Diagnostic Test for Mucopolysaccharidosis. I. Direct Method for Quantifying Excessive Urinary Glysosaminoglycan Excretion

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This direct method for quantifying excessive urinary glycosaminoglycan excretion exploits the specific binding of 1,9-dimethylmethylene blue (DMB). The procedure obviates cumbersome and labor-intensive procedures for separating glycosaminoglycans from other constituents of urine. Pediat-
"creening surmounts quantitative means much treatment fest, four are which polysaccharides.' from urine.)

The present method was also used to assess metabolic correction in a patient with Hurler's syndrome after treatment by bone-marrow transplantation. This qualitative method surmounts the major technical problems of developing mass screening programs for infants, thus offering the potential for earlier diagnosis and treatment of mucopolysaccharidosis diseases.

Additional Keyphrases: neonate • pediatric chemistry • Hurler's syndrome • screening • bone-marrow transplantation

The mucopolysaccharidosis (MPS) storage diseases are inborn errors of glycosaminoglycan metabolism resulting from deficiency of one of the at least 11 different enzymes involved in lysosomal catabolism of these sulfated hetero-
polyglycans.1 The consequent systemic accumulation of heparan sulfate, dermatan sulfate, and (or) keratan sulfate is associated with specific clinical syndromes (Table 1), which recently have been reviewed (1–4). The mildest forms are characterized by debilitating skeletal abnormalities and dwarfism, progressive cardiopulmonary deterioration, and death during childhood or early adulthood. The more severe MPS diseases also result in progressive mental retardation.

MPS disease is first suspected between nine months and four years of age when clinical abnormalities become manifest, and detection of increased glycosaminoglycans in urine confirms the diagnosis. However, recent progress in the treatment of these disorders (5–7) has demonstrated that much earlier therapeutic intervention is crucial, thus providing a strong motivation for presymptomatic diagnosis by means of routine mass screening of all newborn infants.

Current test methods used for diagnosis of these disorders are technically cumbersome, labor-intensive, and require large urine specimens (e.g., 2–10 mL). Furthermore, exist-
ing methods depend on isolation of glycosaminoglycans by precipitation with quaternary ammonium salts such as cetylpyridinium chloride (8, 9) or on separation techniques such as electrophoresis (10) or HPLC (11).

Some abbreviated "screening tests" have also been described, but these have been limited to tests that are applicable to the relatively small number of children presenting with clinical features of MPS disease, and even these abbreviated tests are not practicable for mass screening. In studies evaluating such methods only a few thousands of tests were done in each case (12, 13), typically during several years. The tests are associated with relatively high false-positive and false-negative rates (14). For these reasons, large-scale presymptomatic diagnosis of MPS diseases has not been attempted.

To surmount these problems, we examined the potential for developing a direct dye-binding assay for quantifying urinary glycosaminoglycan that involves no preparatory isolation procedures. By exploiting the metachromatic properties of the histochemical dye 1,9-dimethylmethylene blue (DMB), sulfated glycosaminoglycans can be directly measured in very small urine specimens without cumbersome separatory techniques.

Materials and Methods

DMB dye reagent. For analysis of fresh or frozen-stored urine specimens, a stock solution containing 0.35 mmol of 1,9-dimethylmethylene blue chloride (no. 03610-1; Poly-
siences, Inc., Warrington, PA) per liter, the "10× stock solution," was made by dissolving 122 mg of dye in 10 mL of 95% ethanol, which was then diluted to 1 L with sodium formate buffer (pH 3.5, 0.2 mmol/L). This 10× stock solu-
tion, stored in an amber-colored bottle at room temperature, could be used for up to two months. For analysis of reference solutions or urine specimens, we prepared a 35 μmol/L "1×" dye solution on the day of testing by appropriately diluting the 10× stock solution with the sodium formate buffer.

Collection of urine specimens. Urine specimens, collected from normal individuals or patients with MPS, were either analyzed promptly or stored at −70 °C until assay.

Glycosaminoglycan standards. Reference solutions were prepared from heparan sulfate (from bovine kidney, no. H9637), keratan sulfate (from bovine cornea, no. K3001), chondroitin sulfate type A (from whale cartilage, no. C4134), dermatan sulfate (i.e., chondroitin sulfate type B, from porcine skin; no. C4259), and chondroitin sulfate type C (from shark cartilage, no. C4584), all obtained from Sigma Chemical Co., St. Louis, MO.

DMB dye-binding assay for glycosaminoglycan. For the standard assay, 40 μL of sample (urine specimen from normal individuals, diluted urine specimen from patients with Hurler's or Hunter's syndromes, or glycosaminoglycan standard) was mixed with 1.0 mL of 35 mmol/L "1×" DMB reagent in plastic semi-micro spectrophotometer cuvettes (no. 2410; Stockwell Scientific, Walnut, CA 91789). Absor-
Table 1. Biochemical Nosology of the Mucopolysaccharidosis Diseases (1-4)

<table>
<thead>
<tr>
<th>Name</th>
<th>Defective enzyme</th>
<th>Glycosaminoglycan species in urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucopolysaccharidosis type I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hurler’s syndrome, Scheie’s</td>
<td>α-L-iduronidase</td>
<td>Heparan sulfate, dermatan sulfate</td>
</tr>
<tr>
<td>syndrome, *Hurler–Scheie</td>
<td></td>
<td></td>
</tr>
<tr>
<td>syndrome, and variants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucopolysaccharidosis type II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hunter’s syndrome, mild and</td>
<td>Iduronate sulfate sulfatase</td>
<td>Heparan sulfate, dermatan sulfate</td>
</tr>
<tr>
<td>severe forms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucopolysaccharidosis type III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sanfilippo’s syndrome A</td>
<td>Heparan sulfate sulfamidase</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>Sanfilippo’s syndrome B</td>
<td>N-Acetyl-α-glucosaminidase</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>Sanfilippo’s syndrome C</td>
<td>CoA:α-glucosaminide-N-acetyl-</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>transferase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sanfilippo’s syndrome D</td>
<td>N-Acetyl-glucosamine-6-sulfate</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>Mucopolysaccharidosis type IV</td>
<td>Galactosamine-N-acetyl-6-sulfate</td>
<td>Keratan sulfate</td>
</tr>
<tr>
<td>Marquio’s syndrome A</td>
<td>β-Galactosidase</td>
<td>Keratan sulfate</td>
</tr>
<tr>
<td>Mucopolysaccharidosis type V</td>
<td>N-Acetyl-galactosamine-4-sulfate</td>
<td>Keratan sulfate</td>
</tr>
<tr>
<td>Maroteaux–Lamy syndrome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucopolysaccharidosis type VII</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sly’s syndrome</td>
<td>β-Glucuronidase</td>
<td>Heparan sulfate, dermatan sulfate</td>
</tr>
</tbody>
</table>

*Previously mucopolysaccharidosis type V.

bance at 555 nm was measured within 30 min in a Beckman DU-8 or DU-50 spectrophotometer and compared with that of appropriate standard solutions of chondroitin-6-sulfate. Color was assessed visually under standard indoor fluorescent ceiling lighting.

Carbazole–borate assay for glycosaminoglycan. For comparison, urinary glycosaminoglycan was quantified by the method of Biter and Muir (8) as modified by Di Ferrante (9). A 10-mL aliquot of urine was neutralized with concentrated HCl or 5 mol/L NaOH, precipitated overnight at 4 °C with cetylpyridinium chloride (final concentration 2.5 g/L), and then assayed for uronic acid with the carbazole–borate reagent. The absorbance at 552 nm was measured and compared with that of reference solutions of β-glucuronolactone (no. G8875; Sigma). Glycosaminoglycan content was expressed as grams of uronic acid per liter of urine.

Creatinine assay. Urinary creatinine was measured by a modification of the method of Folin and Wu (15) by mixing 50 μL of urine and 200 μL of distilled water with 1.0 mL of a fivefold dilution of saturated picric acid (no. 925-40; Sigma) and 1.0 mL of a 7.5 g/L solution of sodium hydroxide. After reaction for 20 min, the absorbance at 535 nm was measured and compared with that for creatinine reference solutions of 10, 30, 100, and 150 mg/L concentrations (no. 925-11 and 925-15; Sigma).

Data management and statistical methods. To compute results, we programmed Lotus 1-2-3 version 2A (Lotus Development Corp., Cambridge, MA) to calculate the slope and intercept of the linear portion of standard curves for glycosaminoglycan and creatinine by regression analysis, to calculate glycosaminoglycan and creatinine concentrations of test samples, and to calculate urinary glycosaminoglycan as the ratio glycosaminoglycan/creatinine (milligrams of glycosaminoglycan per gram of creatinine). Lotus 1-2-3 and Cricket Graph version 1.2 (Cricket Software, Malverne, PA) were used for graphic illustrations and calculation of correlation coefficients.

Pharmaceutical formulations. To study potential interference of pharmaceutical coloring agents in the test system, we obtained commonly prescribed pediatric preparations for study, specifically Augmentin (Beecham Laboratories, Bristol, TN), Ceclor (Eli Lilly, Indianapolis, IN), erythromycin (Abbott Laboratories, North Chicago, IL), furosemide (Lyphomed Inc., Melrose Park, IL), morphine sulfate (Winthrop-Brecon, Des Plains, IL), Pedialyte (Ross Laboratories, Columbus, OH), penicillin (Pfizer, New York, NY), Rondue syrup (Ross Laboratories), Septra (Burroughs Wellcome, Research Triangle Park, NC), Tavist (Sandoz, Parsippany, NJ), Triaminic Cold Syrup (Sandoz), Triaminic-DM (Sandoz), and Tylenol drops (McNeil, Fort Washington, PA). Also evaluated were heparin from porcine bowel (1000 USP units/mL; Elkins-sinn, Cherry Hill, NJ) and bovine serum albumin (Sigma).

Results

Standard Assay Conditions

Optimal assay conditions were determined in a series of experiments to study the absorption spectrum of DMB and glycosaminoglycan-DMB (GAG-DMB) complexes under various conditions of glycosaminoglycan concentration, pH, and species of glycosaminoglycan.

Absorption spectrum of DMB and GAG-DMB complexes. To determine the absorption spectrum of DMB and GAG-DMB complexes, various concentrations of heparan sulfate were added to the standard reaction mixture at pH 3.5 and scanned over the range of 400–700 nm afterblanking the spectrophotometer against a cuvette containing 0.2 mol/L formate buffer without dye. Identical spectra were obtained in solutions having a pH of 3.0, 4.0, 4.5, or 5.0 (data not shown).

Saturation of GAG-DMB complex formation. As an initial means of evaluating the potential useful range of glycosaminoglycan concentration, the color and absorbance of various concentrations of heparan sulfate were determined. The visible color changes and increasing absorbance were
observed within the first few seconds after heparan sulfate was mixed with DMB reagent. While reference solutions with concentrations of 60 µg/mL or less retained the blue color of unreacted DMB, solutions with 70 to 90 µg/mL appeared lavender, and samples with 100 to 1000 µg/mL were brilliant pink and were prone to precipitate over time. Increasing heparan sulfate concentrations above approximately 200 µg/mL resulted in no additional increase in absorbance, the maximum being about 0.35 A.

Specificity of GAG-DMB complex formation. Other glycosaminoglycans were then similarly studied. The diagnostically important sulfated glycosaminoglycans (heparan sulfate, keratan sulfate, chondroitin-4-sulfate, dermatan sulfate, and chondroitin-6-sulfate) exhibited the same reaction with the dye. When presented as a semilogarithmic plot, absorbance was seen as a sigmoidal function of glycosaminoglycan concentration (Figure 1), with the same maximal absorbance being reached by glycosaminoglycan concentrations of 200 mg/L or more. Much higher glycosaminoglycan concentrations often resulted in a visible GAG-DMB precipitate and a corresponding decrease in absorbance (data not shown).

Linear range for routine determinations. The relationship between glycosaminoglycan concentration and absorbance of GAG-DMB complexes was examined in the range of concentrations between 1 and 1000 µg/mL. For each of the sulfated glycosaminoglycans, the absorbance of the GAG-DMB complex was, up to a point, linearly proportional to glycosaminoglycan concentration (e.g., heparan sulfate, Figure 2). However, absorbances of <0.025 A were considered unreliable, sample-to-sample variation being considerable owing to the limitations of instrumentation. Therefore, glycosaminoglycan concentration can be determined by direct linear extrapolation from absorbance over the approximate range 10 to 150 µg/mL. By this method, DMB exhibited the same reactivity with each of the different sulfated glycosaminoglycans. This observation contrasts with the previous observation (16) that DMB is less reactive with keratan sulfate.

For routine analysis of urine specimens, a series of reference solutions of chondroitin-6-sulfate (25, 50, 75, . . . , 350 µg/mL) was treated in parallel with test samples, and the upper limit of linearity was re-assessed in each series based on this standard curve. Concentrations exceeding the upper limit, typically 0.30 A, could be exactly quantified by testing an appropriate dilution of the original specimen.

Theoretical Considerations Regarding Application

Comparison with carbazole–borate/cetylpyridinium chloride methodology. The direct DMB method was compared with the conventional procedure of Bitter and Muir (9) as modified by Di Ferrante (9). For this comparison, urine samples extending over the range of normal and pathologically glycosaminoglycan concentrations were analyzed by both methods. Six urine samples were collected from each of four normal individuals (n = 24; "low glycosaminoglycan") and from each of four MPS patients (three with Hurler's syndrome, and one with Hunter's syndrome, n = 24; "high glycosaminoglycan"). We could assay a batch of 10 to 30 samples and calculate the glycosaminoglycan/creatinine ratio by the present method in less than 2 h. Less than 0.1 mL of urine is required for determination of both glycosaminoglycan and creatinine. In contrast, the conventional two-day cetylpyridinium chloride–carbazole–borate method requires overnight precipitation of duplicate urine specimens (10 mL each), followed by sequential washing of precipitated glycosaminoglycan in cold ethanol, drying, and rehydration of the specimen, and finally assay of uric acid content. Results for 48 urine specimens are represented graphically (Figure 3). The present method correctly identified all 24 MPS specimens on the basis of high glycosaminoglycan (range: 117 to 813 mg of glycosaminoglycan per gram of creatinine) and discriminated the 24 specimens from normal adults (range: 6.3–19.0 mg/g). Linear regression analysis indicated a correlation coefficient of 0.764.

Potential interfering substances. Potential interference from some pharmaceutical formulations and protein in urine was assessed by determining the effect of each in the standard assay reaction mixture (Table 2). For each substance, 40 µL of the specified material was added at the indicated concentration in place of the usual urine test...
sample. Albumin was nonreactive with dye at a concentration of 1 mg/mL. In contrast, the anticoagulant heparin, a sulfated glycosaminoglycan, was found to be highly reactive in the assay. Other drugs likely to be administered to infants produced no interference except when formulations containing artificial coloring agents were added to the reaction at full strength.

Clinical application. Five 1-mL urine specimens were obtained from a one-year-old girl with Hurler’s syndrome and, when tested by the direct DMB method, were found to have pathognomonic concentrations of glycosaminoglycan (Figure 4). After bone-marrow transplantation from a normal histocompatible donor, urinary glycosaminoglycan declined, reaching the lowest values 40–60 days after transplantation. Drugs administered to patients during bone-marrow transplantation and excreted in urine did not appear to interfere with quantification of urinary glycosaminoglycan.

Discussion

As originally recommended in the Scriver guidelines (17), institution of mass screening of newborns must be justified on the basis of several pragmatic criteria. Some degree of successful treatment of the MPS diseases has recently been achieved (5–7). Practical, low-cost testing methodology has become a paramount consideration. Despite numerous studies describing putative “screening” tests for MPS diseases, no such test is routinely applied for mass screening of the newborn population. Part of the failure to implement newborn screening can be ascribed to problems inherent in available testing methodologies. Existing tests are labor-intensive and thus prohibitively expensive, and many require large sample volumes (2–10 mL), which cannot be routinely collected from infants.

As one of the most broadly applied metabolic screening procedures, the “Berry spot test” (12, 18) has been widely used for preliminary testing of children in whom the diagno-
strated that DMB could be used to quantify glycosaminoglycan isolated from urine, but their method required large aliquots from 24-h urine specimens and the cumbersome isolation procedure of overnight precipitation with cetylpyridinium chloride and washing with alcohol.

To exploit the sensitivity of a dye-based assay, we explored the specificity of DMB for sulfated glycosaminoglycans in urine. As developed here, with results expressed in terms of urinary creatinine concentration, the direct DMB method is technically simple and therefore both rapid and inexpensive, with precisely quantitative results. Furthermore, the very small sample requirement (<0.1 mL of urine) is appropriate to mass screening of neonates.

In looking for potential interferences, we found that albumin, at concentrations found in urine, is nonreactive with the dye. Heparin, a sulfated glycosaminoglycan, is highly reactive in the assay. However, newborn infants do not receive heparin except in the setting of the newborn intensive-care unit. Other drugs likely to be administered to infants did not interfere except for those containing artificial coloring agents, and then only when added at full strength and not at concentrations expected in urine.

The direct DMB method provides a quantitative test for identifying excessive excretion of the abnormal metabolites pathognomonic of MPS disease. The precisely quantitative nature of the test gives the method additional application in monitoring the response to treatment such as bone-marrow transplantation. To merit widespread implementation for mass screening, the error rates (false positive, false negative) must be relatively small (17), a major problem of existing MPS tests (14). From preliminary results (27), we predict that studies of larger populations of normal infants and MPS patients will permit assignment of limits associated with acceptable error rates.

In summary, this new direct method of quantifying excessive excretion of sulfated glycosaminoglycans is ideally suited to mass screening programs for MPS diseases, and it circumvents the problems of existing methodologies. It requires only a few drops of urine, and many specimens can be assayed in less than 2 h with standard laboratory instrumentation. As a basis for discriminating normal and pathological values, urinary glycosaminoglycan excretion can be expressed in terms of urinary creatinine. Test results of the direct DMB method correlate with those of the standard cetylpyridinium chloride/carbazole–borate method. Based on these observations, ongoing studies are directed toward determining normal and pathological values in representative populations of age-matched normal and affected children (27).

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References


