TISSUE DISTRIBUTION OF ISONIAZID AND ITS METABOLITES IN RATS

YOSHIHARU KANEKO, HIROAKI KUBO, TETSURO TABATA, KENJI MATSUYAMA, ATSUKO NODA AND SADAO IGUCHI*

Faculty of Pharmaceutical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka, 812, Japan

(Received February 20, 1981)

Distribution of isoniazid and its metabolites was observed in the liver, kidney, lung and plasma after the subcutaneous administration of isoniazid to rats. The tissue levels of isoniazid, acetylisoniazid, acetylhydrazine, 1,2-diaceetylhydrazine and hydrazine were determined by mass fragmentography using a gas chromatograph-mass spectrometer equipped with a multiple ion detector-peak matcher. Using the compounds labeled with a stable isotope as an internal standard, namely the isotope dilution method, made it possible to estimate trace amounts of these metabolites in the tissues. The amount of hydrazine was much less than the other hydrazines, but the metabolite which is well known as a mutagen, could be successfully detected in the tissues and plasma. The greater part of free hydrazine is formed through a direct hydrolysis of isoniazid. The isoniazid-hydrorlyzing activity was found to be significantly higher in the liver homogenate. This suggested that hydrazine formation is mainly caused by hepatic hydrolysis.

Keywords—tissue distribution; isoniazid; acetylisoniazid; acetylhydrazine; 1,2-diaceetylhydrazine; hydrazine; gas chromatography-mass spectrometry

Isoniazid (isonicotinic acid hydrazide, INH) has been widely used as a potent antituberculosis drug. Hydrolysis and direct conjugation are the primary pathways in the metabolism of the drug. The major metabolites detected in urine resulting from isoniazid administration to humans are intact INH, pyruvic acid isonicotinoylhydrazone, α-ketoglutaric acid isonicotinoylhydrazone, acetylisoniazid, isonicotinic acid, isonicotinuric acid, acetylhydrazine, and diacetylhydrazine (1,2-diaceetylhydrazine). Since the C-N bond between the aromatic carbonyl and the hydrazine moieties is labile, it is well known that hydrazine can be formed from the hydrolysis of INH to isonicotinic acid, but this compound has not yet been detected in human urine.

Acetylation is the major route of inactivation for INH, and the acetylation polymorphism is now well established. Mitchell et al. revealed that rapid acetylators hydrolyze much more INH to isonicotinic acid and the free hydrazino moiety than do slow acetylators. They have suggested that release of acetylhydrazine, the hepatotoxic hydrazino moiety of INH in man, is responsible for isoniazid liver injury.

On the other hand, pharmacological and biochemical effects of hydrazine have been described, such as hypoglycemia, increased ammonia levels in the blood and spinal fluid, decreased hepatic lipid mobilization, blood dyscrasias and convulsions. While it has been established for a long time that INH induces tumors in various animal species, Banciifiori et al. reported that hydrazine sulfate also induces liver and lung tumors in mice. They suggested that the tumors observed after the administration of INH to the mouse are mainly due to the liberation of hydrazine. This idea was supported by the
work of Braun et al.\textsuperscript{15} on the mutagenicity of INH, suggesting that hydrazine represents the active principle in INH mutagenesis in mice. More recently, Durant and Harris\textsuperscript{16} pointed out the possibility that hydrazine may induce a syndrome similar to systemic lupus erythematosus, presenting the case of the researcher who had used hydrazine sulfate routinely in a laboratory.

So, we developed a method for estimating the hydrazines, such as hydrazine (HZ), acetylhydrazine (AcHZ) and diacetylhydrazine (DiAcHZ) as well as INH and acetylsalicylic acid (AcINH) by gas chromatography-mass spectrometry.\textsuperscript{17} By using this method, we successfully found free hydrazine in the urine of patients on INH-treatment.\textsuperscript{18} The tissue distribution of INH and its metabolites was, therefore, examined to confirm the formation of free HZ, and to elucidate the mechanism of formation of such a harmful metabolite.

MATERIALS AND METHODS

\textit{Chemicals} — The specific reagent grade INH and hydrazine sulfate were purchased from Wako Pure Chemicals Ind. Ltd., the specific reagent grade of benzoic acid hydrazide (BAH) and AcHZ from Tokyo Chemical Ind. Co., Ltd. Hydrazine sulfate-\textsuperscript{15}N\textsubscript{2} (99 atom\%) and acetic anhydride-\textsuperscript{14}N\textsubscript{2} (99 atom\%) were obtained from the British Oxygen Co., Ltd. and Merck Sharp & Dohme Canada Ltd. respectively. AcINH and DiAcHZ were prepared by the known methods.\textsuperscript{19,20} Trideuterocarboxylates of these compounds, \textit{d}_3-\textit{Ac}{\textit{INH}} and \textit{d}_3-\textit{Di}{\textit{AcHZ}}, were synthesized by the same methods using acetic anhydride-\textit{d}_6 instead of acetic anhydride. Trideuterocarboxylhydrzone (\textit{d}_3-\textit{Ac}{\textit{HZ}}) was prepared as the hydrochloride salt by the method of Nelson \textit{et al.}\textsuperscript{5}

\textit{Animal Experiment} — Male, Wistar rats weighing 250—300 g, fasted for 12 h, were given a subcutaneous injection of 40 mg/kg of INH dissolved in 1 ml of saline. Liver, kidney, lung and blood samples were taken at 1, 2 and 4 h after the drug administration.

\textit{HZ Formation in Vitro} — One part of tissues, such as liver and kidney, and four parts of ice cold \textit{Sørensen} buffer (1/15 mol/l, pH 7.4) were homogenized for 2 min in a Potter glass tissue grinder. Lung homogenate was prepared by using a Waring blender at the same dilution. The standard incubation mixture contained 5 ml of the homogenates and 1 ml of INH solution. The final concentration of the drug was fixed at 2.50 × 10\textsuperscript{-2} M. The incubation was performed in a water bath at 37°C by shaking for 30 min. After adding \textit{\textit{N}}\textsubscript{2}-\textit{HZ} solution as an internal standard, the enzymatic reaction was stopped immediately by the addition of 2 ml of 10\% (w/v) trichloracetic acid. The incubation mixture was centrifuged at 3000 rpm for 10 min, and the supernatant was adjusted to pH 7 by the addition of 0.5N NaOH. The amount of HZ was estimated by the method described below. Parallel studies on incubation mixture without the homogenate were also carried out.

\textit{Assay Procedure} — One to two g of the tissue samples, 5 ml of saline and 1 ml of mixed solution containing benzoic acid hydrazide, \textit{d}_3-\textit{Ac}{\textit{INH}}, \textit{\textit{N}}\textsubscript{2}-\textit{HZ}, \textit{d}_3-\textit{Ac}{\textit{HZ}} and \textit{d}_3-\textit{Di}{\textit{AcHZ}} as an internal standard, were homogenized. The enzymatic reaction was stopped by the addition of 4 g of (\textit{NH}_4\textsubscript{2})\textsubscript{5}SO\textsubscript{4}. The homogenate was centrifuged at 6000 × g for 10 min, and the supernatant was filtered to remove protein using a membrane cone (Centriflo F25, Amicon). The filtrate was placed in a centrifuge tube containing 5 g of (\textit{NH}_4\textsubscript{2})\textsubscript{5}SO\textsubscript{4} which was used to salt out the water-soluble metabolites. Half a ml of 5\% (v/v) benzaldehyde ethanol solution was then added to the filtrate. The mixture was shaken for 30 min at room temperature, then extracted twice with 15 ml of ethyl acetate by shaking for 20 min and centrifuging for 10 min. The organic layer was filtered through anhydrous sodium sulfate to remove water, then combined and evaporated to dryness. The residue was trimethylsilylated with N\textsubscript{2}O-bistrimethylsilylacetyamide in pyridine. One μl of this solution was injected into a gas chromatograph-mass spectrometer.

\textit{Gas Chromatography-Mass Spectrometry (GC-MS)} — The instrument used was a Shimadzu
MS 7000 equipped with a multiple ion detector-peak matcher. A glass column, 1.0 m in length and 3 mm i.d., containing 1.5% OV-17 coated onto 80/100 mesh Shimalite W, was used. The operating temperature were as follows: injection port for the DiAcHZ measurement: 150°C and for the others: 250°C; oven for the DiAcHZ measurement: 100°C, AcHZ: 160°C, AcINH: 195°C, HZ and INH: 220°C. Mass spectrometer conditions were as follows: accelerating voltage, 3 kV; ionizing current, 300 μA; ionizing energy, 23 eV; separator temperature, 260°C.

INH and its internal standard BAH, AcHZ, \(d_3\)-AcHA, HZ and \(^{15}\)N\(_2\)-HZ were mixed in aqueous solution with benzaldehyde to form 1-isonicotinoyl-2-benzylidene-hydrazine (IBH), 1-benzoyl-2-benzylidenehydrazine (BBH), 1-acetyl-2-benzylidene-hydrazine (ABH), \(d_3\)-ABH, benzalazine and \(^{15}\)N\(_2\)-benzalazine, respectively. Furthermore, these compounds except benzalazine each were mono- or ditrimethylsilylated (TMS or di-TMS) by the addition of \(\text{N,O-bistrimethylsilylacetamide. Mass fragmentography was employed in the analysis of these derivatives. To determine the amount of HZ, the relative height of the molecular ion peak of benzalazine at \(m/z\) 208(M\(^+\)) to that of \(^{15}\)N\(_2\)-benzalazine at \(m/z\) 210 (M\(^+\)) was measured. In the case of INH, the relative peak height of IBH-TMS at \(m/z\) 282 (M\(^+\)-15) to BBH-TMS at \(m/z\) 281 (M\(^+\)-15) was determined; AcINH, AcINH-di-TMS at \(m/z\) 308 (M\(^+\)-15) to \(d_3\)-AcINH-di-TMS at \(m/z\) 311 (M\(^+\)-15); AcHZ, ABH-TMS at \(m/z\) 219 (M\(^+\)-15) to \(d_3\)-ABH-TMS at \(m/z\) 222 (M\(^+\)-15); DiAcHZ, DiAcHZ-di-TMS at \(m/z\) 245 (M\(^+\)-15) to \(d_3\)-DiAcHZ-di-TMS at \(m/z\) 248 (M\(^+\)-15). More detailed information has been previously described\(^{17,18}\).

RESULTS AND DISCUSSION

Fig. 1 shows the tissue levels of INH after its administration to rats. The subcutaneous injection resulted in a rapid absorption and distribution of the drug to the tissues in which the maximum

![Graph](image1)

**FIG. 1.** Liver (●), Kidney (○), Lung (△) and Plasma (×) Concentrations of INH after Subcutaneous Administration of INH (40 mg/kg) in the Rat.

Each point indicates the mean value of 3—9 rats. The vertical bar represents standard deviation of the mean. * p < 0.01, when compared with the other tissue levels.

![Graph](image2)

**FIG. 2.** Liver (●), Kidney (○), Lung (△) and Plasma (×) Concentrations of AcINH after Subcutaneous Administration of INH (40 mg/kg) in the Rat.

Each point indicates the mean value of 3—9 rats. The vertical bar represents standard deviation of the mean.
TABLE 1.  Concentration of Hydrazines in Tissues after Subcutaneous Administration of Isoniazid (40 mg/kg) in the Rat

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Concentrations in tissues (µg/g or ml)</th>
<th>1 h</th>
<th>2 h</th>
<th>4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylhydrazine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>1.47±0.42</td>
<td>1.66±0.34</td>
<td>1.46±0.48</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>0.25±0.18</td>
<td>1.19±0.17</td>
<td>2.12±0.57</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>1.20±0.77</td>
<td>1.73±0.53</td>
<td>1.73±0.30</td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>1.15±0.85</td>
<td>0.82±0.34</td>
<td>1.85±0.39</td>
<td></td>
</tr>
<tr>
<td>Diacetylhydrazine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>N.D.</td>
<td>0.40±0.14</td>
<td>0.34±0.08</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>N.D.</td>
<td>0.44±0.22</td>
<td>0.37±0.21</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>N.D.</td>
<td>0.39±0.05</td>
<td>0.50±0.06</td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>N.D.</td>
<td>0.24±0.13</td>
<td>0.30±0.07</td>
<td></td>
</tr>
<tr>
<td>Hydrazine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>0.21±0.20</td>
<td>0.52±0.47</td>
<td>0.15±0.08</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>N.D.</td>
<td>0.45±0.26</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>0.18±0.07</td>
<td>0.21±0.10</td>
<td>N.D.</td>
<td></td>
</tr>
</tbody>
</table>

Values represent the mean ± S.D. of 3–9 rats. 
N.D.: not detectable.

**Chart 1. Main Metabolic Pathways of Isoniazid**

The arrows indicate the kind of metabolism; →→: acetylation, →→→: hydrolysis.

levels were attained within 1 h. As shown in Fig. 1, the INH concentrations in the kidney, especially that of 1 h after the drug administration, were higher than those in the other tissues. Roth and Manthei examined the distribution of 14C-labeled INH in mice, and also reported that a higher radioactivity was detected in the kidneys during the first hour. This reflects the early excretion of the drug by this organ and may be due to the active uptake. On the other hand, the lowest level of INH was detected in the liver. The elimination half-lives in the liver, kidney, lung and plasma were 1.47, 0.73, 0.88 and 0.56 h respectively, and the majority of the drug disappeared within 4 h.

The tissue levels of the main metabolite, AcINH are shown in Fig. 2. The level ranged from 10 to 30 µg/g or ml and peaked in 1–2 h. The time lag in the appearance of the metabolite is attributed to the rate-determining step of acetylation. Therefore, significant amounts of AcINH were detectable even if the parent drug had disappeared from the body.

The concentrations of the three hydrazines are summarized in Table I. As shown in Chart 1, AcHZ is formed partly by hydrolysis of AcINH and partly by acetylation of HZ, and was the
major metabolite of these hydrazines. Timbrell and Wright examined the urinary metabolites of rats which were given $^{14}$C-INH at two dose levels, 5.5 mg/kg and 55 mg/kg. They reported that the proportional excretion of AcHZ was not significantly affected by the dose of INH but a much smaller fraction of the dose was excreted as diacetylhydrazine at a higher dose level. From their results, it is assumed that acetylation of AcHZ produced as a metabolite may be inhibited by the parent drug at the high dose (40 mg/kg) used in our experiment. The level of DiAcHZ found in the tissue was relatively low and was undetectable at 1 h after the drug administration. The amount of HZ was also smaller than AcHZ, but the metabolite which is well known as mutagen, was successfully detected in the tissues and plasma.

Deacetylation of acetylated hydrazine derivatives is not a frequently observed metabolic pathway. However, as shown in Chart 1, two probable metabolic routes which produce free HZ can be suggested, i.e., direct hydrolysis of INH and deacetylation of AcHZ. In order to elucidate the mechanism of HZ formation, INH metabolism including an acetyulating process had been previously examined using isolated hepatocytes of rats. As indicated by the arrows in Chart 1, the hydrolysis activity ($1/K_m$) obtained in this cell suspension can be arranged in the following order of magnitude: INH $\rightarrow$ HZ $\rightarrow$ AcINH $\rightarrow$ AcHZ $\rightarrow$ AcHZ $\rightarrow$ HZ; and the acetylation activity: INH $\rightarrow$ AcINH $\rightarrow$ HZ $\rightarrow$ AcHZ $\rightarrow$ AcHZ $\rightarrow$ DiAcHZ. The low extent of deacetylation of AcHZ and the high hydrolysis activity for INH clearly demonstrated that the greater part of HZ was formed through the direct hydrolysis of INH.

Fig. 3 shows the relative activity to hydrolyze INH in the tissue homogenate. Relatively high activity could be found in the liver homogenate, and in the kidney and lung homogenates a little activity was detected. This indicates that hydrazine formation is mainly due to hepatic hydrolysis, that is, the HZ which is formed in the liver may be distributed to the whole body. However, we could not detected HZ in the liver (Table I). In view of the high reducing capacity of HZ, it is not surprising that a significant portion of the hydrazine nitrogen is converted to N$_2$ or NH$_3$. Therefore, we may fail to detect the metabolic intermediate free HZ in the liver with a high metabolic activity.

On the other hand, HZ could be constantly detected in the kidney (Table I). In a further study we found that HZ accumulates in the kidney. The details of this information will be reported in a subsequent publication.

REFERENCES


