Our findings show that the new nonisotopic analog assays are effectively free of interference from variations in concentrations of TBG and albumin, but may still be affected by the presence of autoantibodies to thyroxin. All gave lower mean values in pregnant patients.

The manufacturers' suggested reference ranges also need to be re-assessed for each laboratory that uses these methods. The GPT4 assay provides results that are the least subject to interferences and that also best correlate with the patients' clinical state.

Addendum. Since this study was performed, the reference range suggested for the MFT4 assay has been amended to 10.7 to 23.6 pmol/L. We also believe that the reagents for this kit have been revised.

We thank Amersham Australia Pty. Ltd. for the Amerlite FT, and TBG kits, and Australian Diagnostic Corp. Pty. Ltd. for the Corning MagicLite reagents.

References

The Spot Test Is Not A Reliable Screening Procedure for Mucopolysaccharidoses

J. G. N. de Jong,1 J. J. F. Hasselman,2 A. A. J. van Landeghem,3 H. L. Vader,4 and R. A. Wevers1

To check the reliability of the Ames MPS paper spot test, which is based on the Azure A dye, we sent a series of urine samples to three laboratories where the spot test is part of the metabolic screening for mucopolysaccharidoses. In these laboratories false-negative results ranged between 19% and 35% and false-positive results ranged between 12% and 29% of all samples submitted. In contrast, the quantitative dimethylmethylen blue test (Clin Chem 1989;35:1472–7) detected an increased glycosaminoglycan content in all urine samples from mucopolysaccharidosis patients and gave no false-positive results. In the latter procedure, glycosaminoglycan content is expressed per millimole of creatinine, and age-dependent reference values are used. We conclude that the Ames spot test and other spot tests are unreliable as a screening procedure for mucopolysaccharidoses and should not be used to screen for these diseases.

Mucopolysaccharidoses are a group of lysosomal diseases characterized by storage of glycosaminoglycans (GAGs) in tissues.5 Glycosaminoglycans are long, sulfated sugar chains composed of repeating disaccharide units. Glycosaminoglycans can be subdivided into four groups: chondroitin sulfate, dermatan sulfate, heparan sulfate, and keratan sulfate. During catabolism, GAGs are internalized in the lysosome and degraded into their monomolecular constituents by lysosomal endoglycosidases, exoglycosidases, and exosulfatases. Ten different enzymes are known to play a role in the degradation of GAGs (1).

In mucopolysaccharidosis (MPS), one of the hydrolytic lysosomal exoenzymes is deficient, which results in the accumulation of GAGs in the lysosome. These compounds are highly soluble in water and are in part excreted in the urine. Testing for GAGs in urine generally is used as a screening procedure for the whole group of mucopolysaccharidoses. Such screening procedures may be qualitative (spot tests [2–4]) or quantitative.

1 Laboratory of Pediatrics and Neurology, Institute of Neurology, University of Nijmegen, St. Radboud Hospital, P.O. Box 9101, NL-6500 HB Nijmegen, The Netherlands.
2 Twentheborg Hospital, Almelo, The Netherlands.
3 St. Elisabeth Hospital, Tilburg, The Netherlands.
4 St. Joseph Hospital, Eindhoven, The Netherlands.

Received November 6, 1990; accepted February 11, 1991.

5 Nonstandard abbreviations: DMB, 1,9-dimethylmethylen blue; MPS, mucopolysaccharidosis; GAG, glycosaminoglycan; CPC, cetylpyridinium chloride.
[turbidity measurement tests (5–7), carbazole test (8, 9), and tests based on metachromasia (10, 11)]. Previously (10), we described a reliable and simple new quantitative assay procedure based on the color reaction of 1,9-dimethylmethylene blue (DMB) with the GAGs. We compared this assay with existing quantitative procedures [cetylpyridinium chloride (CPC) turbidity test and carbazole test] and found it to be more reliable and easier to perform. However, qualitative spot tests are also very simple, require minimal equipment, and, therefore, are still popular. Several types of spot tests are used, differing in the color reagent used (Figure 1). The Ames MPS paper spot test is based on the use of the Azure A dye (2); the Berry spot test (3) involves Toluidine Blue O. Alcian Blue is used as a dye in some spot tests (4).

In this investigation, we compared the reliability of the Ames MPS paper spot test with that of the DMB spectrophotometric test. We did this by sending a series of urine samples to various laboratories in the Netherlands where the spot test is part of the routine procedure for screening for these inborn errors of metabolism.

Materials and Methods

In a first approach to test the reliability of the spot test, we sent 76 urine samples (48 normal specimens and 28 from patients with MPS) to laboratory 1. Because this experimental setup did not allow a direct and objective comparison between the spot test and the DMB assay, we devised a second approach. An independent person, who did not take part in the screening for mucopolysaccharidoses in our laboratory, prepared three identical series of 75 urine specimens. Each series contained 26 samples from MPS patients, but this number was not disclosed to the laboratories. All age categories were equally represented in the group of 49 urine specimens from normal subjects. Laboratory 2 and 3 carried out the spot test. One series was analyzed in our laboratory with the DMB assay. Only the sample number, the age of the person, and the creatinine content of the urine were known to the laboratories performing the spot test. The person performing the DMB test knew only the sample number. The calculation of the GAG content per millimole of creatinine and comparison with age-dependent reference values was carried out by a third (independent) person to prevent MPS urine samples from being recognized from the age of the patient or from the creatinine content of the urine.

Spot Test

The spot test was performed with Ames MPS papers (Ames Co., Div. of Miles Labs. Inc., Elkhart, IN) according to the manufacturer's instructions. Spot test results could be negative, dubious, trace, or positive. The latter three were all considered positive results.

Dimethylmethylene Blue Assay

The DMB assay was performed with a Cobas-Fara analyzer (Hoffmann-La Roche & Co. AG, Basel, Switzerland) and research-grade DMB (no. 20335; Serva Feinbiochemica GmbH, Heidelberg, F.R.G.) (10). The measurements were made with three different urine dilutions: 20 μL of sample + 30 μL of diluent (H2O); 10 μL of sample + 40 μL of diluent; and 5 μL of sample + 45 μL of diluent. To each sample was added 250 μL of DMB solution and the absorbance at 520 nm was measured 10 s after mixing. Values were corrected for sample blank and reagent blank. In preparing each series, a set of three heparan sulfate (Sigma Chemical Co., St. Louis, MO; no. H 7640) standards was included: series 1—18, 36, and 72 mg/L; series 2—36, 72, and 144 mg/L; and series 3—72, 144, and 288 mg/L. To calculate the GAG content, we chose the concentration that gave an absorbance value between the values of the first and second standard solutions. For values between 144 and 288 mg/L, we used the results for series 3. For values >288 mg/L, which were found in some urine samples from patients with MPS, the urine was diluted 10-fold before assay.

Urine

The urine samples analyzed were untreated urine portions. The patients were diagnosed by their clinical symptoms and low enzyme activity in leukocytes and cultured fibroblasts. The specific enzyme deficiency determined the particular syndrome involved.

Results

In our initial study, involving 76 urine samples sent to laboratory 1, the percentages of false-negative (21%) and false-positive results (29%) were unexpectedly high. Six of the 28 MPS urine samples and 14 of the 48 normal urine samples were misidentified.

The investigation was extended to two other laboratories (laboratories 2 and 3) to compare in a blind experimental design (see Materials and Methods) the spot test results with the results of the DMB test (10). Results are given in Table 1. The DMB test gave positive results for all MPS urine samples (sensitivity 100%), whereas for all the normal urine samples the GAG content by the DMB test was within the reference range (specificity 100%). With the spot test, however,
laboratory 2 gave negative results for five of the 26 MPS urine samples (sensitivity 81%) and 11 positive results for 49 normal urine samples (specificity 78%). Laboratory 3 missed nine of 26 MPS urine samples (sensitivity 65%) and found positive results for six of 49 normal urine samples (specificity 88%).

Figure 2 shows the GAG concentration as a function of age for the normal and MPS urine samples used in this study. MPS urine samples found negative in the spot test by one or more of the laboratories are indicated.

Discussion

For screening procedures, urine specimens are mostly random (untimed). The quantitative tests demonstrate that, depending on the type of MPS, GAG excretion can vary and will sometimes be only moderately increased (10). Thus, it is essential to establish reference values very carefully. Good results are possible only if GAG excretion is expressed in terms of creatinine excretion and if variations in excretion with age are taken into consideration (10). The results of a spot test, however, depend mainly on the GAG concentration (and perhaps also on the type of accumulating GAG). When a correction is made for creatinine content by applying more sample at lower creatinine values, the area of the spot will be bigger without intensification of the color. It is our experience that application of more sample does not change the results (data not shown).

GAG concentrations measured in normal and MPS urine samples by the DMB assay directly correlate with spot test results, because their principles of color development (metachromasia) are comparable, as are their color reagents: DMB, Azure A (Ames paper spot test), and Touluidine Blue O (Berry spot test) (Figure 1). Measured GAG concentrations varied from 5 to 120 mg/L for normal urine samples, whereas 16 of 34 MPS urine samples had concentrations within this range. Thus, these MPS urine samples could not be discriminated from normal urine samples by the spot test. Indeed, 12 of these 16 urine samples gave negative spot test results, and all falsely negative samples had GAG concentrations in this range (Figure 2A). In contrast, all these urine samples had an increased GAG content when they were measured by the DMB assay and expressed as milligrams of GAG per millimole of creatinine (Figure 2B). We conclude from Figure 2A that the false-negative spot test results are caused by an overlap of GAG concentrations in these urine samples with the concentrations in normal urine samples. We tested only the Ames spot test; however, results for other spot tests will also depend primarily on GAG concentration. Therefore, similarly high percentages of false-positive and false-negative results can be anticipated with other spot tests as well. In the quality-control studies of Brimble et al. (12) and Rattenbury et al. (13), false-positive and false-negative results were obtained from laboratories using the Touluidine Blue and Alcian Blue spot tests.

Huang et al. (14) described a semiquantitative spot test with Ames MPS papers and compared color intensity after application of 50 μL of urine with that of a series of standards. The authors expressed their results per millimole of creatinine and found a good correlation with the carbazole test; they claimed that all MPS patients, except those with type IV MPS (Morquio disease), could be identified when screened with their semiquantitative MPS paper spot test. However, we have not been able to reproduce these findings. In our study, MPS urine samples that gave a negative spot test result showed no color change at all (all trace or dubious results were considered positive). Berry (15), screening 6055 urine specimens for mucopolysaccharide disorders with the Berry spot test, divided the results into negative, positive (+), and strongly (3+) positive. She found MPS patients only in the group with strongly (3+) positive spot test results. Because none of the patients with a single positive (+) spot test result had an MPS disorder, she concluded that only the strongly positive spot tests were significant. She mentioned that she was not aware of any patients with MPS I, II, or III who gave
false-negative results. However, no specimens were included from patients with a clinical diagnosis of Scheie, Hurler–Scheie compound, Morquio, or Maroteaux–Lamy disease. Generally, Scheie, Hurler–Scheie, and Morquio are milder syndromes with, on average, lower concentrations of the storage products in urine (10). In our series, most false-negative results were seen in these groups (Table 2).

Spot tests are simple and require a minimum of equipment. They are still used by many laboratories as an initial screening procedure. Brimble et al. (12) mentioned that of the laboratories in the U.K. participating in a national MPS quality-assurance scheme, eight (24%) still used a spot test. In a comparable quality-assurance exercise in England mentioned by Rattenbury et al. (13), four of 18 laboratories performing GAG tests used the spot test as an initial screening procedure. In their quality-control scheme, Brimble et al. (12) sent a series of five to seven urine samples, including three to five MPS urine samples, to several laboratories. They obtained 26 spot test results (Toluidine Blue or Alcian Blue) from five laboratories. Only 16 were correct; nine were falsely positive, and one was falsely negative. Rattenbury et al. (13) sent to 18 laboratories one MPS urine sample from a three-year-old patient with Sanfilippo's syndrome. Of the 14 responding laboratories, six gave an incorrect result, four of which involved a spot test (Toluidine Blue or Alcian Blue) as an initial screening procedure. Of the laboratories returning a correct result, none used a spot test.

Earlier (10), we described a new quantitative screening procedure, based on DMB dye, and established its reliability for screening for MPS. In the present study, the DMB procedure detected all the MPS patients and gave no false-positive results, whereas unacceptable percentages of false-negative and false-positive results were found with the spot test.

### Table 1. Spot Test and DMB Assay Results for Normal and MPS Urine Samples

<table>
<thead>
<tr>
<th>MPS urine</th>
<th>Normal urine</th>
<th>Sens.</th>
<th>Spec.</th>
<th>FNR</th>
<th>FPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spot test, lab 2</td>
<td>+</td>
<td>21</td>
<td>11</td>
<td>81</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>5</td>
<td>38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spot test, lab 3</td>
<td>+</td>
<td>17</td>
<td>6</td>
<td>65</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>9</td>
<td>43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMB assay</td>
<td>+</td>
<td>26</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0</td>
<td>49</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sens., sensitivity; Spec., specificity; FNR, false-negative rate; FPR, false-positive rate.

### Table 2. Positive Spot-Test Results for Various Types of Mucopolysaccharidoses

<table>
<thead>
<tr>
<th>Lab 1</th>
<th>Lab 2</th>
<th>Lab 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hunter</td>
<td>5/5</td>
<td></td>
</tr>
<tr>
<td>Hurler</td>
<td>2/2</td>
<td>3/3</td>
</tr>
<tr>
<td>Mar-Lam</td>
<td>1/1</td>
<td>1/1</td>
</tr>
<tr>
<td>Morquio A</td>
<td>3/5</td>
<td>2/5</td>
</tr>
<tr>
<td>Scheie</td>
<td>3/5</td>
<td>4/4</td>
</tr>
<tr>
<td>Sanfilippo</td>
<td>9/11</td>
<td>11/13</td>
</tr>
<tr>
<td>Total</td>
<td>22/28</td>
<td>21/26</td>
</tr>
</tbody>
</table>

Mar-Lam, Maroteaux–Lamy.

In conclusion, we consider the spot test to be unreliable and recommend that it no longer be used as an initial screening procedure. Instead, a simple, quantitative, and reliable procedure is available (10).

We thank R. Liebrand-van Sambeek, G. Steenbergen-Spanjers, L. van Woerkom, and H. de Reus for technical assistance; Ir.Th. M de Boo for statistical advice, and B. J. Wevers for carefully correcting the English of the manuscript.

### References