Mass Phenotyping of Isoniazid Inactivators by Automated Determination of Acetylisoniazid in Urine

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An automated procedure is described for phenotyping isoniazid inactivators. The method is based on the color reaction produced by N-acetylisoniazid in aqueous solution, as described by Eidus and Hamilton [Amer. Rev. Resp. Dis. 89, 587 (1964)]. Maximum sampling rate is 60 samples per hour. Results of this procedure correlated well \( r = 0.987 \) with those of a manual phenotyping method.

Additional Keyphrases: AutoAnalyzer • treatment of tuberculosis • screening

It is established that in treatment of tuberculosis, slow inactivators of isoniazid are more prone to side effects from the drug (1–3), whereas fast inactivators respond less favorably to marginal chemotherapy in which this drug is administered intermittently or for a limited period (4–8). Therefore, phenotyping of patients for isoniazid inactivation became important in the initiation of long-term therapy of tuberculosis (9). This laboratory has introduced a simple screening test for identification of slow and fast acetylator (10, 11). To facilitate phenotyping on a large scale, the procedure used for acetylisoniazid determination was modified and adapted to continuous-flow automation. Here, we describe the method and evaluate its performance.

Materials and Methods

Phenotyping Procedure

Principle. A test dose of 10 mg of isoniazid per kilogram body weight is administered orally. After 6 h the subject totally empties the bladder, and 2 h later a urine sample is collected for estimation of isoniazid and N-acetylisoniazid. Acetylisoniazid in the urine is estimated by the color reaction described by Eidus and Hamilton (12); isoniazid is first converted to acetylisoniazid by acetic anhydride and then determined by the same method. The AutoAnalyzer (Technicon Instruments Corp., Tarrytown, N. Y. 10591) procedure is carried out on two aliquots (A and B) of each urine sample. In aliquot A, only the acetylisoniazid excreted in urine is determined, while in aliquot B the total hydrazides—i.e., the acetylisoniazid excreted and the isoniazid acetylated in vitro—are estimated together.

For classification of patients into slow and fast inactivators, the percentage of acetylisoniazid excreted in urine is estimated:

\[
\text{Acetylisoniazid, \%} = \frac{\text{acetylisoniazid in aliquot A (mg/liter)}}{\text{total hydrazides in aliquot B (mg/liter)}} \times 100
\]

Those with a percentage of less than 70 are identified as slow inactivators; those with values of 70 or greater are fast inactivators.

Apparatus. AutoAnalyzer I (Technicon); Sampler II; 60-per-hour cam with a sample/wash ratio of 2:1. The flow diagram and details of the modules are shown in Figure 1.

Reagents. Hydrochloric acid, 1 and 0.15 mol/liter; potassium cyanide, 40 g/liter ("Analar"; British Drug Houses Ltd., Carle Place, N. Y. 11514); and chloramine-T (Eastman Kodak Co., Rochester, N. Y. 14650), 16 g/liter.

Working standards. These are prepared by dissolving acetylisoniazid in hydrochloric acid (0.15 mol/liter) and diluting it with the same acid solution to
Table 1. Protocol for Determination of Acetylisoniazid and Total Hydrizes in Urine

<table>
<thead>
<tr>
<th>Solution</th>
<th>Acetylisoniazid (aliquot A)</th>
<th>Volume, ml</th>
<th>Total hydrazides (aliquot B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>0.50</td>
<td>1.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Distilled water</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>HCl, 1 mol/liter</td>
<td>1.50</td>
<td>1.50</td>
<td>1.50</td>
</tr>
<tr>
<td>Acetic anhydride</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Distilled water</td>
<td>8.00</td>
<td>7.50</td>
<td>6.50</td>
</tr>
<tr>
<td>Final dilution</td>
<td>1:20</td>
<td>1:10</td>
<td>1:5</td>
</tr>
</tbody>
</table>

Fig. 2. Standard curve for N-acetylisoniazid

yield final concentrations of 2.5, 5, 10, 20, 30, 40, 50, 60, 70, and 80 μg/ml. If acetylisoniazid is not available, isoniazid can be converted to it as follows: Dissolve 76.1 mg of isoniazid (equivalent to 100 mg of acetylisoniazid) in 100 ml of distilled water. To 10 ml of this solution, add 0.7 ml of acetic anhydride, mix gently, and dilute to 100 ml in a volumetric flask. From this solution (100 μg of acetylisoniazid per milliliter), the working standards in concentrations indicated above are prepared with HCl (0.15 mol/liter).

Dilution of urine samples and acetylation of isoniazid: Two aliquots are taken from each urine sample and processed according to the dilution scheme indicated in Table 1.

In aliquot A, only the acetylisoniazid excreted in urine is determined. It is acidified and diluted to yield concentrations not exceeding 80 μg/ml. In aliquot B, total hydrazides are determined. Therefore, in addition to acidification and dilution, 50 μl of acetic anhydride is added, to convert free isoniazid present in urine to acetylisoniazid. In general, the same dilution factor is used for both aliquots. Acidification of the samples is an important step. It liberates isoniazid from its hydrazone bonds and ensures the acidity essential for optimal color reaction.

Automated determination of acetylisoniazid: The standards and two corresponding aliquots (A and B) of the samples treated as above are loaded into the sample tray, with a known control in every eleventh cup. The acetylisoniazid in the acidified aliquots reacts with the potassium cyanide and chloramine-T in the volume ratio 1:1:4.83 to produce a stable red color during its passage through the 12-meter delay coil (i.d., 1.6 mm).

Color intensity is measured at 550 nm and recorded as percentage transmittance. At the end of the run, the values are read from a curve prepared from the peaks of standards with concentrations ranging from 2.5 to 80 μg of acetylisoniazid per milliliter.

Results

Analytical Variables

Linearity of absorbance with concentration of working standards: We processed a series of acetylisoniazid solutions (2.5 to 80 μg/ml) in hydrochloric acid (0.15 mol/liter). Absorbanes of the working standards were linearly related to concentration (Figure 2).

Examination of the need for alternating sample cups with distilled water cups: Two series were run with aliquots of acetylisoniazid working standards. In the first series, alternate cups in the sample tray were filled with standard solutions and distilled water; in the second series they were filled only with standards and arranged in an ascending order of concentrations. The presence of the water cups had no effect on results; their elimination did not influence the transmittance, implying that there was no carryover between cups (Figure 3). If water cups are excluded, the number of samples analyzed is doubled, becoming 60 per hour.

Recovery experiments: To 80 urine samples, acetylisoniazid or isoniazid, or both, was added in known concentrations ranging from 10 to 800 μg/ml. The samples were coded and sent to the laboratory without any indication of their concentration. Recov-
ery of acetylisoniazid ranged from 89–107% (mean, 100.5%; SD, 3.0%). Isoniazid, like acetylisoniazid, was quantitatively accounted for in the urine samples, the range being 86–114%, the mean 99.6%, and the SD 5.0%.

Application

Comparison of automated and manual methods in the phenotyping of isoniazid inactivators: Fifty volunteers were given a test dose of 10 mg of isoniazid per kilogram body weight and urine samples were collected in the 6–8-h period as described earlier. Duplicate aliquots of the urine samples were coded with different numbers and dispatched to two laboratories, in one of which the automated technique was used, while the other estimated isoniazid and acetylisoniazid manually by the method of Eidus et al. (13). By both procedures, 18 of the patients were classified as slow and 32 as fast inactivators. The average percentage of acetylisoniazid vs. total hydrazides excreted in the urine by slow inactivators was 43.6% by the automated method, 51.0% by the manual procedure. The corresponding values for fast inactivators were 88.5% and 89.0%. The correlation coefficient for the two methods, calculated on the basis of 50 pairs of observations, was 0.987.

Figure 4 shows the percentage values obtained by manual and automated procedures, and demonstrates the close agreement between the two methods. It also illustrates the clear separation of slow and fast inactivators.

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References


Fig. 3. Strip-chart recordings with (i) and without (ii) distilled water cups between standards arranged in increasing concentrations from 2.5 to 80 µg/ml

Fig. 4. Proportion (in percent) of acetylisoniazid to total hydrazides in urines of 50 subjects, as determined by manual and automated methods.