# The Determination of Isoniazid and its Metabolites Acetylisoniazid, Monoacetylhydrazine, Diacetylhydrazine, Isonicotinic Acid and Isonicotinylglycine in Serum and Urine

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1. A solvent system was devised for the extraction of isoniazid and its metabolites acetylisoniazid, monoacetylhydrazine, diacetylhydrazine, isonicotinic acid and isonicotinylglycine from serum and urine. 2. Specific chemical and fluorimetric methods were developed for the determination of the extracted isoniazid and acetylisoniazid, and chemical methods for the determination of monoacetylhydrazine, diacetylhydrazine, isonicotinic acid and isonicotinylglycine. 3. When applied to serum, these methods were capable of measuring concentrations of down to about 0.005 $\mu$g of isoniazid/ml, 0.05 $\mu$g of acetylisoniazid/ml, 0.2 $\mu$g of monoacetylhydrazine/ml, 0.2 $\mu$g of diacetylhydrazine/ml, 0.02 $\mu$g of isonicotinic acid/ml and 0.1 $\mu$g of isonicotinylglycine/ml. 4. In urine, these methods were capable of measuring concentrations of down to about 0.05 $\mu$g of isoniazid/ml, 0.2 $\mu$g of acetylisoniazid/ml, 1 $\mu$g of diacetylhydrazine/ml, 0.1 $\mu$g of isonicotinic acid/ml and 0.2 $\mu$g of isonicotinylglycine/ml. 5. The stability of these compounds was studied in serum and urine and a method devised to decrease their decomposition in serum.

Isoniazid (isonicotinyl hydrazide) is the most widely used chemotherapeutic agent for the treatment of tuberculosis (Fox, 1968). Numerous studies have demonstrated that the rate at which isoniazid is metabolized in man varies considerably from one individual to another. Subjects can be classified as 'slow' or 'rapid' inactivators of isoniazid according to the rate at which they convert the drug into metabolites that are devoid of antituberculosis activity, and the isoniazid inactivator status of tuberculosis patients can be of clinical importance in certain circumstances (Devadatta *et al.*, 1960; Tuberculosis Chemotherapy Centre, Madras, 1970). The evidence for the biochemical basis and genetic control of isoniazid inactivation has been reviewed by Evans (1968) and Peters (1968).

The most important metabolites of isoniazid that have been identified from their excretion in the urine are: $N^1$-acetyl-$N^2$-isonicotinylhydrazine (acetylisoniazid), isonicotinic acid, isonicotinylglycine, pyruvic acid isonicotinylhydrazone and $\alpha$-oxoglutaric acid isonicotinylhydrazone (Peters *et al.*, 1965a). Monoacetylhydrazine and diacetylhydrazine have been postulated as potential metabolites of isoniazid in man but their formation has not been unequivocally demonstrated.

Peters *et al.* (1965b) have reviewed the methods

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previously employed for the determination of isoniazid and its metabolites, have discussed their often inadequate specificity and have described a specific ion-exclusion column-chromatographic procedure to separate isoniazid and its metabolites before their colorimetric determination. Unfortunately this procedure is rather laborious; further, like many of the other previous methods, it is not sensitive enough to measure the relatively lower concentrations of these compounds that are found in the serum or plasma of subjects receiving therapeutic doses of isoniazid.

Isoniazid can be determined in serum or urine by the colorimetric method of Maher *et al.* (1957), or by the more sensitive fluorimetric procedure of Peters (1960). The most sensitive specific method for the determination of isoniazid in serum is the fluorimetric method of Scott & Wright (1967), but this method cannot be applied directly to the determination of isoniazid in urine.

Venkataraman *et al.* (1968) have described a specific colorimetric method for the determination of acetylisoniazid in urine but their method is inapplicable to serum. Other methods for the determination of acetylisoniazid are based on its conversion by gentle acid hydrolysis into isoniazid (Short, 1961; Peters *et al.*, 1965b) or by vigorous hydrolysis to isonicotinic acid (Belles & Littleman, 1960; Heller *et al.*, 1961) and hydrazine (Maher *et al.*, 1957; Peters, 1960). As a consequence these methods are necessarily

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unspecific unless acetylisoniazid is first separated from isoniazid, isonicotinic acid and isonicotinylglycine, for example by column chromatography (Belles & Littleman, 1960; Heller *et al.*, 1961; Peters *et al.*, 1965*b*).

Peters *et al.* (1965*b*) have described a method for the determination of the acetylated derivatives of hydrazine in urine in which monoacetylhydrazine and diacetylhydrazine are hydrolysed with M-HCl for 24h at 45°C to hydrazine, which is then determined colorimetrically by reaction with *p*-dimethylamino-benzaldehyde.

Isonicotinic acid and isonicotinylglycine can be determined in urine by reaction with cyanogen chloride and barbituric acid, and measurement of the extinction of the respective polymethine dyes at 600 and 620nm (Nielsch & Giefer, 1959*a,b*). Alternatively, the two compounds can be separated by column chromatography before reaction (Peters *et al.*, 1965*b*). However, these methods are not sufficiently sensitive to permit the determination of isonicotinic acid and isonicotinylglycine in the serum.

In the present method, isoniazid, acetylisoniazid, monoacetylhydrazine, diacetylhydrazine, isonicotinic acid and isonicotinylglycine were separated from serum or urine by a solvent extraction procedure, the urine being first treated with acid to convert the pyruvic acid and α-oxoglutaric hydrazones into isoniazid. After extraction, isoniazid was then determined colorimetrically by a modification of the method of Maher *et al.* (1957), or fluorimetrically, by a modification of the Scott & Wright (1967) procedure. Acetylisoniazid was determined colorimetrically after strong acid hydrolysis to hydrazine by a modification of the method of Kelly & Poet (1952) or fluorimetrically, after gentle acid hydrolysis to isoniazid, by using the conditions described by Peters *et al.* (1965*b*). In each case, isoniazid was first destroyed by treatment with nitrous acid. Mono-acetylhydrazine was converted into hydrazine by using the conditions proposed by Peters *et al.* (1965*a*) and then determined in a manner similar to that employed for the colorimetric determination of acetylisoniazid. Diacetylhydrazine was estimated in a similar way, but after prior treatment with nitrous acid to eliminate interference from isoniazid, hydrazine or monoacetylhydrazine. Extracted isonicotinic acid and isonicotinylglycine were determined by a modification of the Nielsch & Giefer (1959*a,b*) method.

## Methods

### Chemicals

Isoniazid, isonicotinic acid and hydrazine sulphate were purchased from BDH Chemicals, Poole, Dorset, U.K. Isoniazid was then recrystallized from butan-1-

ol (m.p., uncorr., 168°C) (Peters, 1960) and isonicotinic acid from water. Acetylisoniazid was synthesized by the method of McKennis *et al.* (1956) and recrystallized from butan-1-ol (m.p. 156°C). Pyruvic acid isonicotinylhydrazone (sodium salt, m.p. 258°C decomp.) was synthesized by the method of Dymond & Russell (1970) and shown to contain the theoretical amount of acid-labile isoniazid. The 1,2-diacetyl-hydrazine (m.p. 133°C) was synthesized by the method of Turner (1947). Isonicotinylglycine and monoacetylhydrazinium fumarate were gifts from Dr. J. H. Peters. Most of the other chemicals were of A.R. grade; the water used was first deionized and then glass distilled.

### Extraction procedure

A 3ml sample of serum, plasma or urine was gently shaken by hand for 10s with 1ml of 4M-sodium acetate and 13ml of butan-1-ol in a stoppered centrifuge tube of about 30ml capacity. Immediately afterwards, 3g of $(NH_4)_2SO_4$ was added and the tube reshaken. Care was taken to avoid shaking too vigorously at this stage as this could cause emulsion formation. The phases were separated by centrifugation and the upper butan-1-ol extract removed with a Pasteur pipette. A 10ml portion of this extract was then shaken for 10s on a vortex mixer with 1 ml of 4M-sodium acetate and 10ml of ethyl acetate–*n*-heptane (1:1, v/v). After centrifugation, isonicotinic acid and isonicotinylglycine were determined colorimetrically in the aqueous sodium acetate extract. Isoniazid, acetylisoniazid, monoacetylhydrazine and diacetylhydrazine were then extracted from the organic phase by shaking 18ml with 4ml of 0.1M-HCl for 10s on a vortex mixer. The volume of 0.1M-HCl used for the extraction can be increased, if necessary, for multiple analyses. For the determination of isoniazid plus its acid-labile hydrazones (referred to hereafter as acid-labile isoniazid) in the urine, 0.3ml of 2M-HCl was first added to 3ml of urine and left for 15min at room temperature (15–20°C). After the addition of 0.3ml of 2M-NaOH, the extraction was done as above.

Suitable standards (prepared in triplicate) consisted of 3ml of water, normal urine (from a 24h collection adjusted to 2.4 litres), and serum and urine standards containing 0.1μg of isoniazid/ml (fluorimetric method), 20μg of isoniazid/ml (colorimetric method), 10μg of acetylisoniazid/ml, 1μg of isonicotinic acid/ml, 2.5μg of isonicotinylglycine/ml, 10μg of monoacetylhydrazine/ml (as the fumarate) or 10μg of diacetylhydrazine/ml respectively. These were prepared from aq. 1mg/ml solutions of these compounds, which could be stored without significant decomposition for at least 2 months at 4°C. Acetylisoniazid was added to serum-plasma immediately before extraction into butan-1-ol.

*Determination of isoniazid and its metabolites*

*Colorimetric determination of isoniazid.* The extinction of 0.5 ml of the 0.1 M-HCl extract was measured at 380 nm before ($A$) and 5 min after ($B$) the addition of 0.05 ml of 2% (w/v) vanillin in ethanol–water (1:3, v/v) by using microcells of 1 cm light-path in a Unicam SP.600 spectrophotometer. The concentration of isoniazid was then calculated by comparison of the function $1.1 \times B - A$ with the value of this function for the standards.

*Fluorimetric determination of isoniazid.* A 1 ml sample of the 0.1 M-HCl extract was pipetted into a small centrifuge tube together with 0.3 ml of freshly prepared salicylaldehyde solution (0.1 ml of salicyl-aldehyde dissolved in 1 ml of ethanol and diluted to 100 ml with water). Extracts containing more than 0.1 μg of isoniazid/ml were first diluted to 0.05–0.1 μg/ml. M-NaOH was added dropwise until the solution was just yellow, and then 10% (v/v) acetic acid until the yellow colour just disappeared. After adding a further 0.1 ml of 10% (v/v) acetic acid, the reaction was allowed to proceed at room temperature for 15 min. A 1 ml sample of a freshly prepared aqueous solution containing 0.5 M-sodium acetate, 0.2% sodium metabisulphite and 0.15 M-NaOH was then added together with 0.04 ml of 2-mercapto-ethanol from a '50-dropper' pipette. The tube was stoppered and then heated for 10 min at 50°C. After cooling in ice, 2 ml of butan-1-ol was added, and after further cooling, the fluorophore was extracted by shaking for 10 s before and after the addition of 2 g of $(NH_4)_2SO_4$. The fluorescence of the ice-cold butan-1-ol extract was then measured as rapidly as possible in an Aminco–Bowman spectro-photofluorimeter at 480 nm, the excitation wavelength being 390 nm.

*Colorimetric determination of acetylisoniazid.* A 1 ml sample of the 0.1 M-HCl extract was pipetted into a small stoppered centrifuge tube together with 0.1 ml of aq. 1% (w/v) $NaNO_2$. Extracts containing more than 5 μg of acetylisoniazid/ml were first diluted to 3–5 μg/ml. After being left for 15 min at room temperature, 0.1 ml of aq. 10% (w/v) ammonium sulphamate solution was added, and after a further 5 min, 0.5 ml of a freshly prepared solution of 3% (w/v) *p*-dimethylaminobenzaldehyde in 9 M-HCl–ethanol (1:1, v/v) was added. The tube was then heated for 1 h at 100°C and after being cooled, 1 ml of butan-1-ol, 5 ml of acetic acid (23%, v/v) and 4 g of $(NH_4)_2SO_4$ were added and the chromophore extracted by agitating for 15 s on a vortex mixer. The extinction of the butan-1-ol extract was then measured at 485 nm.

*Fluorimetric determination of acetylisoniazid.* A 1 ml sample of the 0.1 M-HCl extract was pipetted into a test-tube graduated with one mark at 10 ml. After treatment with 0.1 ml of aq. 1% (w/v) $NaNO_2$, 0.1 ml of 10% (w/v) ammonium sulphamate, and

hydrolysis with 1 ml of 2 M-HCl for 24 h at 45°C, the tube was made to the mark with water and the isoniazid released was determined by the fluorimetric method described previously.

*Colorimetric determination of monoacetylhydrazine.* A 1 ml sample of the 0.1 M-HCl extract was pipetted into a small stoppered centrifuge tube together with 1 ml of 2 M-HCl and incubated for 24 h at 45°C. After cooling the hydrolysate to room temperature, 0.5 ml of a freshly prepared solution of 3% (w/v) *p*-dimethylaminobenzaldehyde in 9 M-HCl–ethanol (1:1, v/v) was added. After 10 min 1 ml of butan-1-ol, 5 ml of acetic acid (23%, v/v) and 4 g of $(NH_4)_2SO_4$ were added, and after agitating for 15 s on a vortex mixer, the $E_{485}$ of the butan-1-ol extract was measured. From this value was subtracted that obtained by treating a further 1 ml of the 0.1 M-HCl extract in the same way but omitting the incubation at 45°C.

*Colorimetric determination of diacetylhydrazine.* A 1 ml sample of the 0.1 M-HCl extract was treated in a manner identical with that used for the colorimetric determination of monoacetylhydrazine with the exception that before incubation at 45°C it was treated with $NaNO_2$ and ammonium sulphamate in the manner described for the determination of acetyl-isoniazid.

*Colorimetric determination of isonicotinic acid and isonicotinylglycine.* A 0.5 ml sample of the aqueous sodium acetate extract was pipetted into a small test-tube together with 0.2 ml of 23% (v/v) acetic acid. Extracts containing more than 1 μg of isonicotinic acid/ml or 2.5 μg of isonicotinylglycine/ml were appropriately diluted with 4 M-sodium acetate before the addition of acetic acid. The reaction was started by the addition of 0.1 ml of freshly prepared 10% (w/v) KCN from a microsyringe and mixed by agitating for 5 s on a vortex mixer. At 15 s, 0.1 ml of freshly prepared 10% (w/v) chloramine-T was added and the mixture reagitated, and at 30 s, 0.5 ml of freshly prepared 1% (w/v) barbituric acid in acetone–water (1:1, v/v) was added. For accuracy it was essential that the timing of these additions was strictly followed. After being left in the dark for 30 min, the extinction was measured at 600 and 620 nm. If, after extraction and reaction the extinction of the isonicotinic acid and isonicotinylglycine standards was $A_1$ and $B_1$, respectively, per μg/ml at 600 nm, and $A_2$ and $B_2$ at 620 nm, and that of the test sample was $T_1$ and $T_2$ at 600 and 620 nm, respectively (the appropriate corrections having been made for blanks), then the concentrations of isonicotinic acid and isonicotinylglycine in the test sample were given by the equations:

Isonicotinic acid
$$= (B_2T_1 - B_1T_2)/(A_1B_2 - A_2B_1) \ \mu g/ml$$
Isonicotinylglycine
$$= (A_1T_2 - A_2T_1)/(A_1B_2 - A_2B_1) \ \mu g/ml$$

*Determination of the accuracy, sensitivity and specificity of the methods*

Isoniazid, acetylisoniazid, monoacetylhydrazine, diacetylhydrazine, isonicotinic acid and isonicotinylglycine were added to blank serum or urine at 0, 20, 40, 60, 80 and 100%, respectively, of the maximum concentrations of the ranges shown in Table 1. Duplicate samples were then extracted and treated by the methods described above and the results obtained were subjected to regression analysis to determine whether there was any significant deviation from a linear relationship between extinction (or fluorescence) and concentration. The standard deviation of the results (as $\pm \mu g$ compound/ml) in each concentration range was also calculated. The recoveries of these compounds from serum or urine were compared with those from aqueous solution. The specificities of the methods were determined by using aqueous solutions containing in 1 ml up to 1 mg of isoniazid, pyruvic acid isonicotinylhydrazone (sodium salt, freshly prepared), acetylisoniazid, hydrazine sulphate, monoacetylhydrazinium fumarate, diacetylhydrazine, isonicotinic acid and isonicotinylglycine respectively.

Because studies on the metabolism of isoniazid are often done on tuberculosis patients who are receiving concomitant treatment with other antituberculosis drugs, the interference of the following compounds was also tested on the determination of isoniazid and its metabolites by the colorimetric and fluorimetric methods described in this paper: streptomycin, kanamycin, capreomycin, cycloserine (10 mg/ml), *p*-aminosalicylic acid, ethambutol, pyrazinamide (1 mg/ml), thiacetazone and ethionamide (100 $\mu g$/ml).

*Stability studies*

To investigate the potential use of these methods for the analysis of samples collected in places distant from the laboratory, the stability of isoniazid and its metabolites was investigated in serum and urine. Isoniazid was added to blank serum and urine containing 10000 units of benzyl penicillin/ml and 500 $\mu g$ of streptomycin/ml (to decrease bacterial contamination) to a concentration of 10 $\mu g$/ml. Quadruplicate 10 ml samples were then stored for 1 week at room temperature (25°C) in screw-capped universal containers. In an exactly analogous experiment, the stability of a set of identical samples was studied after they had been extracted into butan-1-ol at the first stage of the extraction procedure. Similar studies were done on serum and urine containing 5 $\mu g$ of acetylisoniazid/ml, 1 $\mu g$ of isonicotinic acid/ml and 5 $\mu g$ of isonicotinylglycine/ml. Analyses were then performed on all four compounds to assess both the extent and nature of any decomposition.

The breakdown of acetylisoniazid by serum at 37°C was investigated by incubating 2-ml samples (diluted up to 10-fold with water) together with 1 ml of 0.15 M-phosphate buffer, pH 7.4, containing 0.03–0.3 mM-acetylisoniazid, for up to 24 h and measuring the loss of acetylisoniazid and formation of isonicotinic acid and monoacetylhydrazine. In the controls, serum was omitted. The stability of 10 $\mu g$ of monoacetylhydrazine/ml in serum and 10 $\mu g$ of diacetylhydrazine/ml in serum and urine at 37°C was also investigated.

Other studies were also done on the stability of isoniazid and its metabolites in serum and urine when stored for longer periods at −20°C.

## Results and Discussion

*Accuracy, sensitivity and specificity of the methods*

The results obtained are summarized in Tables 1 and 2. Unless otherwise indicated, regression analysis indicated that there was no significant deviation from linearity in the ranges of concentrations tested. The recoveries of isoniazid and its metabolites from serum or urine did not differ significantly from that from aqueous solution. Estimates of the standard deviations and coefficients of variation for the different methods were obtained from the residual terms in the regression analyses of the results obtained when known concentrations of the compounds had been added to normal serum or urine. The sensitivities of the methods described in this paper have been defined as twice the standard deviation of the method in the lower concentration range tested, or double the absolute magnitude of the serum or urine blank where this was significant.

*Colorimetric determination of isoniazid*

The reaction procedure was modified from that originally described by Maher *et al.* (1957) in two respects. First, the extinction of the 0.1 M-HCl extract was measured at 380 nm instead of at 365 nm. In this way, errors due to any inaccuracies in the pipetting of the vanillin reagent, which are of considerable importance at the lower isoniazid concentrations (Scottish Thoracic Society, 1962), were decreased by approx. fivefold. Secondly, the extinction of the 0.1 M-HCl extract was measured both before and after reaction with vanillin, as suggested by Peters *et al.* (1965*b*) in their column chromatographic method. Although significant amounts of material with an extinction at 380 nm were not extracted from the batches of normal serum used in these studies, measuring the extinction of the extract before reaction with vanillin was considered a worthwhile precaution, especially as Berte *et al.* (1959) have shown that with the solvent extraction

Table 1. *Accuracy and sensitivity of methods*

The standard deviations were determined from duplicate estimates in the standard curve. The sensitivity is defined in the text.

| Compound | Method | Fluid | Concentration range of standard curve ($\mu$g/ml) | Standard deviation ($\mu$g/ml) | Coefficient of variation (% mean) | Blank ($\mu$g/ml) | Sensitivity ($\mu$g/ml) |
|---|---|---|---|---|---|---|---|
| Isoniazid | Colorimetric | Serum | 0.2 – 1.0 | ±0.14 | 28 | Nil | 0.3 |
| | | | 2 –10 | ±0.22 | 4.4 | — | |
| | | Urine | 0.2 – 1.0 | ±0.09 | 18 | 0.2 | 0.4 |
| | | | 2 –10 | ±0.18 | 3.6 | — | |
| | Fluorimetric | Serum | 0.02– 0.1 | ±0.0028 | 5.6 | Nil | 0.006 |
| | | | 0.2 – 1.0* | ±0.044 | 8.8 | — | |
| | | Urine | 0.02– 0.1 | ±0.0034 | 6.8 | 0.02 | 0.04 |
| | | | 0.2 – 1.0* | ±0.034 | 6.8 | — | |
| Acetylisoniazid | Colorimetric | Serum | 0.1 – 0.5 | ±0.065 | 26 | Nil | 0.1 |
| | | | 1 – 5 | ±0.089 | 3.6 | — | |
| | | Urine | 0.1 – 0.5 | ±0.067 | 27 | 0.4 | 0.8 |
| | | | 1 – 5 | ±0.086 | 3.4 | — | |
| | Fluorimetric | Serum | 0.1 – 0.5 | ±0.018 | 7.2 | Nil | 0.04 |
| | | | 1 – 5 | ±0.14 | 5.6 | — | |
| | | Urine | 0.1 – 0.5 | ±0.025 | 10 | 0.1 | 0.2 |
| | | | 1 – 5 | ±0.18 | 7.2 | — | |
| Monoacetyl-hydrazine | Colorimetric | Serum | 0.2 – 1.0 | ±0.145 | 29 | Nil | 0.3 |
| | | | 2 –10 | ±0.35 | 7.0 | — | |
| Diacetyl-hydrazine | Colorimetric | Serum | 0.2 – 1.0 | ±0.077 | 15 | Nil | 0.2 |
| | | | 2 –10 | ±0.18 | 3.6 | — | |
| | | Urine | 0.2 – 1.0 | ±0.078 | 16 | 0.4 | 0.8 |
| | | | 2 –10 | ±0.145 | 2.9 | — | |
| Isonicotinic acid | Colorimetric | Serum | 0.02– 0.1 | ±0.010 | 20 | Nil | 0.02 |
| | | | 0.2 – 1.0 | ±0.015 | 3.0 | — | |
| | | Urine | 0.02– 0.1 | ±0.012 | 24 | 0.04 | 0.08 |
| | | | 0.2 – 1.0 | ±0.018 | 3.6 | — | |
| Isonicotinyl-glycine | Colorimetric | Serum | 0.05– 0.25 | ±0.012 | 10 | 0.04 | 0.1 |
| | | | 0.5 – 2.5 | ±0.043 | 3.4 | — | |
| | | Urine | 0.05– 0.25 | ±0.012 | 10 | 0.07 | 0.2 |
| | | | 0.5 – 2.5 | ±0.048 | 3.8 | — | |

\* Significant deviation from linearity.

system of Maher *et al.* (1957) some samples of normal serum gave extracts equivalent to as much as 1 $\mu$g of isoniazid/ml. With urine, the value of this procedure was readily apparent as samples of normal urine yielded 0.1 M-HCl extracts containing material with an extinction at 380nm equivalent to 1.6±0.7$\mu$g of isoniazid/ml, whereas the vanillin-reacting material was only equivalent to 0.2±0.3$\mu$g of isoniazid/ml. Vanillin-reactive material, however, was not extracted from normal serum.

The standard deviation of the method over the whole range of concentrations tested (0–10$\mu$g/ml) was about ±0.16$\mu$g of isoniazid/ml in both serum and urine, and it was concluded that in each case the

method should enable concentrations of down to about 0.3–0.4$\mu$g of isoniazid/ml to be determined. These results may be compared with the standard deviation of ±0.22$\mu$g/ml obtained from standard curves by other workers (Evans *et al.*, 1960*a,b*, 1961) by using the method of Maher *et al.* (1957). A comparison of the results obtained when both the colorimetric and fluorimetric methods were applied to determine the concentrations of isoniazid in samples of serum and urine (G. A. Ellard & P. T. Gammon, unpublished work), indicated that at isoniazid concentrations of less than 4$\mu$g/ml the real errors of the colorimetric method were equivalent to about ±0.3$\mu$g/ml.

Table 2. Specificity of methods

Aqueous standards without prior treatment with 0.1 vol. of 2M-HCl were used. The results are percentage colour or fluorescence of that given by the appropriate compound on a molar basis.

| Methods | Isoniazid | Pyruvic acid isonicotinylglycine | Acetylisoniazid | Isonicotinic acid | Isonicotinylglycine | Hydrazine | Monoacetyl-hydrazine | Diacetyl-hydrazine |
|---|---|---|---|---|---|---|---|---|
| | | | | Compound | | | | |
| Isoniazid (colorimetric) | 100 | 80 | <1 | <0.02 | <0.02 | 1 | <0.5 | <0.1 |
| Isoniazid (fluorimetric) | 100 | 80 | <1 | <0.001 | <0.001 | <0.001 | <0.2 | <0.05 |
| Acetylisoniazid (colorimetric) | 3 | 3 | 100 | <0.02 | <0.02 | <0.02 | <1 | 43 |
| Acetylisoniazid (fluorimetric) | <1 | 1 | 100 | <0.0005 | <0.0005 | <0.0005 | <0.0005 | 0.0005 |
| Monoacetylhydrazine (colorimetric) | 17 | 14 | 29 | <0.1 | <0.1 | <0.1 | 100 | 113 |
| Diacetylhydrazine (colorimetric) | <0.5 | <0.5 | 13 | <0.01 | <0.01 | <0.05 | 1 | 100 |
| Isonicotinic acid* (colorimetric) | <1 | <1 | <0.05 | 100 | 43 | <0.002 | <0.002 | <0.002 |
| Isonicotinylglycine† (colorimetric) | <0.5 | <0.5 | <0.02 | 72 | 100 | <0.002 | <0.002 | <0.002 |

* If isonicotinic acid was determined by measuring at 600nm only.
† If isonicotinylglycine was determined by measuring at 620nm only.

It was calculated from the standard curves that at concentrations of 2–10 $\mu$g of isoniazid/ml in either serum or urine, the errors were equivalent to a co-efficient of variation of about 4%. The accuracy of the method in practice at these isoniazid concentrations was estimated from the results obtained when both the colorimetric and fluorimetric methods were used to determine the plasma isoniazid concentration attained in a group of Skolts and Saame Lapps after intravenous dosage with 5 mg of isoniazid/kg (G. A. Ellard, P. T. Gammon & H. Tiitinen, unpublished work). The results were examined by analysis of variance in which the main comparisons were (1) between the 21 subjects, (2) between the three collection times, and (3) between the two methods of determination. Logarithmically transformed results were used as this gave similar variances for both the slow and rapid isoniazid inactivator groups. The residual term, which can be considered as a combined error term for the two methods, was ±0.192, corresponding to a coefficient of variation of 4.5%, which is similar to the values calculated from the standard serum curves, by using the colorimetric and fluorimetric methods, of 4.4 and 5.6–8.8% respectively (Table 1).

### Fluorimetric determination of isoniazid

This procedure has been modified from that of Scott & Wright (1967) in several respects. First, the reactions were done after solvent extraction instead of on a protein-free filtrate of serum. Secondly, salicylaldehyde was used as its own 'internal' pH indicator (Feigl, 1956) to facilitate the control of the pH that is critical for the subsequent reduction of the N-isonicotinyl-N'-(salicylidene)-hydrazine. Thirdly, (NH₄)₂SO₄ was added to avoid having to centrifuge the butan-1-ol extract of the fluorophore, in which it is unstable.

The sensitivity of this method when applied to serum was to about 0.006 $\mu$g of isoniazid/ml, which is almost identical with that achieved by Scott & Wright (1967) with their original method, and about 50 times greater than that of the colorimetric method. However, our fluorimetric method, unlike that of Scott & Wright (1967), can also be applied to urine. In this case, the sensitivity is to about 0.04 $\mu$g of isoniazid/ml, or about 10 times greater than that of the colorimetric method.

Significant deviations from linearity were encountered with this method when it was applied to the determination of isoniazid in serum or urine at concentrations from 0.2 to 1.0 $\mu$g/ml without diluting the final 0.1 M-HCl extracts before reaction (Table 1). This was shown to be due to incomplete reaction of isoniazid with salicylaldehyde at the higher isoniazid concentrations, as noted by Scott & Wright (1967).

Thus, when isoniazid was treated directly in aqueous solution, $1.0 \mu g$ of isoniazid gave a fluorescence only about 9 times that of $0.1 \mu g$.

### Specificity of the colorimetric and fluorimetric methods for the determination of isoniazid

Neither the colorimetric nor the fluorimetric method for the determination of isoniazid suffered from significant interference from either acetylisoniazid, isonicotinic acid, isonicotinylglycine or diacetylhydrazine (Table 2), because both methods depend on the presence of the free hydrazine group for hydrazone formation. The small contribution made by acetylisoniazid to this method is probably due to some hydrolysis (usually <1%) occurring during the extraction into 0.1 M-HCl. It should be noted that if the 0.1 M-HCl extracts are stored at 4°C before analysis, acetylisoniazid is hydrolysed to isoniazid at a rate of approx. 0.5% per day. Monoacetylhydrazine appears not to react with vanillin under the conditions employed, whereas hydrazine, although reacting to give a compound absorbing significantly at 380 nm, is very poorly extracted in the solvent system used (only about 1%). Although monoacetylhydrazine yields a fluorescent derivative under the conditions used for the fluorimetric determination of isoniazid, maximum fluorescence occurs at 370/430 nm, and at the wavelengths used for the determination of isoniazid (390/480 nm) its fluorescence is only about 1% of that given by isoniazid. Hydrazine, on the other hand, appears not to give rise to a fluorescent derivative (Scott & Wright, 1967).

Gross interference from pyruvic acid isonicotinylhydrazone is encountered and it would appear that its extraction in this solvent system is only slightly inferior to that of isoniazid. In 0.1 M-HCl, both pyruvic and α-oxoglutaric acid isonicotinylhydrazones break down almost instantaneously to isoniazid. Studies in which isoniazid was measured by direct reaction with vanillin demonstrated that the acidification of urine with 0.1 vol. of 2 M-HCl resulted in the complete hydrolysis of pyruvic acid isonicotinylhydrazone within 5 min at 20°C. Similar results were obtained with urine samples from subjects who had taken isoniazid. Treatment of the urine samples with HCl in this way before neutralization and extraction increased their apparent isoniazid content by about 15%. The precise determination of isoniazid together with its acid-labile hydrazones in the urine is therefore preferable to the determination of 'apparent' isoniazid. When applied to the determination of pyruvic acid isonicotinylhydrazone in the urine, this hydrazone yielded its theoretical isoniazid content, but acid treatment had no effect on the determination of acetylisoniazid, isonicotinic acid, isonicotinylglycine, monoacetylhydrazine or diacetylhydrazine.

### Comparison of the colorimetric and fluorimetric methods for the determination of isoniazid

The fluorimetric method for the determination of isoniazid is much more accurate than the colorimetric method at concentrations of less than $1 \mu g/ml$. However, at concentrations of greater than $2 \mu g/ml$, the fluorimetric method is probably slightly less accurate than the colorimetric method. Further, at such concentrations, the 0.1 M-HCl extracts must first be diluted to $0.05-0.1 \mu g$ of isoniazid/ml before reaction with salicylaldehyde. In these circumstances, the colorimetric method is to be preferred because of its greater simplicity. The results obtained when both the colorimetric and fluorimetric methods were applied to determine the isoniazid concentrations of some 120 plasma (or serum) and 85 urine samples were closely similar. The isoniazid concentrations ranged from $0.5-10 \mu g/ml$ in plasma/serum and $20-200 \mu g/ml$ in urine, and gave correlation coefficients of 0.9819 and 0.9660 respectively.

### Colorimetric determination of acetylisoniazid

The conditions used by Kelly & Poet (1952) to convert isoniazid into p-dimethylaminobenzaldazine have been modified in two important respects to permit the specific determination of acetylisoniazid. First, interference from isoniazid has been eliminated by prior treatment of the 0.1 M-HCl extract with nitrous acid, presumably through the conversion of isoniazid into a diazo compound and thence to isonicotinic acid (Nielsch & Giefer, 1959a). Secondly, after conversion of acetylisoniazid into the double Schiff's base, the chromophore was extracted into butan-1-ol in the presence of 23% (v/v) acetic acid and excess of $(NH_4)_2SO_4$ before determining its extinction. This not only increases the sensitivity of the method by concentrating the chromophore, it also eliminates errors that can arise through the marked influence of changes in the concentration of HCl on the absorption spectrum of the chromophore in contrast with its broad absorption in high concentrations of acetic acid (Dambrauskas & Cornish, 1962).

As expected, there was no significant interference in this method from isonicotinic acid and isonicotinylglycine as they did not contain the hydrazine grouping, or from hydrazine and monoacetylhydrazine because of the prior treatment with nitrous acid. However, on a molar basis, diacetylhydrazine gave about 43% of the colour given by acetylisoniazid. The sensitivity of the method was to about $0.1 \mu g/ml$ in serum and $1 \mu g/ml$ in urine, and at concentrations of greater than $1 \mu g/ml$, the method had a coefficient of variation of about 3.5% in both serum and urine (Table 1). The accuracy of the method was also assessed by doing duplicate determinations on urine samples obtained from two subjects up to 7h after

dosage with 250mg of isoniazid (G. A. Ellard & P. T. Gammon, unpublished work). Acetylisoniazid concentrations averaged about $100\,\mu g/ml$ and duplicate errors were equivalent to a coefficient of variation of $1.9\%$.

### Fluorimetric determination of acetylisoniazid

As expected, there was no significant interference in this method from hydrazine and its acetylated derivatives since hydrazine does not react by the fluorimetric method of Scott & Wright (1967), and interference from isoniazid was eliminated through prior treatment of the 0.1 M-HCl extract with nitrous acid. The sensitivity of this method was to about $0.04\,\mu g/ml$ in serum and $0.2\,\mu g/ml$ in urine, and the coefficient of variation at concentrations of greater than $1\,\mu g/ml$ was $6-7\%$ (Table 1).

### Comparison of the colorimetric and fluorimetric methods for the determination of acetylisoniazid

At the concentrations of acetylisoniazid encountered in serum and urine after normal dosage with isoniazid, the increased accuracy, simplicity and rapidity of the colorimetric method, as compared with that of the fluorimetric method, must be set against the interference from diacetylhydrazine in the colorimetric method. After dosage with isoniazid, the major excretion of diacetylhydrazine occurs during the period 6–24h after dosage, and during the first 6h, the excretion of diacetylhydrazine was, on a molar basis, only $2\%$ of that of acetylisoniazid in a slow inactivator and $13\%$ in a rapid inactivator (G.A. Ellard & P. T. Gammon, unpublished work). It is therefore understandable that the colorimetric and fluorimetric methods yielded virtually identical results for the determination of acetylisoniazid in urine samples collected within $6\frac{1}{2}$h of oral dosage (G. A. Ellard & P. T. Gammon, unpublished work), or within 3h of intravenous dosage with isoniazid (G. A. Ellard, P. T. Gammon & H. Tiitinen, unpublished work). In these two studies the results of 83 analyses by both methods gave a correlation coefficient of 0.9877 for the two methods. In such circumstances, the colorimetric method for the determination of acetylisoniazid is obviously the method of choice.

Previously, no method had been devised for the specific determination of acetylisoniazid in the serum, and the method described by Venkataraman et al. (1968) for application to urine, is much less sensitive than the colorimetric and fluorimetric methods described above.

### Colorimetric determination of monoacetylhydrazine

The method has been modified from that previously described by Peters et al. (1965a) for the determination of the acetylated hydrazides in urine, principally by incorporating extraction procedures both before and after reaction with p-dimethylaminobenzaldehyde. Its sensitivity when applied to serum is to about $0.3\,\mu g/ml$, and at concentrations of $2-10\,\mu g/ml$ the method had a coefficient of variation of $7\%$ (Table 1). The specificity of the method is, however, poor, and the contributions due to isoniazid, acetylisoniazid and diacetylhydrazine must be allowed for (Table 2). The method could not be satisfactorily applied to urine, apparently because of the spontaneous reaction of monoacetylhydrazine with components in the urine to give reaction products that were not completely cleaved to hydrazine when incubated for 24h with M-HCl at 45°C.

### Colorimetric determination of diacetylhydrazine

The method has been modified from that described by Peters et al. (1965a) both by incorporating extraction procedures before and after reaction with p-dimethylaminobenzaldehyde and by eliminating interference from hydrazine, monoacetylhydrazine and isoniazid by treating the 0.1 M-HCl extract with nitrous acid. The only metabolite of isoniazid that causes significant interference and needs to be allowed for in the method is acetylisoniazid (Table 2), presumably because $10-15\%$ of this compound is broken down to hydrazine under the conditions used to hydrolyse diacetylhydrazine (Peters et al., 1965b). The sensitivity of the method is to about $0.2\,\mu g/ml$ in serum and $1\,\mu g/ml$ in urine, and the coefficient of variation in both serum and urine at concentrations of $2-10\,\mu g/ml$ is about $3\%$ (Table 1).

### Colorimetric determination of isonicotinic acid and isonicotinylglycine

This method has been considerably modified from that described by Nielsch & Giefer (1959a,b) and subsequently modified by Peters et al. (1965b). By extracting the isonicotinic acid and isonicotinylglycine into 4M-sodium acetate, adjusting the pH to 5.0 with $23\%$ acetic acid, and then treating the solution successively with KCN, chloramine-T and barbituric acid in acetone–water, the sensitivity of the method has been greatly enhanced so that it can now be used to determine the concentrations of isonicotinic acid and isonicotinylglycine present in the serum after normal dosage with isoniazid. The method has also been considerably simplified both in the preparation of the barbiturate reagent and by decreasing the number of reagent additions from six to three. Further, by increasing the concentration of KCN in the final reaction mixture, the precision of the determination of isonicotinylglycine has been greatly improved.

Table 2 shows that neither isoniazid nor any of its other metabolites seriously interfere in the determination of isonicotinic acid and isonicotinylglycine, although it is necessary to measure the extinction of the reaction product at both 600 and 620 nm and to solve the appropriate differential equations to determine the separate concentrations of isonicotinic acid and isonicotinylglycine. The sensitivity of the method was to about $0.02\,\mu g$ of isonicotinic acid/ml and $0.1\,\mu g$ of isonicotinylglycine/ml in serum and $0.1\,\mu g$ of isonicotinic acid/ml and $0.2\,\mu g$ of isonicotinylglycine/ml in urine. At concentrations of greater than $0.2\,\mu g$ of isonicotinic acid/ml or $0.5\,\mu g$ of isonicotinylglycine/ml in either serum or urine, the method had a coefficient of variation of about 3.5% (Table 1). That such accuracy could be achieved in practice was demonstrated by an analysis of variance of the ratios of isonicotinylglycine/isonicotinic acid excreted in the urine determined at hourly intervals in two subjects up to 6 h after dosage with 25 mg of isonicotinic acid (G. A. Ellard & P. T. Gammon, unpublished work). During this period the concentrations of isonicotinic acid and isonicotinylglycine in the urine fell from about 100 to $1\,\mu g$/ml and the residual term gave a coefficient of variation of 4.5%.

## Specificity of the methods with respect to other anti-tuberculosis drugs

The only significant interference encountered in any of the methods was that caused by pyrazinamide to the colorimetric determination of acetylisoniazid, $100\,\mu g$ of pyrazinamide giving an extinction equivalent to $0.6\,\mu g$ of acetylisoniazid.

## Stability studies

Very similar results were obtained when either the colorimetric or fluorimetric methods were used to study the stability of isoniazid or acetylisoniazid in serum or urine. Nearly three quarters of the isoniazid broke down after a week's storage in serum or urine at a concentration of $10\,\mu g$/ml at 25°C. About 10% of the isoniazid that had been broken down had been converted into isonicotinic acid. The stability of isoniazid was, however, considerably increased by prior extraction into butan-1-ol, and after a week, the isoniazid content of the butan-1-ol extracts of serum and urine was 65 and 82%, respectively, of the original. Further studies, however, often failed to demonstrate the instability of isoniazid in urine and it is possible that the destruction encountered in this experiment might have been due to bacterial contamination of the urine, and might have been avoided by adding a crystal of thymol as preservative (P. Venkataraman, personal communication).

When isoniazid was stored in serum at a concentration of $10\,\mu g$/ml for 6 months at −20°C, the breakdown was equivalent to about 4% per month. This may be compared with the study of Peters & Good (1962) who, using the fluorimetric method of Peters (1960), concluded that isoniazid was stable in frozen serum (−18°C) for periods of at least 56 days, or with that of Gangadharam et al. (1961) who concluded, by using a microbiological assay method, that over an 8 week period isoniazid was destroyed in serum at −20°C at the rate of 3.7% per week.

Acetylisoniazid was found to be completely stable in urine. By contrast, it was rapidly broken down in serum, and after a week at room temperature, only 7% could be recovered. About 75% of it had been converted into isonicotinic acid, but isoniazid was not formed. However, extraction from serum into butan-1-ol almost completely protected it from breakdown, 94% being recovered at the end of a week. When acetylisoniazid was stored in serum at −20°C, its breakdown was equivalent to about 3% a week and at least 50% of this breakdown represented conversion into isonicotinic acid. At 37°C, the half-life of acetylisoniazid in the serum was estimated at between 10 and 20 h. The rate of decomposition of acetylisoniazid and the rates of formation of isonicotinic acid and monoacetylhydrazine were proportional to both the amount of serum and to the concentration of acetylisoniazid. Over 80% of the acetylisoniazid broken down was converted into isonicotinic acid and monoacetylhydrazine. This breakdown was decreased to less than 2% by ultrafiltration, and entirely prevented if the serum was first heated for 1 h at 100°C. These results confirm the qualitative evidence of Wenzel (1955), which indicated that acetylisoniazid could be broken down to isonicotinic acid by an enzyme present in serum, and demonstrate the importance of extracting into butan-1-ol as rapidly as possible when determining acetylisoniazid, isonicotinic acid or monoacetylhydrazine in the plasma after giving isoniazid or acetylisoniazid.

The loss of isonicotinic acid on storage for 1 week at room temperature in serum or urine was only about 10% and it could be stored at −20°C for periods of up to a year without significant decomposition. A loss of about 20% isonicotinylglycine was encountered after storage for 1 week at room temperature in serum or urine, but this loss was not due to hydrolysis to isonicotinic acid. Both isonicotinic acid and isonicotinylglycine were completely protected from breakdown by extraction into butan-1-ol.

It was concluded that urine samples and butan-1-ol extracts of serum could be stored for up to 1 month at −20°C before analysis without appreciable decomposition of these four compounds. Finally, monoacetylhydrazine was completely stable when incubated in serum for 24 h at 37°C, as was diacetylhydrazine in both serum and urine.

*Application of these methods*

These methods have been used to measure the concentrations of isoniazid in the serum of guinea pigs after oral dosage with 2–32mg of isoniazid/kg (Dickinson *et al.*, 1968). They have also been used to measure the serum/plasma concentrations of isoniazid, acetylisoniazid, isonicotinic acid and isonicotinylglycine, and the urinary excretion of these compounds together with that of diacetylhydrazine, in studies on the metabolism of orally administered isoniazid, acetylisoniazid, isonicotinic acid, monoacetylhydrazine and diacetylhydrazine in slow and rapid inactivators of isoniazid (G. A. Ellard & P. T. Gammon, unpublished work). These methods have also been employed in the study of the pharmacology of isoniazid given intravenously to subjects from Finnish Lappland (G. A. Ellard, P. T. Gammon & H. Tiitinen, unpublished work).

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