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DIMETHYL METHYLENE BLUE: A TOOL FOR MPS SCREENING

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Abstract: Mucopolysaccharidoses are a group of lysosomal diseases characterized by storage of glycosaminoglycans (GAGs) in tissue and as a consequence in urine. GAGs consist of N- and /or O-sulfate groups and are covalently linked to core proteins to form proteoglycans. During catabolism, GAGs are internalized in lysosome and degraded into their monomolecular constituents by lysosomal endoglycosidases, exoglycosidases, and exosulfatases. In Mucopolysaccharidoses (MPS) one of the hydrolytic lysosomal enzymes is deficient, which results in the accumulation of GAGs in the lysosomes and the resulting syndrome depends on which enzyme is deficient. The present spectrophotometric procedure based on the color reaction with Dimethyl Methylene Blue can be performed directly on untimed urine sample without prior precipitation. We have tested urine samples without preservative of 21 patients with various Mucopolysaccharidoses (eight hunters, four Hurler, two morquio, two scheie, three sanfilippo A and two sanfilippo C) through Dimethyl Methylene Blue assay and compared the result with Cetypyridinium Chloride (CPC) turbidity assay at pH: 4.8 (Citrate buffered) and at pH: 7.0 (Tris buffered). A total 72 normal urine samples were also tested with the CPC turbidity assay and the DMB assays to establish a reference range. GAG content is measured at absorbance 520 nm. Condroitin-4- sulfate standard were used to prepare calibration curves. In Normal subjects the GAG content in the younger age groups was more than the older one. Glycosaminoglycans level in the DMB assay were increased in 21 of 21 patients; whereas GAG's level in the turbidity test at pH 7.0, value were increased in 19 of the 21 patients and at pH 4.0, GAG's content was increased in only 15 of the 21 patients. The Dimethyl Methylene Blue assay procedure is a rapid, sensitive and reliable screening procedure to determine concentration of GAG's for various MPS.

Key Words: Mucopolysaccharidoses, GAG, DMB, Creatinine.

INTRODUCTION

Mucopolysaccharidoses are a group of lysosomal diseases characterized by storage of glycosamino glycans (GAGs) in tissue and as a consequence in urine. Glycosaminogycans (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 /or 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 internalizes in the lysosomes 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 Mucopolysaccharidoses (MPS) one of the hydrolytic lysosomal enzymes is deficient, which results in the accumulation of GAGs in the lysosomes and the resulting syndrome depends on which enzyme is deficient. 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

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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 Ċ; 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% 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 100µl 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.

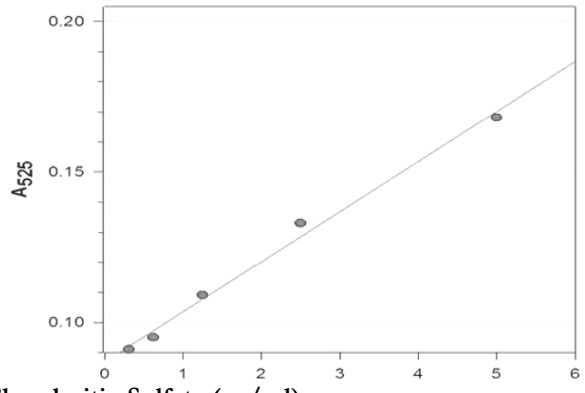
Cetylpyridinium chloride (CPC) Turbidity Assay. Urine samples were added in equal amount of cetylpyridinium chloride (0.1% in citrate buffer pH 4.8). Equal volume of citrate buffer (0.1mol/L pH 4.8) were added and incubated 30 minutes at room temperature. The resulting CPC turbidity was measured in the 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)

Figure 1: Chondroitin Sulfate Standard Graph



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 (r =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

Age (In Year)	CPC Assay			DMB Assay		
	No.	Average	SD	No.	Average	SD
0-1	13	9.28	8.2	16	16.7	7.8
2-5	17	6.21	2.5	15	9.4	2.4
6-10	22	3.31	1.8	18	6.5	1.9
11-15	20	2.9	1.3	23	4.8	1.3

Values in mg Glycosaminogylcon/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.

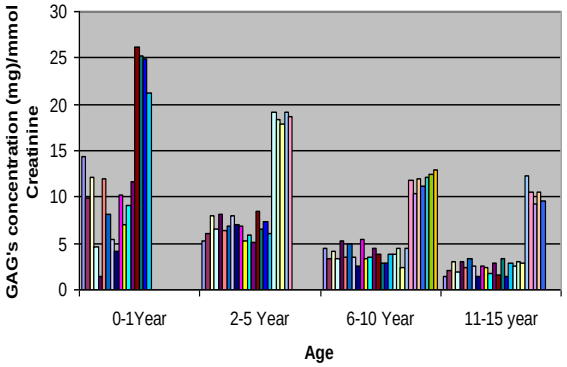


Figure 2: GAG concentration in various age groups in normal and in MPS patients.

In patients

We analyzed urine specimens from 21 mucopolysaccharidoses patients. These patients were screened after clinical symptoms and low enzyme activity

(eight hunters, four hurler, two morquio, two scheie, three sanfilippo A and two sanfilippo C), measuring GAG content by CPC turbidity assay and DMB assay. Figure 1 shows values measured for patients together with values for normal subject urines 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

Syndrome	No.	CPC Assay	DMB Assay
Hunter	8	+	+
Hurler	4	+	+
Morquio	2	-	+
Scheie	2	+	+
Sanfilippo A	3	-	+
Sanfilippo C	2	+	+

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

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 standard which formed stable colored complexes due to its lower molecular mass (13). These complexes remained soluble with normal and patients urine samples.

The quantitative tests demonstrate that depending on the type of MPS, GAG excretion can vary and will sometimes increased moderately. Thus it is essential to established reference values very carefully. The GAG excretion should be expressed in terms of creatinine excretion for good result and the variation of excretion with age group are taken into consideration (14).

Reference value for the DMB as well as CPC turbidity method is established for the various age groups based values for 72 samples of normal urine. We tested reliability of this method on 21 patients with six different mucopolysaccharidoses (including Hunter, Hurler, Morquio Scheie, Sanfilippo A, and Sanfilippo C). The GAG concentration measured by DMB assay in normal and patient urine samples directly correlate with CPC turbidity assay.

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.

REFERENCES

1. Neufeld EF, Meunzer J. The mucopolysaccharidoses. In Scriver CR, Beaudet AL, Sly WS, Valle D (eds). The Metabolic Basis of Inherited Disease, New York: McGraw-Hill, 6th edition.(1989) 1565-1587
2. Berman ER, Vered J, Bach G. A reliable spot test for mucopolysaccharidoses. Clin Chem. 17 (1971): 886-90
3. Berry HK, Spinanger J. A Paper spot test useful in the study of Hurler syndrome.J Lab Clin Med. 55 Jan (1960):136-8.
4. Carson NAJ, Neil DW. Metabolic abnormalities detected in a survey of mentally backward individuals in Northern Ireland. Arch. Dis Child. 37 (1962): 505-13.
5. Steiness IB. Acid mucopolysaccharidoses in urine in gargoylism. Pediatrics. 27 (1961): 112-7.
6. Pennock CA. A modified screening test for Glycosaminoglycon excretion. J Clin Pathol. 22 (1969):379-80.
7. Pennock CA. A review and selection of simple laboratory methods used for the study of Glycosaminoglycon excretion and the diagnosis of the mucopolysaccharidoses. JClin Pathol. 29(1976): 111-23.
8. Di Ferrante NM. The measurement of urinary mucopolysaccharides. Anal Biochem. 21(1967): 98-106.
9. Bitter T, Muir HM. A modified uronic acid carbazole reaction. Anal Biochem. 4(1962): 330-4.
10. Lorincz AE, Hurst RE, Kolodny EH. The early laboratory diagnosis of mucopolysaccharidoses. Ann Clin Lab Sci.12 (1982): 258-66.
11. Farndale RW, Sayers CA, Barrett AJ. A direct Spectrophotometric microassay for sulfated glycosaminoglycons in cartilage cultures. Connect Tiss Res. 9 (1982): 247-8.
12. Whitley CB, Rinnour MD, Draper KA, Dutton CM, Neglia JP. Diagnostic test for mucopolysaccharidoses I. Direct method for quantifying excessive urinary Glycosaminoglycon excretion. Clin Chem 35(1989): 374-9.
13. Farndale RW, Buttle DJ, Barrette AJ. Improved quantitation and discrimination of sulfated glycosaminoglycons by use of DMB. Biochim Biophys Acta 833 (1986): 173-7.
14. De Jong JGN, Wevers RA, Laarakkers C, Poorthuis BJHM. DMB based spectrophotometry of glycosaminoglycons in untreated urine. Clin Chem 35(1989): 1472-7.

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