*Journal of Chromatography*, 230 (1982) 345—352
*Biomedical Applications*
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1256

# STUDIES OF ISONIAZID METABOLISM IN ISOLATED RAT HEPATOCYTES BY MASS FRAGMENTOGRAPHY

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(First received November 23rd, 1981; revised manuscript received February 10th, 1982)

SUMMARY

Isoniazid metabolism in isolated rat hepatocytes was studied by mass fragmentography using single ion monitoring. Isoniazid and its metabolites were determined as the trimethylsilylated derivatives of acetylisoniazid and diacetylhydrazine and of the benzaldehyde hydrazones of isoniazid and acetylhydrazine. Deuterated analogues served as internal standards. Hydrazine was quantitated as benzalazine using $^{15}N$-labeled hydrazine as an internal standard. The method is well suited for the microanalysis of isoniazid metabolites in specificity and reliability to demonstrate the overall pathway of isoniazid metabolism, from which it was clarified that the greater part of hydrazine, a hazardous metabolite of isoniazid, was formed through the direct hydrolysis of isoniazid itself as expected.

## INTRODUCTION

The metabolism of isoniazid (INH), a drug widely used in tuberculosis chemotherapy, has been extensively studied in human and experimental animals, as described in the previous paper [1]. Enzymatic acetylation, hydrolysis and conjugation result in the formation of such diverse metabolites as acetylisoniazid (AcINH), acetylhydrazines, pyruvic hydrazone, isonicotinic acid and isonicotinuric acid. A large portion of INH ingested is excreted into urine as metabolites. Assay techniques established by us, part of which has already been published [1—5], have facilitated the detection and accurate

0378-4347/82/0000—0000/$02.75 

determination of such metabolites in biological fluids. Using the method, we detected constant urinary excretion of free hydrazine (Hz) by patients receiving INH, which drew much attention because of its possible hepatotoxicity and mutagenicity [1]. Although the concentration of the compound found in urine reflects the balance of production and elimination in tissues, the amount of Hz at the site of formation could be higher than that found in the urine. In fact, active formation of Hz, as well as AcINH, from INH in isolated rat hepatocytes has been noted [4]. In relation to this finding, we describe here our assay techniques in detail and the time—course of Hz formation from INH using the rapid and sensitive method.

## EXPERIMENTAL

### *Chemicals*

Collagenase (*Clostridium histolyticum*) was purchased from Boehringer Mannheim (Mannheim, G.F.R.). Bovine serum albumin (demineralized) was the product of Povite Producten, Amsterdam, The Netherlands. Amino acid mixture (without L-glutamine) was obtained from Grand Island Biological Co., Grand Island, NY, U.S.A. $d_3$-AcINH and $d_3$-diacetylhydrazine ($d_3$-DAcHz) were obtained by the acetylation of INH and acetylhydrazine (AcHz) with deuteroacetic anhydride-$d_6$ in deuteroacetic acid-$d_4$. $d_3$-AcHz hydrochloride was synthesized from *tert.*-butylcarbazate (Sigma, St. Louis, MO, U.S.A.) using a modified method of Nelson et al. [6]. All other chemicals were of reagent grade.

### *Gas chromatography—mass spectrometry*

Gas chromatography—mass spectrometry (GC—MS) was carried out on a system comprising a Shimadzu GC-MS 7000, MID-PM. GC separation was performed by using a glass column (1 m × 3 mm I.D.) packed with 1.5% OV-17 on Shimalite W (80—100 mesh). Helium was used as a carrier gas (flow-rate, 30 ml/min). The analytical conditions are listed in Fig. 1.

### *Preparation and incubation of isolated rat hepatocytes*

Isolated hepatocytes were prepared from male Wistar rats, 280—320 g, by the collagenase perfusion method as described by Moldéus et al. [7]. The viability of the cells was 98—99% according to the lactic dehydrogenase latency test and trypan blue exclusion [8]. The hepatocytes were suspended in Krebs-Hensleit buffer, pH 7.4, containing 1% bovine serum albumin, 10 m*M* glucose, amino acid mixture (Gibco), 13 m*M* Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and penicillin (400 IU/ml), and were incubated with a substrate in a rotating round-bottom flask at 37°C under a stream of oxygen—carbon dioxide (95:5). In order to obtain the apparent $K_M$ and $V_{max}$ values, $5 \cdot 10^{-5}$ to $1 \cdot 10^{-3}$ *M* substrates and $4 \cdot 10^6$ cells/ml of hepatocytes were employed. For constructing a time—course of INH metabolism, 0.5 m*M* INH and $7 \cdot 10^6$ to $8 \cdot 10^6$ hepatocytes/ml were used.

### *Sample preparation and extraction*

After incubation for a certain time period, 4-ml aliquots of the mixture were transferred to a test tube with 4 ml of phosphate buffer solution, pH 6.0,

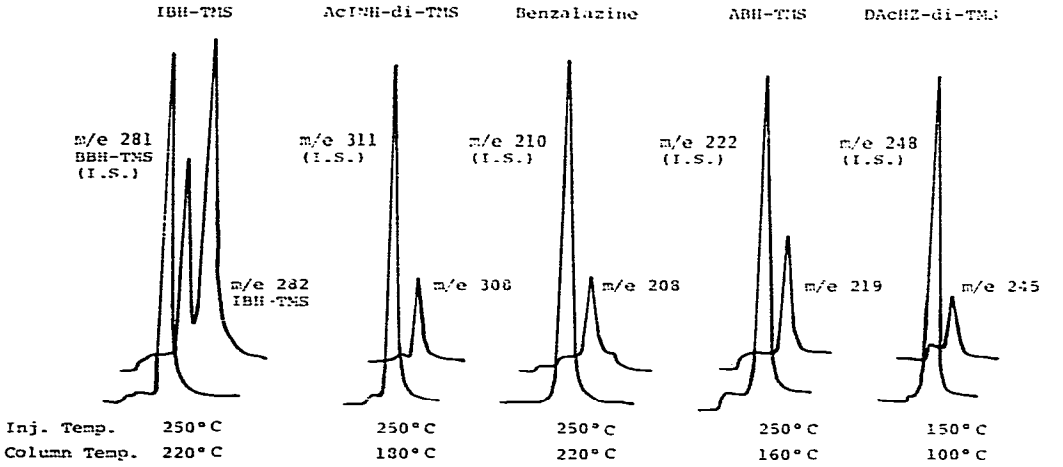


Fig. 1. Mass fragmentograms of derivatives of INH and its metabolites. Column: 1.5% OV-17 on Shimalite W (80—100 mesh), 1 m × 3 mm glass column. MS conditions: accelerating voltage, 3 kV; ionizing current, 60 $\mu$A; ionizing energy, initial 20 eV, jump 70 eV; separator temperature, 250°C.

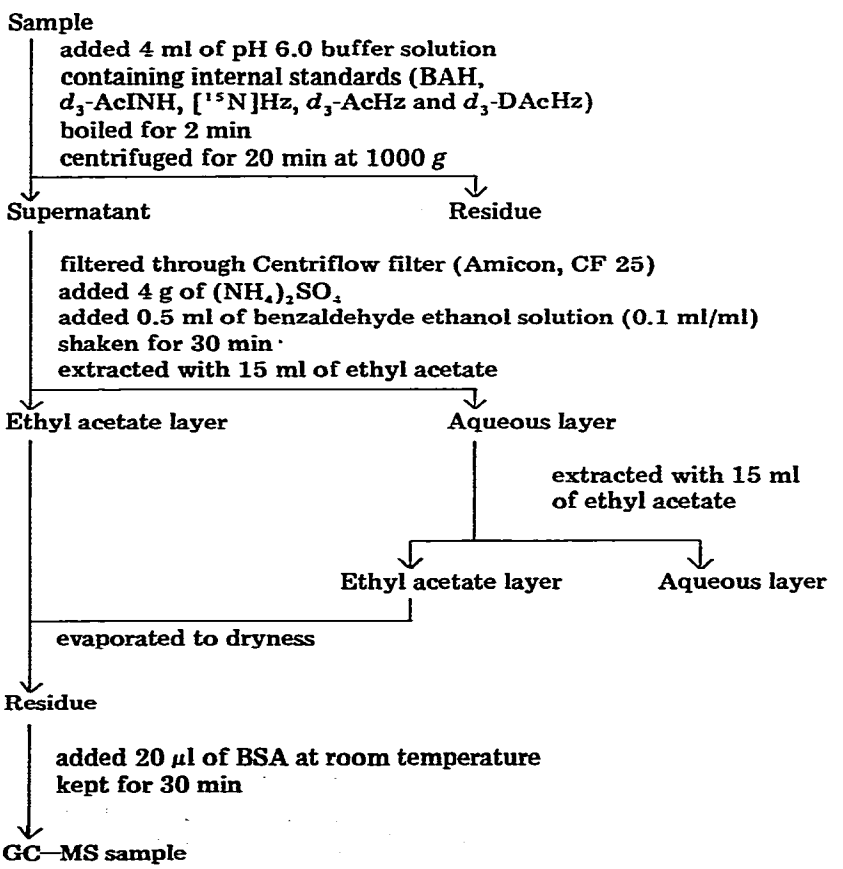


Fig. 2. Sample preparation for mass fragmentography of INH and its metabolites.

containing [$^{15}$N]Hz (2 μg/ml), $d_3$-AcINH (10 μg/ml), $d_3$-DAcHz (2 μg/ml), $d_3$-AcHz (1 μg/ml) and benzoic acid hydrazide (BAH, 50 μg/ml) as internal standards. The mixed solution was boiled for 2 min to terminate the reaction. After cooling on ice, the tubes were centrifuged for 20 min at 1000 *g* and the supernatants were filtered through Centriflow filters (Amicon, CF 25). The filtrates thus obtained were extracted with ethyl acetate as shown in Fig. 2.

*Derivatization for GC—MS*

As shown in Fig. 3, INH, BAH, AcHz, $d_3$-AcHz, Hz and [$^{15}$N]Hz were derivatized with benzaldehyde to give 1-isonicotinoyl-2-benzylidene-hydrazine (IBH), 1-benzoyl-2-benzylidene-hydrazine (BBH), 1-acetyl-2-benzylidene-hydrazine (ABH), $d_3$-ABH, benzalazine and [$^{15}$N]benzalazine, respectively. Further derivatization with N,O-bistrimethylsilylacetamide (BSA) was necessary for IBH, BBH, AcINH, $d_3$-AcINH, ABH, $d_3$-ABH, DAcHz and $d_3$-DAcHz prior to the GC injection to give the corresponding trimethylsilylates as shown in Fig. 4.

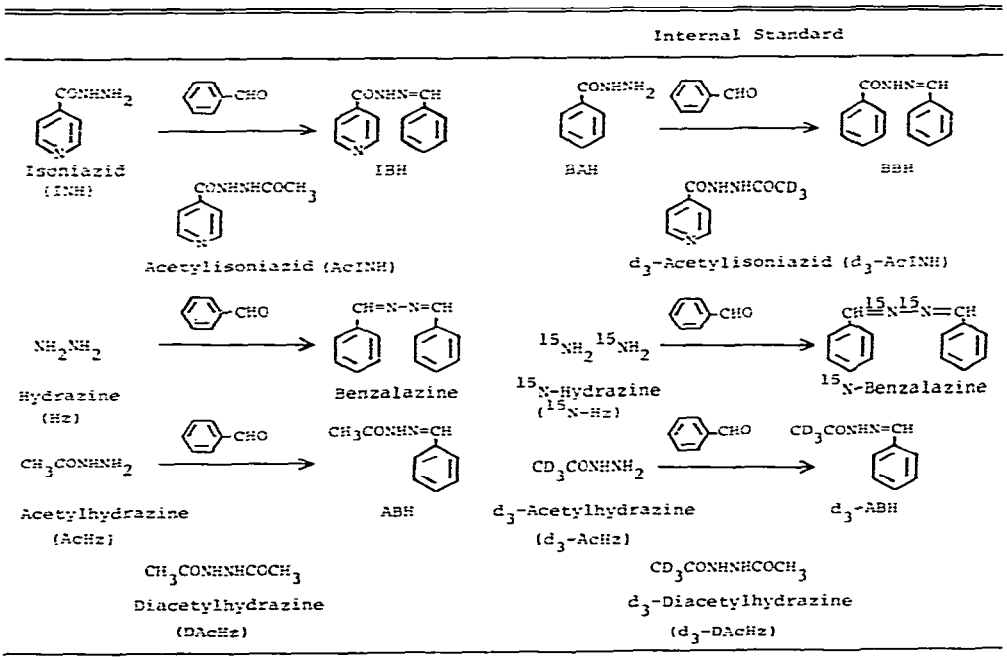


Fig. 3. Derivatization employed for GC—MS.

## RESULTS AND DISCUSSION

*Determination of INH and its metabolites by mass fragmentography*

INH and its metabolites were determined by GC—MS. For mass fragmentography using single ion monitoring, the ions at *m/e* 282 (281), 308 (311), 208 (210), 219 (222) and 245 (248) were selected for INH, AcINH, Hz, AcHz and DAcHz, respectively, with the internal standard in parentheses.

Each metabolite was determined successfully as follows.

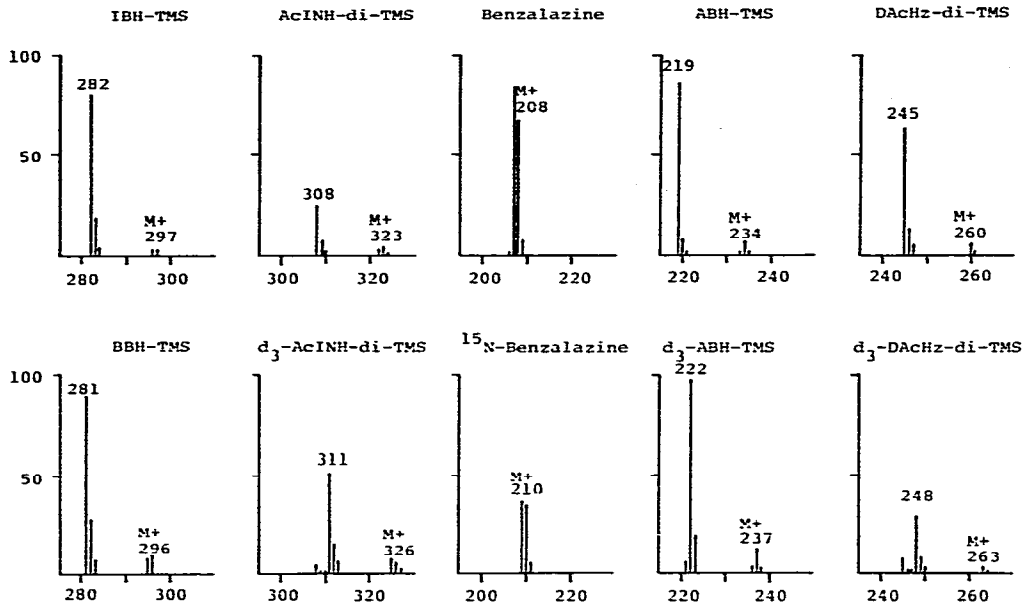


Fig. 4. Mass spectra of derivatives of INH and its metabolites.

(1) Intact INH. The present assay improved the detection limit by a factor of 10 compared to that in the previous method using GC [1].

(2) Hz. The same method that has already been reported was employed [1].

(3) AcINH and DAcHz. In comparison with the GC method used [1] the present assay in which acid hydrolysis was unnecessary prior to the extraction is very simple. Reliable data were obtained by using $d_3$-AcINH and $d_3$-DAcHz as internal standards.

(4) AcHz. In the previous work [1] the ion peaks at *m/e* 162 for ABH derived from AcHz and at *m/e* 133 for [$^{15}$N]benzalazine derived from [$^{15}$N]Hz (internal standard) were employed for monitoring. However, the method is not suitable for the accurate determination of AcHz, because the difference in mass range of the two peaks is more than 15%. The problem was solved by using $d_3$-AcHz as an internal standard.

Table I indicates the accuracy of determination of INH and its metabolites. The assay method is very reliable for the microdetermination, since the values of the regression coefficient of all compounds were distributed around 1.00. It is very important for the assay that calibration curves are made every time the experiment is performed and that the standard samples are treated by the same procedure as shown in Fig. 2.

### *$K_M$ and $V_{max}$ values of INH metabolism in isolated rat hepatocytes*

Since the isolated hepatocyte system catalyses sequential drug metabolizing reactions including phase I and II under conditions similar to those in vivo and different from those in rat liver homogenate (S-9 mixture), the system could serve as a suitable model for investigating INH metabolism.

Isolated rat hepatocytes were incubated with each substrate (INH, AcINH, AcHz or Hz) and the product formed was determined by mass fragmentography

TABLE I

ACCURACY OF DETERMINATION OF INH AND ITS METABOLITES BY MASS FRAGMENTOGRAPHY

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

| Compound | Concentration range of calibration curve (μg/ml) | Regression coefficient | Standard deviation (μg/ml) |
|---|---|---|---|
| Isoniazid | 2.0—10.0 | 0.99 | ± 1.550 |
| Acetylisoniazid | 0.8—4.0 | 1.00 | ± 0.391 |
| Hydrazine | 0.08—0.40 | 0.99 | ± 0.062 |
| Acetylhydrazine | 0.4—2.0 | 0.99 | ± 0.310 |
| Diacetylhydrazine | 0.4—2.0 | 0.98 | ± 0.438 |

TABLE II

APPARENT $K_M$ AND $V_{max}$ VALUES OF EACH METABOLIC PATHWAY OF INH IN ISOLATED RAT HEPATOCYTES AT 37°C

Substrate concentration: $5 \cdot 10^{-5}$ to $1 \cdot 10^{-3}$ *M*.

| Metabolic pathways | $K_M$ (m*M*) | $V_{max}$ (nmole per min per $4 \cdot 10^6$ cells) |
|---|---|---|
| Hydrolysis | | |
| INH → Hz | 0.19 | 1.8 |
| AcINH → AcHz | 0.38 | 1.1 |
| AcHz → Hz | 0.88 | 11.8 |
| Acetylation | | |
| INH → AcINH | 0.03 | 1.5 |
| Hz → AcHz | 0.16 | 2.4 |
| AcHz → DAcHz | 0.28 | 6.0 |

as mentioned above. Table II indicates the apparent $K_M$ and $V_{max}$ values of INH metabolic routes which were calculated using Lineweaver—Burk plots.

In the case of hydrolysis, the $K_M$ values indicate that the reaction from INH to Hz ($K_M$ = 0.19 m*M*) takes place easier than that from AcINH to AcHz ($K_M$ = 0.38 m*M*) and that from AcHz to Hz ($K_M$ = 0.88 m*M*). Therefore, Hz might be directly formed by a simple hydrolysis of INH itself, and Hz formation from AcHz seems to be negligible.

As for acetyl conjugation, it is concluded that AcINH formation from INH ($K_M$ = 0.03 m*M*) takes place predominantly as expected, in comparison with the formation of AcHz from Hz ($K_M$ = 0.16 m*M*) and with that of DAcHz from AcHz ($K_M$ = 0.28 m*M*).

*Time—course of INH and its metabolites in isolated rat hepatocytes*

In order to study a definite pathway of INH metabolism, especially for Hz formation, an additional experiment was performed. Isolated rat hepatocytes were incubated with 0.5 m*M* INH, and the amounts of AcINH, Hz, AcHz and DAcHz formed were determined. INH remaining unchanged in the system was

also quantitated simultaneously at 0, 10, 20 and 30 min. Representative data of the percentage INH eliminated and the metabolites formed are listed in Table III. Fig. 5 indicates the average values of three experiments. AcINH was formed in a linear fashion with time at the rate of about 0.3 nmol per $10^6$ cells per min. AcHz and DAcHz were also formed, though the amounts were much smaller than that of AcINH. For Hz, 1.92 and 3.95 nmol per $10^6$ cells were detected at 5 and 10 min. Particularly interesting is the fact that as much Hz was produced as AcINH after 10 min of incubation, and the Hz produced began to decrease from 10 min and disappeared at 15 min after incubation.

TABLE III

PERCENTAGE INH ELIMINATION AND METABOLITE FORMATION IN ISOLATED RAT HEPATOCYTES

Initial concentration of INH = 0.5 m*M*. Number of liver cells = $8 \cdot 10^6$ cells/ml.

| Products | Incubation time (min at 37°C) | | | |
|---|---|---|---|---|
| | 0 | 10 | 20 | 30 |
| INH | 100.0 % | 79.2 % | 77.8 % | 71.4 % |
| AcINH | 0.0 | 4.1 | 6.8 | 10.3 |
| DAcHz | 0.0 | 0.6 | 0.9 | 1.6 |
| AcHz | 0.0 | 0.2 | 0.5 | 0.8 |
| Hz | 0.0 | 8.4 | 2.7 | 0.8 |

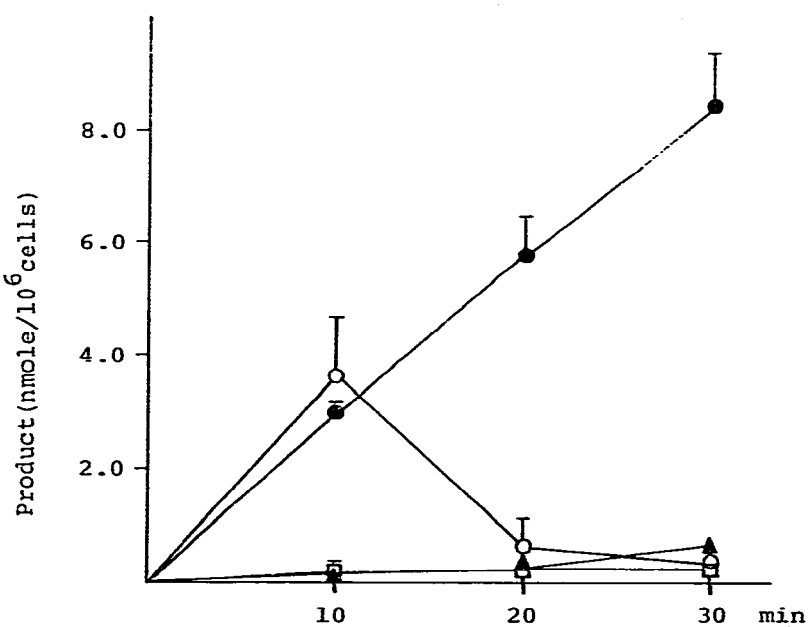


Fig. 5. Time—course of INH metabolism in isolated rat hepatocytes. Isolated hepatocytes were incubated with 0.5 m*M* INH and the metabolites formed were determined as described in the text. Vertical bars represent standard errors of the means. (●—●) AcINH, (○—○) Hz, (▲—▲) AcHz, (□—□) DAcHz.

It is already known that the hydrolytic process of INH is inhibited by AcINH [5]. Therefore, if the rate of Hz degradation remains unaltered, the detectable Hz could decrease as AcINH accumulates. We examined the influence of AcINH on Hz formation from INH in a rat liver homogenate system; the time—course indicated inhibition of Hz formation from INH ($5 \cdot 10^{-4}$ *M*) by AcINH ($2 \cdot 10^{-4}$ *M*) from 5 min after incubation. Further experiments are in progress and the details will be reported soon.

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, and also by a grant from the Shimabara Science Promotion Foundation, Japan.

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