Microbiological Test for Leucine, Valine, and Isoleucine Using Urine Sample Dried on Filter Paper

Helen K. Berry, Carolyn Scheel, and Joy Marks

Urine specimens dried on filter paper squares were obtained from 700 mentally retarded children. A microbiological assay was used to screen the specimens for the presence of valine, leucine, and isoleucine. Three specimens gave positive tests for all three amino acids. A generalized aminoaciduria was observed in two specimens, and in the third, elevation of valine and leucine/isoleucine was confirmed by paper chromatography. The use of these screening procedures for early detection of children with maple sugar urine disease is suggested.

The convenience and reliability of collecting urine specimens on filter paper for preliminary testing and subsequent chromatographic evaluation has been described (1). However, the paper chromatographic evaluation of large numbers of urine specimens is both time consuming and exacting. We have, therefore, examined the possibility of using a more convenient method of assay of the filter-paper urine samples which would extend the usefulness of the filter paper method in large-scale screening programs. The ease, specificity, and precision of microbiological assay suggested the combination of these procedures with the filter-paper sampling method, particularly in screening for conditions in which a relatively large increase in certain amino acids might be expected. Two such conditions are "Hartnup disease" and "maple sugar urine disease" in which valine, leucine, and isoleucine are excreted (2, 3), in large amounts as compared to trace amounts in normal urines (4). The assay procedures, using Lactobacillus arabinosus 17-5 as test organism, for these three amino acids were modified for use with the filter paper urine specimens. The procedures after standardization were applied to the screening of urine specimens from the mentally retarded children.
specimens from approximately 700 mentally retarded children as part of a program for the detection of metabolic disorders in such children.

Experimental Methods

Small pieces of filter paper saturated with urine and dried were incubated in each of three special media inoculated with the test organism. Since the organism requires valine, leucine, and isoleucine for growth, absence of these amino acids was indicated by failure to grow. Each of the special media lacked one of these amino acids, so that no bacterial growth occurred unless the urine supplied adequate amounts of the amino acid in question.

Preparation of Assay for Inoculation

For each specimen, ½-in. paper squares (containing approximately .025 ml. urine) were cut and placed in clean, dry bacteriological culture tubes (13 × 100 mm). Each sample was tested in each assay medium. The culture media (Difco Leucine Assay, Valine Assay, or Isoleucine Assay Broth) were prepared by suspending 5.25 gm. of dried broth in 100 ml. distilled water and heating to boiling as recommended by the manufacturer. After 2 ml. of the appropriate broth was placed in each tube, they were capped or plugged with cotton and autoclaved for 15 min. at 15 lb. pressure, 121°, and allowed to cool before use. Three 10-ml. pipets and one 5-ml. and 50 ml. 0.9% saline were autoclaved at the same time.

Stock solutions of each amino acid were prepared to contain 1 mg./ml. of the L-isomer. For standards, 0.0005, 0.001, 0.002, 0.004, and 0.005 ml. of each stock solution was placed on individual ½-in. squares of filter paper. The standards were placed in tubes and broth added for the appropriate test.

Preparation of Inoculum

_Lactobacillus arabinosus_ was maintained on Difco Micro Assay Culture Agar. The culture was streaked onto a blood agar plate once a month to maintain purity. The inoculum was prepared by making a transfer from the agar stab to a tube containing 10 ml. Difco Micro Inoculum broth and incubating for 48 hours at 30°. The inoculum was centrifuged for 15 min. at medium speed. The broth was removed with a sterile pipet and the cells washed twice in 10 ml. sterile saline. After the final centrifugation the cells were resuspended in 5 ml. sterile saline. One loopful of the cell suspension was used immediately to inoculate sterile unknowns and standards. The tubes were incubated at 30°. The test for leucine was read at 24 hours and those for valine and isoleucine after 48 hours incubation.
Results

Urine specimens from approximately 700 mentally retarded children under 6 years of age were tested. Five specimens gave positive tests for valine, 4 for leucine, and 12 for isoleucine. Only 3 specimens gave positive tests indicating the presence of all three amino acids. In the standard tubes containing known amounts of the amino acids, the minimum amount of L-amino acid required for growth of the test organism was 1 μg. in each case. This corresponded to a minimum detectable urinary concentration of 40 μg./ml. for leucine, valine, or isoleucine. The mean values for urinary excretion of valine and leucine/isoleucine (not separated) by 225 children, measured by paper chromatography, were 12 μg./ml. leucine/isoleucine and 7 μg./ml. valine (4).

The addition of an artificial urine containing 30 gm. urea and 15 gm. sodium chloride per 100 ml. did not inhibit growth in the tubes containing standards. Thymol crystals in the urine specimen did not inhibit growth, nor did salicylate metabolites.

Urine specimens that gave positive results in the microbiological assay were eluted for paper chromatographic testing. Two of the specimens that gave positive tests in all three microbiological procedures showed a generalized aminoaciduria including cystine, tyrosine, phenylalanine, glycine, alanine, and glutamine in addition to valine, leucine, and isoleucine. Chromatographic examination of the third specimen confirmed the presence of valine and leucine/isoleucine. Other amino acids did not appear to be elevated. The specimen also gave a questionably positive test with ferric chloride reagent, suggesting the presence of keto acids. Further testing of this patient was impossible since the original urine sample was exhausted and the child had died. Results of chromatographic testing of those specimens showing only elevation of either valine, leucine, or isoleucine were less clear-cut. In some instances the concentration of the amino acid in question, as judged chromatographically, was below the minimum amount thought necessary for a positive microbiological test. It is known that alpha-keto acids may replace the corresponding alpha-amino acids for growth of certain microorganisms. Therefore, the alpha-keto analogs of valine and leucine were tested for their ability to support bacterial growth in our test. They were found to do so at levels of 2 μg. and 5 μg., respectively, for leucine and valine. The keto-analog of isoleucine (α keto, β-methyl valeric acid) was not available.

Efforts to detect these keto acids in the small amount of urine on the test paper were unsuccessful. Further investigation of the substances responsible for the positive tests in these instances is necessary.
Table 1. Comparison of Paper Chromatographic and Microbiological Tests for Valine, Leucine, and Isoleucine

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Nature of disorder</th>
<th>Valine conc. (µg./ml.)</th>
<th>Leucine/isoLeucine conc. (µg./ml.)</th>
<th>Leucine assay*</th>
<th>Isoleucine assay*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lowe's disease</td>
<td>380</td>
<td>++ + + +</td>
<td>+</td>
<td></td>
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<tr>
<td>2</td>
<td>Hartnup disease</td>
<td>150</td>
<td>++ + + +</td>
<td>+ + + +</td>
<td>++ + +</td>
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<tr>
<td>3</td>
<td>Renal tubular defect</td>
<td>430</td>
<td>++ + + +</td>
<td>++ + +</td>
<td>+</td>
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<tr>
<td>4</td>
<td>Glycogen storage disorder</td>
<td>30</td>
<td>+</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

*++ is equivalent to 1 µg. in test solution (approximately 25 µl urine) or at least 40 µg./ml. ++ ++ + is equivalent to 5 µg. in test solution or at least 100 µg./ml.

Several specimens giving a negative microbiological test were also examined chromatographically and, as expected, showed no increase in valine, leucine, or isoleucine.

Urine specimens previously shown to contain large amounts of valine and leucine/isoLeucine were also tested. The results of paper chromatographic and microbiological testing of these specimens are compared in Table 1. Although the microbiological test is proposed as a qualitative screening procedure rather than a quantitative test, the amounts of the amino acids present in the urine specimens are in the same concentration range using either method.

The microbiological test described above proved an effective means of screening large numbers of urine specimens for the presence of valine, leucine, or isoleucine. The test was simple, relatively rapid and inexpensive (2 cents per sample per complete test) as compared to a chromatographic evaluation. These procedures, combining the simplicity of sample collection from young children with a relatively simple assay should be useful if applied to urine specimens from infants as a means of detecting metabolic disorders such as "maple sugar urine" disease at an early age. The use of urine specimens dried on paper greatly simplifies the collection of urine specimens from infants or older children. The fact that the alpha-keto acids also support growth of the test organisms enhances the value of the screening methods, since these substances may be present in increased amounts in urine of children with "maple sugar urine" disease prior to the appearance of increased amounts of the corresponding amino acids (6).

References