A Reliable Spot Test for Mucopolysaccharidoses

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A new spot test for detecting excessive excretion of mucopolysaccharides in urine has been used with a group of 17 patients. Reliability of the test was evaluated by correlating clinical findings with qualitative and quantitative analyses of urinary mucopolysaccharides. Normal controls, with 3.8 to 10.0 mg of uronic acid in this form per liter of urine, gave negative spot tests; all cases of clinically diagnosed mucopolysaccharidoses (MPS) I, II, and III (Hurler's, Hunter's, and Sanfilippo's syndromes, respectively) gave strongly positive ones. Two patients (female siblings) with Morquio's syndrome (MPS IV) also gave positive spot tests, which were somewhat less intense than those given by patients with MPS I, II, or III. The test for Morquio's syndrome, positive with fresh urine, was essentially negative with samples at $-26\, ^\circ C$ for three months or longer. A group of seven patients having various degrees of skeletal deformities, mental retardation, dwarfism, or corneal opacities, but not fitting the presently accepted classification of mucopolysaccharidoses, gave negative spot tests, and chemical analyses of their urinary mucopolysaccharides showed normal patterns of excretion.

Additional Keyphrases: acid mucopolysaccharides • glycoproteins • McKusick classification • screening test • normal values • DEAE-Sephadex chromatography • dialysis • effects of storage of urine

The excretion of urinary MPS by normal individuals varies from approximately 5 to 25 mg/liter (or 2 to 10 mg of MPS-bound uronic acid per liter of urine), with the principal MPS component being chondroitin sulfate (1-3). However, in cases of genetically determined disorders of MPS metabolism, excretion may rise as much as 40-fold (4-7). Six syndromes are now recognized as belonging to this group of inborn errors of MPS metabolism. They are, according to McKusick's classification (5): MPS I (Hurler's syndrome); MPS II (Hunter's); MPS III (Sanfilippo's); MPS IV (Morquio's); MPS V (Scheie's); and MPS VI (Maroteaux-Lamy). Clinical features of these disorders include various degrees of dwarfism, skeletal deformities, mental retardation, and corneal opacities. Biochemically, they are distinguishable from one another by specific patterns of abnormal urinary MPS. For example, both heparan sulfate and dermatan sulfate are excreted in MPS I, II, and V, while in MPS III and VI only heparan sulfate or dermatan sulfate, respectively, are found in abnormal amounts (4-7); individuals with Morquio's syndrome (MPS IV) excrete excessive amounts of keratan sulfate and chondroitin sulfate, probably as peptide complexes (8, 9).

Precise identification and quantitative estimation of urinary MPS is technically difficult and very time-consuming, partly because of the many purification steps required to isolate them and partly because the MPS are highly heterogeneous both in molecular size and in chemical composition (1-3, 10-12). Therefore detailed analyses of urinary MPS are not done in most clinical laboratories. Instead, a number of simplified procedures have been developed to detect excess MPS in the urine. The simplest, the Alcian blue (13) or toluidine blue (14) spot tests, are often difficult to interpret and are not considered reliable by

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2 Nonstandard abbreviations used: MPS, mucopolysaccharides (glycosaminoglycans), mucopolysaccharidoses (with roman numerals); C/O, carbazole/orcinol (ratio of results by these methods); Tris, tri(hydroxymethyl)aminomethane.

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many investigators (16–17). Other tests, based on the turbidity produced by the interaction of mps with albumin, cetylpyridinium chloride, or cetyltrimethylammonium bromide, must be carried out under carefully controlled conditions of temperature, time, pH, and ionic strength if false positives are to be avoided (17–19). Quantitative measurement of the cetylpyridinium chloride-precipitable mps (2) is probably the most reliable method for analyzing urinary mps, but is too time-consuming either for routine use or as a potential screening procedure (16–17). More consistent values may be obtained with many of the tests cited above by relating urinary mps and creatinine excretions (19, 20). However, care must be taken to ensure that collection of 24-h samples is complete, to avoid errors resulting from daily fluctuations in the excretions of both mps and creatinine (21). In view of the present lack of a straightforward and reliable method for detecting excess urinary mps, the test to be described should be of considerable usefulness.

Methods

Patients

Twenty-four-hour urine samples as well as casual samples were collected from 17 individuals. Five (Group A) served as controls; another five (Group B) had been diagnosed, on the basis of clinical, genetic, and radiological data, as having one of the six types of mucopolysaccharidoses; and seven (Group C) were patients with hereditary connective-tissue disorders, who showed various degrees of skeletal deformities, mental retardation, dwarfism, or corneal opacities, but did not fit into the McKusick classification.

Procedures

Spot test. The “mps test-papers” we used were prepared by the Ames Co. (Division of Miles Laboratories, Inc., Elkhart, Ind. 46514), and consist of specially treated paper impregnated with Azure A. This cationic dye forms purple metachromatic complexes with polyanions such as mps, and is commonly used for their histochemical detection (22).

Either fresh casual samples of urine or 24-h samples that had been refrigerated during collection, but had no added preservatives, were used. Preliminary trials showed that results with either were similar in the spot test. Hence, for convenience and ease of handling, casual samples were used in preference to 24-h samples. If the urine is cloudy, it should be filtered or centrifuged first.

A drop of the sample is placed in the middle of the test paper. After 3 min it is transferred to a Petri dish, which is then filled with a liberal amount of wash solution consisting of 20 ml of methanol, 0.1 ml of glacial acetic acid, and 200 ml of distilled water. The paper is rinsed by gentle agitation for 10–20 min, then removed and blotted until dry. Positive tests are distinctly purple in the area where the drop of urine had been applied. Negative tests show no coloration, except for the pale-blue background of the washed paper. A faint blue spot may occasionally be seen in urines with high pigment content. However, if the spot is not distinctly purple, it should be considered negative.

Analyses of urinary mps. One-tenth of the total volume of each 24-h sample was used for chemical analysis. In the first step, samples were dialyzed against two changes of phosphate buffer (5 mmol/liter, pH 7) in Visking cellophane tubing that had been heated for 24 h at 90°C. This treatment, by decreasing the pore size of the casing (23), minimizes loss of low-molecular-weight mps (24). The dialyzed samples were then applied to short columns of DEAE-Sephadex, A-50, Medium, prepared in sintered-glass funnels and previously equilibrated with the same buffer. They were washed first with 100 ml of buffer and then successively with 70 ml of each of the following: 0.20M NaCl, 1.0M NaCl, and 2.0M NaCl, all in the phosphate buffer. The first two fractions (i.e., the buffer wash and the 0.20M NaCl) contained only glycoproteins and in most cases were not analyzed further; the last two were concentrated, dialyzed, and then digested with “Pronase” (Calbiochem, La Jolla, Calif. 92037) for 7 h in 0.1M Tris buffer, pH 7.8, 37°C. The mps were precipitated with three volumes of ethanol and analyzed for uronic acid, hexosamine, and sulfate by methods described previously (25). Glucosamine and galactosamine were estimated on a Beckman Model 120 B Amino Acid Analyzer after hydrolysis in 2N HCl at 100°C for 12 h. In all cases except the two diagnosed as Morquio’s syndrome (mps IV), 95% or more of the urinary mps were found in the 1.0M NaCl eluate. In the two cases of Morquio’s syndrome about three-quarters of the keratan sulfate was found in this fraction, the rest in the 2.0M NaCl eluates.

Results

Results of the spot test and the patterns of urinary mps excretion in the 17 individuals are given in Table 1. The mps were not fractionated into their individual components, but they can be characterized by use of the following criteria: (a)
the C/O ratio, (b) the ratio of galactosamine to glucosamine, and (c) the ratio of sulfate to hexos- 
amine. In addition, the daily excretion of all \( \text{MPS} \) except keratan sulfate can be calculated from 
the uronic acid content.

**Group A (Normal Individuals)**

In normal individuals (Group A), the excretion of uronic acid (carbazole method) varied from 1.2 
to 4.9 mg/24 h, or 3.8 to 10.0 mg/liter of urine. All C/O ratios were between 1.15 and 1.33. The 
principal amino sugar was galactosamine, and the ratio of sulfate to hexosamine was about 0.9. In 
the light of our present knowledge (3, 11), these data show that two-thirds or more of the \( \text{MPS} \) 
excreted by normal individuals is chondroitin sulfates; the remainder consists of small amounts 
of heparan sulfate, nonsulfated \( \text{MPS} \), dermatan sulfate, and keratan sulfate (1, 3, 11, 20, 26, 27). 
The last column in Table 1 shows that all of the spot tests for the urines in Group A were negative.

**Group B (Mucopolysaccharidoses)**

All of the patients in Group B gave positive spot tests, the color being more intense for \( \text{MPS} \) I, 
II, and III than for \( \text{MPS} \) IV (Morquio’s). All were excreting uronic acid in amounts varying from 7 
to 36 mg/24 h, or 19 to 105 mg/liter of urine. Quantitative and qualitative data on the pattern 
of urinary excretion of \( \text{MPS} \) we found in these syndromes agree well with results reported else- 
where in which different isolation procedures were used (4, 6, 7). Cases 6–8, which fit the clinical 
picture of Hurler–Hunter syndromes (\( \text{MPS} \) I and II), excrete an excess of both dermatan sulfate and 
heparan sulfate, the former contributing to the subnormal C/O ratio. However, the magnitude of 
the differences in ratios of galactosamine to glucosamine in the two syndromes were not as great as 
those reported by Kaplan (6). Patients 9 and 10 (Sanfilippo’s, \( \text{MPS} \) III) were excreting mainly 
heparan sulfate, as shown by the high C/O ratios and the excess of glucosamine. Cases 11 and 12 are 
with clinical and radiological features of Morquio’s syndrome (\( \text{MPS} \) IV). The uronic acid 
excretions were two- to threefold that for the normal controls, but not as great as in \( \text{MPS} \) I–III. 
This in itself would not establish Morquio’s syndrome in these patients, but measurements of 
anthrone-positive material in the combined 1.0 and 2.0 mg/24 h NaCl eluates showed excretion rates of 2.4 
and 1.9 mg/24 h, as compared to about 0.2 to 0.5 

### Table 1. Analysis of Urinary MPS in 17 Individuals

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age, yr</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Uronic acid</th>
<th>Ratios</th>
<th>Spot-test results</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>mg/24 h</td>
<td>C/O</td>
<td>Galactosamine/glu-</td>
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<td></td>
<td></td>
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<td>mg/l</td>
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<td>Group A</td>
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<tr>
<td>1</td>
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<td>F</td>
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<td>1.15</td>
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<tr>
<td>2</td>
<td>5</td>
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<td>1.21</td>
<td>75:25</td>
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<tr>
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<td>4</td>
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<td>Normal</td>
<td>2.3</td>
<td>1.17</td>
<td>65:35</td>
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<tr>
<td>4</td>
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<td>1.25</td>
<td>63:37</td>
</tr>
<tr>
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<td>30</td>
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<td>4.9</td>
<td>1.33</td>
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<td>0.90</td>
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<td>10</td>
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<td>1.32</td>
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<td>1.27</td>
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<tr>
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<td>19</td>
<td>F</td>
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<td>6.8</td>
<td>1.11</td>
<td>44:56</td>
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</tbody>
</table>

* 1.0 NaCl eluates from DEAE-Sephadex after digestion with “Pronase” and ethanol precipitation.

b Carbazole method.

e Denotes siblings.
most laboratories have little choice but to freeze the samples until chemical analyses can be performed. Nevertheless, it was still considered important to find out whether freezing and (or) storage had any effect on the intensity of the metachromasia given by the MPS test-papers. For this purpose urine samples that had been collected previously from patients with MPS I, II, and III and stored at −26°C for periods of six months to two years were compared with fresh samples (or samples stored less than a month) from the same patients. In all cases there was no difference in the intensity of the metachromatic color on the MPS test-papers (Figure 1). In contrast to this, urine samples of patients with MPS IV, which had been positive at the time of collection, gave essentially negative tests after three months of storage at −26°C (Figure 1). This completely unexpected finding strongly suggests that, to detect cases of Morquio’s syndrome by this spot test, it must be performed on fresh urine samples.

Group C (Unclassified)

The patients in Group C represent a heterogeneous group, all of whom were suspected of having one of the mucopolysaccharidoses. Cases 13 and 14 had marked skeletal dysplasias, suggestive of Morquio’s syndrome. Nevertheless, the spot tests were negative and chemical analyses showed that both the level and pattern of urinary MPS were normal. Patient 15 was a suspected (though not proven) case of Gm1-gangliosidosis. This syndrome is not accompanied by a supernormal excretion of MPS (28), although abnormal keratan sulfate-like substances in the urine may give a weakly metachromatic reaction with cationic dyes (29). In our case the spot test was negative, and the urinary MPS were normal.

Cases 16 and 17 were siblings displaying gross skeletal abnormalities, severe arthritic changes, stunted growth, and peripheral corneal opacities. The clinical findings were in many respects similar to those described by McKusick (30) and others (31–34), and the names “pseudo-Hurler polydystrophy” or “mucopolysaccharidosis without mucopolysacchariduria” have been proposed for such cases. The spot tests were negative for these two patients’ urines, and although the total amount of MPS excreted per 24 h was slightly greater than normal, it was still considerably less than that found in any of the mucopolysaccharidoses. Further examination of the 1.0M NaCl eluates in these patients revealed the presence of an abnormal glycoprotein containing sialic acid, fucose, and neutral sugar (Berman, unpublished data). The latter could have contributed substantially to the color produced in the carbazole reaction, as shown in previous studies in this laboratory (25). Therefore, the slight excess of urinary MPS found in these patients may be only an artifact caused by the neutral sugars present in the abnormal glycoprotein component. These substances—i.e., glycoproteins—do not interact with cationic dyes such as Azure A, as borne out by negative spot tests with urines from patients 16 and 17.

Discussion

Diagnosis of individuals affected with one of the six types of inherited disorders of MPS metabolism (5) is based on clinical, radiological, and laboratory data; the latter always show an increased excretion of urinary MPS. For each of the mucopolysaccharidoses there is a characteristic pattern of urinary excretion (4, 6, 7) which, although essential to a precise diagnosis, is too complex and time-consuming to ascertain as a routine laboratory procedure. Although a number of simplified procedures are available for detecting excess excretion only, without identifying the individual MPS, these are often difficult to interpret (15–17) and in many cases give false positives except under carefully controlled conditions (17–19).

The newly developed spot test described here is simple and rapid; a purple color is observed only in the urines of those individuals with clinically diagnosed mucopolysaccharidoses who are excreting more than 19 mg of MPS-bound uronic acid per liter of urine. Normal controls (Group A), who excrete 4 to 10 mg of MPS-bound uronic acid, gave negative tests. Hence the least MPS-bound uronic acid that can be detected is about 20 mg/liter, the concentration found in the two cases of Morquio’s syndrome (MPS IV). The color given in this range is less intense than it is for cases of MPS I, II, and III, for whom the excretion is at least threefold greater.

On the basis of the present data, we cannot predict whether all cases of MPS IV (Morquio’s syndrome) will give a positive test with MPS test-papers, since keratan sulfate and (or) chondroitin sulfate are not always excreted in great excess.
(9). The most decisive factor may be the time that the test is made; fresh urine samples are the most desirable, although those stored for less than a month should also be satisfactory.

The individuals in Group C were studied because they showed some clinical features of mucopolysaccharidoses; however, they could not be assigned to any of the six types classified by McKusick (5). The spot test was negative and the urinary excretion of MUR was normal for all of this group. Some of them must therefore represent separate disease entities, at present not clearly defined, but possibly belonging to a group of syndromes (30–34), some of which are characterized by the excretion of abnormal glycoproteins.

Urinary samples from patients with other types of diseases have also been examined with MPS test papers but since complete analyses of the MPS were not made, the data have not been given in Table 1. These patients included four with Tay-Sachs disease (GM₂-gangliosidosis, Type I), two with infantile Gaucher’s disease and one with the adult form, three (siblings) with the Batten type of juvenile amaurotic idiocy, two cretins, three cases of megalocornea, six patients with unusual types of corneal dystrophies, and three cases of achondroplasia. All of the spot tests were negative. A brother and sister with glycogen storage disease, Type III, were examined on four different occasions and, although the spot tests were essentially negative, occasionally a faint blue-purple could be detected. However, this color was easily distinguishable from the bright purple metachromasia found in all cases of mucopolysaccharidoses (Table 1).

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References


