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DETERMINATION OF ISONIAZID AND ITS HYDRAZINO METABOLITES, ACETYLISONIAZID, ACETYLHYDRAZINE, AND DIACETYLHYDRAZINE IN HUMAN PLASMA BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A gas chromatographic—mass spectrometric assay for isoniazid and its hydrazino metabolites in human plasma was developed. The trimethylsilyl derivatives of diacetylhydrazine and acetylisoniazid and of the benzaldehyde hydrazones of acetylhydrazine and isoniazid were separated on a 1% OV-17 column and quantitated by single ion monitoring using a LKB 9000 mass spectrometer. Deuterated analogues served as internal standards. The method is well suited for the determination of the hepatotoxic hydrazino metabolites of isoniazid in human plasma following an oral therapeutic dose of isoniazid.

INTRODUCTION

Isoniazid is widely used for the treatment and prophylaxis of tuberculosis. Unfortunately, isoniazid therapy is not without risk. Up to 2.3% of isoniazid recipients will develop clinically overt hepatitis, which has a mortality of about 10% [1]. Older patients, alcoholics and patients on concomitant drug therapy such as rifampin appear to be at a higher risk [2, 3]. Animal studies suggest that it is the metabolic activation of the isoniazid metabolite acetylhydrazine, and possibly diacetylhydrazine, that causes the isoniazid-induced liver injury [1]. If similar mechanisms are responsible for isoniazid hepatotoxicity in man, a kinetic analysis of the hydrazino metabolites of isoniazid might explain why the patient populations mentioned above are more susceptible to the toxic effects of isoniazid. Unfortunately, such an analysis has not been possible with current assays. We have, therefore, developed a specific and sensitive gas chro-

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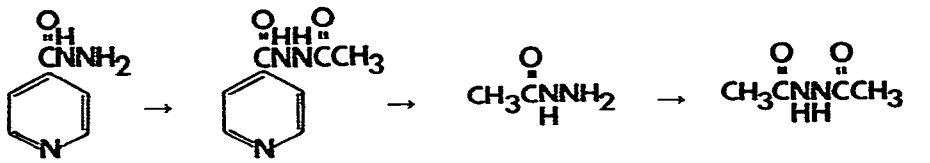

Fig. 1. Structures of isoniazid and its hydrazino metabolites.

matographic—mass spectrometric (GC—MS) method for the simultaneous measurement of isoniazid, acetylisoniazid, acetylhydrazine and diacetylhydrazine in human plasma (Fig. 1).

MATERIALS AND METHODS

Synthesis of internal standards

d_6-1,2-Diacetylhydrazine was synthesized by refluxing 6.7 mmole of hydrazine hydrate (Fisher Scientific, Pittsburgh, PA, U.S.A., as an 85% solution) with 20 mmole d_6-acetic anhydride (Merck, Sharp and Dohme Isotopes, Rahway, NJ, U.S.A.) for 90 min. After distillation of the volatile substances in vacuo, the residue was recrystallized from methanol—diethyl ether (40% yield, m.p. 139—139.5°C). Homogeneity was confirmed by thin-layer chromatography on silica gel with *n*-butanol—ethanol—0.4 *N* ammonium hydroxide (4:1:1), $R_F = 0.56$. d_3-Acetylhydrazine was synthesized according to Nelson et al. [4].

d_3-Acetylisoniazid was synthesized according to Fox and Gibas [5] using d_3-acetic anhydride (Merck, Sharp and Dohme Isotopes) and tetrahydrofuran instead of acetic anhydride and glacial acetic acid.

Isoniazid d_6-benzaldehyde was synthesized by mixing equimolar amounts of isoniazid with d_6-benzaldehyde (Merck, Sharp and Dohme Isotopes) in methanol at 35°C. The hydrazone was crystallized twice, first from methanol and then from ethyl acetate.

Reference compounds

Isoniazid, diacetylhydrazine, acetylhydrazine and benzaldehyde were obtained from Aldrich (Milwaukee, WI, U.S.A.). Acetylisoniazid, [^{14}C] acetylisoniazid and [^{14}C] acetylhydrazine were synthesized as described previously [4, 6]. [^{3}H] Diacetylhydrazine was prepared like the deuterated analogue using [^{3}H]-acetic anhydride (New England Nuclear, Boston, MA, U.S.A.). [^{3}H] Isoniazid was purchased from Amersham Corporation, Arlington Heights, IL, U.S.A.

Sample preparation

For the determination of the concentrations of hydrazino metabolites in human plasma following an oral dose of 300 mg of isoniazid, venous blood was drawn into heparinized tubes. The plasma was immediately separated and stored at −20°C until the time of the assay which took place within five days. For the generation of standard curves between 0.02 and 2 μg of acetylhydra-

zine, diacetylhydrazine and acetylisoniazid and 0.5—15 μg of isoniazid were added to pooled, citrated plasma.

Each of the internal standards (20 μl of 0.1 mg/ml in methanol) was added to 1 ml of plasma, except for isoniazid d_6-benzaldehyde. The plasma was then extracted with 10 ml of methylene chloride to remove lipids interfering with the GC—MS assay. After centrifugation, the aqueous layer was added to 10 μl of benzaldehyde containing 10 μg isoniazid d_6-benzaldehyde. Under frequent shaking, the solution was reacted for 20 min at room temperature to form the benzaldehyde hydrazones of acetylhydrazine and isoniazid. Thirty milliliters of ethyl acetate were then added. While mixing the sample on a vortex mixer, 2 g of anhydrous sodium sulfate powder were slowly added. The ethyl acetate was decanted and evaporated under a stream of nitrogen in a water bath of 40°C. After transfer of the sample into Reactivials, 30 μl of BSTFA (both from Pierce, Rockford, IL, U.S.A.) were added, and the sample was reacted for 1 h at 80°C. A 1—5 μl aliquot was then injected onto the column.

GC—MS assay

A LKB 9000 gas chromatograph—mass spectrometer equipped with a LKB 9020 peak matcher was used. The trimethylsilyl derivatives were separated on 1% OV-17 in a glass column, 1.8 m × 2 mm I.D., using helium, 30 ml/min, as the carrier gas and a temperature program which ran from 90—270°C at 10°C/min. The MS analysis was performed under the following conditions: electron energy 20 eV, accelerating voltage 3.5 kV, electron multiplier 2400 V. A full scan spectrum was obtained of each compound and each internal standard in individual runs to confirm its identity and to determine the major ion closest to the molecular ion for single ion monitoring. For the simultaneous measurement of the metabolites and the corresponding deuterated internal standard, the accelerating voltage was increased periodically and the mass of the selected ion of the metabolite and the mass of the ions of the internal standards 3 or 6 mass units, respectively, greater than the metabolite ion were monitored alternatively. In order to correct for slight drifts of the mass marker during a day's run, the sweep generator modulated the magnetic field with an amplitude chosen to cover ± 0.2 mass units on both sides of the monitored ion.

For quantitation, the peak height ratio of the selected ions of each metabolite and its deuterated analogue were compared with a computer fitted standard curve which was run each day.

Recovery studies were performed with radioactively labeled analogues that were carried through the extraction and derivatization procedures. To assess the yield of hydrazone formation plasma samples spiked with [^{3}H]isoniazid were chromatographed after derivatization and extraction on Avicel F (Analtech, Newark, DE, U.S.A.) using the solvent system described above. Bands containing isoniazid (R_F 0.45) and isoniazid—benzaldehyde—hydrazone (R_F 0.85) were scraped off, eluted with methanol and counted by liquid scintillation spectrometry.

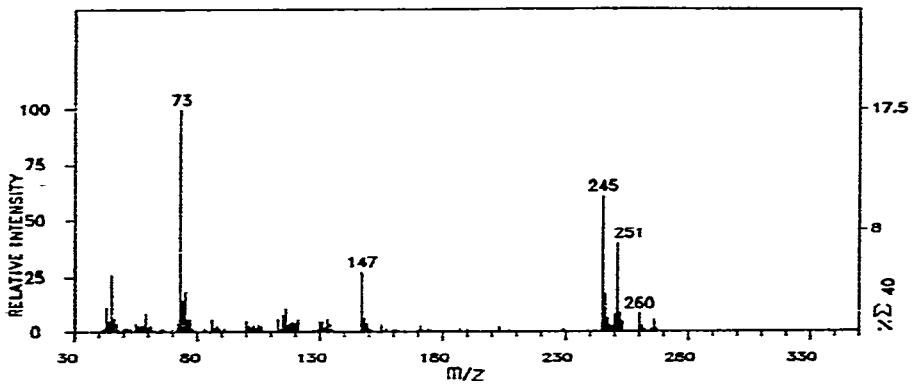

Fig. 2. Mass spectrum of the trimethylsilyl derivatives of diacetylhydrazine and the corresponding internal standard, d_6-diacetylhydrazine. For conditions see text. The ions at *m/z* 245 and 251 were monitored for quantification.

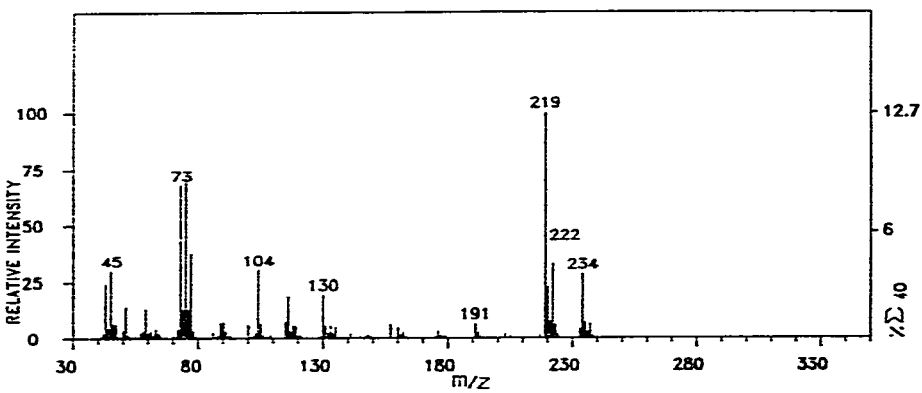

Fig. 3. Mass spectrum of the trimethylsilyl derivative of acetylhydrazine benzaldehyde hydrazone and the corresponding internal standard d_3-acetylhydrazine. The ions at *m/z* 219 and 222 were monitored for quantification.

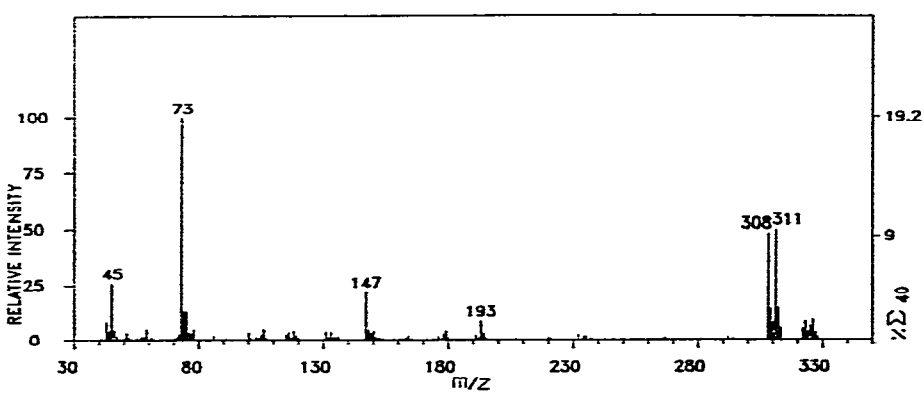

Fig. 4. Mass spectrum of the trimethylsilyl derivative of acetylisoniazid and the corresponding internal standard d_3-acetylisoniazid. The ions at *m/z* 308 and 311 were monitored for quantification.

RESULTS

Full scan spectra of the measured compounds and the corresponding internal standards are shown in Figs. 2—5. The spectra of synthetic standards and of isoniazid metabolites formed in man were identical. For the quantitative analysis by single ion monitoring the most abundant ion, M^+-15, was chosen (Fig. 6). Each of the internal standards appears shortly before the corresponding metabolite. This isotope effect results in a characteristic peak configuration for each

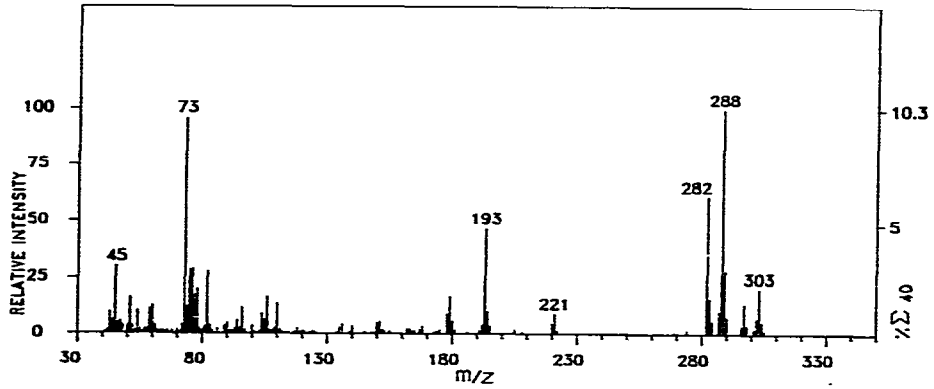

Fig. 5. Mass spectrum of the trimethylsilyl derivative of isoniazid benzaldehyde hydrazone and the corresponding deuterated internal standard. The ions at *m/z* 282 and 288 were monitored for quantification.

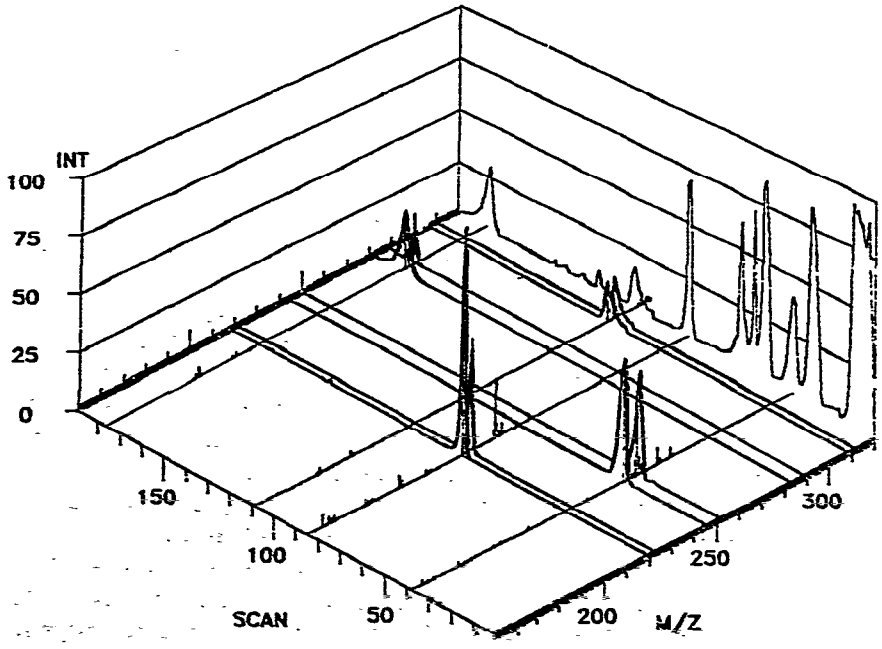

Fig. 6. Total ion current and single ion tracings of a standard mixture of diacetylhydrazine (*m/z* 245), acetylhydrazine (*m/z* 219), acetylisoniazid (*m/z* 308) and isoniazid (*m/z* 282) and their deuterated internal standards. For GC—MS conditions see text.

metabolite which, together with the retention time, allows a ready identification of each peak of interest. The recoveries of the sample preparation and the precision of the method are shown in Table I. Thin-layer chromatography of plasma extracts demonstrated that an average of 90.3% of the isoniazid is derivatized to the benzaldehyde hydrazone. The peak ratios of the metabolites and their corresponding internal standards are proportional over the concentration range encountered in plasma of slow and rapid acetylators following an oral therapeutic dose of 300 mg isoniazid (Fig. 7).

TABLE I

RECOVERY OF SAMPLE PREPARATION AND PRECISION OF METHOD

Recovery was measured with radioactively labeled compounds as described in Materials and methods.

	Recovery (%)	Coefficient of variation (%) of three determinations at indicated concentrations				
		0.05 μg/ml	0.1 μg/ml	0.5 μg/ml	1 μg/ml	5 μg/ml
Isoniazid	76	—	11	8	5	4
Acetylisoniazid	71	—	9	5	6	—
Acetylhydrazine	70	8	11	6	3	—
Diacetylhydrazine	64	9	5	6	4	—

Following the addition of either α-ketoglutarate acetylhydrazone or pyruvate acetylhydrazone to pooled plasma, no free acetylhydrazine was found under the chosen conditions indicating that the assay does not measure hydrazones of acetylhydrazine circulating in plasma.

DISCUSSION

Based on the extensive animal studies from this laboratory, the toxicologically important metabolites of isoniazid are acetylhydrazine and diacetylhydrazine [1]. A number of methods has been proposed to measure these metabolites. Earlier colorimetric and fluorometric assays of uncertain specificity have been used to quantitate the urinary levels of these metabolites but are insufficiently sensitive to quantitate plasma concentrations [7]. More recently published GC methods for acetylhydrazine and diacetylhydrazine, although more specific, are also not sensitive enough to measure these metabolites in plasma [8, 9]. Our highly specific GC—MS assay, on the other hand, is clearly sensitive enough to accurately determine acetylhydrazine and diacetylhydrazine plasma concentrations in the range of 0.01—2 μg/ml following an oral therapeutic dose of isoniazid. Our assay is least sensitive for the parent compound isoniazid itself, mainly due to the long and slow temperature program which is required to analyze the more volatile metabolites. Under the described chromatographic conditions, however, the method is sensitive enough to accurately determine the disappearance rate of isoniazid which is present in high enough concentrations for the first few hours after its ingestion. The sensitivity of the assay for

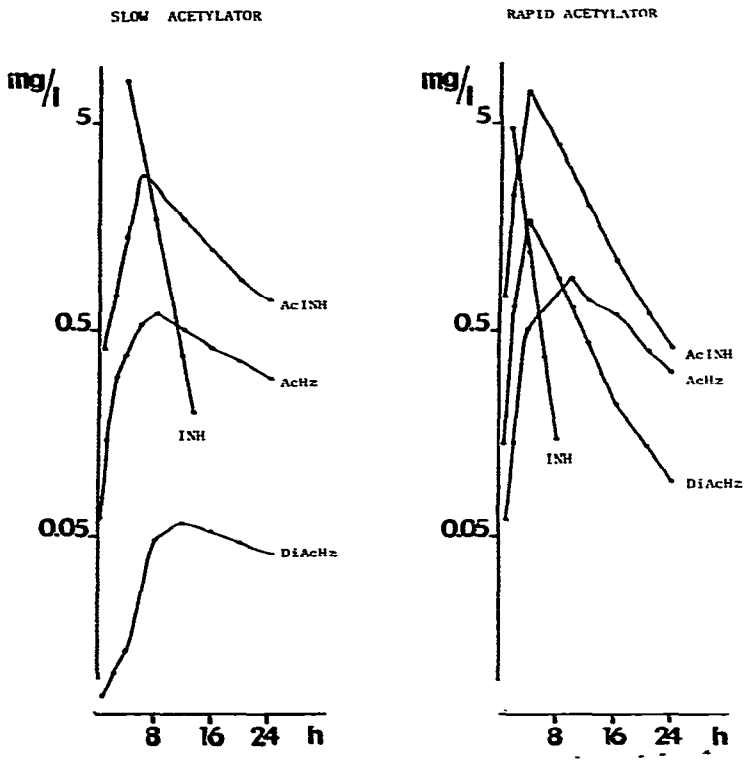

Fig. 7. Concentrations of isoniazid (INH), acetylisoniazid (AcINH), acetylhydrazine (AcHz), and diacetylhydrazine (DiAcHz) in the plasma of a patient with slow acetylator phenotype and one with rapid acetylator phenotype following the oral administration of 300 mg isoniazid.

isoniazid can be markedly increased if the temperature program of the GC separation is started at 200°C. Under these conditions, however, acetylhydrazine and diacetylhydrazine appear in the solvent front and can not be measured.

After acid hydrolysis of urinary hydrazones, the urinary excretion of metabolites can be followed with the identical procedure. Thus, the assay allows a complete kinetic analysis of the toxicologically important metabolites of isoniazid and may help elucidate factors determining the variable susceptibility of patients to isoniazid liver injury.

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