Test for Mucopolysaccharidoses: Simple Method for Quantitative Estimation of Urinary Glycosaminoglycans

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A simple method is described for measurement of urinary glycosaminoglycans (GAG) in normal and pathological urine: a fixed fraction of the 24-h urine is passed over a small plug of ECTEOLA-modified cellulose and the GAG are eluted with a few milliliters of NaCl solution (3 mol/liter) and measured as hexuronic acid. Because results are compared for equal aliquots of complete 24-h urines, GAG are quickly detected, thus providing means for a chemical diagnosis of mucopolysaccharidosis. The procedure requires less than 1 h, and several tests may be conveniently run simultaneously. When urine specimens collected from normal children or children affected by mucopolysaccharidosis were analyzed, the results obtained with the proposed procedure agree well with those of more elaborate, widely accepted methods.

Additional Keyphrases ECTEOLA-cellulose • glucuronic acid • colorimetry

The rubric "mucopolysaccharidosis" embraces a heterogeneous group of diseases, which have been described and classified from clinical, genetic, and biochemical points of view into at least six distinct entities (Hurler, Hunter, Sanfilippo, Morquio, Scheie, and Maroteaux–Lamy syndromes) (1). Such diseases are now well known as disorders of GAG metabolism (1). Although the diagnosis of mucopolysaccharidosis is usually possible on clinical examination, the analysis of urine for excessive excretion of GAG is more than helpful in confirming that diagnosis.

Several procedures have been described for providing a reliable laboratory diagnosis of mucopolysaccharidosis, and the commonly used simple screening tests for detection of excess GAG excretion have been recently reviewed and discussed (2). Although these semiquantitative screening tests are often subject to "false positive," "false negative," (3) or contradictory (4) results, they are widely used in busy clinical laboratories, and the more complex, time-consuming, quantitative procedures are only used to confirm positive screening results. Because of this unsatisfactory situation, a new, acceptable, simple method for estimation of urinary GAG is required (5). I describe here a simple procedure for measurement of urinary GAG in normal and pathological urine, which aims at providing a routine method for chemical diagnosis of mucopolysaccharidosis. The proposed procedure is a time-saving modification of the ECTEOLA column method for the measurement of urinary GAG described by Di Ferrante (5). It requires less than 1 h for complete determination, and several tests may be conveniently run simultaneously.

Materials and Methods

Materials

ECTEOLA-modified cellulose (MN 2100; Macherey, Nagel & Co., Duren, Germany; 0.35 mEq/g capacity) was successively washed with NaOH (2.0 mol/liter), distilled water until neutral, HCl (2 mol/liter), distilled water until neutral, and saline (NaCl, 9.0 g/liter). Before use, it was suspended in saline by magnetic stirring.

Molded-plastic, disposable 10-ml syringe.

Reagents

Sodium tetraborate·10 H2O, 25 mmol/liter of sulfuric acid, relative density 1.8.

Carbazole, 1 g/liter of absolute ethanol. Stable for 12 weeks at 4°C in the dark.

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1 Nonstandard abbreviations used: GAG, glycosaminoglycan (s); ECTEOLA, epichlorohydrin triethanolamine.

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Glucuronic acid standards of 5–50 μg/ml in distilled water. Stable for two weeks at 4°C.

Specimens

Urine samples (24-h) obtained from normal children and from patients with mucopolysaccharidosis, or who were mentally or physically retarded, were kept at 4°C during the collection period and frozen, after their volume had been recorded, until they were analyzed. When more than a few hours was required for samples to be transferred to the laboratory, a few crystals of thymol were added as a preservative. Urinary sediments, if present, were separated by filtration.

Procedure

Transfer a volume of the thawed specimen corresponding to 0.025 of the 24-h volume into a 25-ml volumetric flask, and bring to volume with distilled water. The resulting solution contains a thousandth of the 24-h urine per ml. If the volume of the specimen is more than one liter, dilute an aliquot to 50 ml, with a final concentration of 0.002 of the 24-h volume per milliliter.

Into the bottom of the plastic barrel of the commercial disposable syringe, pack (by use of a glass rod) a well-compressed plug of wet glass wool to a depth of 0.5 cm and vertically fix the barrel, by means of a rubber stopper with hole of suitable diameter, into the neck of a Pyrex filter flask, the side-arm of which is connected to a water aspirating pump. Fill the barrel with the suspension of ECTEOLA in saline and pack a 15–20 mm column of resin on the glass wool under water aspiration, using 30–40 ml of saline. Finally, remove the suction just before the fluid reaches the top of the resin and apply 3 ml of the diluted urine (or 6 ml if the dilution was to 50 ml) to the resin, and allow it to filter through under gravity. Column and solutions are used at 26°–28°C. After the sample has drained into the resin, wash the column with 2 ml of the saline. After this wash has drained through, fill the barrel with saline, restore water aspiration, and wash the resin with 100 ml of saline at one time, then release the suction just after the liquid reaches the top of the column. Remove the barrel from the rubber stopper and set the needle in place. The needle allows the barrel to stand vertically at the top of an accurately graduated 15-ml conical tube. Layer 2 ml of NaCl solution, 3.0 mol/liter, on the resin and permit about 0.5 ml of liquid to drop from the needle into the graduated tube by applying gentle pressure on the syringe plunger. Five minutes later, if any residual 3.0 molar NaCl is left at the top of the resin, push it into the resin by a further gentle pressure on the plunger. After a further 5 min, push the plunger down firmly and tightly compress resin and glass wool, in order to collect the maximum volume of liquid into the graduated tube. Record this volume.

Uronic acid is then determined on 1 ml (in duplicate) of the collected fraction by the borate-carbazole method of Bitter and Muir (6), as modified by the adoption of the “one-step” technique of Sajdera described by Di Ferrante et al. (7): Place 5 ml of sulfuric acid–sodium tetraborate reagent in test tubes and cool them in ice-water. Carefully overlay 1.0 ml of sample (or standard) on the acid and gently shake the tubes, with constant cooling. Then close them with glass stoppers and shake vigorously, with cooling. Remove the stoppers, beginning with those of the sample, to facilitate the elimination of the hydrochloric acid that is generated. Add 0.2 ml of the carbazole reagent, again shake the stoppered tubes, heat them for 15 min in a vigorously boiling water bath, and cool to room temperature in running tap water.

Read the absorbance at 522 nm and multiply the value for absorbance of the sample by the number of milliliters collected from the syringe, and divide the product by three, to obtain the absorbance value corresponding to 0.001 of the 24-h volume. From this value, express the results as micrograms of uronic acid per 0.001 of the 24-h volume (which corresponds to the same number of milligrams of uronic acid per 24 h).

Results

Results obtained with the proposed procedure are presented in Table 1, where they are compared with those obtained for the same urine specimens by the precipitation and column methods described by Di Ferrante (5). Results are expressed as milligrams of hexuronic acid/24 h.

Discussion

The data presented in Table 1 clearly demonstrate the reliability of the proposed test to (a) immediately distinguish between normal and pathological urinary GAG excretion, and (b) correctly evaluate the amount of urinary GAG in both normal and pathological specimens.

Indeed, when the absorbances shown in Table 1 for patients with mucopolysaccharidosis are compared with those for either normal or mentally or physically retarded children, a striking difference is noted; they are more than 10-fold greater than the rest, with the sole exception of urines from patients with Morquio’s syndrome. The difference is obviously a predictable consequence of the theoretical basis of the proposed test, which, by comparing equal fractions of the whole 24-h urine volume, reflects whether the daily excretion of GAG

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Table 1. Measurement of Urinary GAG in Normal Children and in Children with Mucopolysaccharidosis or in Mentally or Physically Retarded Patients

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex, Age</th>
<th>Diagnosis</th>
<th>( A_{\text{um}} \text{ of 0.001} ) alliquot (mean)</th>
<th>Proposed test mean ±SD</th>
<th>Hexuronic acid, mg/24 h</th>
<th>Other procedures (%)</th>
<th>Precipitation%</th>
<th>Column</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M, 20m</td>
<td>Normal</td>
<td>0.048</td>
<td>3.7 ± 0.2 [3]</td>
<td>2.7 ± 0.0 (1.37)</td>
<td>4.1 ± 0.2 (0.90)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>M, 20m</td>
<td>Normal</td>
<td>0.039</td>
<td>3.0 ± 0.2 [2]</td>
<td>3.8 (0.79)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>M, 20m</td>
<td>Normal</td>
<td>0.030</td>
<td>2.3 [1]</td>
<td>2.2 (1.04)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>M, 15m</td>
<td>Normal</td>
<td>0.064</td>
<td>4.9 ± 0.2 [3]</td>
<td>3.8 (1.29)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M, 3y</td>
<td>Sanfilippo's</td>
<td>0.474</td>
<td>36.5 ± 0.6 [4]</td>
<td>30.3 ± 1.0 (1.20)</td>
<td>29.7 ± 0.0 (1.22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>M, 3y</td>
<td>Sanfilippo's</td>
<td>0.512</td>
<td>39.3 ± 0.7 [3]</td>
<td>33.8 ± 0.5 (1.16)</td>
<td>35.6 (1.10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>F, 6y</td>
<td>Sanfilippo's</td>
<td>0.500</td>
<td>38.4 ± 1.2 [3]</td>
<td>35.7 ± 1.4 (1.07)</td>
<td>30.7 (1.25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>M, 7y</td>
<td>Sanfilippo's</td>
<td>0.340</td>
<td>26.1 ± 0.1 [2]</td>
<td>24.7 ± 0.0 (1.05)</td>
<td>29.1 (0.89)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>M, 5y</td>
<td>Hunter's</td>
<td>0.482</td>
<td>37.0 ± 0.2 [3]</td>
<td>29.7 ± 0.2 (1.24)</td>
<td>33.4 (1.10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>F, 12y</td>
<td>Morquio's</td>
<td>0.183</td>
<td>14.0 ± 0.4 [6]</td>
<td>15.7 ± 0.1 (0.89)</td>
<td>10.3 ± 0.1 (1.35)</td>
<td></td>
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</tr>
<tr>
<td>11</td>
<td>F, 13y</td>
<td>Morquio's</td>
<td>0.156</td>
<td>12.0 ± 0.4 [6]</td>
<td>15.6 ± 0.1 (0.76)</td>
<td>12.6 ± 0.3 (0.95)</td>
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<td></td>
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<tr>
<td>12</td>
<td>F, 30y</td>
<td>Marfan's</td>
<td>0.053</td>
<td>3.9 ± 0.5 [3]</td>
<td>3.1 ± 0.2 (1.25)</td>
<td>3.4 (1.14)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>M, 5y</td>
<td>Chondroosteodystrophy</td>
<td>0.029</td>
<td>2.2 ± 0.2 [3]</td>
<td>0.8 ± 0.0 (2.75)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>M, 20m</td>
<td>Phys. retarded</td>
<td>0.016</td>
<td>1.2 ± 0.1 [3]</td>
<td>0.84 (1.42)</td>
<td>1.2 ± 0.1 (1.00)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>M, 7m</td>
<td>Ment. retarded</td>
<td>0.022</td>
<td>1.7 ± 0.1 [4]</td>
<td>0.9 ± 0.0 (1.88)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>F, 3y</td>
<td>Chondrodystrophy</td>
<td>0.060</td>
<td>4.6 [1]</td>
<td>4.8 (0.96)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figures in square brackets are numbers of tests performed. Figures in parentheses are ratios between mean values obtained with the proposed test and mean values obtained with precipitation or column method (5).

* Results of the proposed procedure are compared with those obtained on the same urine specimens with Di Ferrante's methods.

* Most of the precipitation and column tests were made in duplicate, and the mean value is reported as ± the simple deviation from the mean.

in urine is normal or excessive. It is therefore clear that a simple comparison between the red color yield of the sample and that of 10 μg of standard glucuronic acid permits the detection of excess GAG excretion. Measurement of absorbance gives quantitative confirmation.

In view of the above-mentioned theoretical basis, the proposed procedure is diagnostically reliable only when applied to complete 24-h urines. In any case, incomplete urine collection is a considerable source of error for any quantitative study of urinary GAG, as pointed out by Di Ferrante and Lipscomb (8).

The data presented in Table 1 demonstrate that, when urine specimens collected from normal children or children with mucopolysaccharidosis (cases 1 to 11) are analyzed as described, the results agree well with those obtained with the more elaborate, widely accepted methods described by Di Ferrante. The results of the proposed procedure are, however, usually higher, and for children with mucopolysaccharidosis the differences from Di Ferrante's values range between −10% and +25%. On the contrary, more remarkable differences were found when urines obtained from children mentally or physically retarded were analyzed (cases 13 to 15).

The extremely low absorbances obtained on these specimens may only be responsible to a certain extent for the difference, because in the normal cases (cases 1 to 4) similar values of absorbance yielded results comparable with those of Di Ferrante. However, case 14 may provide an explanation; in this case a remarkable difference (confirmed on different 24-h urine collections; unpublished data) was found between the results obtained with the precipitation method and those obtained with the column method, while the latter was in perfect agreement with the proposed test. This is not surprising, because the proposed procedure is a modification of the column method and consequently maintains the same characteristics. The remarkable differences found between the results of the proposed procedure and those of the precipitation method thus probably result from those inconveniences of the latter method that induced Di Ferrante to develop the alternative column method (8).

The reproducibility of the proposed procedure is clearly demonstrated in Table 1. Although the procedures described by Di Ferrante show less remarkable deviation from the mean, especially for specimens containing a normal amount of GAG, the statistical range of error found for the pro-
posed test is quite satisfactory.

As far as the method adopted for estimating uronic acid is concerned, absorbancies at 522 nm were lower than those obtained with either the Bitter and Muir method (6) or Sajdera's technique as described by Di Ferrante et al. (7), who used 0.5 ml rather than 1.0 ml of sample. In the present procedure, the volume of 1.0 ml was chosen in order to immediately obtain an absorbance close to that relative to 0.001 of the 24-h volume. In effect, in the tested solution collected from the syringe, which has a volume of about 3.0 ml, the GAG concentration is close to 0.001 part per milliliter, like the starting diluted urine solution. However, any other method of estimating uronic acid may be used, with obviously comparable results.

An estimation of uronic acid does not detect keratan sulfate, which is present in excess in urine obtained from patients with Morquio's syndrome. However, because almost equal amounts of chondroitin sulfate (mainly C) and keratan sulfate are excreted in Morquio's syndrome, and because the excretion of carbazole-positive materials (chondroitin sulfate) in Morquio's syndrome is usually greater than that found in normal subjects, although less than that found in the other mucopolysaccharidoses (chondroitin sulfate B and heparan sulfate) (9), the proposed procedure can also detect excess GAG excretion in Morquio's syndromes, as confirmed by analysis of cases 10 and 11 (Table 1). However, when a diagnosis of Morquio's has to be confirmed, the anthrone reaction for hexoses (10) may be usefully performed on the fraction collected from the syringe. Results of the anthrone reaction, performed on specimens 10 and 11 and expressed as milligrams of hexoses per 24 h, agreed perfectly with those obtained by Di Ferrante's precipitation method, but were slightly greater than those obtained by his column method.

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References