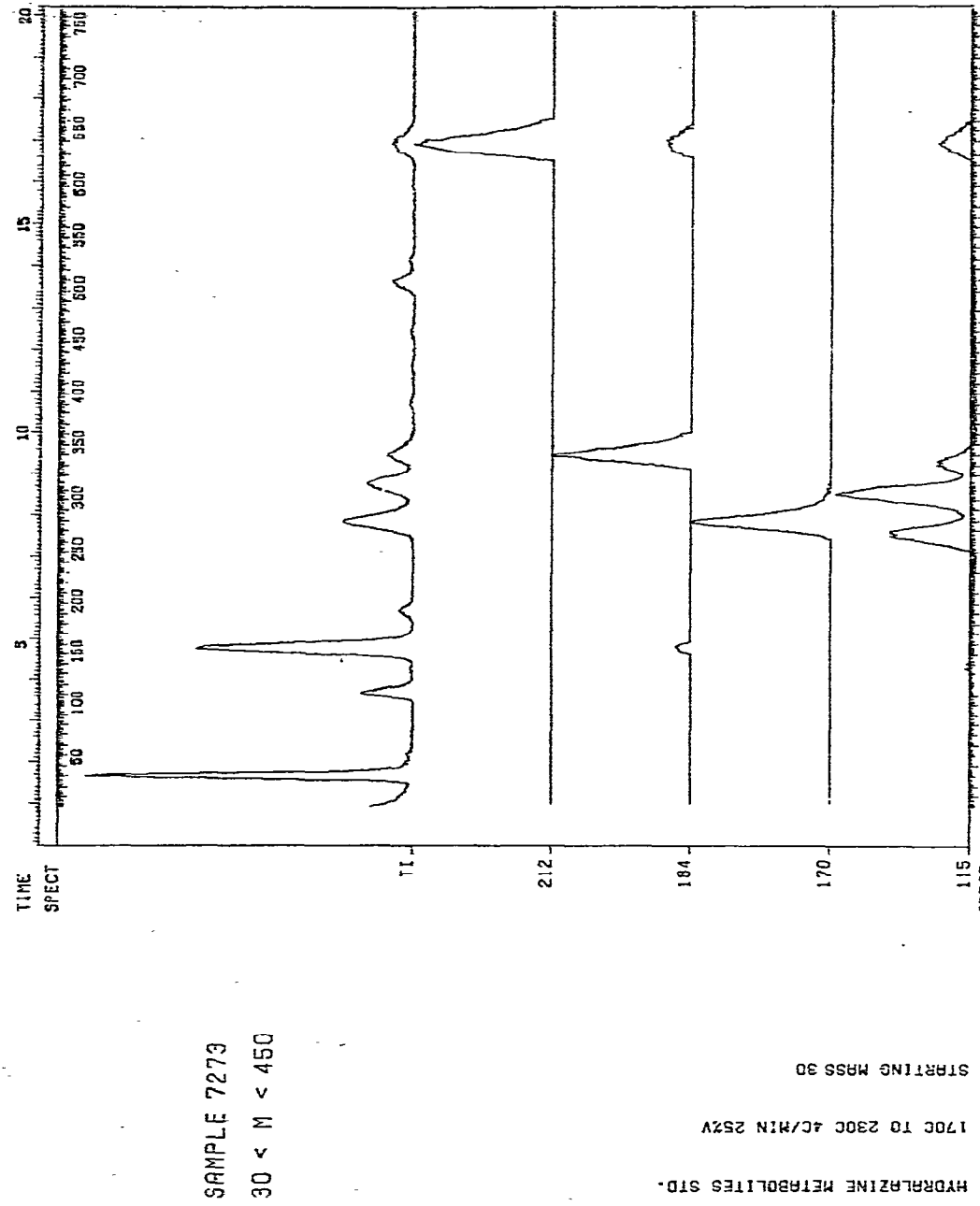


Fig. 10. SIM for standards: *m/e* 212, 184, and 115 for pyruvate, TMS ester; *m/e* 184 and 115 for MTP; *m/e* 170 and 115 for TP; *m/e* 115 at a retention time of 8.9 min for tetrazolo-P.

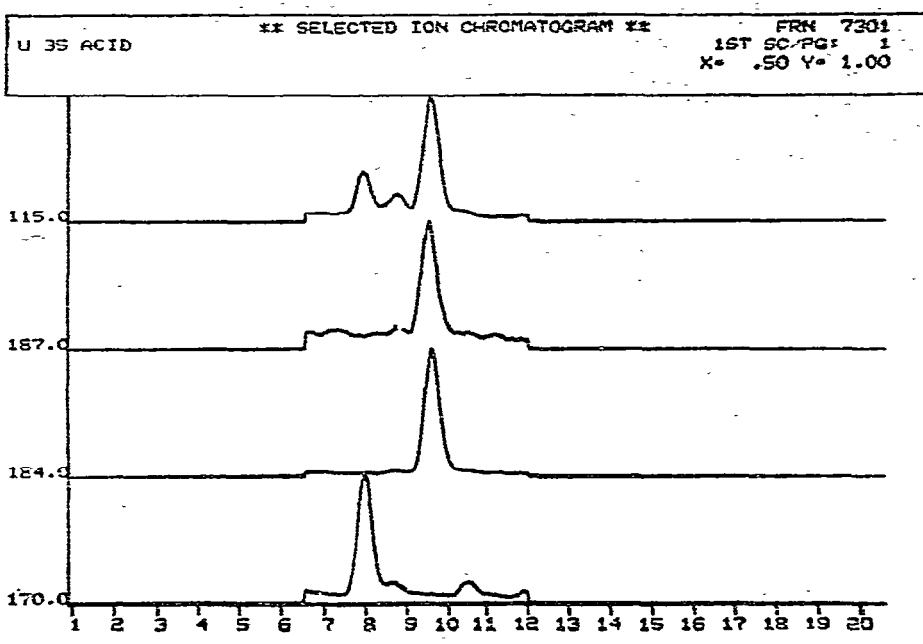


Fig. 11. SIM for rat urinary extract; *m/e* 115 for tetrazolo-P; *m/e* 187 and 115 for d_3-MTP; *m/e* 184 and 115 for MTP; *m/e* 170 and 115 for TP.

likely occurring ions due to this compound were monitored: $M^{+\cdot}$ at *m/e* 272; M — CH_3^+ at *m/e* 257; M — TMS-O^+ at *m/e* 183 and *m/e* 115. Indeed, positive responses were found at two retention times: 14.8 and 18.8 min. The full-scan mass spectrum

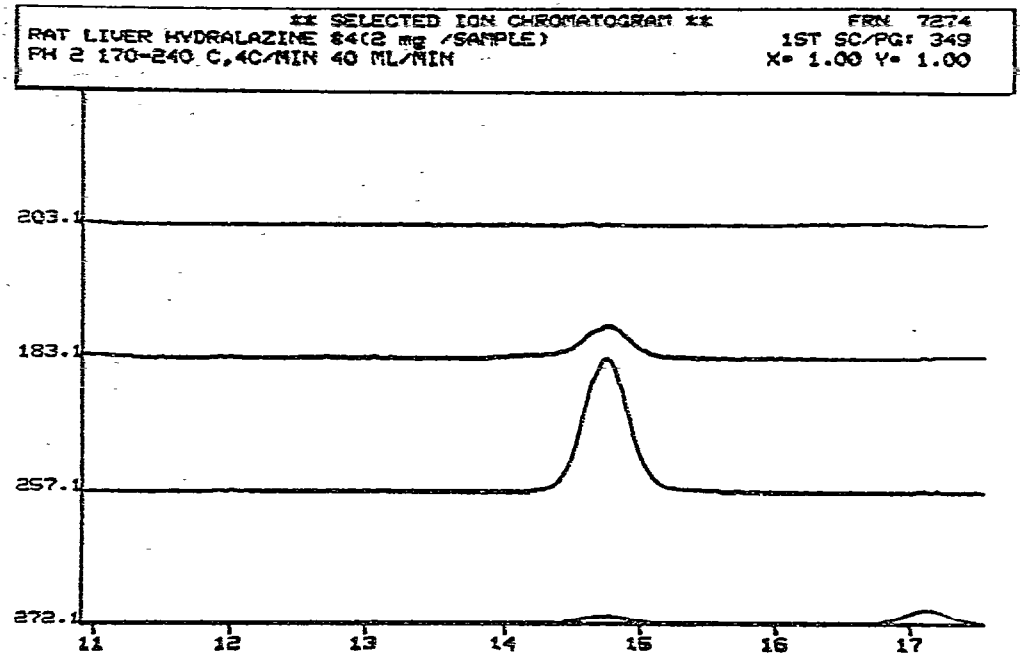
TABLE III

QUANTITATIVE ANALYSIS OF LIVER HOMOGENATE SAMPLES

(a) No cofactors added; (b) with cofactors added (glucose-6-phosphate, NADPH, NADH).

Compound	*Recovered (%)*	
	a	*b*
P*	0.2	1.6
Phthalazinone*	7.0	4.5
Parent drug*	0.4	0.2
MTP*	69.5	70.8
TP-CH_2OH	12.2	9.5
TP-COOH	0.1	0.6
TP*	6.8	5.1
"Ring-OH-MTP"	3.1	8.0
Acetonide*	0.8	0.6
Pyruvate*	0.1	0.1
	100.2	100.0

* For these compounds standards were synthesized and their retention times on GC and their mass spectra matched these standards.



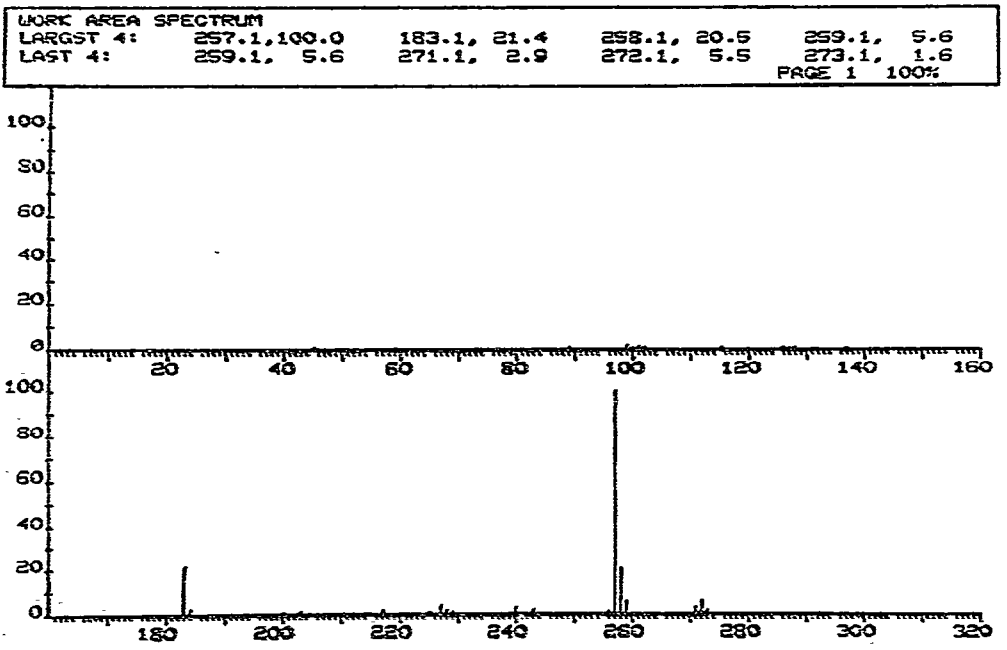


Fig. 12. Mass spectrum of the compound with retention time 14.8 min tentatively identified as 3-hydroxymethyl-TP, TM ester.

TABLE IV

QUANTITATIVE ANALYSIS OF URINARY EXTRACT

Compound	*%*
P*	6.9
Phthalazinone*	3.4
Parent drug*	2.0
MTP*	31.8
TP-CH_2OH	4.3
TP-COOH	0.2
TP*	20.5
"Ring-OH-MTP"	29.5
Acetonide*	0.8
Pyruvate*	0.6
	100.0

* For these compounds standards were synthesized and their retention times on GC and their mass spectra matched these standards.

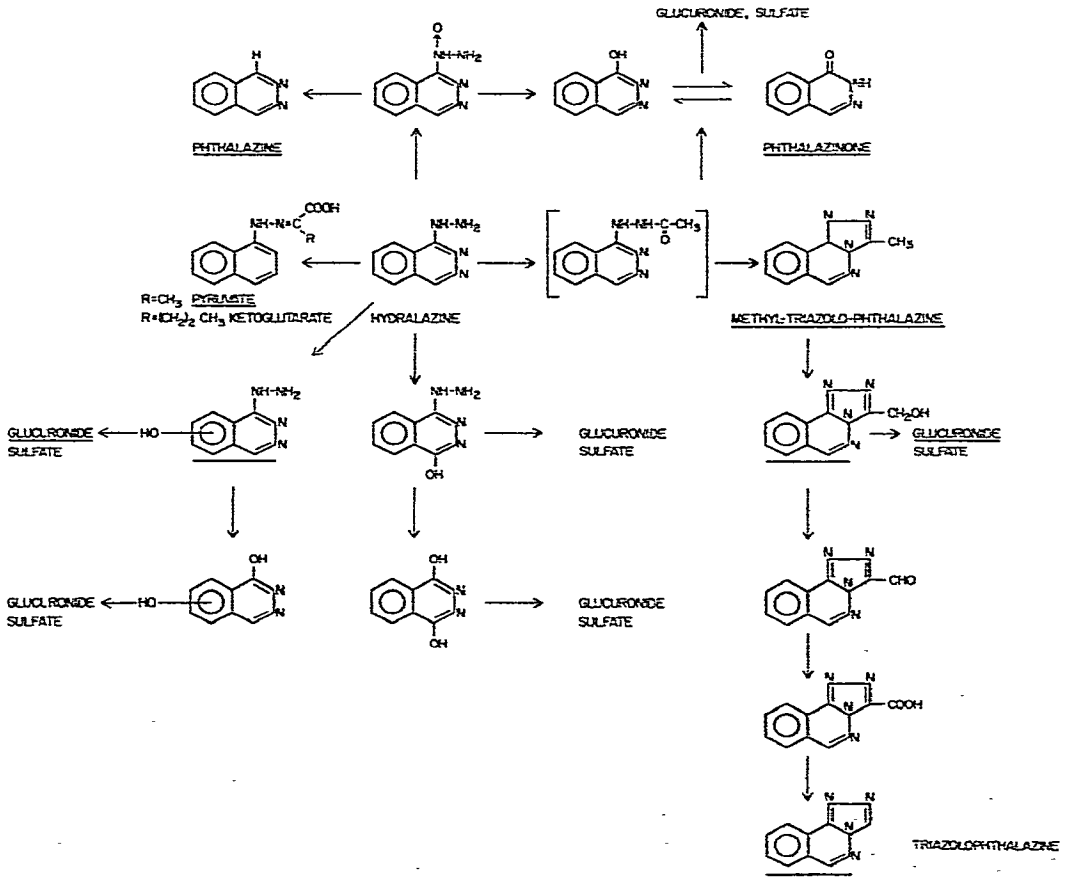


Fig. 13. Metabolic scheme of hydralazine. Underlined compounds have been reported in the literature previously.

of the GC peak at 14.8 min is shown in Fig. 12. The peak at 18.8 min gives a similar mass spectrum, but *m/e* 272 is the base peak of the spectrum. We assume therefore that the first peak represents 3-hydroxymethyl-TP, whereas the second one may represent a ring-hydroxylated MTP. However, further investigation is still needed, especially synthesis of the appropriate standard will be necessary.

Table III shows a quantitative analysis of a liver homogenate sample. MTP was found to be the major metabolite. Extensive further metabolism of this product is observed, as indicated by the oxidation products TP-CH_2OH, TP-COOH, and the decarboxylation product of TP-COOH, TP. In addition, significant amounts of "ring-hydroxylated MTP" were found. M-Carboxyl-TP was tentatively identified by the occurrence of ions *m/e* 286 ($M^{+\cdot}$), *m/e* 271 ($M - CH_3^+$), and *m/e* 197 (M — TMS-O^+), however, no *m/e* 115 was observed for this compound. Synthesis of the appropriate standard will therefore be necessary to clearly identify this product.

Analysis of a single rat urine sample is shown in Table IV. The most striking difference observed, as compared to the rat liver homogenate, is the lower ratio of MTP *versus* TP-CH_2OH and "ring-hydroxylated MTP". The higher percentage of P is most likely due to decomposition of excreted parent drug and/or the hydrazones with pyruvic acid and acetone. The decomposition takes place spontaneously at the pH of distilled water[21] (usually about 5.8–6.4) and is even more pronounced at more basic pH values[3].

Fig. 13 represents the metabolic scheme for hydralazine which takes into account fall the metabolic products reported in the literature, those described in this report, and those expected but not yet identified. (Underlined compounds represent those reported in the literature.)

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