Determination of Isoniazid and Metabolites in Biological Fluids

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Abstract □ Methods were developed for the determination of isoniazid and some of its metabolites in biological fluids. Isoniazid is determined in whole blood by a fluorometric procedure, and isoniazid and hydrazones, acetylisoniazid, isonicotinic acid, and isonicotinuric acid are determined in urine by colorimetry; each method is specific and accurate. Data are presented on these compounds following intravenous doses of isoniazid to both rapid and slow inactivator subjects of isoniazid.

Keyphrases □ Isoniazid and metabolites—fluorometric and colorimetric procedures for determination in biological samples □ Antituberculosis drugs—determination of isoniazid and metabolites in biological fluids □ Fluorometry—determination, isoniazid and metabolites in biological fluids □ Colorimetry—determination, isoniazid and metabolites in biological fluids

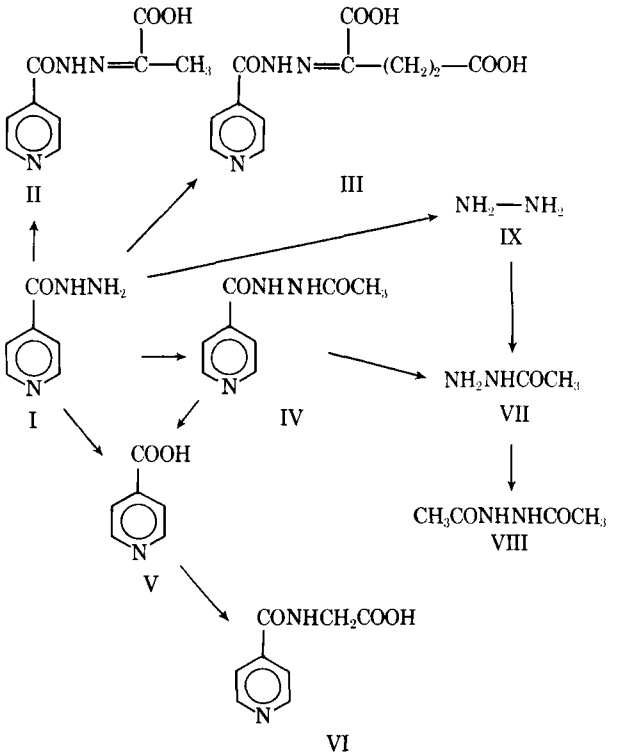
The synthesis of isoniazid (isonicotinic acid hydrazide) was first reported in 1912 (1), but it was not until the early 1950's that three independent research groups simultaneously discovered the marked antituberculosis activity of this compound (2). Presently, isoniazid is considered to be the most effective of the commonly employed antituberculosis drugs (3). Numerous methods have been reported for determining isoniazid and its metabolites in biological fluids (4, 5).

The major urinary excretion products resulting from isoniazid administration to humans are intact isoniazid (I), pyruvic acid isonicotinoylhydrazone (II), α-ketoglutaric acid isonicotinoylhydrazone (III), acetylisoniazid (IV), isonicotinic acid (V), isonicotinuric acid (VI), acetylhydrazine[1] (VII), and 1,2-diacetylhydrazine[1] (VIII) (4, 6). The pathways proposed[1] for the metabolism of isoniazid in humans are shown in Scheme I. Hydrazine (IX) was presumed to be formed from the hydrolysis of isoniazid to isonicotinic acid, but this compound was not detected. Examination of the literature indicates other metabolic routes may exist. Ziporin *et al.* (7, 8) reported an elevation of blood ammonia following isoniazid administration. Additionally, *in vitro* studies in which isoniazid was converted by animal tissue preparations into isonicotinic acid and hydrazine indicated that the hydrazine was subsequently converted to ammonia (9–13).

Schmidt (14) reported that pyruvic acid isonicotinoylhydrazone and α-ketoglutaric acid isonicotinoylhydrazone were cleaved promptly in pH 6–6.5 buffer to free isoniazid and the corresponding keto acids. Taking cognizance of the lability of the hydrazones, Schmidt suggested that their cleavage back to isoniazid in the body is possible. The direct conversion of isoniazid to isonicotinuric acid is suggested by the experiments of Wenzel (15–17). Incubation of isoniazid with glycine, with or without human serum, resulted in the production of isonicotinuric acid. Incubation of isonicotinic acid with glycine under the same conditions did not produce isonicotinuric acid.

Humans have been classified as either rapid or slow inactivators of isoniazid, and this capacity to inactivate (metabolize) isoniazid is a permanent hereditary characteristic (18). Approximately half of the Caucasian and American Negro population are rapid isoniazid inactivators and the other half are slow inactivators. Plasma half-lives in rapid inactivators range from 45 to 80 min; in slow inactivators, the half-lives range from 140 to 200 min. Isoniazid inactivator status results from the capacity to acetylate isoniazid (6).

The purpose of this investigation was to develop specific and accurate methods for the determination



Scheme I—Metabolism of isoniazid in humans

[1] G. A. Ellard and P. T. Gammon, to be published.

of isoniazid and some of its metabolites in biological fluids. In most instances, modifications of previously published methods were used. However, critical details of the methodology were found to be lacking, as was verification of the accuracy of methods in the presence of potentially interfering substances. Methods are reported here for the determination of isoniazid in blood as well as isoniazid plus hydrazones, acetylisoniazid, isonicotinic acid, and isonicotinuric acid in urine. Total isonicotinoyl compounds present in urine were determined by measurement of isonicotinic acid after strong acid hydrolysis. Verification of the utility of these methods is presented.

EXPERIMENTAL

Materials—Isonicotinic acid hydrazide[2], isonicotinic acid[2], barbituric acid[3], and vanillin[2] were purchased. Pyruvic acid isonicotinoylhydrazone and α-ketoglutaric acid isonicotinoylhydrazone were prepared by the method of Wenner (19), acetylisoniazid was prepared by the method of Fox and Gibas (20), isonicotinuric acid was prepared by a modification of the method used by Rohrlich (21) for the preparation of nicotinuric acid, acetylhydrazine was prepared by the method of Kost and Sagitullin (22), and 1,2-diacetylhydrazine was prepared by the method of Turner (23). All compounds were recrystallized or otherwise purified to constant melting point, and elemental analyses were obtained. Verification was obtained that the compounds were greater than 99% pure (24).

The following were used without further purification: chloramine-T[2], potassium cyanide (purified grade)[4], salicylaldehyde (from bisulfite addition compound)[2], 2-mercaptoethanol[2], *p*-dimethylaminobenzaldehyde[2], and hydrazine dihydrochloride[2].

The isobutanol[2] was extracted with equal volumes of 0.10 *N* HCl, 0.10 *N* NaOH, and water.

Apparatus—A fluorescence spectrophotometer[5] equipped with a xenon lamp[6] was used. A spectrophotometer[7] using 1.3-cm (0.5-in.) o.d. × 10.5-cm (4-in.) length colorimeter tubes was used for all colorimetric determinations. When filled with a colored solution absorbing at 600 nm, the absorbance from any two tubes differed by no more than 2.0%. A pH meter[8] was also utilized.

Methods—In each urine assay, standard solutions were prepared in water and carried through the experimental procedure. Water was also carried through the procedure and provided a blank. Unless otherwise stated, for each urine assay the standards and blanks prepared in water and urine resulted in identical calibration curves. For each assay, freshly prepared standards were run concomitantly with the unknowns. Metabolite interferences are reported on a molar basis. Representative calibration curves are described in each section; in every case, the intercept of the regression lines did not differ significantly from zero at the 5% level of significance (30).

Determination of Sum of Isoniazid and Its Hydrazones in Urine—The sum of isoniazid and its hydrazones in urine was determined, since it was demonstrated that the amount of free isoniazid in urine most probably does not reflect the amount of free isoniazid excreted by the kidney. Pyruvic acid, α-ketoglutaric acid, and many other keto-containing molecules are very reactive with isoniazid, forming hydrazones virtually spontaneously in aqueous solutions. Although these hydrazones may be preformed within the body, they are also very labile to hydrolysis at various urinary pH's. When using a concentration of pyruvic acid isonicotinoylhydrazone that might normally occur in the urine, approximately 30% of the pyruvic acid isonicotinoylhydrazone in 0.067 *M* pH 8.00 phosphate buffer was hydrolyzed to isoniazid in 30 min at room temperature (initial concentration of pyruvic acid isonicotinoylhydrazone was 49.7 μmoles/ml). When a freshly collected urine (pH 5.9) was spiked with an amount of pyruvic acid isonicotinoylhydrazone that might normally be present after an isoniazid dose, 35 min at room temperature resulted in approximately 25% hydrolysis to isoniazid. On the basis of these experiments, it seems likely that pyruvic acid isonicotinoylhydrazone in the urinary bladder would undergo hydrolysis. Hence, a measurement of intact urinary isoniazid would not reflect the actual amount excreted from the renal tubules. Therefore, it was decided to measure the sum of isoniazid and its hydrazones in urine.

The method involves the reaction between isoniazid and vanillin to form a colored hydrazone and is modified from the procedures of Peters (25) and Deeb and Vitagliano (26). In this method, a weak acid hydrolysis, modified from the procedures of Hughes *et al.* (27) and Short (28), is used to quantitatively split pyruvic acid isonicotinoylhydrazone and α-ketoglutaric acid isonicotinoylhydrazone to isoniazid.

Urine is diluted so as to contain approximately 4 μg/ml of isoniazid plus hydrazones (expressed in terms of isoniazid), and 5.00 ml is mixed with 1.00 ml of 0.200 *N* HCl in a culture tube. The capped tube is placed in a 52° water bath for 60 min and then cooled to room temperature. One milliliter of 1.00 *N* HCl and 1 ml of 10.0% (w/v) solution of vanillin in 95% ethanol are added. After 10 min the absorbance is determined at 365–370 nm against a blank. The yellow color of urine does add slightly to the absorbance, and a correction for each sample is necessary. For example, a 2 in 10 dilution of blank urine would have an absorbance of approximately 0.3. For many determinations, a common dilution is 3–5 in 100. Consequently, a second sample of diluted urine is also carried through the procedure, but 1.00 ml of 95% ethanol is used in place of the 1.00 ml of vanillin reagent. The absorbance of this solution is measured against its blank, and this absorbance is subtracted from the original absorbance value. Beer's law is obeyed ($r \geq 0.999$) for initial concentrations of isoniazid up to 8 μg/ml ($A \approx 0.35$). Isonicotinic acid, isonicotinuric acid, and 1,2-diacetylhydrazine do not interfere with this method, whereas 1% of the acetylisoniazid present is detected as isoniazid; presumably this results from its hydrolysis. Acetylhydrazine interferes to the extent of 1.8%, while hydrazine (not a urinary metabolite) interferes to the extent of 242%. Substances were present in blank urines that did react as apparent isoniazid, and these substances were equivalent, in a 24-hr period, to approximately 6 mg isoniazid. For a rapid isoniazid inactivator receiving a 700-mg dose of isoniazid, this interference would introduce an error of approximately 3.7%.

Determination of Acetylisoniazid in Urine—A modification of the Peters (29) extraction method is used; acetylisoniazid is extracted from diluted urine into an organic solvent mixture and is subsequently reextracted into dilute hydrochloric acid. Acetylisoniazid is separated from other hydrazino compounds by a modification of the ion-exchange chromatography procedure of Belles and Littleman (30). Unfortunately, the complete metabolic pathways of isoniazid were not known at that time, and the original ion-exchange method was developed solely to separate isoniazid and acetylisoniazid. The possibility that other isoniazid metabolites eluted with the acetylisoniazid was not determined. This is of critical importance because of the potential interference of such metabolites in the subsequent quantitation of acetylisoniazid.

In the present method, urine is diluted so as to contain approximately 50 μg acetylisoniazid/ml, and 3.00 ml is pipeted into a 90-ml prescription bottle containing 3.2 g ammonium sulfate. A 1.00-ml quantity of 0.100 *N* NaOH and 40.0 ml of organic extraction solvent (3:7 isopentyl alcohol–1,2-dichloroethane, water saturated) are added, and this mixture is vigorously shaken for 30 min followed by centrifugation. Thirty-five milliliters of the organic phase (top) is reextracted for 30 min with 6.00 ml of 0.100 *N* HCl and then separated by centrifugation. A 4.00-ml aliquot of the aqueous layer (top) is gently pipeted onto the ion-exchange column.

Prior to use, the resin[9] is soaked in 0.100 *N* HCl for at least 12 hr and then placed into columns. Disposable columns[10], glass barrel, 0.70 cm i.d., cut from 30 to a length of 15.0 cm, were used.

[2] Eastman.
[3] Aldrich.
[4] Mallinckrodt.
[5] Perkin-Elmer model 203.
[6] Perkin-Elmer model 150.
[7] Bausch and Lomb Spectronic 20.
[8] Corning model 10.

[9] AG 50W-X4, 100–200 mesh, hydrogen form, analytical grade cation-exchange resin, Bio-Rad Laboratories, Richmond, Calif.
[10] Bio-Rad.

The resin slurry is added to the column to a height of 3.0 cm, and 25 ml of 0.100 *N* HCl is passed through; if necessary, the resin bed height is then readjusted to 3.0 cm. Immediately prior to use, the 0.100 *N* HCl above the resin bed is permitted to fall to the level of the bed. Columns are prepared no sooner than 3 days prior to the assays, and 0.100 *N* HCl is permitted to remain on top of the resin bed during storage.

Just as the 4 ml of hydrochloric acid extract runs to the level of the resin bed, 4.00 ml of 0.100 *N* HCl is gently pipeted onto the column, and this is also permitted to run through. This hydrochloric acid wash does not elute acetylisoniazid but does remove substances that interfere with the assay. As soon as the 0.100 *N* HCl level runs to the level of the resin bed, a 25-ml volumetric flask is placed under the column to collect effluent. Immediately thereafter, elution solution is run through the column until the 25-ml flask is full. The elution solution consists of an aqueous solution of 0.100 *N* HCl, 0.200 *M* LiCl, 0.1 *M* KCl, and 10.0% (v/v) 95% ethanol. Reservoir caps for the columns, provided by the manufacturer, may be placed on top of the glass barrels to provide for a greater volume and pressure head for the elution solution (these caps hold approximately 7 ml). No attempt is made to control flow rate; however, a rate of approximately 1.5 ml/min is achieved with a maximum pressure head. Four milliliters of the 25.0 ml effluent is added to a calibrated test tube containing 1.00 ml of a freshly prepared solution of Ehrlich's reagent. Ehrlich's reagent is prepared fresh by adding 0.60 g *p*-dimethylaminobenzaldehyde to 20.0 ml of 10.0 *N* HCl. The uncapped tube is placed in a boiling water bath for 2.0 hr, thereby quantitatively converting acetylisoniazid to hydrazine. The solution is cooled to room temperature and made up to a volume of 5.0 ml with 0.100 *N* HCl, and the absorbance is measured at 450 nm. Beer's law is obeyed ($r \geq 0.999$) up to initial acetylisoniazid concentrations of 100 μg/ml ($A \approx 0.92$).

Acetylisoniazid is quantitatively eluted from the column, and the amount recovered in the 25 ml effluent is approximately 54% of that present in the initial 3.00 ml diluted urine. This recovery is a consequence of nonquantitative extraction and the use of aliquots (Fig. 1).

Isoniazid, pyruvic acid isonicotinoylhydrazone, α-ketoglutaric acid isonicotinoylhydrazone, isonicotinic acid, isonicotinuric acid, and 1,2-diacetylhydrazine do not interfere with this determination. Although isoniazid and the hydrazones are extracted, they are not eluted from the column with this procedure. Isonicotinic acid and isonicotinuric acid cannot interfere, since they have no hydrazine moiety. 1,2-Diacetylhydrazine is extracted but elutes prior to the addition of the elution solution. When urines were spiked with hydrazine and acetylhydrazine, these compounds interfered to the extent of approximately 7.8 and 9.0%, respectively. Whereas hydrazine is not found in urine after isoniazid administration, acetylhydrazine is definitely present (5). However, one can estimate the magnitude of this possible error; it will be shown that it results in a maximum error of 5%.

Determination of Isonicotinic and Isonicotinuric Acids in Urine—This two-component colorimetric analysis, modified from the method of Nielsch (31, 32) and Nielsch and Giefer (33), is based upon polymethine dye formation (33). Both isonicotinic acid and isonicotinuric acid form colored dyes: by measuring absorbance at two wavelengths, isonicotinic acid as well as isonicotinuric acid may be simultaneously determined. The original method is modified so as to achieve a greater degree of accuracy and to accommodate the rapid processing of large numbers of samples.

Acetate buffer is prepared according to Nielsch and Giefer (33), which on dilution with 4 parts water yields a solution of pH 5.0. Barbiturate buffer contains approximately 2% barbituric acid adjusted to pH 5.0 with sodium hydroxide. On standing, this solution develops a precipitate which is removed by filtration or decantation before use.

Urine is diluted so that the concentration of isonicotinic acid plus ½ isonicotinuric acid is approximately 10–15 μg/ml; this concentration may not exceed 20 μg/ml. To a 25-ml volumetric flask under a hood, the following are added with shaking in the following sequence: (*a*) 1.25 ml acetate buffer (pH 5.0); (*b*) 0.50 ml of 2% aqueous KCN, prepared fresh under a hood; (*c*) 5.0 ml of 1.0% chloramine-T trihydrate, prepared fresh; (*d*) 0.50 ml of 8.6% (v/v) acetic acid; (*e*) 6.50 ml barbiturate (pH 5.0) buffer; (*f*) 2.50 ml acetone; and (*g*) 1.00 ml unknown solution. By using automatic pipeting devices, 15–20 samples may be processed simultaneously; each reagent is added to all flasks before proceeding to the next reagent. The volume of each flask is brought to 25.0 ml with water, and the flasks are placed in the dark for 1.0 hr. Absorbances are determined at 590 and 625 nm.

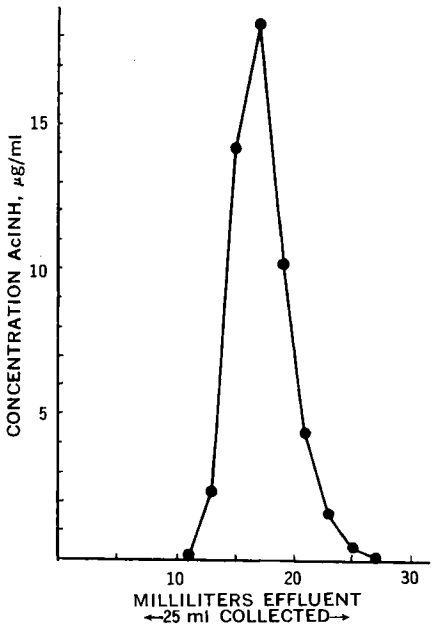


Figure 1—*Elution chromatogram for acetylisoniazid (AcINH).*

An important presumption in the method presented here is that the absorbances from the two components be additive; this presumption was shown to be valid. The absorbance maxima for the polymethine dyes of isonicotinic acid and isonicotinuric acid are 600 and 615 nm, respectively. The wavelengths of 590 and 625 nm were selected because they provided greater accuracy than 600 and 615 nm. To minimize errors, it is important to make all absorbance measurements at one wavelength setting before proceeding to the second setting, particularly since the wavelength settings on the spectrophotometer used are not precisely reproducible. Wavelength settings were selected to provide absorptivities similar to those calculated from Table I; these nominal settings would not be expected, however, to differ by more than about 5 nm from those reported here. Additionally, greater accuracy is achieved when the microgram per milliliter concentration of isonicotinic acid plus ½ isonicotinuric acid is 10 or above. The ratio of isonicotinic acid to isonicotinuric acid in a sample should be less than 4 and greater than 0.25 or accuracy in the determination of the lesser component is significantly reduced. Standard solutions of isonicotinic acid (10, 15, and 20 μg/ml) and isonicotinuric acid (20, 30, and 40 μg/ml) are carried through the procedure. Isonicotinic acid and isonicotinuric acid concentrations are determined by solving two simultaneous equations with two unknowns (33). Table I shows typical data from calibration curves.

Isoniazid, acetylisoniazid, pyruvic acid isonicotinoylhydrazone, α-ketoglutaric acid isonicotinoylhydrazone, hydrazine, acetylhydrazine, and 1,2-diacetylhydrazine do not interfere with this determination.

Determination of Total Isonicotinoyl Compounds in Urine (Total Isonicotinic Acid)—Total isonicotinic acid includes isonia-

Table I—Absorbances from Isonicotinic Acid and Isonicotinuric Acid from Typical Calibration Curves[a]

Absorbance[b]			
Isonicotinic Acid (590 nm)	Isonicotinic Acid (625 nm)	Isonicotinuric Acid (590 nm)	Isonicotinuric Acid (625 nm)
0.410 (0.9999)	0.103 (0.9991)	0.120 (0.9997)	0.135 (0.9994)

[a] Concentration of each component was 10 μg/25 ml final solution. [b] Values in parentheses indicate correlation coefficient of calibration curve.

zid, pyruvic acid isonicotinoylhydrazone, α-ketoglutaric acid isonicotinoylhydrazone, acetylisoniazid, isonicotinic acid, and isonicotinuric acid and is determined by a modification of the method of Peters (25). The principle of the method is to convert quantitatively all the aforementioned compounds to isonicotinic acid by strong acid hydrolysis. The isonicotinic acid is subsequently measured colorimetrically. The original Peters procedure utilizes hydrochloric acid as an acid catalyst. As will be discussed subsequently, this method consistently underestimates total isonicotinoyl compounds in urine.

In the modified procedure, urine is diluted so as to contain approximately 30–120 μg isonicotinoyl compounds/ml (expressed in terms of isonicotinic acid). Two milliliters of this solution is added to a 5-ml ampul containing 2.00 ml of 10.0 *N* H_2SO_4, and the ampul is sealed. The ampul is placed in an autoclave at 125–130° (27 psig) for 3.0 hr, and 1.00 ml of hydrolysate is neutralized with 5.00 ml of 1.00 *N* NaOH. Alternatively, 2.00 ml of hydrolysate may be neutralized with 5.00 ml of 2.00 *N* NaOH. An appropriate aliquot of the neutralized solution is subsequently used for isonicotinic acid determination. The colorimetric method is the same as that described for isonicotinic acid and isonicotinuric acid, except that the wavelength is 600 nm. One milliliter each of isonicotinic acid solutions (5, 10, 15, and 20 μg/ml) is used to prepare standards. Beer's law is obeyed ($r \geq 0.999$) up to 20 μg/25 ml final volume ($A \approx 0.88$).

With this procedure, all isonicotinoyl compounds are quantitatively converted to isonicotinic acid. While hydrochloric acid is entirely suitable for hydrolysis of aqueous isonicotinoyl solutions, it does not result in accurate quantitation of total isonicotinoyl compounds when urinary constituents are present; in such situations, recoveries of approximately 90–95% are obtained. The reason for this anomaly is unclear, since urines spiked with isonicotinic acid are not affected. One possibility is that a product other than isonicotinic acid is formed during the hydrolysis period.

The use of an autoclave is essential; placing these ampuls in a 130° oven frequently resulted in violent explosions.

Determination of Isoniazid in Whole Blood—The method used was modified from that of Scott and Wright (34) and is based upon formation of the isoniazid–salicylaldehyde hydrazone. The reduced form of the hydrazone, extracted into isobutanol, is highly fluorescent. Bisulfite is used to react with excess salicylaldehyde, thus making it nonfluorescent. Unfortunately, the method presented here requires precise handling of samples and completion of the total assay within 24 hr.

The salicylaldehyde–acetic acid reagent, zinc sulfate solution, and barium hydroxide solution are prepared fresh according to Scott and Wright (34). The acetate buffer consists of 0.120 *N* NaOH and 0.385 *M* sodium acetate; sufficient acetic acid or sodium hydroxide is added to this buffer so that 1.30 ml of buffer, 1.50 ml of water, and 0.40 ml of salicylaldehyde–acetic acid reagent result in a solution of pH 5.60 ± 0.05.

A 0.5-ml quantity of freshly drawn, heparinized blood is immediately placed in an acid-washed tube (Teflon cap) containing 15.0 ml of distilled water. The blood is permitted to hemolyze for about 1 min, after which time 1.00 ml of the zinc sulfate solution is added. The tube is vigorously shaken by hand for 5 sec; then 1.00 ml of barium hydroxide solution is added. After shaking vigorously for 5 sec, the mixture is immediately filtered through Whatman No. 1 filter paper into a thick-walled Pyrex culture tube (acid washed, Teflon-lined cap) resting in an ice water bath. The clear, colorless filtrate is immediately frozen by immersing in a dry ice–isopropanol mixture for 2 min and stored at −18°. Frozen filtrates gradually deteriorate; therefore, within 24 hr from the time of freezing, the filtrate is thawed and brought to room temperature in a 50° water bath; 1.50 ml of filtrate is mixed with 0.40 ml of salicylaldehyde–acetic acid reagent in an acid-washed tube with a Teflon-lined cap and is kept at room temperature for 20 min. After that reaction period, 1.35 ml of 2-mercaptoethanol–bisulfite buffer is added and the capped tube is placed in a 50° water bath for 10 min. The 2-mercaptoethanol–bisulfite buffer is prepared fresh by adding 1.92 ml of 2-mercaptoethanol (stench!) to 50.0 ml of acetate buffer containing 77.0 mg of sodium bisulfite (total volume approximately 51.92 ml).

The tube is cooled to room temperature by immersing in an ice water bath for 15 sec, and 4.00 ml isobutanol (water saturated) is added. The tube is vigorously shaken by hand for 5 sec to extract the reduced form of the hydrazone into the isobutanol. The tube is subsequently placed in an ice water bath for 5 min and is centrifuged for 5 min at 2400 rpm (without prior chilling, the heat generated by centrifugation causes turbidity). The top isobutanol layer is removed and fluorescence is determined (activation wavelength 392 nm, emission wavelength 478 nm). With the instrument used, the blank is set to 0 units and the standard of maximum concentration is set to 100 units. With a wide concentration range of unknowns, several calibration curves of limited range must be prepared (*e.g.*, 0.05–1 μg isoniazid/ml blood and 1–30 μg isoniazid/ml blood). In the examples just cited, linear curves were obtained with correlation coefficients of greater than 0.999. The lower limit of sensitivity is approximately 0.01 μg isoniazid/ml blood, whereas the upper limit of measurability is greater than 30 μg isoniazid/ml blood. Standards are prepared by having the requisite amount of isoniazid in the 15.0 ml of distilled water and adding 0.50 ml of blood.

Blood must be obtained from the subject receiving the isoniazid, and this should be drawn just prior to injection. In this way, standards are prepared immediately before the start of the experiment and carried through the procedure with the samples.

Isonicotinic acid, isonicotinuric acid, hydrazine, acetylhydrazine, 1,2-diacetylhydrazine, and acetylisoniazid do not interfere with this determination. The hydrazones α-ketoglutaric acid isonicotinoylhydrazone and pyruvic acid isonicotinoylhydrazone interfere to the extent of 1.1 and 7.8%, respectively. The potential for interference from hydrazones will be discussed.

RESULTS AND DISCUSSION

Potentially Interfering Substances in Assay Procedures—The assay of acetylisoniazid in urine requires one to hydrolyze the compound to hydrazine. The presence of acetylhydrazine in the sample can lead to an error since it also results in the formation of hydrazine. An estimation of the error resulting from the acetylhydrazine interference was calculated from the data of Ellard and Gammon[1]. These investigators administered 190 mg monoacetylhydrazinium fumarate orally to a slow isoniazid inactivator subject and recovered 44.5% of the dose as 1,2-diacetylhydrazine in the urine; although urinary acetylhydrazine could not be determined accurately, the maximum amount that could be excreted was 55.5% of the oral dose. This information may be applied to the data reported elsewhere (24).

After intravenous infusion of 670 mg of isoniazid to Subject B (rapid acetylator), equimolar cumulative amounts of acetylisoniazid and isonicotinic acid derivatives (isonicotinic acid and isonicotinuric acid) were excreted in the urine. Since isonicotinic acid and isonicotinuric acid do not contain a hydrazine moiety, their former hydrazine content would necessarily have to be accounted for elsewhere. For this calculation, it was assumed that these hydrazine moieties were solely converted to acetylhydrazine and 1,2-diacetylhydrazine. If the 55.5% figure of Ellard and Gammon is used, the ratio of cumulatively excreted acetylisoniazid–acetylhydrazine is 1:0.555. Since only 9.0% of acetylhydrazine is detected as acetylisoniazid, 0.05 mole (0.555 × 0.09) acetylhydrazine is detected as acetylisoniazid for each mole of acetylisoniazid actually excreted. This indicates that the contribution of acetylhydrazine to the acetylisoniazid assay results in a maximum 5% error. In rapid acetylator subjects, more acetylhydrazine is converted to 1,2-diacetylhydrazine, and the error would be even smaller. Conversion of the hydrazine moieties to ammonia would reduce the error to a greater extent.

Another measure of the error in the acetylisoniazid determination is provided by the cumulative urinary excretion data after intravenous administration of acetylisoniazid to a slow acetylator volunteer (Subject A) who received 587 mg by constant infusion over 60 min; urine pH was maintained at 8.0 ± 2.0 by administration of sodium bicarbonate (35), and urine was collected for 34 hr. The cumulative excretion of acetylisoniazid, isonicotinic acid, and isonicotinuric acid was 586 mg. Total isonicotinoyl compounds accounted for 585 mg. If there were a significant interference from acetylhydrazine in the acetylisoniazid determination, the sum of the urinary components would exceed total isonicotinoyl compounds; this did not occur.

In the assay of isoniazid in whole blood, there is a potential for interference from hydrazones that are converted in the processing into isoniazid. To quantitate the interference from hydrazones in these experiments, a study was undertaken with Subject A (slow

Table II—Experimental Trial of Urinary Assay Methods

Compound	Percent of Theoretical Amount Found	
	Rapid Inactivator Urine	Slow Inactivator Urine
Isoniazid plus hydrazones	99.6	98.0
Acetylisoniazid	96.9	97.0
Isonicotinic acid	95.6	97.9
Isonicotinuric acid	101	95.4
Total isonicotinoyl compounds	98.8	98.8

acetylator) with urine pH maintained at 8 by sodium bicarbonate administration (35). A dose of 670 mg isoniazid was infused at a constant rate over 5.48 min. Blood was drawn at approximately 50-min intervals for 550 min; assays were performed on each sample for isoniazid by the fluorometric method and for isoniazid plus hydrazones by a modification (24) of the vanillin colorimetric method of Deeb and Vitagliano (26). Isoniazid hydrazones were first hydrolyzed to isoniazid in the latter method. The concentrations at each time point were analyzed by the t test for paired observations (36); at a 5% level of significance, the concentrations were not identical, indicating that hydrazones were present in blood. By assuming that pyruvic acid isonicotinoylhydrazone was the only hydrazone in blood, the concentration of isoniazid and pyruvic acid isonicotinoylhydrazone in each sample was calculated by solving two equations with two unknowns:

V = 100% isoniazid concentration + 100% pyruvic acid isonicotinoylhydrazone concentration (Eq. 1)

F = 100% isoniazid concentration + 7.8% pyruvic acid isonicotinoylhydrazone concentration (Eq. 2)

where V = concentration determined by vanillin colorimetric method, and F = concentration determined by fluorometric method.

The two coefficients of 100% in Eq. 1 indicate that 100% of the isoniazid and 100% of the pyruvic acid isonicotinoylhydrazone are detected by the vanillin colorimetric method. The coefficients of 100 and 7.8% in Eq. 2 indicate that the fluorometric method measures 100% of the isoniazid present but only 7.8% of the pyruvic acid isonicotinoylhydrazone. Since these are two simultaneous equations with two unknowns, isoniazid and pyruvic acid isonicotinoylhydrazone concentrations are solvable.

The maximum concentration of pyruvic acid isonicotinoylhydrazone in any sample was 19.9% of the concentration of isoniazid present. The interference in the fluorometric method would be 7.8% of that value, for an approximate overall 1.6% error in the isoniazid blood level. The average blood level of pyruvic acid isonicotinoylhydrazone was calculated at 4.65% of the isoniazid blood level (average error of isoniazid by fluorometry approximately 0.36%). Russell (37) reported a similar finding in subjects receiving isoniazid; in five subjects, hydrazone blood levels averaged 15.5% of the isoniazid blood levels.

The original Scott and Wright (34) method was modified only slightly, in hope of eliminating the slight interference from the hydrazones. The hydrazones, however, interfere slightly in both methods, and this interference has been shown to be virtually insignificant due to the low levels of hydrazones in blood. The Scott and Wright (34) procedure or possibly the modification of Ellard *et al.* (5) are consequently preferred over the present method due to the instability problem inherent in the present method.

Experimental Trial of Urine Assay Methods—The data of Peters *et al.* (6) were utilized to prepare spiked urines containing isoniazid, pyruvic acid isonicotinoylhydrazone, α-ketoglutaric acid isonicotinoylhydrazone, acetylisoniazid, isonicotinic acid, isonicotinuric acid, and 1,2-diacetylhydrazine (at the time of these studies, acetylhydrazine had not been established as a urinary metabolite). 1,2-Diacetylhydrazine was added in equal molar concentration to the sum of isonicotinic acid and isonicotinuric acid. The urines were prepared in such a manner as to mimic what might be expected in the 24-hr urines of rapid and slow isoniazid inactivators who were administered a 1.00-g iv isoniazid dose; a urine flow rate of 1.00 ml/min was assumed.

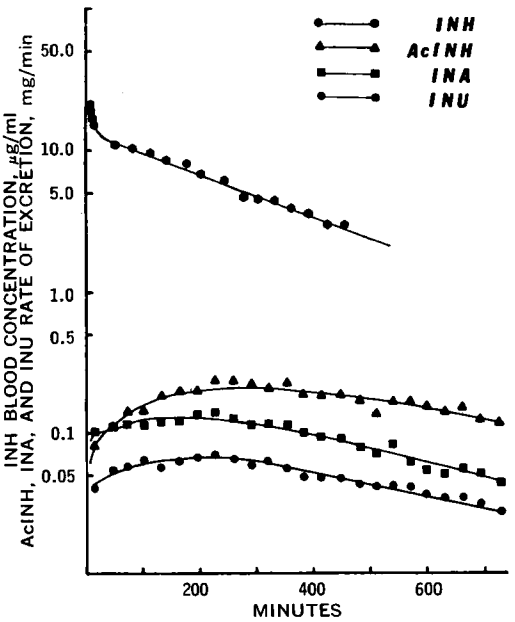


Figure 2—*Isoniazid blood levels and rates of urinary excretion of acetylisoniazid (AcINH), isonicotinic acid (INA), and isonicotinuric acid (INU) following intravenous administration of isoniazid (INH) to slow inactivator Subject A (all compound amounts corrected to correspond to mole equivalents of INH).*

The rapid inactivator urine contained the following molar percentages: isoniazid, 3.6; pyruvic acid isonicotinoylhydrazone, 2.9; α-ketoglutaric acid isonicotinoylhydrazone, 1.4; acetylisoniazid, 43.8; isonicotinic acid, 27.1; isonicotinuric acid, 21.3; and 1,2-diacetylhydrazine, 48.4. The slow inactivator urine contained the following molar percentages: isoniazid, 12.7; pyruvic acid isonicotinoylhydrazone, 13.9; α-ketoglutaric acid isonicotinoylhydrazone,

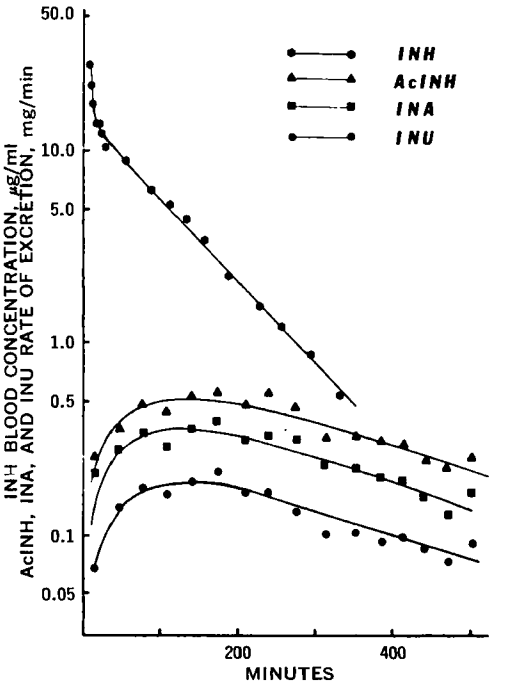


Figure 3—*Isoniazid blood levels and rates of urinary excretion of acetylisoniazid (AcINH), isonicotinic acid (INA), and isonicotinuric acid (INU) following intravenous administration of isoniazid (INH) to rapid inactivator Subject B (all compound amounts corrected to correspond to mole equivalents of INH).*

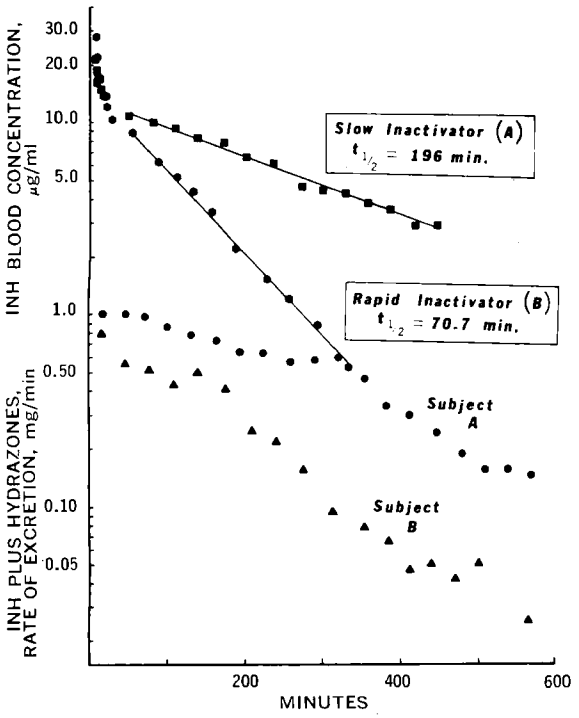


Figure 4—*Isoniazid blood levels and rates of urinary excretion of isoniazid (INH) plus hydrazones in rapid and slow inactivator subjects following intravenous isoniazid administration (all compound amounts corrected to mole equivalents of INH).*

6.9; acetylisoniazid, 34.3; isonicotinic acid, 18.1; isonicotinuric acid, 14.2; and 1,2-diacetylhydrazine, 32.3. Each urine assay was performed on these samples. On the basis of these results (Table II), it was concluded that the asays are valid. Acetylhydrazine interferes somewhat in the acetylisoniazid determination, as previously discussed.

Additionally, it was demonstrated from urine collected after isoniazid administration that these urine assays gave identical results when the urine was assayed shortly after collection and after 6.0 months of storage at −18°.

Isoniazid Administration Studies—Isoniazid was administered intraveneously to two human subjects in apparent good health. Subject A (Caucasian male, age 29 years, weight 69.0 kg, height 165 cm) was a slow inactivator who received 681 mg infused at a constant rate over 5.40 min. Subject B (Caucasian male, age 50 years, weight 80.0 kg, height 181 cm) was a rapid inactivator who received 670 mg infused at a constant rate over 5.20 min. Urine pH was maintained at pH 8.0 ± 0.2 in each study by oral administration of sodium bicarbonate (35) to eliminate the possibility of fluctuations in urinary excretion rates due to changes in urinary pH.

Figures 2 and 3 show isoniazid blood curves as well as rates of excretion curves for acetylisoniazid, isonicotinic acid, and isonicotinuric acid for Subjects A and B, respectively. Figure 4 compares the isoniazid blood curves to the rates of excretion curves for the sum of isoniazid and hydrazones. The slow inactivator subject had an isoniazid half-life of 196 min compared to 70.7 min for the rapid inactivator. The rapid decline of isoniazid blood concentrations during the first 50 min occurred during the so-called distributive phase. In separate experiments, without urine pH control, Subject A received 1.00 g isoniazid orally and Subject B received 350 mg isoniazid orally. The method of Scott and Wright (34) was used to determine isoniazid serum levels, and the half-lives were 148 and 69.9 min for Subjects A and B, respectively.

The validity of the urinary assay methods was verified in the two intravenous isoniazid administration studies by comparing the sum of isoniazid plus hydrazones, acetylisoniazid, isonicotinic acid, and isonicotinuric acid as determined by each individual method to total isonicotinoyl compounds, determined subsequent to strong acid hydrolysis. Theoretically, these results should be equal (Table III), and good agreement is observed.

Table III—Comparison of Sum of Urinary Isoniazid plus Hydrazones, Acetylisoniazid, Isonicotinic Acid, and Isonicotinuric Acid as Determined by Each Method to Total Isonicotinoyl Compounds Determined Subsequent to Strong Acid Hydrolysis

Minutes after Start of Injection	Sum of Isoniazid plus Hydrazones, Acetylisoniazid, Isonicotinic Acid, and Isonicotinuric Acid, (mg)	Total Isonicotinoyl Compounds, mg	Difference[a], %
Subject A (Slow Inactivator)			
35.5	44.45	50.1	+11.3
59.1	30.44	33.7	+9.67
88.5	37.75	39.9	+5.39
116.0	32.27	32.0	−0.844
150.0	38.71	38.1	−1.60
181.3	34.76	33.6	−3.45
208.7	28.41	27.1	−4.83
243.5	37.21	36.2	−2.79
276.3	32.59	33.2	+1.84
305.0	27.92	27.3	−2.27
339.3	33.69	32.0	−5.28
370.3	26.76	26.6	−0.602
396.0	17.18	16.5	−4.12
430.2	21.24	20.0	−6.20
466.0	20.23	20.0	−1.15
496.0	14.26	13.5	−5.63
524.0	11.32	10.4	−8.85
554.0	13.38	13.1	−2.14
586.0	13.18	12.7	−3.78
613.0	11.202	12.2	+8.18
648.0	12.82	11.6	−10.52
677.0	11.396	10.6	−7.51
710.0	10.68	9.25	−15.46
747.0	10.074	9.44	−6.72
Subject B (Rapid Inactivator)			
32.0	42.1	45.9	+8.28
62.0	39.9	43.3	+7.85
94.0	48.1	51.8	+7.14
126.0	42.3	43.4	+2.53
158.0	49.9	51.7	+3.48
195.0	57.7	59.9	+3.67
227.0	38.5	38.8	+0.773
259.0	40.5	39.7	−2.02
296.0	39.4	42.1	+6.41
335.0	29.0	29.6	+2.03
373.0	28.0	28.7	+2.44
400.0	17.9	18.5	+3.24
426.0	16.5	17.1	+3.51
458.0	17.3	17.2	−0.581
486.0	13.0	12.5	−4.00
516.0	16.8	17.0	+1.18

[a] Calculated from:

$$\frac{\left(\begin{array}{c}\text{total isonicotinoyl}\\ \text{compounds}\end{array}\right) - \left(\begin{array}{c}\text{sum of isoniazid plus}\\ \text{hydrazones, acetylisoniazid,}\\ \text{isonicotinic acid, and}\\ \text{isonicotinuric acid}\end{array}\right)}{\text{(total isonicotinoyl compounds)}} \times 100$$

Pharmacokinetic analyses will be reported at a later date.

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Interconversion of Ampicillin and Hetacillin *In Vitro*

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Abstract □ The conversion of hetacillin to ampicillin in aqueous solution was studied by hydroxylamine assay, IR analysis, and ^{14}C-labeling methods. Each technique indicates a half-life of at least 4 hr for hetacillin at physiological pH. The interconversion of ampicillin and hetacillin in aqueous solutions can be controlled by the concentration of added acetone. Excess acetone leads to the formation of hetacillin in aqueous solutions of ampicillin. A scheme for this interconversion is proposed, which accounts for the production of a Schiff base intermediate and penicillenic acid.

Keyphrases □ Hetacillin and ampicillin interconversion in aqueous solution—mechanism, effect of acetone concentration, determination by hydroxylamine assay, IR analysis, and ^{14}C-labeling □ Ampicillin and hetacillin interconversion in aqueous solution—mechanism, effect of acetone concentration, determination by hydroxylamine assay, IR analysis, and ^{14}C-labeling

Ampicillin and hetacillin degrade to different products in aqueous solution. Concentrated solutions of ampicillin form polymers while similar solutions of hetacillin do not produce polymers of the ampicillin type but lead instead to a penicillenic acid with a characteristic UV absorbance peak at 317 nm (1). In a previous report (2), the finding of different degradative pathways for ampicillin and hetacillin in solution indicated that hetacillin could not be rapidly and completely converted to ampicillin and acetone