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Identification of Hydrazine derived from Hydralazine in Experimental Animals[1)]

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The liberation of hydrazine from hydralazine was demonstrated in rabbit urine, in rat plasma and in a rat enzyme system. The identification of hydrazine in rabbit urine was performed by comparison of the gas chromatogram and mass spectrum of its benzalazine derivative with those of an authentic sample. Small amounts of hydrazine extracted from rat plasma and the rat enzyme system were detected by mass fragmentography using ^{15}N-hydrazine.

Keywords——hydrazine formation from hydralazine; rabbit urine; rat plasma; rat liver enzyme system; determination by GC and GC–MS

Methods to determine unambiguously the concentration of hydrazine (HZ), a metabolite of isoniazid (INH), in human urine and animal plasma were recently reported.[1a,3,4)] We also found that HZ formation took place enzymatically through nucleophilic hydrolysis of an acid hydrazide moiety of INH itself; the details will be reported shortly.

On the other hand, hydralazine (1-hydrazinophthalazine; 2,3-benzodiazin-1-hydrazine; 1-(2H)-phthalazinone hydrazone; HP), which is a potent peripherally acting vasodilator used to treat essential hypertension, has a reactive hydrazino group available for nucleophilic substitution in the same way as INH, since HP is considered to be an aza-analog of acid hydrazide as shown in Chart 1.

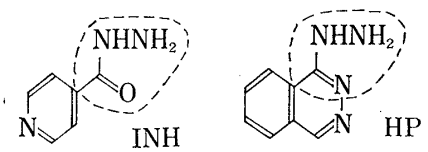

Chart 1

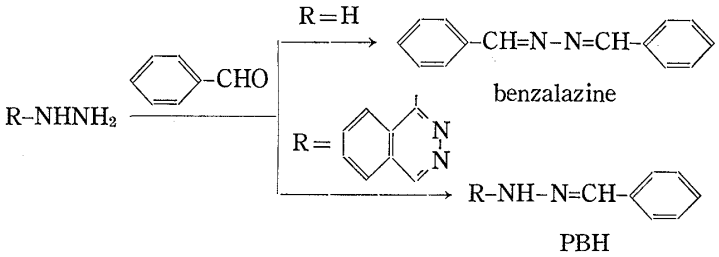

Chart 2

1) Part II of "Quantitative Determination of Hydrazines derived from Isoniazid in Patients". *a*) Part I: A. Noda, T. Goromaru, K. Matsuyama, K. Sogabe, K.Y. Hsu, and S. Iguchi, *J. Pharm. Dyn.*, **1**, 132 (1978); *b*) This work was presented at the 98th Annual Meeting of the Pharmaceutical Society of Japan, Okayama, April 1978; *c*) This work was supported by a Grant-in-Aid for Cancer Research from the Fukuoka Anticancer Association, Japan.

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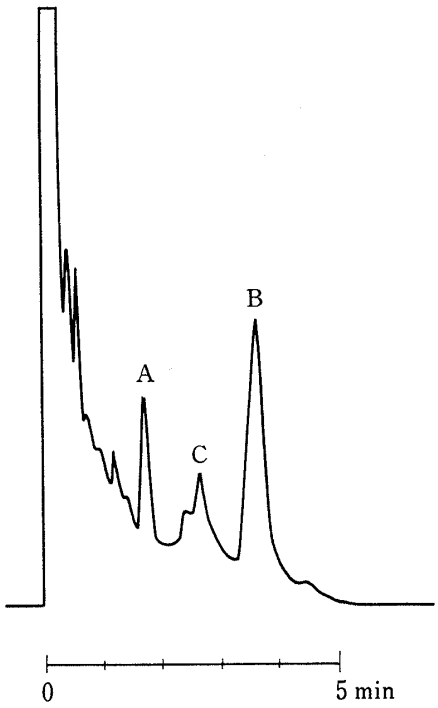
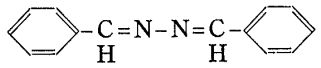


GC conditions
1.5% OV-17 on Shimalite-W;
3 mm × 1 m glass column;
column temp., 220°; inj. temp., 250°;
carrier gas, N_2, 40 ml/min; FID
A:Benzalazine



B: MTP (R=CH_3)
C: Tri-P (R=H)

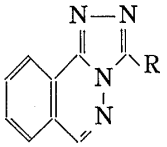


Fig. 1. Gas Chromatogram of the Extract of 24 hr Urine from a Rabbit after Oral Administration of HP–HCl (214.2 mg)

Intact HP was chromatographed after derivatization to PBH using another similar column packed with 1.5% OV-1 on Shimalite-W (retention time: 2.3 min at 240° column temp).

Although many studies on the fate of HP in man and experimental animals have been carried out,[5–8] no work has been reported on the identification of HZ derived from HP. In this paper, we report the identification of HZ in biological fluids by means of gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). HZ and intact HP were derivatized to benzalazine and 1-phthalazino-2-benzylidene-hydrazine (PBH) by reaction with benzaldehyde for GC.

Urine samples were collected from a male albino rabbit during 24 hr following the oral administration of HP hydrochloride (HP–HCl) as an aqueous solution after fasting for 16 hr. The main metabolites in rabbit urine were identified as HZ, 3-methyl-*s*-triazolo[3,4-*a*]phthalazine (MTP), *s*-triazolo[3,4-*a*]phthalazine (Tri-P) and intact HP, as shown in Fig. 1; the mass spectrum of each compound obtained on GC–MS was coincident with that of an authentic sample (Fig. 2).

As shown in Fig. 3a, HZ was also detected by means of mass fragmentography using ^{15}N-hydrazine in blood plasma obtained from rat heart. A minor amount of HZ derived from INH has been similarly determined in human urine.[1a] Furthermore, HZ was formed enzymatically upon incubation of HP–HCl with a rat microsomal fraction obtained from the liver by the centrifugation at 9000 ***g*** (s-9 mixture) (Fig. 3b).

HZ itself is a well-known carcinogen as well as mutagen.[1a] Significant mutagenicity was observed with HP itself, with or without s-9 mixture[1b] by Ames' method with a slight

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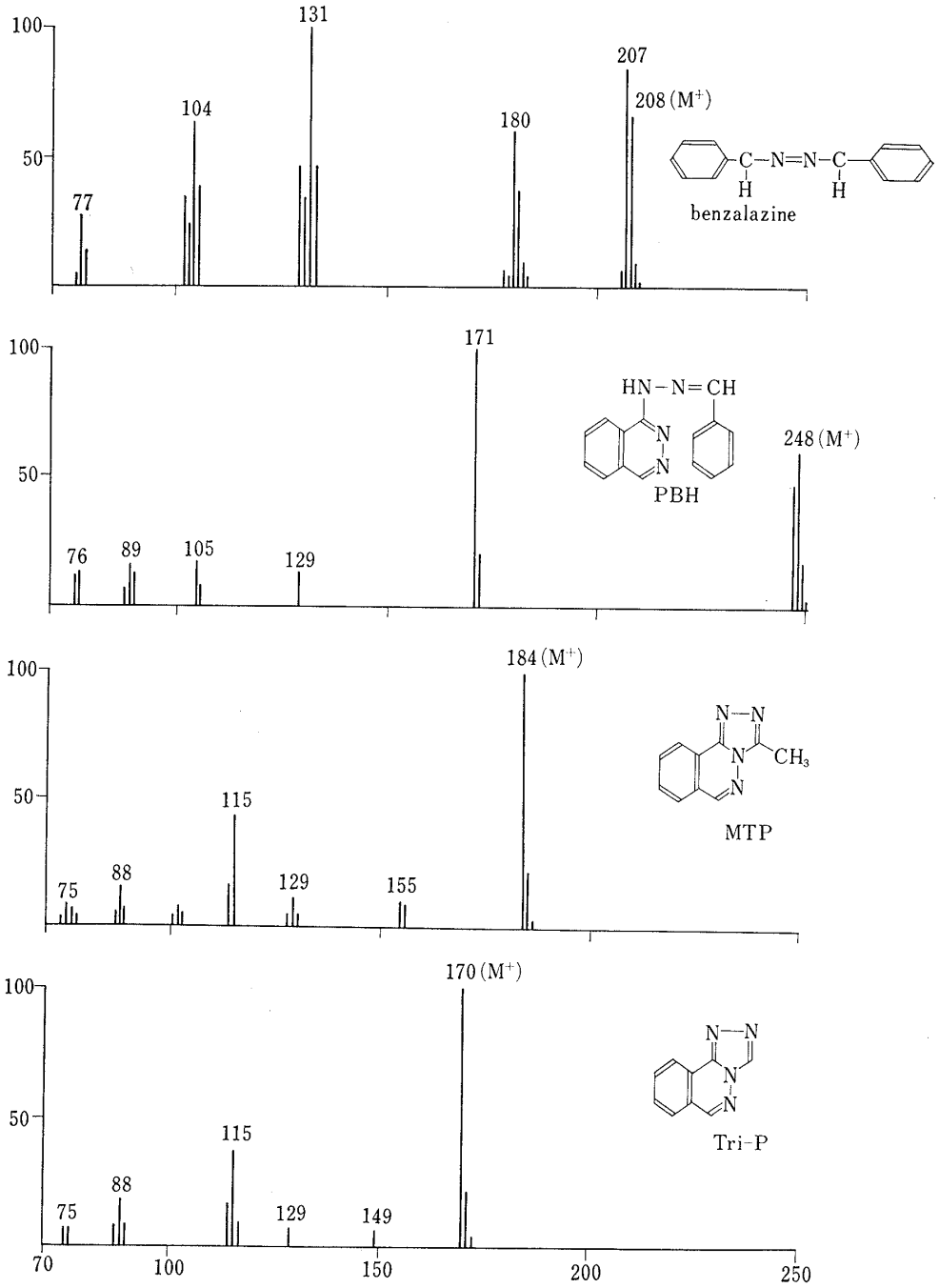

Fig. 2. Mass Spectra of Benzalazine, PBH, MTP and Tri--P

modification; this method is based on back mutation from histidine auxotrophy (His^-) to prototrophy (His^+) in a strain of *Salmonella typhimurium* TA 100.[9,10]

Considering that successive administration of hydralazine to patients is practiced for long periods, it is clearly desirable to examine the fate of this drug in man in detail.

Experimental

Chemicals——HP–HCl and HZ sulfate were purchased from Tokyo Chemical Ind. Co., Ltd. All other reagents were of chemical grade.

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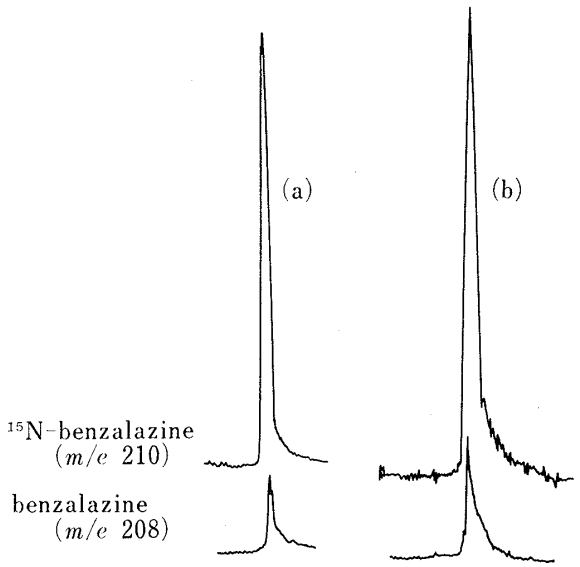

Fig. 3. Mass Fragmentograms of the Benzaldehyde Derivative of Hydrazine (Benzalazine, *m/e* 208) derived from Hydralazine

(a) Extract of blood plasma obtained from rat heart, (b) Extract after incubation in an enzyme system of rat liver. ^{15}N-benzalazine (*m/e* 210) synthesized from ^{15}N-hydrazine and benzaldehyde was used as an internal standard.

40 ml of urine
↓ adjust to pH 6
add 2 ml of ethanolic benzaldehyde
(0.1 ml/ml ethanol)
shake for 30 min
add $(NH_4)_2SO_4$
extract with 40 ml of CH_2Cl_2
dry over Na_2SO_4
↓
CH_2Cl_2 layer
↓ evaporate
residue
↓ add 100 μl of CH_2Cl_2
GC or GC–MS sample

Chart 3. Sample Preparation for Gas Chromatographic Detection of Hydrazine and Other Metabolites derived from Hydralazine

Gas Chromatography (GC)——For GC analysis, a Shimadzu GC-4CM-PF gas chromatograph with a hydrogen flame ionization detector was used. Metabolites of HP were assayed on a glass column (1 or 2 m × 3 mm inner diameter) packed with 1.5% OV-17 on Shimalite-W (80—100 mesh). The temperature of the injection port and the detector was 250°, while that of the column oven was 220°. Nitrogen was used as a carrier at a flow rate of 40 ml/min.

Gas Chromatography-Mass Spectrometry (GC-MS)——GC–MS analysis was carried out on a combined system, consisting of a JGC-20K gas chromatograph and JEOL JMS-100 mass spectrometer. A glass column (1 m × 2 mm inner diameter) packed with 2% OV-17 on Shimalite-W (170—200 mesh) was used. The flow rate of helium carrier gas was 40 ml/min. GC–MS operating conditions were as follows; ionizing energy, 23 eV; accelerating voltage, 3 kV; ionizing current, 300 μA; separator, 240°; ion source, 250°; column temp, 220°.

Animal Treatment——A male albino rabbit weighing 3 kg and male Sprague-Dawley rats weighing 200—230 g were employed after fasting for 16 hr prior to experiments. Urine samples of the rabbit were collected for 24 hr following the oral administration of HP–HCl (214.2 mg) as an aqueous solution. Blood from the rat heart was taken in a heparinized centrifuge tube 1 hr after the oral administration of HP–HCl (20 mg/body weight).

Extraction Procedure——The sample preparation procedure for GC–MS analysis is shown in Chart 3. Forty ml of urine was adjusted to pH 6.0 with hydrochloric acid or sodium hydroxide, then an ethanolic solution of benzaldehyde was added. After shaking for 30 min, the mixture was treated with ammonium sulfate followed by extraction with 40 ml of CH_2Cl_2. The extract was dried over anhydrous sodium sulfate and the solvent was removed. The resulting residue was injected into the GC column.

Fate of Hydralazine *in Vitro*——(i) Preparation of Liver Homogenate Fraction: Male Sprague-Dawley rats weighing 200—230 g were fasted for a minimum of 12 hr prior to decapitation. The liver was removed and homogenized with a motor-driven Teflon-glass homogenizer in four volumes of phosphate buffer solution (pH 7.4). The homogenate was centrifuged for 20 min at 9000 ***g*** and the supernatant was decanted. The fresh supernatant was distributed in 10 ml portions into 50 ml conical flasks, quickly frozen in dry ice, and stored at −30° in a freezer.

(ii) Incubation: NADPH (12.5 μmol), glucose-6-phosphate (250 μmol), nicotinamide (1250 μmol), glucose-6-phosphate dehydrogenase (10 units) and HP (0.1 mmol), were added to the homogenate fraction (10 ml, equivalent to 2.5 g of original liver). The mixture was shaken for 30 min at 37° in a water-bath. The enzyme was inactivated by the addition of 1 ml of 3 M trichloroacetic acid. Extraction was performed by the method described above.

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