INTRODUCTION

Mucopolysaccharidoses are a group of lysosomal diseases characterized by storage of glycosaminoglycans (GAGs) in tissue and as a consequence in urine. Glycosaminoglycans (GAGs) are a major component of the extracellular matrix (ECM) of the tissue and are long unbranched polysaccharides containing a repeating disaccharides unit. GAGs consist of N- and O-sulfate groups and are covalently linked to core proteins to form proteoglycans. The most common GAGs are hyaluronic acid, dermatan sulfate, chondroitin sulfate, heparin, heparan sulfate and keratin sulfate. Hyaluronic is unique among the GAGs in that it does not contain any sulfate and is not found covalently attached to protein as a proteoglycan (it is, however, a component of non-covalently formed complexes with proteoglycan in the ECM).

GAGs are highly negatively charged molecules that impart high viscosity to tissues and liquid. Along with the high viscosity comes low compressibility, which makes these molecules ideal for examples as a lubricant (synovial fluid), for joints. GAGs and proteoglycans play an important role in cellular adhesion, growth, migration, and differentiation. They are also involved in regulation of the enzymes and tissue remodeling in response to injury, for example, or tissue destruction in disease such as rheumatoid arthritis. During catabolism, GAGs are internalized in 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)

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. These screening procedures may be qualitative (2-4) or quantitative (5-9). Several type of assay have been developed and reported in the past.

The spot tests were used as a screening test is very simple, qualitative but less sensitive, and there is a chance of false positive and false negative results depending on the types of mucopolysaccharidoses (2, 3, 10).

The measurement of glycosaminoglycans through turbidity tests depends on the interaction of GAGs with cationic detergents (like cetylpyridinium chloride). Although these tests are quantitative but their sensitivity depends on the types glycosaminoglycans. False negative results reported for certain types of mucopolysaccharidoses (5, 6, 7).

Farndale et al., (11) and Whitley et al., (12) reported a spectrophotometric based quantitative method for the detection of glycosaminoglycans concentration using DMB chloride to stain sulfated GAGs.

Here we have developed a similar procedure for the screening of mucopolysaccharidoses and comparing the result with those obtained with the CPC turbidity test. No screening based data have been published from Indian subcontinent.

Materials and Methods

Cetylpyridinium chloride (CPC), Heparan sulfate, Chondroitin sulfates were from sigma Chemicals Company St. Louis, MO. 1, 9- Dimethymethylene blue chloride was a
product of Serva Feinbiochemica GmbH, Heidelberg, F.R.G. Turbidity was measured with a hyland laser Nephelometer PDQ (Travelon, Brussels, Belgium). A total 72 normal specimens and 21 from patients with MPS under age of 15 years. All urine samples were stored at -20°C; No preservative were used.

DMB Assays

In this procedure, 100µL of urine sample or standard solution were added to 500µL of DMB solution (6.96mg DMB dye dissolved in 2ml of 95% of ethanol and 0.1M format buffer pH 3.5 added up to 100ml); then 0.1 ml of SDS solution added and vortex to dissolve the precipitant. The 50 µL of 200 mg/ml solution of BSA was added and mixed gently. Then 1.0 ml of ethanol (95% w/v) was added in it and mix gently and allowed to stand in an ice bucket for 20 minutes. Centrifuged it at (5000 rpm/15 minutes) and decant/siphon off the supernatant. The precipitate was dissolved with 100 µL of normal saline. Then 1 ml of protein based DMB dye reagent was added and mixed to allow to develop metachromasia for 10 minutes. Measured the absorbance at 520 nm against reagent blank. Correct the measured values for reagent blank and sample blank absorbance (11, 12).

In this assays, serial dilution of 10 µL/ml heparan sulfate and chondroitin sulfate standards was prepared by mixing 1.0ml of standard with 1.0ml of normal saline in a separate tube to make 5 µL/ml solution This procedure was repeated to make 2.5, 1.25, 0.625, 0.3125 µL/ml. Cetylpyridium chloride (CPC) Turbidity Assay. Urine samples were added in equal amount of cetylpyridium chloride (0.1% in citrate buffer pH 4.8). Equal volume of citrate buffer (0.1mol/L pH 4.8) were added and incubated 30 minutes at room temperature. The resulting CPC turbidity was measured in the nephelometer. Chondroitin sulfate and heparan sulfate standards were used to prepare calibration curves.

RESULTS

Dimethylmethelene Blue Assay

In the assay (100µL of sample or standard solution added to 500µL of DMB solution), chondroitin sulfate and heparan sulfate gave a linear calibration curve for 0.3125 to 5.0µL concentration. (Fig.1)

We were used chondroitin sulfate and heparan sulfate both as standard because sometimes the color of chondroitin sulfate was not stable. But in the patients and controls urine samples the color of DMB- GAG complex was stable for at least 25 minutes.

A total 72 normal urine samples was tested with the CPC turbidity assay and the DMB assays, the results of which reflects a good correlation (t =0.945) between these two procedures.

The following relationship was found between GAG concentration (µg/ml) measured by the CPC turbidity assay and the GAG concentration (µg/ml) measured by the DMB chloride assay: [CPC] = 0.994+0.268 [DMB]. The intercept does not deviate significantly from zero, and the slope does not deviate significantly from 1.

GAG content in normal subjects:

Reference values were established for the CPC assay and the DMB assay by measuring GAG concentration in a series of untimed (random) urine specimen from 72 normal subjects. In figure: 2 GAG content is expressed as milligram per millimole of creatinine. These normal subjects were grouped by age.

The GAG content in the younger age groups was more than the older one. Therefore groups were selected as indicated in Table 1; and for each of these the averages and standard deviation were determined.

Table 1: Average value and Standard Deviation for GAGs in Normal Urine

<table>
<thead>
<tr>
<th>Age (In Year)</th>
<th>CPC Assay</th>
<th>DMB Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Average</td>
</tr>
<tr>
<td>0-1</td>
<td>13</td>
<td>9.28</td>
</tr>
<tr>
<td>2-5</td>
<td>17</td>
<td>6.21</td>
</tr>
<tr>
<td>6-10</td>
<td>22</td>
<td>3.31</td>
</tr>
<tr>
<td>11-15</td>
<td>20</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Values in mg Glycosaminoglycon/mmol creatinine
GAG values for normal subjects were also analyzed by linear regression. GAG content and the age correlated after logarithmic transformation for the CPC turbidity assays and the DMB assay.

Figure 1: Chondroitin Sulfate Standard Graph

Figure 2: GAG concentration in various age groups in normal and in MPS patients.

In patients

We analyzed urine specimens from 21 mucopolysaccharidoses patients. These patients were screened after clinical symptoms and low enzyme activity
In 21 of the 21 mucopolysaccharidoses patients with the DMB assays the GAG/Creatinine ratio was increased (Table 2). This shows better result than with the other methods we had done; like in the turbidity assay some morquio and sanfilippo, A patients showed normal values (14). This could be because of formation of insoluble complexes with the cationic detergent CPC (Table 2).

After establishing these methods we conclude that the DMB assay as described is the more faster and sensitive method and it is a reliable procedure for the detection of all types of mucopolysaccharidoses.

DISCUSSION

In our procedure, small volume of urine can be measured GAGs concentration without prior precipitation and this could be done in untimed urine sample. And the assays is not hampered by instability of reagents or by insoluble complexes formed during the assays. While using the condroitin sulfate standards in the determination of GAG with DMB assay there is a problem in the solubility of the colored complex but this is not while using the heparan sulfate standards in the determination of GAG with CPC turbidity assay. In our procedure, small volume of urine can be measured GAGs concentration without prior precipitation and this could be done in untimed urine sample. In the CPC turbidity assay and DMB assay the measurement of urinary mucopolysaccharides. Figure 1 shows values measured for patients together with values for normal subject urine as a function of age. Table 2 shows whether the patient’s values were increased with respect to the established normal values for each of the two assay procedures.

Table 2: GAG content in Urines from MPS Patients

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>CPC Assay</th>
<th>DMB Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hunter</td>
<td>8</td>
<td>+</td>
</tr>
<tr>
<td>Hurler</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>Morquio</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Scheie</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>Sanfilippo A</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Sanfilippo C</td>
<td>2</td>
<td>+</td>
</tr>
</tbody>
</table>

Positive (+) result means the patients value exceeds the normal average plus the SD calculated for the corresponding age group.

REFERENCES