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DETERMINATION OF HYDRAZINE METABOLITES OF ISONIAZID IN HUMAN URINE BY GAS CHROMATOGRAPHY

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SUMMARY

A method is described for the determination of isoniazid, acetylisoniazid, acetylhydrazine, diacetylhydrazine and hydrazine in urine. Isoniazid, acetylhydrazine and hydrazine are reacted in aqueous solution with *p*-chlorobenzaldehyde to form hydrazones. Following the addition of appropriate internal standards, these hydrazones are then extracted into an organic solvent and determined by gas chromatography using a nitrogen-sensitive detector. Acetylisoniazid and diacetylhydrazine are determined similarly after hydrolysis to isoniazid and acetylhydrazine, respectively.

INTRODUCTION

As hydrazine compounds are widely used as therapeutic agents, rocket fuels and herbicides, a method for the measurement of these compounds in aqueous solutions has wide applications. Isoniazid, the most commonly used anti-tubercular drug, is a hydrazine derivative, and recent studies[1–3] have shown that the hepatotoxic side effect of the drug may be due to the toxic metabolite acetylhydrazine. It was, therefore, important to measure this metabolite and also diacetylhydrazine and hydrazine in the urine of human subjects given isoniazid, to further define the mechanism of hepatotoxicity of the drug.

There have been many studies of the metabolism of isoniazid in man using a variety of assay techniques[4–7]. Although many of these different methods for the determination of isoniazid and its metabolites previously described are based on sensitive microbiological or colorimetric techniques, specific methods for the determination of acetylhydrazine and hydrazine in the presence of isoniazid and its metabolites were not previously available[7]. Radiochemical methods, although having both sensitivity and specificity, are often not applicable to studies in humans.

The gas chromatographic (GC) method for isoniazid and acetylisoniazid determination recently described[8] was only sensitive to 50 μg/ml of isoniazid, which is probably not sufficiently sensitive for analysis of human urine following therapeutic

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doses of the drug. Therefore a GC assay has been devised to determine acetylhydrazine, diacetylhydrazine and hydrazine in urine. This method may also be used to measure isoniazid and acetylisoniazid in the same urine samples and so determine the acetylator phenotype of the subject.

EXPERIMENTAL

Reagents

p-Chlorobenzaldehyde and hydrazine hydrate were obtained from BDH (Poole, Great Britain), *p*-bromobenzaldehyde from Aldrich (Gillingham, Great Britain), isoniazid from K & K Labs. (Plainview, N.Y., U.S.A.), acetylhydrazine (hydrochloride and free base) and diacetylhydrazine from Aldrich (Milwaukee, Wisc., U.S.A.). Isoniazid, acetylhydrazine hydrochloride and diacetylhydrazine were recrystallised from methanol–diethyl ether before use.

General method

Paper chromatography (PC) was carried out by descending development using Whatman No. 3 paper in butan-1-ol–ethanol–0.5 *M* ammonia (4:1:1).

Nuclear magnetic resonance spectroscopy (NMR) was carried out on a Varian instrument at 60 MHz using hexadeuterated dimethyl sulphoxide as solvent. Chemical ionisation mass spectrometry (CI–MS) was carried out on a VG Micromass 16F instrument, using a direct-insertion probe at an electron energy of 100 eV, an ionising current of 200 μA and an ion source temperature of 200° (isobutane reactant gas). Electron impact mass spectrometry (EI-MS) was performed on a Finnigan 3200 instrument by direct insertion probe using an ionisation current of 330 μA and electron energy of 25 eV.

GC was carried out on a Perkin-Elmer F17 instrument fitted with a nitrogen–phosphorus detector. The column, 2 m × 1.75 mm I.D. glass, was packed with 10% OV-17 on Gas-Chrom Q, 100–120 mesh (Applied Science Labs.). For chromatography of the derivatives the detector and injector temperatures were set at 300° with a nitrogen carrier gas flow-rate of 35 ml/min and an oven temperature of 250° for acetylhydrazine and hydrazine derivatives and 300° for the isoniazid derivative. For determinations of hydrazine alone, when *p*-bromobenzaldehyde azine was used as an internal standard, an oven temperature of 300° was used. The nitrogen detector setting used was 6.0, giving a rubidium bead temperature of approximately 600°.

Syntheses

Acetylisoniazid. This metabolite was synthesised by acetylation of isoniazid with acetyl chloride as previously described[1] and recrystallised from methanol–diethyl ether.

α-Ketoglutarate and pyruvate isonicotinoylhydrazones. These metabolites were synthesised as previously described[1,9] by reaction of the sodium salts of α-ketoglutaric acid or pyruvic acid with isoniazid in aqueous propan-2-ol. The purity of the recrystallised products was established by PC in two solvent systems as described previously[1].

α-Ketoglutaric acid acetylhydrazone. This compound was synthesized by mixing equimolar amounts of α-ketoglutaric acid and acetylhydrazine in toluene–methanol. After stirring at room temperature for 60 min the hydrazine crystallised out. The

compound was recrystallised from ethanol and gave a m.p. of 160° (no literature value). PC (R_F 0.027) showed the compound to be homogenous and separable from acetylhydrazine and α-ketoglutarate. NMR: 2.18δ (s,3H); 10.86δ (s,1H); 12.06δ (s,1H); 2.5δ (m,4H). CI-MS: 203 (MH+); 185 (M−18); 157 (M−45).

Pyruvic acid acetylhydrazone. This compound was synthesized by reacting equimolar amounts of acetylhydrazine and sodium pyruvate in toluene. After stirring for 60 min the hydrazone crystallised out. The pyruvic acid acetylhydrazone crystallised from an aqueous solution of the sodium salt on addition of conc. HCl. The hydrazone was recrystallised from ethanol and gave a m.p. of 186°–188° (literature[10] value 186°). PC revealed two spots (R_F 0.07 and 0.2) presumably the *syn* and *anti* isomers previously reported for pyruvate hydrazones[12]. These two spots were separable from acetylhydrazine and pyruvate NMR: 2.18δ (s,3H); 2.05δ (s,3H); 10.65δ (s,1H). CI-MS: 145 (MH+); 100 (M−44).

p-Chlorobenzaldehyde acetylhydrazone. Equimolar amounts of acetylhydrazine hydrochloride and *p*-chlorobenzaldehyde were heated (60°) in methanol for 15 min. The yellow crystals of *p*-chlorobenzaldehyde azine formed as a by-product on cooling to room temperature, were removed by filtration. On cooling the mother liquor to 0° white crystals appeared which were filtered off and recrystallised from methanol. GC of the product revealed one peak with a retention time of 2 min at 250°. EI-MS: 186 (M+, parent ion cluster, relative intensity 3:1); 153; 137 (M−59, base peak); 43.

p-Bromobenzaldehyde acetylhydrazone. This compound, used as the internal standard for acetylhydrazine determination, was prepared as described for the *p*-chlorobenzaldehyde derivative using *p*-bromobenzaldehyde as the reagent. GC of the recrystallised product revealed one peak with a retention time of 3 min at 250°. EI-MS: 240 (M+, parent ion cluster, relative intensity 1:1), 197; 181 (M−59, base peak); 43.

p-Chlorobenzaldehyde azine. Equimolar amounts of *p*-chlorobenzaldehyde and hydrazine hydrate were heated in methanol (60°) for 1 h. Immediately on cooling to room temperature yellow crystals separated which were filtered off and recrystallised from methanol. GC of the product revealed one peak with a retention time of 9.4 min at 250° and 2.2 min at 300°. EI-MS: 276 (M+, parent ion cluster, relative intensity, 9:6:1); 165 (M−111, base peak); 138; 111.

p-Bromobenzaldehyde azine. This compound, used as the internal standard for hydrazine determinations when isoniazid was not present, was synthesised in the same manner as described for the *p*-chlorobenzaldehyde derivative, using *p*-bromobenzaldehyde as the reagent. GC of the recrystallised product revealed one peak with a retention time of 4.1 min at 300°. EI-MS: 364 (M+, parent ion cluster, relative intensity, 1:2:1); 209 (M−155, base peak); 182; 155.

p-Chlorobenzaldehyde isonicotinoylhydrazone. Equimolar amounts of isoniazid and *p*-chlorobenzaldehyde in methanol were heated for 1 h at 60°. After cooling for 24 h at −20°, white crystals separated. These were filtered off and recrystallised from methanol. The compound was examined by GC and gave one peak with a retention time of 4 min at 300°. EI-MS: 259 (M+, parent ion cluster, relative intensity, 3:1); 224; 122; 106 (M−153, base peak); 78.

p-Bromobenzaldehyde isonicotinoylhydrazone. This derivative, used as internal standard for isoniazid determinations, was prepared as described for the *p*-chlorobenzaldehyde derivative by using *p*-bromebenzaldehyde as the reagent. GC of the

recrystallised product gave one peak with a retention time of 5.4 min at 300°. EI-MS: 303 (M+, parent ion cluster, relative intensity, 1:1); 224; 122; 106 (M−197, base peak); 78.

Assay

The procedure for measuring the compounds in urine involved the reaction of those compounds with a free hydrazine nitrogen, that is acetylhydrazine, isoniazid and hydrazine, with *p*-chlorobenzaldehyde (Fig. 1), the addition of an appropriate internal standard and their subsequent extraction into methylene chloride. The diacetylhydrazine and acetylisoniazid remaining in the urine were then subjected to hydrolysis to acetylhydrazine and isoniazid, respectively, and then determined in the same manner (Fig. 2).

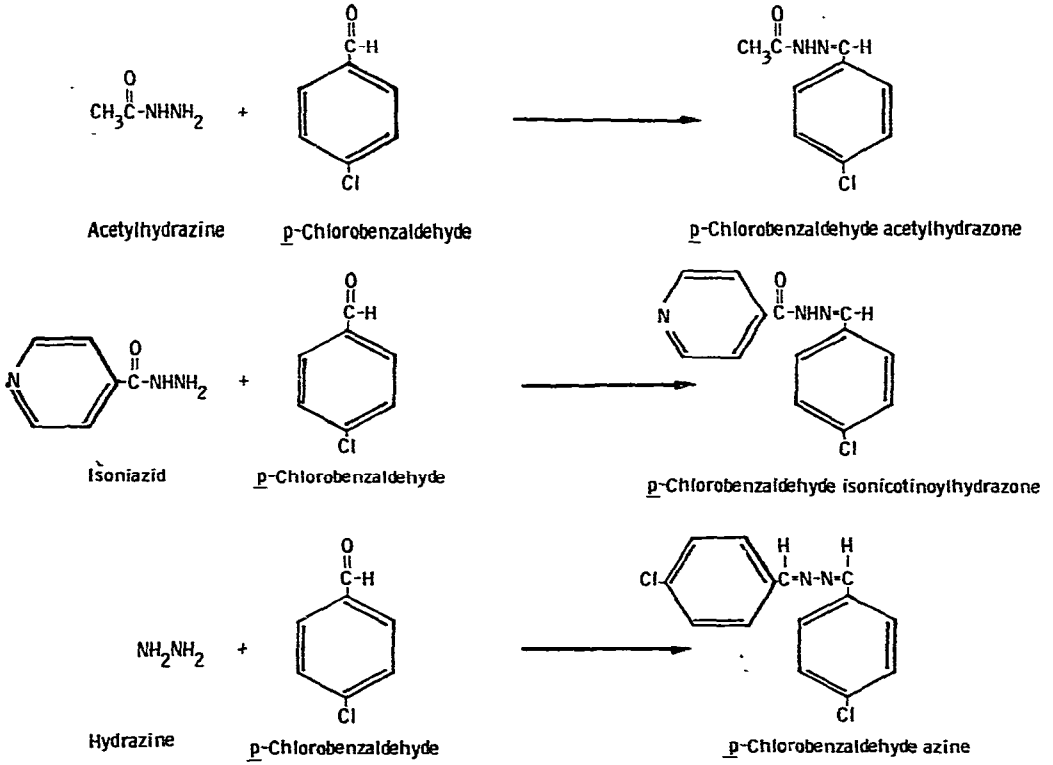

Fig. 1. Derivatisation reactions employed in the assay procedure.

Determination of acetylhydrazine, hydrazine and isoniazid. Duplicate urine samples (25 ml) and blank urine were adjusted to pH 3 (conc. HCl) and extracted with methylene chloride (3 × 50 ml). This initial extraction was found to remove contaminating substances present in urine. *p*-Chlorobenzaldehyde (30 mg) in methanol (1 ml) was added to each of the extracted urine samples and these were allowed to react for 30 min at room temperature. After the reaction the internal

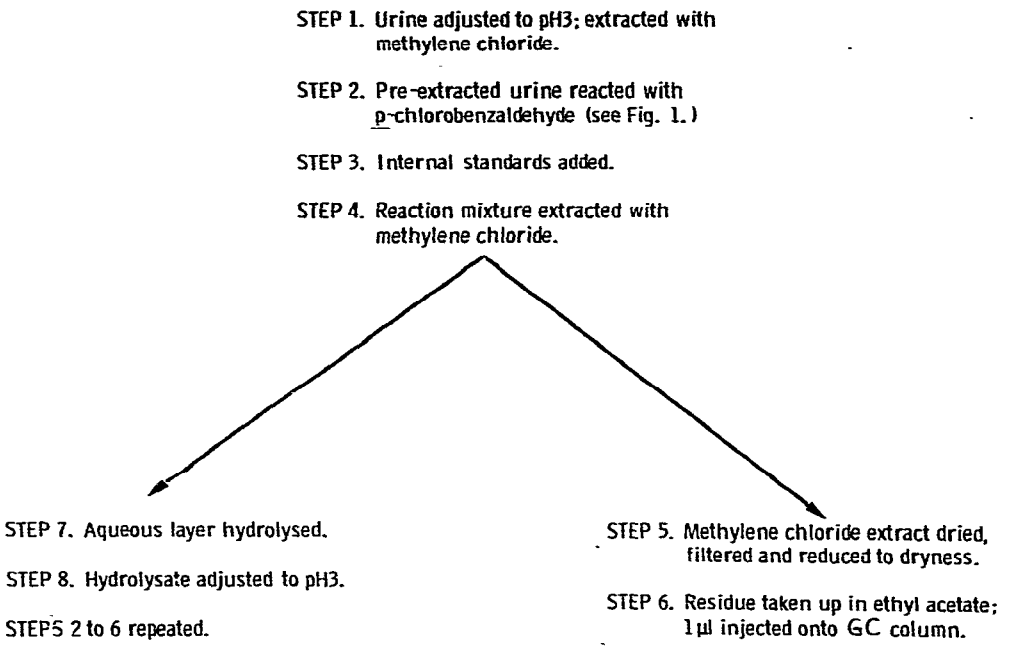

Fig. 2. Assay procedure.

standards, *p*-bromobenzaldehyde acetyl hydrazone (1 mg)* in ethyl acetate (0.1 ml) and *p*-bromobenzaldehyde isonicotinoyl hydrazone (1 mg) in ethyl acetate (1.0 ml) were added to each sample. The samples were then extracted with methylene chloride (3 × 50 ml) and the aqueous layer retained for the subsequent determination of acetylisoniazid and diacetylhydrazine. The methylene chloride extracts were dried over anhydrous sodium sulphate, filtered and reduced to dryness by rotary evaporation. The residues were taken up in approximately 3 ml of ethyl acetate.

A portion (1 µl) of the ethyl acetate extract was injected onto the GC column with an oven temperature of 250°, and the peak heights of the *p*-chlorobenzaldehyde derivatives of acetylhydrazine and hydrazine and of the internal standard were recorded. For the determination of isoniazid a second injection (1 µl) was made with an oven temperature of 300° and the peak heights of the *p*-chlorobenzaldehyde derivative of isoniazid and of the internal standard were recorded (Fig. 3).

For the preparation of standard curves, duplicate urine samples (25 ml) were spiked with acetylhydrazine hydrochloride, hydrazine hydrate or isoniazid and carried through the above procedure.

Determination of diacetylhydrazine and acetylisoniazid. The aqueous samples remaining from the above procedure were centrifuged (10 min at 1250 *g*) to remove the small amount of methylene chloride emulsion remaining after the previous

* *p*-Bromobenzaldehyde acetylhydrazone was also used as an internal standard for hydrazine determination when isoniazid was present, as the retention time of the *p*-chlorobenzaldehyde isonicotinoylhydrazone was almost identical to that of *p*-bromobenzaldehyde azine. However, the latter could be used as an internal standard for hydrazine determination when isoniazid was not present.

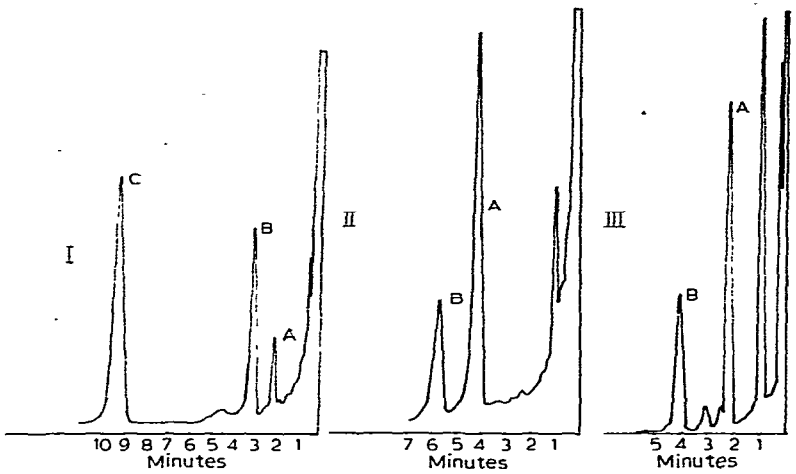

Fig. 3. Chromatograms of extracts of urine containing (I) acetylhydrazine and hydrazine, (II) isoniazid, (III) hydrazine. I: peak A, *p*-chlorobenzaldehyde acetyl hydrazone; B, *p*-bromobenzaldehyde acetyl hydrazone (internal standard); C, *p*-chlorobenzaldehyde azine (oven temp., 250°). II: A, *p*-chlorobenzaldehyde isonicotinoyl hydrazone; B, *p*-bromobenzaldehyde isonicotinyol hydrazone (internal standard) (oven temp., 300°). III: A, *p*-chlorobenzaldehyde azine; B, *p*-bromobenzaldehyde azine (internal standard) (oven temp., 300°.

determinations. HCl (11.4 *M*; 0.6 ml) was added to 22 ml of the aqueous layer and the sample was heated for 24 h at 45°. After this treatment the sample was adjusted to pH 3 with 5 *M* NaOH and reacted with *p*-chlorobenzaldehyde as previously described. The remaining procedure was identical to that described for acetylhydrazine, hydrazine and isoniazid determination (Fig. 2). For the preparation of standard curves, duplicate urine samples were spiked with diacetylhydrazine or acetylisoniazid and carried through both procedures.

RESULTS AND DISCUSSION

The method described is simple, specific and sensitive. The calibration curves (Fig. 4) constructed from spiked urine samples indicate that for isoniazid, acetylisoniazid, acetylhydrazine, diacetylhydrazine and hydrazine, there is a linear relationship between the peak height of the internal standard and that of the derivative. The standard curves for hydrazine indicate that the *p*-bromobenzaldehyde acetylhydrazone is a suitable internal standard for the measurement of hydrazine when isoniazid and acetylhydrazine are present.

The accuracy and sensitivity of the method are shown in Table I. Standard curves were fitted by computer-assisted regression analysis and the estimates of sensitivity are the minimum concentrations detectable by the method described.

None of the compounds interfered with the isoniazid, acetylhydrazine or hydrazine determinations when added to urine samples. Acetylhydrazine was found to contribute 4% to the diacetylhydrazine determination and isoniazid contributed 8% to the acetylisoniazid determination. However, in urine from humans dosed with isoniazid, greater amounts of diacetylhydrazine and acetylisoniazid are excreted than

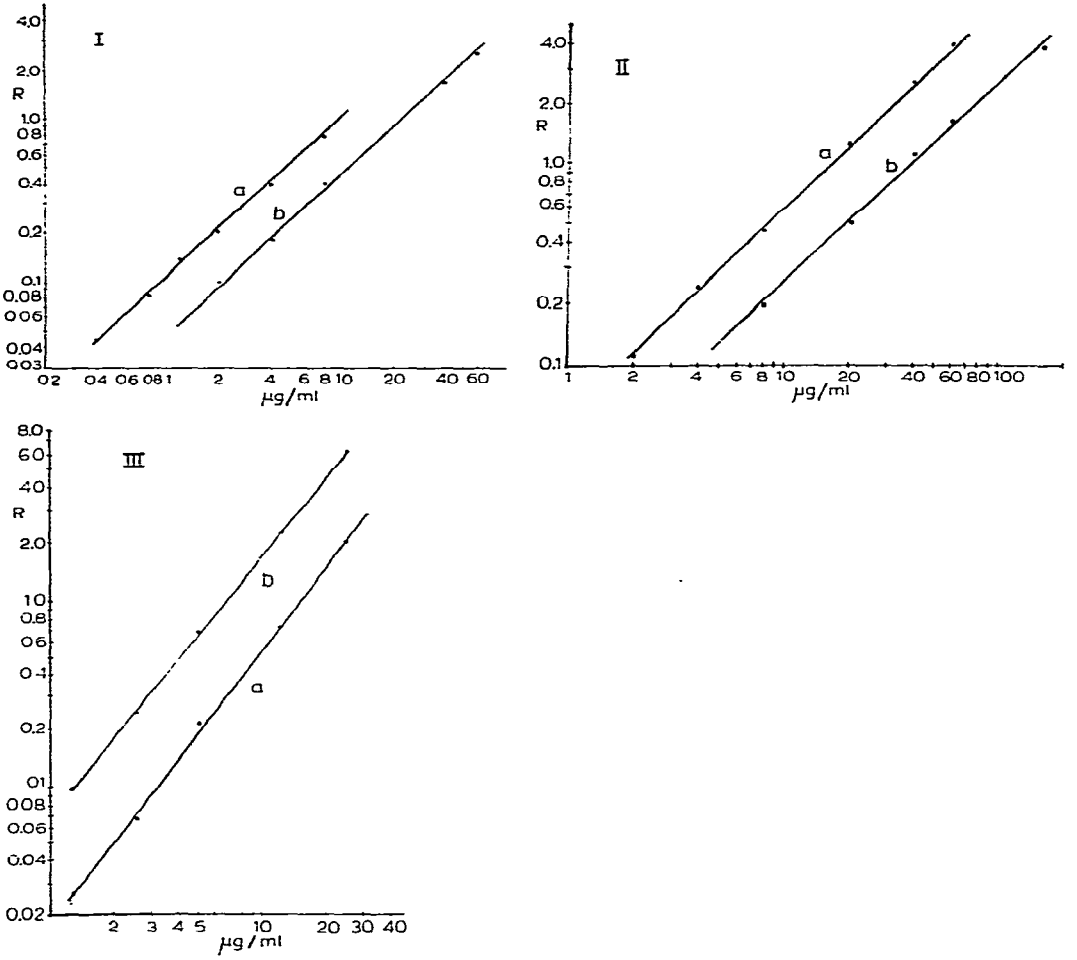

Fig. 4. Calibration curves for measurement of metabolites in urine. R = ratio of peak height of derivative to peak height of internal standard. I: (a) acetylhydrazine (concentration of free base) and (b) diacetylhydrazine. II: (a) isoniazid and (b) acetylisoniazid. III: hydrazine (concentration of free base) using (a) *p*-bromobenzaldehyde acetyl hydrazone and (b) *p*-bromobenzaldehyde azine as internal standards.

TABLE I

ACCURACY AND SENSITIVITY OF THE METHOD

Compound	*Conc. range of standard curve (μg/ml)*	*Standard error of curve*	*Regression coefficient*	*Standard deviation (μg/ml)* *	*Sensitivity (μg/ml)* **
Acetylhydrazine	0.4–8.0	±0.028	0.99	±0.042	0.4
Isoniazid	2–60	±0.049	0.99	±0.44	2
Acetylisoniazid	8–160	±0.058	0.99	±1.6	2
Diacetylhydrazine	2–60	±0.048	0.99	±0.27	0.4
Hydrazine (curve a, Fig. 4)	1.28–25.6	±0.061	0.99	±0.12	0.4
Hydrazine (curve b, Fig. 4)	1.28–25.6	±0.062	0.99	±0.4	0.4

* Standard deviation calculated for lowest two points on standard curve.
** Sensitivity as defined in the text.

of acetylhydrazine and isoniazid, respectively. Therefore this interference is not a significant problem. Acetylisoniazid did not produce measurable amounts of acetylhydrazine after hydrolysis.

Measurement of selected concentrations of the hydrazine compounds in water gave identical values to those carried out in urine. Isoniazid and acetylhydrazine are partially excreted in the urine as labile hydrazones of α-ketoglutaric acid and of pyruvic acid[11,12] and urine spiked with known amounts of the synthetic α-ketoacid hydrazones were found to yield the theoretical amounts of isoniazid and acetylhydrazine respectively when carried through the procedure.

Although the assay for isoniazid and acetylisoniazid is less sensitive than that for acetylhydrazine, diacetylhydrazine and hydrazine it is sufficiently sensitive to determine the levels of isoniazid and acetylisoniazid in the 0–24-h urine of humans given therepeutic doses of isoniazid. It was devised to do this at the same time as measuring acetylhydrazine, diacetylhydrazine and hydrazine. The sensitivity of the assay could however be increased by reducing the final volume of ethyl acetate if necessary. Thus, using one urine sample this method yields two extracts in which it is possible to measure acetylhydrazine, hydrazine, diacetylhydrazine, isoniazid and acetylisoniazid and the levels of these compounds in the urine of human volunteers given isoniazid have now been determined[13].

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REFERENCES

1 J. R. Mitchell, U. P. Thorgiersson, M. Black, J. A. Timbrell, W. S. Snodgrass, W. Z. Potter, D. J. Jollow and H. R. Keiser, *Clin. Pharmacol. Ther.*, 18 (1975) 70.
2 J. R. Mitchell, H. J. Zimmerman, K. G. Ishak, U. P. Thorgiersson, J. A. Timbrell, W. S. Snodgrass and S. D. Nelson, *Ann. Intern. Med.*, 84 (1976) 181.
3 S. D. Nelson, J. R. Mitchell, J. A. Timbrell, W. S. Snodgrass and G. B. Corcoran, *Science*, 193 (1976) 901.
4 J. Lloyd and D. A. Mitchison, *J. Clin. Pathol.*, 17 (1964) 622.
5 J. H. Peters, K. S. Miller and P. Brown, *J. Pharmacol. Exp. Ther.*, 150 (1965) 298.
6 H. G. Boxenbaum and S. Riegelman, *J. Pharm. Sci.*, 63 (1974) 1191.
7 G. E. Ellard, P. T. Gammon and S. M. Wallace, *Biochem. J.*, 126 (1972) 449.
8 M. Frater-Schröder and G. Zbinden, *Biochem. Med.*, 14 (1975) 274.
9 L. C. Dymond and D. W. Russell, *Clin. Chim. Acta*, 27 (1970) 513.
10 C. D. Hurd and R. I. Moni, *J. Amer. Chem. Soc.*, 77 (1955) 5359.
11 J. A. Timbrell, unpublished results, 1977.
12 V. Zamboni and A. Defrancheschi, *Biochim. Biophys. Acta*, 14 (1954) 430.
13 J. A. Timbrell and J. Wright, unpublished results, 1977.