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Research Article

Quantitative and Qualitative Analysis of Urinary Glycosaminoglycans (GAGs)

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Abstract

Objective: Urine Glycosaminoglycans (GAGs) by one dimensional electrophoresis; A simplified technique for mucopolysaccharide screening: Our study of 128 Indian children.

Design: Retrospective study.

Setting: The study was carried out at the institute after taking institutional ethics committee approval and patient/guardian written informed consent.

Participants: 128 clinically suspected to have mucopolysaccharide disorder within an age group of 6 months to 12 years.

Methods: Quantitative and qualitative analysis of glycosaminoglycans followed by confirmative lysosomal enzyme study.

Results: Total 105/128 cases (82%) were found to have elevated level of total glycosaminoglycans; while 23 (18%) of them showed normal glycosaminoglycan with normal GAGs excretion pattern. Ninety four of 105 cases had showed high glycosaminoglycans with abnormal excretion pattern and all of them were confirmed to have enzyme deficiency for various mucoplysaccharide disorders. Among these, Hunter syndrome (MPS-II) was the most common 27.7% followed by Morquio-A syndrome (MPS-IVA) in 26.6%, Sanfilippo-A (MPS-IIIA) in 15.95%, Hurler syndrome (MPS-I), Maroteaux lamy (MPS-VI) and Sanfilippo-B (MPS-IIIB) in 9.6% for each one. Moreover, 11/105 (10.47%) patients with false positive glycosaminoglycan levels, 5 were found to be normal by electrophoresis and enzymes study. Remaining 6 patients were positive for lysosomal storage disorders other than mucopolysaccharide disorder (two patients affected with I-cell disease, two patients with GM1 gangliosidosis, one with Sialic acid storage and one with Fucosidosis).

Conclusions: The combined approach of glycosaminoglycans quantitative and one-dimensional electrophoresis is 100% sensitive and 68% specific technique to reach at the specific diagnosis of mucopolysaccharide disorder. It also provide clue to the diagnosis of other lysosomal storage disorders.

Abbreviations:

LSDs	:	Lysosomal Storage Disorders
GAGs	:	Glycosaminoglycans
MPS	:	Mucopolysaccharidosis
KS	:	Keratan Sulfate
CS	:	Chondroitin Sulfate
DS	:	Dermatan Sulfate
HS	:	Heparan Sulfate
PGs	:	Proteoglycans
DMB	:	Dimethylmethylene Blue
4-MU	:	4-Methylumbelliferone

Keywords: Glycosaminoglycans (GAGs); Lysosomal Storage Diseases (LSDs); Mucopolysaccharidosis (MPS)

Introduction

Mucopolysaccharidoses (MPS) are a group of seven different type and eleven subtypes of lysosomal storage diseases (LSDs). They are the most common storage disorders with overlapping and easily identifiable phenotype with an overall collective incidence of 1 in 25,000 live births [1]. They occur due to deficient activity of a specific lysosomal enzyme required to degrade glycosaminoglycans (GAGs) known as Keratan sulfate (KS), Chondroitin Sulfate (CS), Dermatan Sulfate (DS) and Heparan Sulfate (HS) [2].

Every MPS disorder has a characteristic accumulation of GAGs in the lysosomes and Extra Cellular Matrix (ECM) in different tissues of the body like brain, lung, liver, spleen, heart, kidney, joints, bone, eyes and skin. Due to this accumulation, most MPSs are clinically indistinguishable with signs and symptoms of mental retardation, abnormal skeletal development, hearing and visual impairment, aggressive behavior, coarse facial features, hepatosplenomegaly, umbilical or an inguinal hernia, short stature during infancy or in early childhood [3]. Even though it is possible to narrow down the type of MPS by careful analysis of clinical features, overlaps do exist between MPS I and VI. Though, mild cases of Hunter syndrome and Sanfilippo syndrome cannot be differentiated based on clinical analysis alone. Hence the differential diagnosis of MPS disorders is a difficult task for the clinicians without confirmative enzymes study. To narrow down the window of enzyme study, it is imperative to perform screening test that can point towards a specific type of MPS disorders, minimizing the cost of enzymes study. Several methods have been established to measure and differentiate GAGs as a screening test for MPS disorders. That include Dimethylene Blue (DMB) and alcian blue spectrophotometric method [4] followed by Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), Enzyme-linked Immunosorbent Assay (ELISA), Liquid Chromatography-Mass Spectrometry (LC-MS) to detect different type of GAGs in urine and blood [5-7]. All these techniques demand expensive instrument and skill; limiting its widespread use. Here we describe the use of simple one-dimensional electrophoresis screening that can easily differentiate excretion of different GAGs mainly KS/CS/DS/HS providing the first line of information to the clinician to decide on the specific enzyme activity to be measured for confirmative diagnosis.

Methods

This is a retrospective study carried out in 128 children referred to our center during a period of December 2008 to May 2016. All referrals were mainly by geneticists, pediatricians and metabolic specialists from different parts of the country. Subjects under study were within the age range of 6 months to 12 years and were clinically suspected to have MPS disorder. The most common clinical features were- short stature, facial dysmorphism, joint stiffness, skeletal abnormalities, hepato/hepatosplenomegaly, corneal clouding, cardiac involvement and progressive neuroregression with behavioral problems. The study has included only those cases with the presence of any of the above-mentioned signs and symptoms.

10 to 15 ml random urine samples without any preservatives were collected in a sterile container for quantitative and electrophoresis study of Glycosaminoglycans (GAGs). In all subjects, 4 ml of blood was collected in sodium heparin/ EDTA vacutainer for confirmatory enzyme study from leukocytes and/or plasma as per the flow chart is shown in Table S1. Urinary GAG was determined by Dimethylmethylene Blue (DMB) dye method [4]. This was carried out by centrifugation at 2000 rpm for 5 minutes at room temperature and residues were discarded. Urine creatinine concentration was measured by a bio-chemistry analyzer using commercially available kit. For GAG assay, 100 µg/ml Chondroitin sulfate (type A, sodium salt from the bovine trachea, Sigma C8529) was used as a standard. 100 µl distilled water and 20 µl of the sample or standards were added to 1 ml DMB dye, mixed properly and the color measured immediately at 520 nm in the spectrophotometer. GAGs concentration was expressed in terms of mg/mmol of creatinine. Qualitative analysis of GAGs was carried out by one-dimensional electrophoresis.

Disease name	Enzyme deficiency
Hurler disease (MPS-I)	α-Iduronidase
Hunter disease (MPS-II)	α-iduronidate-sulfatase
Sanfilippo syndrome-A (MPS-IIIA)	Heparan sulphamidase
Sanfilippo syndrome-B (MPS-IIIB)	N-Acetyl-α-D-glucosaminidase
Morquio A (MPS-IVA)	β-galactosidase-6-sulfate-sulfatase
Morquio B (MPS-IVB)	β-galactosidase
Maroteaux lamy syndrome (MPS-VI)	Arylsulfatase-B
Sly Syndrome (MPS-VII)	β-glucuronidase
GM1 gangliosidosis	β- galactosidase
Fucosidosis	α-Fucosidosis
Mannosidosis	α-Mannosidosis
Mucolipidosis-II/III (I-cell disease)	Multiple enzyme raised in plasma
Sialic acid storage disorders	Free and Total N-Acetyl neuraminic acid level
	(Free NANA and Total NANA)

TableS1: Lysosomal Storage Disorders (LSDs) associated with dysmorphic features.

Prior to electrophoresis, pre-treatment of urine was performed essentially by the method described by Hopwood and Harrison (1982) with some modifications. The calculated amount of urine used was centrifuged at 2200 rpm for 10 min at 25˚C to remove debris. An equal volume of CPC/citrate buffer was added to urine samples, mixed and incubated in the water bath at 37˚C for 30 minutes. The resulting CPC-GAGs complex was separated by centrifuging at 2200 rpm for 10 minutes. The supernatant was decanted and the centrifuge tube drained inverted for 30 minutes. The pellet was dissolved in 150 µl 2M lithium chloride and mixed with 800µl ethanol followed by vortexing it at full speed for 5 min. until pellet is well mixed. The mixture was then transferred into 1.5 ml micro-centrifuge tube, let stand for 5 minutes at 25˚C, centrifuge at 2200 rpm for 10 minutes at 25˚C. The supernatant was decanted and the pellet containing GAGs was dried by inverting the microcentrifuge tube on a tissue paper. 20-30 µl of 0.05% phenol red was added to the pellet, vortexed vigorously to dislodge the pellet and dissolve the GAGs. The dissolved GAGs sample was stored at -20˚C for electrophoresis study.

One-dimensional electrophoresis was carried out using 0.1 M barium acetate buffer and cellulose acetate sheet. Cellulose acetate sheet was initially immersed in 0.1 M barium acetate buffer, pH 5.0 and 3 mm Whatman paper was applied to form wicks and the system was pre-electrophoresed for 30 minutes at 60 V constant voltages. By using a plastic comb, 0.15 mg of each standard and 3 µl of GAGs were loaded onto the cathode side of cellulose acetate plate which had been pre-soaked in 0.1 M aqueous barium acetate, pH 5.0 for 10 minutes. Electrophoretic pattern of GAGs; Keratan Sulfate (KS), Chondroitin Sulfate (CS), Dermatan Sulfate (DS) and Heparin Sulfate (HS) were visualized by staining the cellulose acetate sheet with alcian blue dye followed by distaining with 5% acetic acid.

An external quality control system was set up with ERNDiM quality control program for MPS electrophoresis. Six unknowns affected and/or normal samples were received from ERNDiM quality control program; the samples were run with test batches where possible. All our testing complied with the ERNDiM results. All the samples that are screened positive by GAGs study were processed for enzyme analysis from leukocytes and/or plasma using 4-MU specific substrates based on excretion pattern as shown in Table 1 [8-10]. Those that were negative by GAG study were also investigated for various MPS disorders as shown in Supplementary Table S1.

Total cases	**Qualitative GAG analysis by electrophoresis**				**Disease**	**Enzyme name**	**Enzyme in affected proband nmol/hr/mg protein**
(n=94)							
	KS	**CS**	**DS**	**HS**			
Positive cases of MPS					MPS I	α- iduronidase	5.2 - 12.9
(n = 94)	-	+	+++	++	(n = 9)		(N.R.: 32-70 nmol/hr/mg)

with raised GAG					MPS II	α- iduronidate-sulfatase	3.0 - 6.0
confirmed					(n = 26)		(N.R.: 600-1616 nmol/4 hr/ml)
diagnosis by enzyme	-	++	-	+++	MPS IIIA	Heparan sulphamidase	0.17- 0.25
analysis					(n = 15)		(NR : 1.2-4.5 nmol/17 hr/mg)
					MPS IIIB	N-Acetyl-α-D-glucosaminidase	5.0 – 20.0
					(n = 10)		(NR : 300–600 nmol/17 hr/ml plasma)
	++	+++	-	-	MPS IVA	β- galactosidase-6-sulfate-sulfatase	0.25-2.1
					(n = 23)		(N.R.: 15-32 nmol/17 hr/mg)
	-	+++	-	-	MPS IVA		
					(n = 2)		
	-	++	+++	-	MPS VI	Aryl sulfatase B	0.00 - 0.1
					(n = 4)		(NR : 0.6 - 8.0 nmol/ hr/mg)
	-	-	+++	-	MPS VI		
					(n = 5)		

Table 1: GAG Excretion pattern obtained by electrophoresis and confirmative enzyme study in MPS disorders.

Results

Out of 128 children investigated, 105 (82.0%) were found to have elevated level of total GAG compared to age-matched healthy controls; while 23 (18%) of them showed normal GAGs as shown in Table 1. Further upon electrophoresis study, 23 children with normal GAG levels showed normal GAG excretion pattern, which was consistent with that of quantitative results. Of 105 children with elevated GAGs, 94 (89.5%) showed abnormal GAG excretion pattern by electrophoresis. Based on this, confirmatory lysosomal enzyme study was carried out. Eleven patients (10.5%) with borderline elevated GAGs showed normal excretion of GAGs with excess of CS on electrophoresis. Six of them were found to have storage disorders other than MPS. Details of patients with confirmatory enzymes study with GAGs electrophoresis is shown in Table 2.

	Age range				
	0.5 - 1	**1 - 2**	**2 - 5 Years**	**5 - 9 Years**	**9 - 12 Years**
Normal patients					
Normal patients with normal GAG (n=23)	12 - 13.4 (n=3)	4.8 - 11.9 (n=6)	4.7 - 9.8 (n=5)	4.9 - 7.0 (n=7)	3.8 -4.4 (n=2)
Normal patients with raised GAG (n=5)	16.2 (n=1)	13.0-15.2 (n=2)	-	10.5 (n=1)	-
Patients affected with MPS disorders					
MPS-I (n=10)	-	17.2 -49.3 (n=2)	15.1 - 61.3 (n=5)	-	-
MPS-II (n=26)	17.0 - 36.9 (n=2)	15.5 - 42.7 (n=6)	22.8 - 52.6 (n=11)	11.0 - 57.1 (n=7)	-
MPS-IIIA (n=15)	18.3 - 28.3 (n=2)	20.1 - 39.2 (n=2)	9.5 -24.1 (n=4)	7.8 - 27.7 (n=6)	8.8 (n=1)
MPS-IIIB (n=9)	-	-	18.5 - 45.3 (n=2)	10.2 - 38.4 (n=5)	9.6 - 17.8 (n=2)
MPS-IVA (n=25)	18.1 - 22.1 (n=2)	15.2 - 41.2 (n=8)	9.6 - 14.6 (n=8)	9.9 - 22.4 (n=4)	7.2 - 10.1 (n=3)
MPS-VI (n=9)	50.7 (n=1)	24.1 - 39.8 9n=3)	20.8 -30.5 (n=3)	37.7 - 50.9 (n=2)	-
Patients affected with other storage disorders					
GM1 (n=2)	18.1 (n=1)	14.1 (n=1)	-	-	-

I-cell disease (n=2)	13.9 (n=1)	-	12.4 (n=1)	-	-
Sialic acid storage disorders (n=1)	-	12.0 (n=1)	-	-	-
Fucosidosis (n=1)	-	-	-	-	4.9 (n=1)

Table 2: GAGs concentration of normal individual, affected with various MPS disorders and other LSDs.

All 94 patients with abnormal GAG excretion pattern (as shown in Table 2) were found to be affected with various MPS. Those with excess DS with moderate HS and CS pattern were confirmed to have Hurler/Hunter disease as the most common manifestation. Those with only excess DS with moderate CS pattern were shown to have Maroteaux Lamy syndrome (MPS-VI) and those with excess HS with moderate CS pattern were confirmed to have Sanfilippo syndrome (MPS-III). Excess KS and CS pattern was seen in 25 patients with Morquio-A syndrome (MPS-IVA). Among all, highest number of patients were found with Hunter syndrome (MPS-II) (26/94) and Morquio A syndrome (MPS-IVA) (25/94) followed by Sanfilippo A (MPS-IIIA) (15/94), Hurler syndrome (MPS-I) (10/94), Maroteaux Lamy (MPS VI) (9/94) and Sanfilippo-B (MPS-IIIB) (9/94) (Table 1). While we are unaware of the exact number of Sanfilippo C and D as present study was unable to carry out confirmatory enzyme study.

Of eleven patients with borderline raised GAG levels, 5 were found to be normal by electrophoresis and enzyme study. Of the remaining six children two had shown excess of CS with mildly raised DS/HS and were found to have I-cell disease. Four children with the only excess of CS were investigated for other lysosomal storage disorders that include two with GM1 gangliosidosis, one with Sialic acid storage and one with Fucosidosis as shown in Table 1. Remaining 23 patients with normal GAGs by quantitative and electrophoresis was also investigated for all MPS disorders and was found to be normal.

Discussion

Of several aforementioned advanced techniques to measure urinary GAG, present study was carried out by a simple technique of one-dimensional electrophoresis for the diagnosis of MPS disorders.

This method separates a mixture of GAGs (DS, HS, CS, and KS) into its individual components. Excess of DS with moderate amount of CS and mild HS was seen in MPS-I, II and VI. In our study MPS-VI patients had shown excess of DS with absence of any HS. This suggests that GAG electrophoresis pattern can distinguish MPS-I, II from MPS-VI. HS was consistently the predominant component and CS the minor component in all MPS-III patients confirms that excess of HS excretion is the specific marker for MPS-III. Though KS is difficult to separate from CS [11], one can see a clear pattern of KS with CS in all patients with MPS-IVA. None of our patients were found to have MPS-IVB.

Two children with mild elevated GAG had shown excess excretion of CS with mild HS and were found to have I-cell disease. This could be explained by the fact that in mucolipidosis type II/III there is an overall deficiency of all lysosomal enzymes likely to be influencing breaking down of GAGs [12]. Moreover, two patients with borderline raised GAGs with moderately excess excretion of CS in GAG electrophoresis pattern were confirmed to have GM1 gangliosidosis. Both these children were presented with mild to moderate dysmorphic face, cherry red spot, Mongolian spot on back, mild skeletal deformity and hepato/hepatosplenomegaly. This could be due to deficiency of β-Galactosidase that might have influenced on breaking down of GAGs like KS/CS and showing excess of CS in electrophoresis [13]. While in one patient with borderline elevated GAG was found to have Sialic acid storage disorders and Fucosidosis one each. It is likely to be due to presence of GAG-derived oligosaccharides present in urine showing excess of GAG with normal electrophoresis results [14]. It can be observed from these cases that in borderline raised GAGs with excess CS with mild DS/HS; other Lysosomal Storage Disorders (LSDs) like mucolipidosis and oligosaccharide disorders cannot be ruled out and need to be further investigated.

To conclude, present study demonstrates the usefulness of GAG quantitative and one-dimensional electrophoresis study as a firsttier biomarker study for MPS disorders with 100% sensitivity and 68% specificity. This approach can reduce the overall cost for enzyme study as a confirmative diagnosis for MPS disorders.

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Conflict of Interests

All authors in the manuscript declare that they have no conflict of interests (Financial or Nonfinancial).

Consent

Informed written consent was obtained from all the participants for publication of their clinical details. A copy of the written consent is available for review by the editor of this journal.

Ethics

Present study under submission has been approved by the institutional ethics committee [FRIGE's Institute of Human Genetics] wide approval number FRIGE/IEC/5/2010 dated 7th March, 2010. This process is in accordance with the Helsinki Declaration.

Authors' Contributions

Planned and designed the experiments: JS, MM. Clinical analysis: SN, IP, MK. Enzyme analysis: MM, RB. Wrote the first draft of the manuscript: JS, MM. Made critical revisions and approved final version: JS and FS. All authors reviewed and approved of the final manuscript.

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