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## Note

# Analysis of isoniazid, acetylhydrazine and [$^{15}N_2$]acetylhydrazine in serum by capillary gas chromatography–ammonia chemical ionization mass spectrometry

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Isoniazid remains the drug of choice for the treatment and prophylaxis of tuberculosis, in spite of its hepatotoxicity [1]. Interindividual differences in the disposition of the potentially toxic metabolite acetylhydrazine and interactions between isoniazid and acetylhydrazine that both compete for detoxification by acetylation might explain the high incidence of hepatotoxicity associated with isoniazid therapy [2–4]. In order to elucidate further the mechanism of toxicity in humans, the disposition of acetylhydrazine and the effect of isoniazid on the detoxification of this metabolite require study. However, the characterization of interactions between isoniazid and acetylhydrazine requires the administration of labelled acetylhydrazine since acetylhydrazine is a metabolite of isoniazid and its kinetics are very complex owing to its formation from isoniazid via acetylisoniazid [3].

In order to study the effects of isoniazid on the disposition of acetylhydrazine we have developed a sensitive gas chromatographic–mass spectrometric (GC–MS) method that would permit the analysis of isoniazid and acetylhydrazine in serum and the simultaneous determination of two species of acetylhydrazine, namely acetylhydrazine formed from isoniazid and [$^{15}N_2$]acetylhydrazine administered intravenously.

## EXPERIMENTAL

### *Chemicals*

All reagents were of analytical grade. Isoniazid was obtained from Fluka (Buchs, Switzerland), acetylhydrazine and diacetylhydrazine from Aldrich (Steinheim,

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F.R.G.) and hydrazine sulphate from Merck (Darmstadt, F.R.G.). Isoniazid and diacetylhydrazine were recrystallized from methanol–diethyl ether before use. Acetylhydrazine fumarate was prepared by addition of hot ethanolic solutions of fumaric acid and acetylhydrazine in a molar ratio of 1:2 and recrystallization from hot ethanol. Acetylisoniazid was synthesized according to Mitchell et al. [5]. All hydrazines and hydrazones were stored in a desiccator.

*Synthesis of deuterated internal standards*

Three deuterated internal standards for GC–MS analysis were prepared by adding [$^2H_6$]benzaldehyde (98% deuterium, Merck Sharp and Dohme, Montreal, Canada) in 20 ml of methanol in a 1:1 molar ratio to isoniazid and acetylhydrazine or in a 1:2 molar ratio to hydrazine, respectively. The solutions were stirred for 1 h at 60°C [6] and evaporated to dryness, and the deuterated hydrazones were recrystallized twice from ethyl acetate.

Benzaldehyde hydrazones of isoniazid, acetylhydrazine and hydrazine were synthesized in the same way, using benzaldehyde to obtain reference spectra.

*Synthesis of [$^{15}N_2$]acetylhydrazine*

In order to generate free hydrazine [7], 3.78 mmol of [$^{15}N_2$]hydrazine sulphate (95% $^{15}N$, Cambridge Isotope Labs., Woburn, MA, U.S.A.) in 20 ml of methanol were gassed for 10 min with ammonia under stirring and cooling in ice. The suspension was then gassed with nitrogen at 35°C for 15 min. Acetylation was performed by a transacetylation reaction [8]. Methyl acetate (5.8 mmol) and five drops of water were added since the acetylation proceeds via hydrazine hydrate. Subsequently the suspension was refluxed under an argon atmosphere for 8 h at 92°C. The reaction mixture was then evaporated, and the residue was stirred in 30 ml of chloroform for 10 min. The suspension was filtered, and the filtrate was evaporated, solidified under a stream of nitrogen and dried in a desiccator for 72 h. The yield of [$^{15}N_2$]acetylhydrazine was 59.5%. An ammonia chemical ionization mass spectrum of the compound as its benzaldehyde derivative is depicted in Fig. 1. A $^1H$ NMR spectrum in chloroform showed proton resonances at 2.0 ppm (singlet, 3H from $CH_3CO$), 3.9 ppm (singlet, 2H from $NH_2$) and 7.0 ppm (multiplet, 1H from –NH).

*Extraction of serum samples*

Serum samples were stored under argon at −70°C until analysis. To 2.5 ml of serum were added 0.1 ml of 1 *M* hydrochloric acid and 1 g of ammonium sulphate in a 20-ml screw-capped PTFE-lined glass tube. The mixture was vortexed and centrifuged at 2500 *g* for 20 min, and 1.5 ml of the supernatant were transferred to 1 ml of 0.5 *M* sodium citrate buffer (pH 6). Then 5 ml of dichloromethane were added to extract lipids interfering with the analysis. After centrifugation, 2 ml of the aqueous phase were derivatized with 0.1 ml of benzaldehyde in methanol (0.1 ml/ml) while shaking for 1 h at room temperature [9]. Following the addition of the three internal standards, [$^2H_6$]benzaldehyde isonicotinoylhydrazone (50 nmol), [$^2H_6$]benzaldehyde acetylhydrazone (10 nmol) and

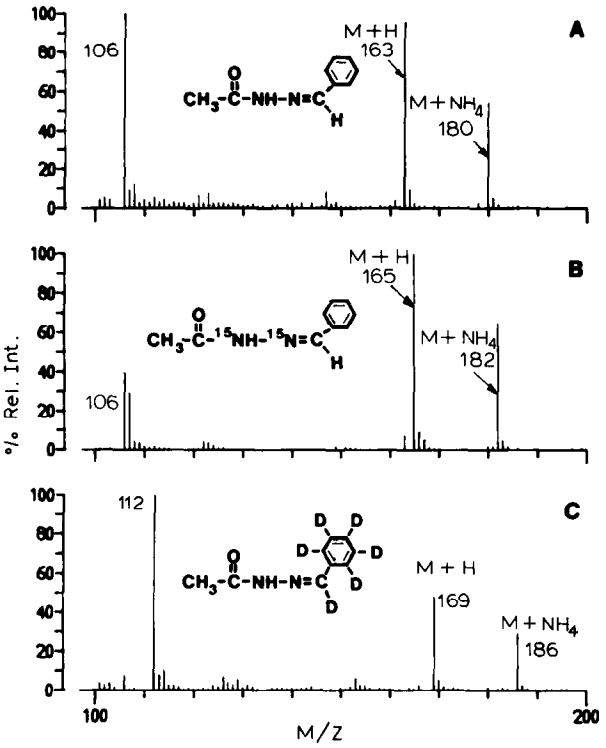


Fig. 1. Ammonia chemical ionization mass spectra of benzaldehyde acetylhydrazone (A), benzaldehyde [$^{15}N_2$]acetylhydrazone (B) and the internal standard [$^2H_6$]benzaldehyde acetylhydrazone (C). The M+1 quasimolecular ions *m/z* 163, 165 and 169 were used for quantification.

[$^2H_{12}$]benzaldehyde azine (10 nmol) in 0.1 ml of ethylacetate, the sample was extracted twice with 6 ml of dichloromethane.The dichloromethane phases were combined, dried with sodium sulphate and evaporated at 40°C under a stream of nitrogen. The residue was dissolved in 0.1 ml of ethyl acetate and 1 $\mu$l was injected into the GC–MS system.

*Capillary GC–ammonia chemical ionization MS*

Capillary GC–MS was performed on a Finnigan 1020 instrument. A 15 m×0.32 mm I.D. glass capillary column was persilanized with 1,3-divinyl-1,1,3,3-tetramethyldisilazane and coated with OV-1701vi (Ohio Valley, Marietta, OH, U.S.A.; 0.4% static coating; 0.32 $\mu$m film thickness). The stationary phase was immobilized with 1% dicumyl peroxide [10]. A fast temperature programme was used (from 80°C at 20°C per min to 260°C). The electron energy was 60 eV. The ion-source pressure of the chemical ionization source was 0.43 Torr of ammonia (99.995%) as reagent gas.

Selected-ion monitoring was carried out at the following masses corresponding to M+1: *m/z* 163 (benzaldehyde acetylhydrazone); *m/z* 165 (benzaldehyde [$^{15}N_2$]acetylhydrazone); *m/z* 169 ([$^2H_6$]benzaldehyde acetylhydrazone, internal standard); *m/z* 209 (benzaldehyde azine); *m/z* 211 (benzaldehyde [$^{15}N_2$]azine); *m/z* 221 ([$^2H_{12}$]benzaldehyde azine, internal standard); *m/z* 226

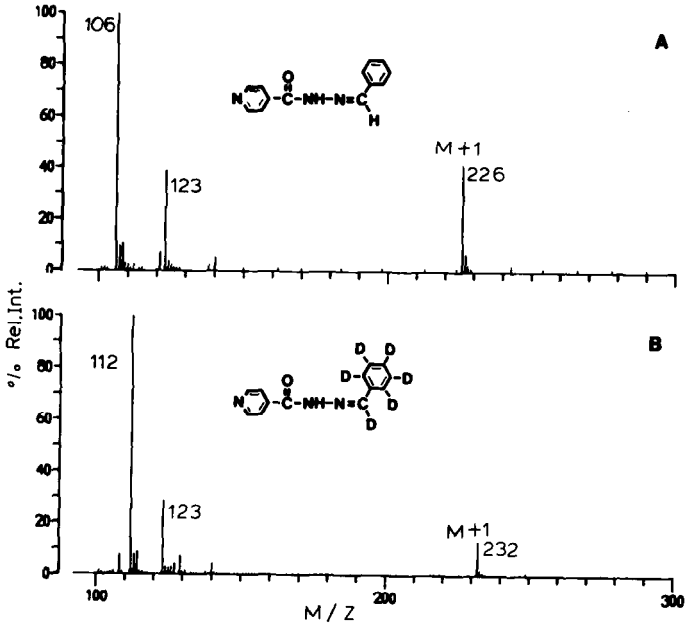


Fig. 2. Ammonia chemical ionization mass spectra of benzaldehyde isonicotinoylhydrazone (A) and [$^2N_6$]benzaldehyde isonicotinoylhydrazone (B) yielding quasimolecular ions with the difference of 6 atomic mass units.

(benzaldehyde isonicotinoylhydrazone); *m/z* 232 ([$^2H_6$]benzaldehyde isonicotinoylhydrazone, internal standard).

*Calibration*

Mixtures of the hydrazines in pooled, drug-free serum (isoniazid 5–60 nmol/ml, acetylhydrazine fumarate 0.1–3 nmol/ml, hydrazine sulphate 0.2–3 nmol/ml) were stored in portions under argon at −70 °C and used for calibration.

## RESULTS AND DISCUSSION

The ammonia chemical ionization mass spectra of the isoniazid derivatives (Fig. 2A and B) show intense quasimolecular ions at *m/z* 226 (benzaldehyde isonicotinoylhydrazone) and *m/z* 232 ([$^2H_6$]benzaldehyde isonicotinoylhydrazone, internal standard), which allowed quantification by multiple-ion monitoring. Fragmentation took place between the two nitrogen atoms of the hydrazine moiety yielding ions at *m/z* 106, 123 and 112, 123, respectively.

Three corresponding ammonia chemical ionization mass spectra of unlabelled benzaldehyde acetylhydrazone, benzaldehyde [$^{15}N_2$]acetylhydrazone and the internal standard [$^2H_6$]benzaldehyde acetylhydrazone are depicted in Fig. 1A, B and C, respectively. Benzaldehyde acetylhydrazone (Fig. 1A) yields only three intense ions at *m/z* 180 (M+18; M+$NH_4$), *m/z* 163 (M+H; used for quantification) and *m/z* 106 (protonated benzimino moiety). The quasimolecular ions

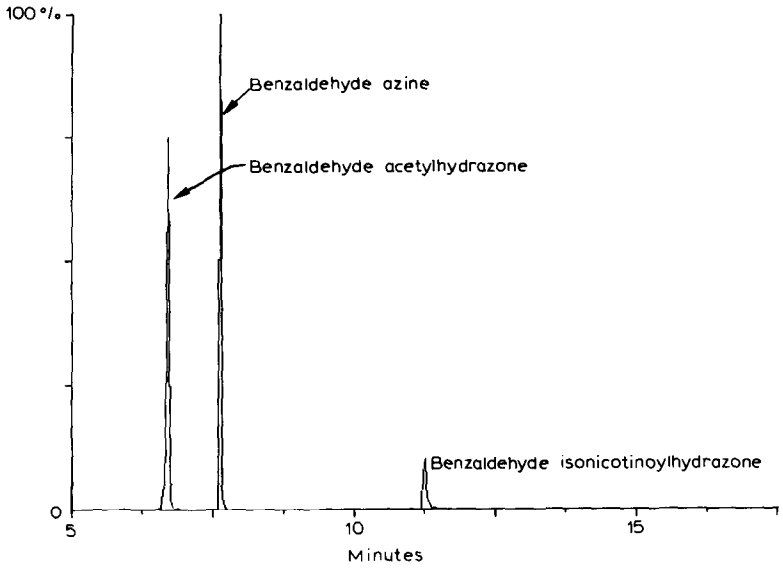


Fig. 3. Reconstructed ion chromatogram of isoniazid and its metabolites in a plasma sample from a healthy volunteer 2 h after oral administration of 300 mg of isoniazid. Analysis was performed in the multiple-ion detection mode monitoring eight ions.

of benzaldehyde [$^{15}N_2$]acetylhydrazone (Fig. 1B) are 2 mass units higher: *m/z* 182 (M+18), *m/z* 165 (M+H; base peak, used for quantification). The ions of the deuterium-labelled internal standard (Fig. 1C) are 6 mass units higher than the unlabelled compound, showing peaks at *m/z* 112, 169 and 186.

A reconstructed ion chromatogram of a serum extract 2 h after oral administration of 2.2 mmol of isoniazid and intravenous injection of 0.1 mmol of $^{15}N$-labelled acetylhydrazine is depicted in Fig. 3. The eight ions described above were monitored in the multiple-ion detection mode. Therefore, only three peaks are seen corresponding to the compounds of interest. The use of a highly inert capillary column [10] allows analysis without silylation of the benzaldehyde hydrazones, in contrast to the previously published method [9]. The three compounds elute within 12 min from the capillary column, resulting in a high throughput of samples.

The use of deuterated compounds as internal standards for GC–MS analysis corrects for losses during the most critical steps, the extraction after derivatization and the GC–MS analysis. Calibration curves obtained with spiked serum samples were linear ($r > 0.99$) within the concentration ranges given above, indicating reliable reproducibility of the steps not corrected for by the internal standards. Reproducibility of the method was further tested using a pool of serum samples of healthy volunteers after a single dose of isoniazid orally. Coefficients of variation for intra-day reproducibility ($n = 8$) were 5.0% for isoniazid ($21.6 \pm 1.1$ nmol/ml) and 4.1% for acetylhydrazine ($3.1 \pm 0.1$ nmol/ml).

The serum concentration–time curves of isoniazid and acetylhydrazine in a healthy slow acetylator are shown in Fig. 4. On one day, 7.4 mg (100 μmol) of

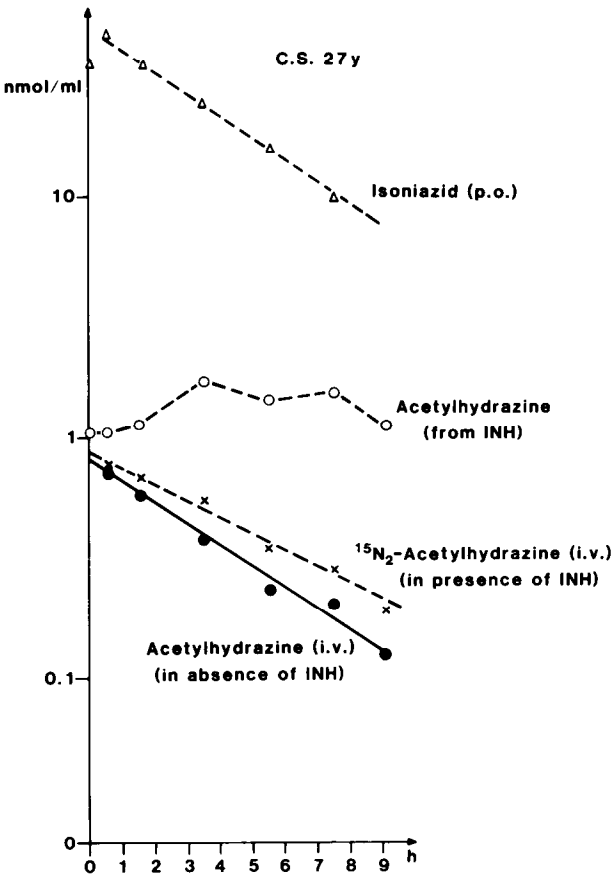


Fig. 4. Serum concentration in a healthy male volunteer (slow acetylator phenotype) of acetylhydrazine (—) following intravenous administration of acetylhydrazine and of [$^{15}N_2$]acetylhydrazine (- - -) following the ingestion of 300 mg of isoniazid orally and the intravenous administration of [$^{15}N_2$]acethylhydrazine. Acetylhydrazine (- - -) is formed from isoniazid. In contrast to [$^{15}N_2$]acetylhydrazine the time course of acetylhydrazine from isoniazid depends on the rate of formation from isoniazid via acetylisoniazid and the rate of elimination of acetylhydrazine. The lower rate of disappearance of [$^{15}N_2$]acetylhydrazine (- - -) compared with the disappearance of acetylhydrazine (—) in the absence of isoniazid indicates that isoniazid inhibits the acetylation and thus the detoxification of acetylhydrazine.

acetylhydrazine fumarate were injected intravenously. One week later the study was repeated. The same subject ingested 300 mg (2.19 mmol) of isoniazid dissolved in water, and 100 $\mu$mol of [$^{15}N_2$]acethylhydrazine were injected intravenously 90 min later. The dose of acetylhydrazine injected intravenously corresponds to ca. 1/5 of the amount of acetylhydrazine generated from a single dose (300 mg) of isoniazid and was, therefore, considered safe. In contrast to the acetylhydrazine formed from isoniazid, intravenously administered acetylhydrazine disappeared monoexponentially from the circulation. The half-life of acetylhydrazine was very similar to the half-life of isoniazid, which suggests that the acetylation of acetylhydrazine is subject to the same acetylation polymorphism as the acetylation of isoniazid.

Since acetylhydrazine is a metabolite of isoniazid, a labelled analogue is required to study the effect of isoniazid on the detoxification of acetylhydrazine. For this purpose we chose the non-radioactive [$^{15}N_2$]acetylhydrazine. The data presented in Fig. 4 demonstrate that isoniazid delays the elimination of acetylhydrazine, probably by competing for acetylation.

The GC–MS assay of isoniazid and its hydrazine metabolites presented here thus provides the tools to investigate the complex interactions between the parent compound isoniazid and its metabolites which may be of clinical relevance.

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