MPS Screening Methods, the Berry Spot and Acid Turbidity Tests, Cause a High Incidence of False-Negative Results in Sanfilippo and Morquio Syndromes

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Because of differences in the types and quantities of glycosaminoglycans (GAGs) in various mucopolysaccharidoses (MPSs), MPS screening tests, including the Berry spot and acid turbidity tests, are not specific or sensitive enough for the preliminary diagnosis of MPS. A false-negative result is common. We analyzed urine samples collected from 492 patients who were examined for inborn errors of metabolism using the Berry spot and acid turbidity (qualitative and quantitative) tests. Of those, 48 MPS patients (seven with MPS I, 17 with MPS II, nine with MPS III, 11 with MPS IV, and four with MPS VI) underwent preliminary differentiation between MPS types by two-dimensional electrophoresis (2D-EP), and were confirmed by enzymatic assay. Approximately 21.0% and 7.1% of the 492 samples showed positive reactions in the Berry spot and acid turbidity tests, respectively. Of these, a total of 35 samples

with MPS types I, II, and VI showed strong positive reactions in both tests. Five patients with Sanfilippo (MPS III) and six patients with Morquio (IV) syndromes showed false-negative results in both tests. In our study, approximately 13.8% (68 in 492 samples) samples showed a positive reaction in the Berry spot test but a negative one in the acid turbidity test, for unknown reasons. The Berry spot and acid turbidity tests are used extensively for the preliminary diagnosis of MPS in Asia; however, the possibility of a misdiagnosis of MPS type III and IV with both tests should be kept in mind. For accurate diagnosis and confirmation of MPS, the 2D-EP method and enzymatic assay are recommended. They provide high sensitivity, specificity, and efficiency in diagnosing MPS. *J. Clin. Lab. Anal.* 16:253–258, 2002. © 2002 Wiley-Liss, Inc.

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INTRODUCTION

The mucopolysaccharidoses (MPSs), a group of lysosomal storage disorders, are caused by a deficiency of specific enzymes, resulting in an inability to degrade glycosaminoglycans (GAGs), which then accumulate abnormally in cells. At least six MPS types are classified according to clinical manifestations, enzyme deficiency, and the GAG affected (1–4). Ordinarily, a preliminary diagnosis of MPS is made by first performing non-enzymatic screening tests, including the MPS spot test (5–7), acid turbidity test (7,8), dimethylmethylene blue (DMB) method (9), and electrophoresis (10–11). Once the MPS type is determined, an enzymatic assay with

fluorogenic, chromogenic, or radioactive substrates is used to measure the specific enzyme activity in leukocytes to confirm the diagnosis of MPS.

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Because of the complexity of GAG content in various MPSs, accurate diagnosis with simple MPS screening methods, such as the Berry spot and acid turbidity tests, is difficult to achieve. The Berry spot test is based on metachromatic staining of urinary hexuronic acid with toluidine blue, in which the mean concentration of hexuronic acid in urine specimens with a positive reaction is 113 ± 67 mmol/L (6). The acid albumin turbidity test is based on the interaction of urinary uronic acid with albumin, followed by acid precipitation. The test is sensitive to as little as $28 \mu\text{g/mL}$, and it may be affected by pH, temperature, time, and ionic strength (8,12). Both of these screening tests have high incidences of false-negative or false-positive results (8,13). The DMB method is clinically applicable for urinary GAG estimation, but the DMB ratio (GAG mg/mmol creatinine) is age-dependent, and varies from one MPS type to another. The DMB ratio is used as a reference for MPS diagnosis but not for determining certain MPS types (1,2). Currently, the two-dimensional electrophoresis (2D-EP) method is thought to be the most effective for MPS differential diagnosis (14). GAG types, including chondroitin sulfate (CS), keratan sulfate (KS), heparan sulfate (HS), and dermatan sulfate (DS), are well separated by 2D-EP in two different buffer systems. The electrophoretic pattern of the GAG content is the basis of MPS type determination (10,15).

The Berry spot and acid turbidity tests have both been used as first-line screening tests for MPS since the 1960s; however, the reliability of both tests is controversial. At present, both are still widely used in most clinical laboratories in Asia for MPS diagnosis because they are easy to do and provide rapid results. To reduce the possibility of misdiagnosis of MPS using non-enzymatic methods, these tests should be compared with more reliable methods.

METHODS AND MATERIALS

Urine samples were randomly collected from 492 children in the out-patient department of MacKay Memorial Hospital, Taipei, Taiwan. Of these, 48 were confirmed with MPS by fluorimetric enzyme assay (16–19); seven with Hurler (MPS I), 17 with Hunter (II), nine with Sanfilippo (III), 11 with Morquio (IV), and four with Maroteaux-Lamy (VI). Approximately 10–20 mL of urine were stored at -20°C before the MPS screening procedures were performed. The average age of the 492 children was 5.7 years (± 3.7); of the Hurler patients, 11.7 (± 2.6); of the Hunter patients, 5.6 (± 3.2); of the Sanfilippo patients, 7.8 (± 3.0); of the Morquio patients, 9.0 (± 3.5); and of the Maroteaux-Lamy patients, 13.1 (± 3.5).

Berry Spot Test (Toluidine Blue Test)

This test was modified and performed based on the procedures described by Berry and Spinanger (5). Ten microliters of a urine sample, negative control, and positive standard solution containing 0.05, 0.1, and 0.5 mg/mL chondroitin sulfate (CS; Sigma Chemical Co., St. Louis, MO) were applied as separate spots on Whatman (Clifton, NJ) no. 1 filter paper. When it was completely dry, the paper was impregnated with toluidine blue O solution (40 mg/dL at pH 2.0). The density of the purple metachromatic pattern conformed to the standards for determination of CS concentration.

Acid Albumin Turbidity Test (Quantitative Test)

This test was performed according to the method of Carter et al. (20). All of the reagents, including 0.1 gm/dL bovine albumin in 0.1 M acetic buffer (pH = 3.75) and 0.3 M phosphate-citrate buffer (pH = 5.5), were prepared in-house. A 1.0 mL urine sample, 0.5 mL phosphate-citrate buffer, and 8.0 mL acid albumin solution were placed in a tube, mixed, and allowed to stand at room temperature for 10 min. The resulting turbidity was measured with a spectrophotometer at a wavelength of 600 nm. A calibration curve with CS concentrations ranging from 2.8 to $140 \mu\text{g}$ uronic acid/mL was also prepared. The turbidity was proportional to the concentration of uronic acid in the urine sample.

DMB Method for GAG Estimation

This procedure was performed according to method reported by de Jong et al. (9). GAGs were determined quantitatively in urine by reaction with DMB, and the color was measured at a wavelength of 520 nm. The DMB ratio was obtained by dividing the GAG concentration in mg/L by the urinary creatinine, and was expressed as mg/mmol creatinine. A urine sample ($50 \mu\text{L}$) and 1 mg/L heparan sulfate standard ($10 \mu\text{L}$) were both diluted with deionized water to $500 \mu\text{L}$. Exactly 2.5 mL of reagent solution (10 volumes of 55 mM formic acid containing 31 mM DMB with 1 volume of 2 M Tris) was added to each sample, mixed, and read immediately at 520 nm.

2D-EP

This procedure was performed according to the method described by Hopwood et al. (10). Alcian blue reagent (containing 500 mg/dL Alcian blue solution, 2 M MgCl_2 , and 0.5 M sodium acetate buffer) was added to 2 mL centrifuged urine and allowed to stand for 2 hr for formation of MPS-AB complex. Then, 0.2 M sodium

chloride and methanol were added to dissolve the complex, followed by 0.1M sodium carbonate and water to dissociate the MPS-AB, and, finally, four volumes of ethanol to the clear supernatant and reprecipitate the MPS. The MPS precipitate was dissolved with adequate amounts of water based on the DMB value in mg/L. A 2- or 3- μ L aliquot of GAG solution was applied to a cellulose acetate sheet (Sartorius AG, Goettingen, Germany), and electrophoresis (50 volts) was carried out in pyridine-acetic acid buffer in the first direction and in barium acetate buffer in the second direction for 1 and 3 hr, respectively. The GAG electrophoretic patterns were visualized by staining with Alcian blue reagent for 30 min and then decolorizing with 5% acetic acid. A positive control sample prepared from Hunter syndrome was used only in the second-direction EP. The positive control EP pattern from cathode to anode was HS, DS, and CS, successively.

RESULTS

The limit of detection of the Berry spot test was 0.1 mg/mL, at which concentration a light purple metachromatic pattern was distinctly observed (Fig. 1). Concentrations of hexuronic acid in standards (chondroitin sulfuric acid) with 1+ to 3+ reactions were 0.1, 0.25, and 0.5 mg/mL, respectively. At concentrations of 1.5 μ g/dL of uronic acid, the acid albumin turbidity test apparently had a negative reaction, i.e., no turbidity was detected. At concentrations between 1.6 and 3.3 μ g/dL, a slightly turbid reaction became visible (weak reaction: “ $\pm$ ”). At concentrations >3.3 μ g/dL, the turbidity test was positive from 1+ to 4+ (3.9~141.6 μ g/dL).

Of the 492 samples, approximately 21.0% (103 samples) and 7.1% (35 samples) showed positive

reactions in the Berry spot and acid turbidity tests, respectively. Of these, 35 (7.1%) showed all positive reactions in both tests. Patients with Hurler, Hunter, and Maroteaux-Lamy syndromes all revealed positive reactions in both tests, ranging from 1+ to 4+. Of the nine children with Sanfilippo syndrome and the 11 with Morquio syndrome, approximately 55.6% (five patients) and 54.5% (six patients) had negative reactions with both tests. In our study, about 13.8% samples (68 of 492 samples) revealed a positive reaction in the Berry spot test, but a negative one in the acid turbidity test. The reason for this is unknown.

The reference GAG values for the DMB method were determined from 78 MPS-unaffected children. The DMB ratio ranged from 7.1 to 54.3 mg/mM creatinine (30.7 ± 23.6), and the average urine creatinine value was $2.8 (\pm 3.1)$ mmol. The average DMB ratio for Hunter syndrome was $54.3 (\pm 23.3)$ mg/mM creatinine, which was markedly greater than it was for the Hurler (39.6 ± 11.8), Maroteaux-Lamy (35.8 ± 5.7), Sanfilippo (34.5 ± 17.8), and Morquio (33.4 ± 11.0) syndromes. The urinary creatinine levels in MPS I, II, III, IV, and VI were 4.75, 5.89, 4.86, 4.21, and 4.03 mM, respectively.

2D-EP clearly separated the GAGs and differentiated between the various types of MPS (Fig. 2). For normal urine (Fig. 2a), the major GAG content was CS, demonstrated by a discrete spot on the celagram. For the Hurler and Hunter syndromes (Fig. 2b and c), the EP patterns were similar, showing CS, DS, and HS. In addition to quantitative differences in GAG content, the primary discriminating pattern was an upward spread portion of heparin extending from HS in Hunter syndrome. The particular EP patterns observed for Sanfilippo, Morquio, and Maroteaux-Lamy were HS/CS, KS/CS, and DS/CS, respectively (Fig. 2d and f).

Fig. 1. The metachromatic pattern of the Berry spot test. Chondroitin sulfate is used as the positive standard in 0.05, 0.1, and 0.5 mg/mL, in which the metachromatic pattern is reported as $\pm$, +, and 3+, respectively.

Two-Dimensional Electrophoretic Patterns for Various MPS Types

Fig. 2. The electrophoretic patterns of the affected glycosaminoglycans in various MPSs: (a) normal control person with CS expressed, and MPS positive control (HS, DS, and CS); (b) Hurler syndrome (HS, DS, and CS); (c) Hunter syndrome (HS, DS, and CS); (d) Sanfilippo syndrome (HS and CS); (e) Morquio syndrome (KS and CS); and (f) Maroteaux-Lamy syndrome (DS and CS). CS = chondroitin sulfate; HS = heparan sulfate; DS = dermatan sulfate; KS = keratan sulfate.

DISCUSSION AND CONCLUSIONS

In a normal person, the urinary GAG concentration ranges from 7.1 to 54.3 mg/mM creatinine (DMB ratio). The GAG content is thought to be chondroitin sulfate, and it will exhibit negative reactions in the Berry spot and acid turbidity tests, respectively. In our study, 68 samples that unusually showed positive in the Berry spot test, but negative in the acid turbidity test is considered to be abnormal; however, the pathological occurrence cannot be explained at this point.

The traditional Berry spot and acid turbidity tests were uniformly positive for CS and DS, but not for HS and KS. HS gave a weak reaction in the acid turbidity test, and KS showed a faint staining pattern in the Berry spot test. Based on our findings, we believe these tests are not sufficiently specific for detecting all suspected MPSs, especially for Sanfilippo and Morquio variants. Since the primary GAGs affected in Sanfilippo and Morquio are HS/CS and KS/CS, respectively, a false-negative result on screening is quite likely. According to the results reported by de Jong et al. (9) and Brimble et al. (14), the spot and turbidity tests were unreliable for detecting abnormal GAG excretion. In addition, Ratterbury et al. (21) reported that the traditional GAG tests, such as the toluidine blue spot and acid albumin tests, were not successful in detecting the Sanfilippo sample, and missed the abnormalities in the MPS interlaboratory surveys. This may have been due to the reduction of the GAG amount in the Sanfilippo sample, or to the specificity of whatever GAG method was selected in the individual laboratories.

The age- and urine creatinine-dependent results of the DMB/CRE method make it unsuitable for screening, even though it provides useful reference values. The higher DMB/CRE ratio found in Hunter syndrome in this study was thought to be closely related to the younger ages of our patients with that syndrome. We found no significant differences in urinary creatinine between the five MPS types. The DMB/CRE ratio should be considered useful only for estimating the amount of GAG in a sample, not as a biochemical method for MPS diagnosis.

We consider the 2D-EP method to be the best screening method for MPS. It separates the various GAG components, which is the basis of an MPS differential diagnosis. Even though 1D-EP has heretofore been the method of choice for biochemical classification of MPS, it is being replaced by 2D-EP in many MPS screening laboratories. The old method involves a cumbersome procedure and often gives equivocal results. Ordinarily, it takes 2 days to complete the test and interpret the results. Once the MPS class has been determined, the assay to determine the specific

enzyme deficiency involved can be performed. In our study, the results of subsequent enzymatic assays in every case confirmed the MPS classification by 2D-EP. In addition to its use in the clinical evaluation of children with suspected MPS, 2D-EP is also useful for prenatal diagnosis (22). In our study, the cell-free amniotic fluid samples obtained from pregnant mothers (five with Hunter syndrome, one with Sanfilippo, and one with Morquio) at high risk of having a child with MPS were also analyzed. All of the samples with suspected Hunter syndrome showed normal 2D-EP patterns (CS and hyaluronic acid (HA)). The two with the Sanfilippo or Morquio were abnormal (CS/KS/HA or CS/HS/HA were clearly observed). All of the 2D-EP results were confirmed by enzymatic assay (23,24) and molecular analysis (25,26).

In conclusion, the non-enzymatic MPS screening tests, such as the Berry spot and acid albumin turbidity tests, are not specific or sensitive enough for the preliminary diagnosis of MPS. They may give false-negative results and lead to misdiagnosis of MPS. In particular, both tests produced false-negative results in more than 50% of Sanfilippo patients (five of nine patients) and Morquio patients (six of 11 patients). The DMB/CRE ratio is a reference value for GAG content, but it cannot determine the specific MPS type. 2D-EP, on the other hand, provides good separation of GAG contents and precise typing of MPS. To ensure the accuracy of specific MPS enzymatic assays, an accurate and reliable screening method for MPS determination is necessary. 2D-EP is the method of choice for the preliminary diagnosis of MPS.

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