QUANTITATIVE DETERMINATION OF HYDRAZINES DERIVED FROM ISONIAZID IN PATIENTS. I*

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(Received December 14, 1977)

Hydrazine formation took place in man after the oral administration of isoniazid. The successive excretion of free hydrazine, one of the well-known mutagens, was observed in the urine of the patients on INH-treatment. The intact INH and the other metabolites, acetyl-INH, acetyl-hydrazine and diacetylhydrazine, were also analyzed by gas chromatography-mass spectrometry.

Keywords—patient on INH-treatment; the metabolites in urine; quantitative analysis; by GC and GC-MS; successive excretion of free hydrazine

In the early 1950's, three independent research groups simultaneously discovered the marked antitubercular activity of isoniazid (INH).¹⁾ Today, this drug still remains in the front rank of antituberculosis drugs. Numerous works in different fields were published on this drug; metabolism in man and in different animals, its mode of action against mycobacteria, its therapeutic use, its toxicity, *etc.*

The qualitative studies on INH metabolism in the human body were reported in 1953 through the work of Wolff *et al.*²⁾ and Hughes *et al.*³⁾ Quantitative data have been added since. Important progress in the field of clinical pharmacogenetics has been made in the course of the last 10 years. The studies on the variations of INH metabolism in relation to species and on phenotyping of INH inactivators by Evans *et al.*⁴⁻⁸⁾ are of particular interest.

The known urinary products resulting from INH administration to humans are intact INH, pyruvic hydrazone, α -keto-glutaric hydrazone, acetyl-INH, isonicotinic acid, isonicotinuric acid, monoacetylhydrazine (acetylhydrazine) and 1, 2-diacetylhydrazine (diacetylhydrazine).⁹⁾ Free hydrazine is presumed to be formed from the hydrolysis of INH to isonicotinic acid, but this compound had not been detected in humans.

Since 1962, Biancifiori and his coworkers¹⁰⁻¹⁵⁾ examined the relation of INH and allied compounds to carcinogenesis in some species of small animals. They reported that carcinogenicity was caused by the hydrazine moiety of INH, and that hydrazine sulfate had been shown to lead to lung and liver tumors in rats but INH itself appeared to be non-carcinogenic in species other than the mouse.

In 1975, Mitchell *et al.* suggested that the high

* Previous report: S.Iguchi, T.Goromaru, A.Noda, K.Matsuyama, and K.Sogabe: Quantitative determination of hydrazines derived from isoniazid in man. *Chem. Pharm. Bull.* (Tokyo), 25, 2796 (1977). b) This work was partly presented at 97th meeting of Pharmaceutical Society of Japan, Tokyo, April 1977; at 37th International Congress of Pharmaceutical Sciences, Hague, September 1977; at 9th Symposium of Drug Metabolism and Action, Kumamoto, November 1977. This work was supported by the Grant-in-Aid for Scientific Research from the Ministry of Education.

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incidence of INH hepatitis in rapid acetylators might be related to acetylhydrazine which was a metabolite of INH *via* acetyl-INH, since the studies in animals showed acetylhydrazine to be converted to a potent acylating agent that produced liver necrosis.¹⁶⁾ In 1976, they also defined a mechanism in acetylhydrazine toxicity, *i.e.* the entire acetyl group was trapped by cysteine, thereby eliminating ketene as the reactive acylating agent formed during the oxidation of acetylhydrazine by liver microsomes.¹⁷⁾ However, they did not touch on free hydrazine itself yet.

In 1976, Braun *et al.*¹⁸⁾ reported the mutagenicity of INH, in which metabolic degradation product of hydrazine itself was determined in blood specimens from mice after subcutaneous injection of INH. INH and its metabolites were analyzed photometrically after thin layer chromatography. The results obtained by them suggested that free hydrazine represents the active principle in INH mutagenesis in mice.

In view to the importance of the problem as to whether hydrazine is formed from INH in humans, we have been conducting studies on the analytical method of INH and the fate of INH in man since 1975, and the results reported recently.¹⁹⁾

Techniques for estimating INH and the metabolites by fluorometry or microbiological methods were also developed during the last ten years. However, the methods available were insufficient to detect the various kinds of metabolites in minute quantities. The use of gas chromatograph analysis is the most convenient method in measuring the low concentration of various metabolites in the biological fluid. A few studies on gas chromatography (GC) of INH have been reported,^{20,21)} but these are not adaptable to examine the hydrazine moiety of INH in biological samples. Recently, Timbrell *et al.* reported an excellent method for determining the hydrazine metabolites of INH in human urine by GC.²²⁾

At almost the same time, we also succeeded in the estimation of hydrazines as well as INH and acetyl-INH in human urine by using a similar technique.²³⁾ The method established by us is better than others in accuracy and sensitivity, because

we used some of the unique techniques, *i.e.* (i) the derivatization into trimethylsilylate after the hydrazone formation with benzaldehyde on GC, (ii) the utilization of benzoic acid hydrazide (BAH) as an internal standard on GC of intact INH, acetyl-INH and diacetylhydrazine, (iii) the utilization of ¹⁵N-hydrazine as an internal standard on mass fragmentography of free hydrazine and monoacetylhydrazine. Some of our studies have already been published.^{24,25)}

By using the method developed by us, we examined the actual state of INH metabolism in the patients on INH treatment.

MATERIALS AND METHODS

Chemicals—The specific reagent grade of INH and hydrazine sulfate were purchased from Wako Pure Chemicals Ind. Ltd., the specific grade of acetylhydrazine and benzoic acid hydrazide from Tokyo Chemical Ind. Co., Ltd. Acetyl-INH and diacetylhydrazine were prepared by the known methods.^{26,27)}

Gas Chromatography (GC)—INH, acetyl-INH and diacetylhydrazine were assayed on a glass column (1 m×3 mm inner diameter) packed with 1.5% OV-17 on Shimalite W (80—100 mesh) in Shimadzu Model GC-4BM-PF gas chromatograph equipped with a hydrogen flame ionization detector. Temperature of the injector, detector, and column oven was set at 250°, 250° and 220°, respectively. Carrier gas was nitrogen (flow rate 20 ml/min).

Gas Chromatography-Mass Spectrometry (GC-MS)—The instrument used was Model JMS-D100 mass spectrometer equipped with a JGC-20K gas chromatograph. The glass column used (1 m×2 mm inner diameter) was packed with 1.5% OV-17 on Shimalite W (80—100 mesh). Temperature of the injector was 230° and that of the column oven was 185°. Mass spectrometer conditions were as follows; accelerating voltage, 3 kV; ionizing current, 300 μ A; ionizing energy, 23 eV; separator temperature, 230°.

Assay Procedure—*i) Intact INH:* To 10 ml of urine sample adjusted to pH 6, was added 0.5 ml of aqueous benzoic acid hydrazide (BAH) solution

CHART 1. *Derivatization Reaction employed on GC or GC-MS*

10 ml of urine
 add 0.5 ml of BAH aqueous solution (0.8 mg/ml)
 adjust to pH 6.0
 add 0.5 ml of benzaldehyde ethanol solution (0.1 ml/ml)
 shake for 30 min
 extract with 10 ml of CH₂Cl₂

CH₂Cl₂ layer aqueous layer

extract with 10 ml of CH₂Cl₂

CH₂Cl₂ layer aqueous layer

residue

add 100 μ l of pyridine and 100 μ l of N,O-bis(trimethylsilyl)acetamide (BSA)
 room temp., 30 min
 gas chromatographic sample

CHART 2. *Sample Preparation for Gas Chromatographic Determination of INH*

(0.8 mg/ml) as an internal standard and 0.5 ml of ethanolic benzaldehyde solution (0.1 ml/ml). After the mixture was shaken for 30 min, 1-isonicotinoyl-2-benzylidene-hydrazine (IBH) and 1-benzoyl-2-benzylidene-hydrazine (BBH) were formed as shown in Chart 1. These products were extracted twice with 10 ml of CH₂Cl₂. The combined extracts were dehydrated with anhydrous sodium sulfate and evaporated to dryness. One μ l of the solution, which was obtained after trimethylsilylation of the residue with 100 μ l of N, O-bis(trimethylsilyl)acetamide in 100 μ l of pyridine at room temperature for 30 min, was injected into the gas chromatograph as shown in Chart 2. The gas chromatogram of the urinary extract was shown in Fig. 1.

According to the metabolic scheme proposed by Ellard and Gammon (10), INH conjugates with α -ketoglutaric acid and pyruvic acid. Therefore, our data of INH in Table III included the amounts of INH-hydrazones which were hydrolyzed at pH 6.

FIG. 1. Gas Chromatogram of Urine Extract after INH Administration (Subject H1)

ii) *Acetyl-INH and Diacetylhydrazine*: Ten ml of urine was initially treated with 1 ml of conc. HCl for 24 hr at 37°.* The hydrolyzed sample containing INH and hydrazine was analyzed as IBH and benzalazine by the same method used in the assay of INH. The values of diacetylhydrazine obtained from gas chromatogram were apparent, because they included the amounts of monoacetylhydrazine and free hydrazine. Therefore, the amount of diacetylhydrazine in Table III was calculated by subtracting the quantities of monoacetylhydrazine and free hydrazine obtained in the mass fragmentography from the apparent amount of diacetylhydrazine.

iii) *Hydrazine and monoacetylhydrazine*: Mass fragmentography was employed in the analysis of these two metabolites by using ¹⁵N-hydrazine as an internal standard because only a minute quantity is present in urine. Twenty five µg of ¹⁵N-hydrazine and 0.5 ml of benzaldehyde were added to 10 ml of urine sample (pH 6). After the mixture was shaken for 30 min, 1-acetyl-2-benzylidene-hydrazine (ABH), benzalazine and ¹⁵N-benzalazine were formed. These products were extracted twice with 10 ml of CH₂Cl₂. The combined extracts were dehydrated with anhydrous sodium sulfate and evaporated to dryness. The residue was subjected to GC-MS analysis like that of CH₂Cl₂ solution shown in Chart 3. The mass spectra of ABH, benzalazine and ¹⁵N-benzalazine are shown in Fig. 2. In order to measure the amount of free hydrazine, the height of the molecular ion peak of benzalazine at *m/e* 208 was compared with ¹⁵N-benzalazine at *m/e* 210. In the case of acetylhydrazine, the height of the molecular ion peak of ABH at *m/e* 162 was compared with the highest peak (base ion peak) of ¹⁵N-benzalazine at *m/e* 133 (—CH=N—N=CHC₆H₅). The details are described in the previous report.

Accuracy of the Method—In the case of INH and acetyl-INH, the degree of accuracy has already been reported in the previous paper.¹⁹⁾ The data of hydrazine, acetylhydrazine and diacetylhydrazine are shown in Table I.

* Acetyl-INH was hydrolyzed not into isonicotinic acid, but into INH under the present condition.

FIG. 2. Mass Spectra of ABH, Benzalazine and ¹⁵N-Benzalazine

CHART 3. Sample Preparation for GC-MS Determination of Free Hydrazine and Monoacetylhydrazine

Mutagenesis Test of INH and Its Metabolites in the Plate — Mutagenicity was tested according to Ames' method.²⁸⁾ Reaction mixture contained 0.1 ml of an overnight nutrient broth culture of *Salmonella typhimurium* TA-100 (*hisG46 uvrB rfa/pKM₁₀₁*), 0.1 ml of the compound (INH, acetyl-INH, hydrazine, acetylhydrazine, diacetylhydrazine and 2-acetylaminofluorene) to be tested, and 0.5 ml of the S-9 fraction (9000g supernatant of liver homogenates from rats). The mixture was incubated at 37° for 30 min, and poured together with 2 ml of soft-agar into a petri dish containing 30 ml of minimal-glucose agar medium. The plates were incubated in a dark at 37° for 2 days and his⁺ colonies were counted. The results are summarized in Table II. It was concluded that hydrazine was the only compound to show a marked mutagenicity.

RESULTS AND DISCUSSION

INH and its metabolites in urine specimens from patients and healthy volunteer were measured by the present method. High sensitivity and sufficient accuracy of the method were clearly shown in Table I. The total amounts of hydrazines in urine excreted during the 8 hr period after the oral administration of INH are summarized in Table III. The group P consists of the patients on INH-treatment, while H1 consists of the healthy adult male volunteer in our laboratory. To the healthy volunteer, an aqueous solution of INH (100 mg) was administered orally once in the morning after 12 hr fasting. Since five patients except P6 were given 200 mg each of INH in powdered form twice

TABLE I. Accuracy of Determination of Hydrazines

Compound	Conc. range of calibration curve (µg/ml)	Standard error of curve (µg/ml)	Regression coefficient	Standard deviation (µg/ml)
Hydrazine ^{a)}	0.5 — 2.5	±0.012	0.99	±0.027
Acetylhydrazine ^{a)}	0.8 — 5.6	±0.097	0.99	±0.257
Diacetylhydrazine ^{b)}	8.0 — 40.0	±0.356	0.99	±0.796

a) Measured by GC-MS.

b) Measured by GC.

Each value was obtained from the results of the experiments performed five times.

TABLE II. *Mutagenesis Tests activated by Rats Liver Homogenates (S-9 Mixture)*

	10 mg/ml	1 mg/ml	0.5 mg/ml	0.1 mg/ml	0.01 mg/ml
Hydrazine	death ^{a)}	+	—	—	—
Acetylhydrazine	—	—	—	—	—
Diacetylhydrazine	—	—	—	—	—
Isoniazid	—	—	—	—	—
Acetylisoniazid	—	—	—	—	—
2-Acetyl-amino-fluorene			++	+	—

—: colonies on the plate were less than 100,

+: more than 200,

++: more than 600,

a): no colonies because of killing by high concentration of hydrazine.

a day after meal (in the morning and in the evening) every day, the urine collection was started immediately after the administration of INH in the morning. However, P6 was given daily dose of 300 mg of INH twice a day after meal (in the morning and in the evening).

As shown in Table IV, P1 was given 400 mg/day of INH without any other drug, while the other patients (P2—P6) have taken INH together with 450 mg/day of Rifampicin and 1000 mg/day of Ethambutol. Neither of these drugs interfered with the analysis of INH and its metabolites. The urine samples were collected at 0—2, 2—4, 4—6 and 6—8 hr after the administration of INH, and were analyzed immediately. The most remarkable observation was the excretion of free hydrazine, one of the well-known mutagens, in every subject. Free hydrazine was detected even in the urine collected at 0—2 hr after the administration of INH in which the concentration of free hydrazine was less than 0.05% to the dose as shown in H1. In the case of patients, the percent excretion of the amount of free hydrazine to the administered dose could not be calculated, because their urine gave a positive value even at 0 hr (Table III) due to the successive excretion for more than 12 hr. The individual difference was remarkable in the excreted amount of hydrazine; *i.e.* 1258 μ g for P6,

173—503 μ g for P2—P5, and 147 μ g (0.6% to the dose amount) for the healthy volunteer. The individual difference among the patients (P1—P5) was also observed in the excreted amount of acetylhydrazine (186—2186 μ g) and in that of diacetylhydrazine (11.9—32.3 mg).

It is interesting to note that the patient P5 who excreted a remarkable amount of acetylhydrazine had a medical history of the temporary increase of transaminase two months before this experiment (two weeks after the beginning of INH therapy). There might be some relationships between the observation obtained from P5 mentioned above and Mitchell's suggestion that the release of acetylhydrazine in man is responsible for INH liver injury. According to our examination, the healthy volunteer in this experiment was so-called "rapid inactivator."^{*} In the case of patient, phenotyping of slow and rapid inactivators in the present data does not seem to be significant, because the patients have taken not only INH but also a few kinds of drugs successively. Therefore, we did not determine the phenotype for the patients.

The results of the mutagenesis test of INH, acetyl-INH, and hydrazines by using Ames' method (*Salmonella typhimurium*)²⁸⁾ are shown in Table II. As was expected, hydrazine was positive in mutagenesis test, while INH, acetyl-INH,

* unpublished data.

TABLE III. *Excreted Amounts of INH and Its Metabolites in Human Urine during the 8 hr Period after Oral Administration of INH*

	Subj.	Control ^{a)}	0—2hr	2—4hr	4—6hr	6—8hr	Total amount		
							c)	(% to dose)	
INH ^{b)} (mg)	P1	0.0	0.3	0.4	0.5	1.4	2.6	2.6	1.3
	P2	0.0	1.1	9.5	17.5	18.2	46.3	6.3	23.2
	P3	0.0	6.8	0.6	0.3	0.0	7.7	7.7	3.9
	P4	0.0	6.4	2.3	0.0	0.0	8.7	8.7	4.4
	P5	0.0	5.6	4.4	0.0	0.0	10.0	10.0	5.0
	P6	2.1	5.4	7.8	4.4	2.4	20.0	20.0	—
	H1	0.0	1.9	1.0	0.0	0.0	2.9	2.9	2.9
Acetyl-INH (mg)	P1	9.3	18.6	11.3	10.0	23.4	63.3	48.4	—
	P2	0.0	5.5	22.6	25.4	24.5	78.0	59.7	—
	P3	1.8	26.6	13.1	11.1	8.6	59.4	45.5	—
	P4	2.4	29.0	26.5	18.8	17.9	92.2	70.6	—
	P5	13.3	32.3	20.9	22.2	28.7	104.1	79.7	—
	P6	13.9	27.7	35.0	24.6	12.8	100.1	76.6	—
	H1	0.0	0.9	20.0	15.1	10.0	46.0	35.2	35.2
Diacetylhydrazine (mg)	P1	4.6	6.9	2.8	2.1	6.0	17.8	21.0	—
	P2	2.3	0.3	0.9	4.6	6.1	11.9	14.1	—
	P3	3.9	8.6	5.9	5.7	5.5	25.7	30.4	—
	P4	2.8	9.7	7.3	6.8	8.5	32.3	38.1	—
	P5	2.5	4.4	3.4	6.9	4.7	19.4	22.9	—
	P6	6.0	5.0	2.2	2.0	1.7	10.9	12.9	—
	H1	0.0	0.3	0.4	0.5	0.2	1.4	1.7	1.7
Acetylhydrazine (μg)	P1	31	53	22	34	77	186	344	—
	P2	10	5	60	112	105	282	522	—
	P3	28	546	316	328	409	1599	2963	—
	P4	15	338	475	428	465	1706	3158	—
	P5	57	472	480	1032	202	2186	4047	—
	P6	270	250	250	310	590	1400	2592	—
	H1	0	36	77	104	83	300	555	0.6
Free hydrazine (μg)	P1	33	51	33	26	143	253	1083	—
	P2	13	4	46	110	76	236	1010	—
	P3	14	190	127	86	100	503	2153	—
	P4	23	69	78	49	59	255	1092	—
	P5	7	39	44	64	26	173	741	—
	P6	263	355	332	295	276	1258	5386	—
	H1	0	11	28	44	64	147	629	0.6

P: Tuberculous patient, H: Healthy volunteer.

a) Sampling just before administration.

b) Each value of INH includes the amounts of INH-hydrazones (pyruvic hydrazone, α-ketoglutaric hydrazone) which were hydrolyzed under our experimental condition (pH 6.0).

c) Each value indicates the converted value into INH.

TABLE IV. Dose Schedule to Subjects

Subject	Age	Sex	Administration period (month)	Prescription	Dosage form
P1	76	M	6	INH 400 mg/day (at 9 AM, 9 PM after meal)	Powder
P2	26	F	4	INH 400 mg/day	Powder
P3	27	M	5	Ethambutol 1000 mg/day } (at 9 AM, 9 PM after meal)	
P4	16	M	2	Rifampicin 450 mg/day (at 7 AM before breakfast)	
P5	19	M	3	INH 400 mg/day	Powder
				Ethambutol 1000 mg/day } (at 9 AM, 9 PM after meal)	
				Lysozyme 150 mg/day } (at 9 AM after meal)	
				Glutathione 300 mg/day } (at 9 AM after meal)	
P6	37	M	5	Rifampicin 450 mg/day (at 7 AM before breakfast)	Powder
				INH 600 mg/day	
				Ethambutol 1000 mg/day } (at 9 AM, 9 PM after meal)	
				Rifampicin 450 mg/day (at 7 AM before breakfast)	
HI(R)	57	M		INH 100 mg (before breakfast)	Solution

(R): rapid acetylator, M: male, F: female.

acetylhydrazine and diacetylhydrazine gave a negative test even when S-9 fraction was used.

In this paper, we could not refer to the problem of the individual difference on the excretion and metabolism of INH, because the number of subject was insufficient for the epidemiological study. It is the purpose of this paper to report the finding that the formation of free hydrazine, one of the well-known mutagens, takes place in man, and was slowly excreted into the urine after the oral administration of INH.

Acknowledgement The authors thank professor Dr. H. Ibayashi and the staffs in 3rd department of Internal Medicine, Kyushu University Hospital, for collection of urine specimens from patients on INH treatment. Thanks are also due to Dr. N. Otsuji in the department of microorganism of our faculty for providing information for the mutagenesis test.

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