HYDRAZINE FORMATION FROM IPRONIAZID

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After the administration of iproniazid to rabbits and rats, a harmful metabolite, hydrazine, was detected in urine and plasma by means of gas chromatography and gas chromatography-mass spectrometry. The amount of excreted hydrazine from iproniazid was much more than that from isoniazid.

**Keywords**—iproniazid; hydrazine formation; animal urine and plasma; oral, intravenous and subcutaneous administration; GC and GC–MS analyses

Iproniazid (IPN) which is an N-isopropyl derivative of isoniazid (INH) was originally synthesized as an antitubercular agent in 1951 but utilized as an antidepressant because of its pharmacological effect of MAO inhibition. However, it has no longer been used, since a report concerning its hepatic injury to patients was published in 1960. Nelson et al. have studied on the factors of hepatotoxicity induced by IPN as well as INH. Recently, they reported that monoacetylhydrazine from INH and isopropylhydrazine (Isop-HZ) from IPN might be the inducers of hepatic injury. However, they do not mention at all about a harmful metabolite, hydrazine (HZ) itself. In 1977, we succeeded in detection and estimation of HZ from the urinary extracts of patients on INH and hydralazine-treatment. The observations are very significant from the standpoint of safety in medication, because HZ is well-known for inducing hepatic injury, mutagenesis and carcinogenesis.

After the administration of IPN to rabbits and rats, HZ was also detected in the urine and plasma by means of gas chromatography (GC) and gas chromatography-mass spectrometry (GC–MS). Examination of a relationship between a drug structure and a harmful metabolite is indispensable for improving a drug in question. Therefore, we compared the excreted amount of HZ derived from IPN with that derived from INH, and discussed the mechanism of HZ formation in this paper.

**MATERIALS AND METHODS**

Chemicals — The specific reagent grade of IPN phosphate, benzoic acid hydrazide (BAH) and methylhydrazine sulfate were purchased from Tokyo Chemical Ind. Co., Ltd., INH and HZ sulfate from Wako Pure Chemical Ind., Ltd., and 15N-HZ sulfate (99.8 atom %) from the British Oxygen Co., Ltd. Isop-HZ sulfate was prepared from IPN. Anal. Calcd for C₈H₁₀N₂·H₂SO₄: C, 20.93; H, 6.98; N, 16.28. Found C, 20.81; H, 6.73; N, 16.33. 1-Isopropyl-3,5-dimethylpyrazole (IDP) was synthesized by the modified method for 3,5-dimethylpyrazole from HZ. MS $m/e$: 138 (M⁺). Colorless oil. Picrate was prepared, mp 133–135° (from AcOEt). Anal. Calcd for C₆H₁₄N₂·C₆H₃N₃O₇: C, 45.78; H, 4.63; N, 19.07. Found C, 45.90; H, 4.73; N, 18.95.

GC and GC–MS — The same instruments reported in the previous paper were employed for the analyses.

**Animals** — (a) Male albino rabbits weighing 2.7–3.3 kg were used after fasting for 16 hr prior
to the experiments. Water was maintained *ad libitum*. (b) Male Wistar rats weighing 200—250 g were used.

**Dosing** — (a) Oral Administration: IPN phosphate (303.3 mg/body weight) or INH (150 mg/body weight) was dissolved in 40 ml of distilled water and administered to rabbits by using a catheter.

(b) Intravenous Administration: IPN phosphate (202.1 mg/body weight) or INH (100 mg/body weight) was dissolved in 2 ml of sterile water for injection and administered to rabbits through the ear vein. The period of dosing was more than 7 days in rabbits cases (a) and (b).

(c) Subcutaneous administration: IPN phosphate (309.5 mg/kg) was given to rats by injection.

**Sampling** — (a) Rabbit: Urine was collected for 24 hr after dosing. Blood samples were taken from marginal vein at 2, 4, 6 and 8 hr after dosing by using a heparinized syringe.

(b) Rat: Blood samples were taken from the vena cava inferior at 0.5, 1, 2 and 4 hr after dosing by using a heparinized syringe under anesthesia for 3 min, respectively.

**Assay Procedure** — (a) Intact IPN: 0.5 ml of aqueous BAH solution (2.4 mg/ml) was added to 10 ml of urine sample as an internal standard. The pH of the mixture was adjusted to 6.0. After the addition of ammonium sulfate (5 g), the mixture was extracted twice with 10 ml of CH₂Cl₂. The combined extracts were dried over anhydrous sodium sulfate and concentrated to 100 µl. One µl was injected into the gas chromatograph. The chromatogram is shown in Fig. 1.

(b) Isop-HZ: 0.5 ml of aqueous methylhydra-

FIG. 1. *Gas Chromatogram of Iproniazid excreted in Rabbit Urine*  
GC conditions: 1% XE-60 on Chromosorb W (60—80 mesh), 3mm x 1m glass column, column temp.: 175°; inj. temp.: 210°; carrier gas: N₂, 40 ml/min, FID.  
Peak 1: benzoic acid hydrazide (BAH), 2: iproniazid.

FIG. 2. *Gas Chromatogram of 1-Isopropyl-3,5-dimethylpyrazole extracted in Rabbit Urine*  
GC conditions: 1.5% OV-17 on Shimalite W (80—100 mesh), 3mm x 1m glass column, column temp.: 70°; inj. temp.: 150°; carrier gas: N₂, 40 ml/min, FID.  
Peak 1: 1,3,5-trimethylpyrazole (TMP), 2: 1-isopropyl-3,5-dimethylpyrazole (IDP).
zine solution (0.4 mg/ml) was added to 10 ml of urine sample as an internal standard. The pH of the solution was adjusted to 6.0. Then 0.5 ml of ethanolic acetylacetone solution (0.1 ml/ml) was added. After shaking the mixture for 30 min, 1-isopropyl-3,5-dimethylpyrazole (IDP) and 1,3,5-trimethylpyrazole (TMP) formed were extracted twice with 10 ml of ether. The combined extracts were dehydrated and concentrated to 100 μl. One μl of the solution was injected into the gas chromatograph. The gas chromatogram is shown in Fig. 2.

(c) INH and HZ: Two of these compounds were assayed by the methods described in the previous paper.7)

(d) Metabolites from IPN in Plasma: Two ml of plasma was used. After deproteinization with trichloroacetic acid (10% solution), the volume was adjusted to 10 ml using a phosphate buffer solution (pH 6.0). The metabolites from IPN were also assayed by the same methods mentioned above [(a)–(c)].

RESULTS

Although the assay of IPN by GC has been well investigated, no adequate methods have been established. In 1976, Sagher et al. quantified IPN in patients' urine.10) In their method, a concentrated alkaline solution was used for extraction. From the results of our experiments, the -CONH group in IPN was so easily hydrolyzed to Isop-HZ in an alkaline medium that Sagher's method is not recommendable for IPN analysis. We think the GC method established by us is an excellent one without causing decomposition of IPN. In case of IPN determination, the accuracy and the precision were sufficient in three trials, i.e. regression coefficient, 0.99; standard error, ±0.006 (mg/ml); standard deviation, ±0.029 (mg/ml) in the range of 0.1–0.5 mg/ml.

As to derivatization of the hydrazino group in INH, some hydrazines and hydralazine, benzaldehyde has been used in our laboratory.7–9) However, because this reagent is not available for derivatization of alkylhydrazines to yield various kinds of derivatives,11) we tried to establish another method. It is well-known that HZ and methylhydrazine react with acetylacetone to yield the corresponding pyrazole quantitatively.12) Therefore, we used this reaction for determining Isop-HZ. Isop-HZ reacted with acetylacetone to yield IDP quantitatively, which is stable to heating, nonvolatile and easily extracted from an aqueous solution. On GC, a linear relationship was obtained in the calibration curve of IDP derived from Isop-HZ using TMP derived from

\[
\begin{align*}
\text{CH}_3\text{-CH-NH-NH}_2 & \quad + \quad \text{O} \quad \text{O} \\
\text{CH}_3 & \quad \text{CH}_2\text{-C-CH}_2\text{-C-CH}_3 \\
\text{Isop-HZ} & \quad \rightarrow \\
\text{CH}_3 \quad \text{N} \quad \text{N} \\
\text{CH}_3 \quad \text{CH} \quad \text{CH}_3 \\
\text{IDP} & \quad \text{O} \quad \text{O} \\
\text{CH}_3 \quad \text{C-CH}_2\text{-C-CH}_3 \\
\text{methylhydrazine} & \quad \rightarrow \\
\text{(I.S.)} & \\
\text{CH}_3 \quad \text{N} \quad \text{N} \\
\text{CH}_3 \quad \text{CH}_3 \\
\text{TMP} &
\end{align*}
\]
methylhydrazine as an internal standard. The accuracy and the precision were as follows; regression coefficient, 0.99; standard error, ±0.015 (μg/ml); standard deviation, ±0.023 (μg/ml) in the range of 20—100 μg/ml. After the addition of acetylacetone to the urine extract of IPN-treated rabbits, Isop-HZ was determined as IDP. The mass spectrum of IDP is shown in Chart 1.

HZ formed from IPN in rabbits was confirmed as benzalazine after the derivatization of HZ with benzaldehyde.

The total amounts of metabolites excreted in the urine samples during 24 hr after the oral administration of IPN to 5 rabbits are summarized in Table I. Every rabbit excreted Isop-HZ (1.5—2.6% of the dose) and HZ (2.3—5.5% of the

CHART 1. Mass Spectrum of 1-Isopropyl-3,5-dimethylpyrazole (IDP)

<table>
<thead>
<tr>
<th></th>
<th>Iproniazid</th>
<th>Isoniazid</th>
<th>Isopropylhydrazine</th>
<th>Hydrazine</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1</td>
<td>3.61</td>
<td>3.38</td>
<td>4.24</td>
<td>10.70</td>
<td>21.93</td>
</tr>
<tr>
<td>No. 2</td>
<td>10.36</td>
<td>2.53</td>
<td>2.91</td>
<td>8.12</td>
<td>23.92</td>
</tr>
<tr>
<td>No. 3</td>
<td>10.66</td>
<td>2.93</td>
<td>5.16</td>
<td>8.48</td>
<td>27.23</td>
</tr>
<tr>
<td>No. 4</td>
<td>2.86</td>
<td>2.49</td>
<td>4.95</td>
<td>7.06</td>
<td>17.36</td>
</tr>
<tr>
<td>No. 5</td>
<td>15.59</td>
<td>7.31</td>
<td>3.17</td>
<td>4.46</td>
<td>30.53</td>
</tr>
</tbody>
</table>

Dose: Iproniazid phosphate 303.30 mg/body weight.
Each value was determined by calculation from the converted value into iproniazid.
dose), but the individual differences were remarkable. Since both hydrazines are harmful metabolites, the observation described above affords a very important information.

Fig. 3 indicates the amounts of IPN and its metabolites in rat plasma after subcutaneous administration. The amount of INH which is one of the IPN metabolites was higher than that of Isop-HZ. The result coincided with Koechlin's report\textsuperscript{13} that deisopropylation of IPN is the main route. It is interesting that the plasma concentration of HZ was higher than that of Isop-HZ. Based on our observation that only a little amount of HZ can be derived from INH, HZ formation from IPN might occur mainly from another course, i.e. the deisopropylation of Isop-HZ. The same observation was obtained in the rabbits study as described below.

Elucidation of the relationship between the drug structure and HZ formation is indispensable for improving the antitubercular agent including the hydrazino group. After an equal mol of IPN or INH was administered orally or intravenously to the same rabbit by the cross over method, the amounts of HZ formed were determined respectively. As shown in Table II, the excreted amount of HZ in the urine collected for 24 hr from IPN-treated rabbits was significantly higher than that from INH-treated rabbits. In order to examine the plasma concentration of HZ, mass fragmentography was performed by the same method described in the previous paper.\textsuperscript{7} As shown in Fig. 4, the plasma concentration of HZ from IPN

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{The Plasma Concentration of (A) Iproniazid, (B) Isoniazid, (C) Hydrazine and (D) Isopropylhydrazine after Subcutaneous Administration of Iproniazid in 6 Rats Dose amount: 200 mg/kg. Results are expressed as means± S.E.}
\end{figure}

\begin{table}[h]
\centering
\caption{Excreted Amounts of Hydrazine by Rabbit in 24-hr Urine after Administration of Iproniazid and Isoniazid}
\begin{tabular}{lccccc}
\hline
          & \multicolumn{5}{c}{Excreted amount (mg)} \\
          & NO. 1       & NO. 2       & NO. 3       & NO. 4       & NO. 5       \\
\hline
Iproniazid & \textit{p.o.} & 1.91        & 1.46        & 1.51        & 1.26        & 0.79        \\
           & \textit{i.v.} & 0.85        & 0.77        & 0.71        & 0.67        & 0.67        \\
Isoniazid  & \textit{p.o.} & 1.05        & 0.82        & 0.74        & 0.57        & 0.68        \\
           & \textit{i.v.} & 0.39        & 0.37        & 0.34        & 0.36        & 0.59        \\
\hline
\end{tabular}
\end{table}

was also higher than that from INH. Comparing each AUC of HZ from 0 to 8 hr among four rabbits, HZ amount from IPN was 3–6 times more than that from INH.

DISCUSSION

From our study on the metabolism of INH in man, it was clarified that about 25–70% of the dose was excreted in the urine as acetyl-INH, and that HZ formation took place by the direct hydrolysis of INH itself. Ellard et al. showed that the isonicotinic acid formed after INH administration was indirectly obtained by the hydrolysis of acetyl-INH, and that another metabolite, monoacetylhdydrazine, was easily acetylated to diacetylhdydrazine. Therefore, the amount of

FIG. 4. The Plasma Concentration of Hydrazine (HZ) after Oral Administration of Iproniazid (IPN) and Isoniazid (INH) in 4 Rabbits

Dose amount : iproniazid phosphate: 303.3 mg/body weight
isoniazid: 150.0 mg/body weight
HZ formed from intact INH is thought to be minor.

The IPN metabolism must be mainly controlled by oxidation as shown in Chart 2. Since IPN cannot accept acetylation, it was metabolized to INH (route A) to follow HZ formation (route C). Otherwise, Isop-HZ which was derived from IPN on hydrolysis (route D) gave also HZ good in yield (route E).

Using INH or Isop-HZ as a substrate, the amount of HZ formed in the rat liver 9000 g supernatant was determined. In the system, the amount of HZ derived from Isop-HZ was much more than that derived from INH.15) According to Nelson et al.,5) most of Isop-HZ from IPN was further metabolized to propane, carbon dioxide and acetone. Although in their experiments HZ was to be formed markedly in the metabolic pathway, they did not detect HZ at that time in animals administered with IPN. Consequently, HZ derived from IPN and INH must be similar to monoacetylhydrazine and Isop-HZ in inducing hepatic injury.

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CHART 2. Routes of Hydrazine Formation from Iproniazid
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