Reliability of a Visual Test for the Rapid Detection of Mucopolysaccharidoses: GAG-Test[®]

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Mucopolysaccharidoses (MPS) are a group of lysosomal storage disorders, characterized by the deficiency/absence of one of the enzymes involved in the intralysosomal degradation of glycosaminoglycans (GAGs). The quantitative determination of urinary GAGs using dimethylmethylene blue (DMB) shows high reliability. However, the logistics and staff for this method are not always available in primary care centers. Sending urine samples to reference laboratories increases the cost and delays the diagnosis. Thus, the aim of this article is to develop and evaluate a simple and low-cost visual test (GAG-test[®]) for the screening of urine samples from patients under suspicion of suffering from MPS. The purpose is to narrow down the number of samples to be

assayed through the quantitative method. A measure of 50 µl urine was added to 2 ml DMB solution. A color change from dark blue to purple indicates an excess of GAGs. The quantitative analyses showed a significant difference between controls' and patients' concentrations ($P < 0.05$). After optimization of the composition, positive and negative results obtained with the qualitative test were able to discriminate between normal urines and those from patients suffering from mucopolysaccharidosis. Therefore, GAG-test[®] has proved to be a useful tool for the prior diagnosis of patients suffering from mucopolysaccharidosis, reducing the number of individuals with whom investigations should be continued. J. Clin. Lab. Anal. 25:179–184, 2011. © 2011 Wiley-Liss, Inc.

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INTRODUCTION

Mucopolysaccharidoses (MPS) are a heterogeneous group of multisystem progressive lysosomal storage disorders, characterized by the deficiency/absence of one of the 11 enzymes involved in the lysosomal catabolism of glycosaminoglycans (GAGs). Depending on the carbohydrate composition of the polysaccharide chain and the sulfation pattern, the GAGs are grouped into different classes, including heparan sulfate (HS), dermatan sulfate, keratan sulfate (KS), and chondroitin sulfate (CS) (1). These molecules are structurally complex sulfated heteropolysaccharides composed of repeating disaccharide moieties, whose structure is constituted by a hexosamine (D-glycosamine or D-galactosamine) and by a hexuronic acid (D-glucuronic or D-iduronic), or, in KS, galactose instead of hexuronic acid. These enzyme deficiencies lead to the systemic accumulation of undegraded

or partially degraded GAGs within all cell lysosomes and extracellularly in various tissues and organs of the body, resulting in a wide range of symptoms and signs: coarse faces, progressive neurological deterioration, multiple bone dysplasias, short stature, macrocephaly, and cardiorespiratory damage (2). The high degree of overlap of the phenotypes among MPS and between

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MPS and other lysosomal or nonlysosomal disorders makes clinical diagnosis highly problematic.

According to the literature, MPS can be classified as MPS I (Hurler (MPS IH), Hurler-Scheie (MPS IHS) or Scheie disease (MPS IS)), MPS II (Hunter disease), MPS III (Sanfilippo A, B, C, or D disease), MPS IV (Morquio disease, types A or B), MPS VI (Maroteaux disease), and MPS VII (Sly disease) (3). MPS IH, II, III, and VI result in increased urinary GAG excretion (4). However, MPS IS, ISH, IV, and VII do not produce an excess of urinary GAGs, and may even fall within the reference interval for healthy individuals.

Several methods for the determination of total GAGs in urine are described in the literature. The Berry spot test is based on the ability of toluidine blue to attach to GAGs. It may, therefore, be considered a simple and rapid procedure; however, it is not quantitative (5). In the cetylpyridinium chloride test, GAGs are separated by precipitation and then quantified, but the method is tedious and time consuming (6). The Alcian blue test entails the formation of an insoluble complex that, after separation, is dissociated and measured spectrophotometrically, but it is very time consuming and particles are difficult to harvest (7). Finally, in the uronic acid-carbazole reaction, uronic acid is precipitated with cationic reagents; but this methodology is not commonly used (8), because it does not detect the keratan sulfaturia of MPS IV and it is potentially dangerous, owing to the use of concentrated sulfuric acid.

An easier and more widespread procedure for the quantitative determination of urinary GAGs is their spectrophotometric determination with dimethylmethylene blue (DMB) as a dye (9–11). According to the previously published studies, this method is the most reliable in terms of false-negative results (12–14), although it is not totally free of false negatives in particular in types of MPS where the elimination of GAGs is less conspicuously increased (MPS IS, ISH, IV, and VII). In any case, these methods are not able to distinguish the kind of GAG excreted and, as a result, enzymatic or separation methods are required in order to reach a correct diagnosis.

Despite the fact that the DMB assay is a reliable procedure for the determination of urinary GAGs, it requires logistical support (spectrophotometer), chemical products, and specialized staff that are not generally available in primary care centers. As a result, urine samples are sent to reference laboratories, causing an increase in the total cost and time of the diagnosis, which can be carried out by various methods (15–17). However, a definitive diagnosis of MPS can only be achieved using enzyme and genetic analysis, but it is not feasible to use these methods for screening all the samples from patients under suspicion of MPS. Hence, a simpler selective

screening procedure, to be applied along with clinical signs, needs to be devised in order to narrow down the list of potential patients to be assayed.

We present a simple and rapid colorimetric test (GAG-test[®]), based on the color change of the DMB solution when the complex between GAGs and DMB is formed. The GAG-test[®] could be offered in any primary care center, owing to its low cost and no need for additional facilities or staff, to confirm or discard a suspicion of MPS. Several studies on the treatment of these disorders indicate the need for early therapeutic intervention.

The aim of this article is to evaluate the reliability of a qualitative visual test (GAG-test[®]) for the screening of urine samples from patients under suspicion of MPS. The purpose is to narrow down the number of samples to be analyzed through the quantitative method. Only those samples with a positive result in the GAG-test[®] would be further studied. For this purpose, sensitivity, specificity, and likelihood ratios (both positive and negative) of the test were calculated and compared with previously published values. The limitation of this test is that it is applicable for the screening of MPS IH, II, III, and VI, but not for attenuated MPS (IS, ISH, IV and VII), because they do not necessarily produce high excretion of GAGs.

MATERIALS AND METHODS

Patients

Urine samples from healthy patients ($n = 319$, age ranging from 1 month to 87 years) were analyzed in order to obtain reference values of GAGs. Pathological urine samples were obtained from patients suffering from MPS without any clinical treatment ($n = 23$; 1 Hurler, 15 Hunter, 7 Sanfilippo; age ranging from 1 to 31 years). In both cases, qualitative and quantitative assays were used.

According to the literature, several diseases other than MPS (leukemia, rheumatic arthritis, diabetes, obesity, etc.) may also give rise to an abnormally high GAG excretion in urine (3). This fact, as well as the presence of the anticoagulant heparin, a sulfated GAG, and blood or hemoglobin (18,19) may interfere with the measurement of urinary GAGs and lead to false-positive results. Furthermore, patients suffering from proteinuria (11) and from Morquio, Sly, Scheie, or Hurler-Scheie diseases can produce false-negative results. Bearing this in mind, urines of controls or patients with any of these features were excluded from the study.

The study protocol was approved by the ethics committee of our hospital and all patients' parents or tutors gave written informed consent for this study.

Apparatus

Measurements were performed using a Hewlett Packard 8453 Diode Array Spectrophotometer (Agilent Technologies, Madrid, Spain). Samples were quantified in Hellma quartz cells (Hellma Hispania, Badalona, Spain). The pH value was measured with an Eutech pHScan WP1, 2 (Eutech Instruments Pte Ltd., Singapore).

Reagents and Solutions

DMB (research grade) was purchased from Serva (Heidelberg, Germany). Ethanol (p.a.), sodium formate, and formic acid (p.a.) were acquired from Merck (Barcelona, Spain). Chondroitin 6-sulfate 90% from shark cartilage (Sigma, Madrid, Spain) was used to prepare calibration standards.

DMB solution (for both qualitative and quantitative procedures) was prepared with 10.66 mg of DMB, 3.33 ml ethanol, 1.33 g sodium formate, and 1.33 ml formic acid. The pH value was adjusted to 3.75 by adding solid sodium formate and the flask made up to 1 l with distilled water. The solution proved to be stable for at least 10 months at room temperature. For chondroitin-6-sulfate, a standard solution containing 100 mg/l was prepared in distilled water. This solution was stored in an amber bottle at 5°C and proved to be stable for at least 6 months.

Qualitative Assay Procedure

The GAG-test[®] was prepared by adding 2 ml DMB solution to a 2.5 ml colorless vial, which was sealed and stored at 4°C until use.

Urine was obtained from the first micturition in the morning and processed by centrifugation at 2,000g for 5 min at 4°C. If the assay was not immediately performed, urine samples were stored at -30°C.

A volume of 50 µl of urine was added to the vial. The mixture was shaken and the resulting color observed. If the urinary GAG concentration was elevated, as occurs in the case of patients suffering from MPS, and the solution turned from dark blue to purple, it was considered as a positive result. If the urinary GAG concentration was low and there was no color change, it was considered as a negative result. Finally, if the color of the GAG-test[®] changed to violet, it was considered as an ambiguous result.

Quantitative Assay Procedure

The quantitative determination was performed by the method previously reported by our group (20). Briefly, a blank solution is prepared by mixing 500 µl water and 2.5 ml DMB solution. To quantify urinary GAGs, 100 µl urine, 400 µl water, and 2.5 ml DMB solution are added in a quartz cell. The mixture is shaken and allowed to

stand for 5 min. Afterwards, the absorption spectrum is recorded between 400 and 800 nm. The difference between the maximum and the minimum absorption (approximately 520 and 595 nm, respectively) is recorded. Finally, the value is corrected with regard to the amount of creatinine (Crn), determined by an automated analyzer using the Jaffe method. The final result was expressed in mg GAG/mmol Crn or mg GAG/l.

Statistical Analysis

The SPSS software version 16 (SPSS Inc., Chicago, IL) was used to perform the statistical analysis. Descriptive statistics are presented as mean, SD, and range. Statistically significant differences between groups were analyzed using the Student's *t*-test. The Pearson test was used to evaluate relationships between continuous variables. All probability values are two-tailed and the level of significance required was $P < 0.05$.

RESULTS

GAG-Test[®] Cut-Off Limits

As described above, the reaction between GAGs and DMB produces a complex which changes the color of the DMB solution from dark blue to purple.

We proceeded to calculate the concentration of GAGs that produced different colors of the solution (blue, violet, purple). For that purpose, we added 50 µl of different solutions of chondroitin-6-sulfate (25–250 mg/l) to test tubes containing 2 ml DMB solution. It was observed that if the GAG concentration was below 100 mg/l, the color remained dark blue and the test was considered negative. The range from 100 to 200 mg/l was considered ambiguous, because there was a full range of intermediate colors. Finally, a GAG concentration above 200 mg/l made the solution turn purple and was taken to represent a positive result. The addition of a more concentrated solution did not produce any further color change.

GAG-Test[®] as a Diagnostic Tool for MPS

A series of 319 unaffected controls were included in the study in order to determine their urinary GAG concentration values that would represent the reference values. These individuals were divided into two groups. Patients whose GAG-test[®] turned out to be negative ($n = 304$), age range 1 month to 87 years, presented a mean value of GAG concentration of 58.5 ± 29.2 mg/l (3.4–118.6), and a GAG/Crn value ranging from 0.5 to 41.5 mg GAG/mmol Crn. In the case of the ambiguous results ($n = 15$), patients' age ranged from 8 months to 15 years and presented a mean value for the GAG concentration of 152.4 ± 37.4 mg/l (104.6–199.8) and a

Fig. 1. GAG concentration in control and patients urine samples.

GAG/Crn value ranging from 6 to 14.2 mg GAG/mmol Crn. As reported in previous studies, we found a substantial decline in GAG concentration with age, particularly during the first months of life (Fig. 1) (21). There was a significant positive correlation between the age and the GAG concentration, expressed both as mg GAG/mmol Crn and as mg GAG/l ($r = -0.493$, $P < 0.001$ and $r = -0.176$, $P = 0.002$, respectively). GAG concentration ranges for the negative and ambiguous results were consistent with the experiments carried out with chondroitin-6-sulfate. The data were transformed logarithmically and standard deviations calculated on the basis of this transformed data. The upper action limits (expressed both as mg GAG/mmol Crn and as mg GAG/l) are shown in Table 1.

Patients' urinary GAGs concentration ranged from 177.4 to 1,025.9 mg/l and the GAG/Crn ranged from 15.6 to 80.7 mg GAG/mmol Crn. The results of GAG-test[®] for this group were positive in all cases. No age effect was seen within the range of values for this group, expressed both as mg GAG/mmol Crn ($r = -0.233$; $P = 0.385$) and mg GAG/l ($r = 0.333$; $P = 0.208$). These individuals showed GAG concentration (mg GAG/mmol Crn) above the predetermined action limits (Table 1; Fig. 1). Moreover, the cut-off values to the change of color in the visual test (see previous section) are in accordance with the concentration values of GAGs obtained for healthy volunteers and patients.

The statistically significant differences between control and patient groups were subsequently analyzed. Therefore, GAG-test[®] is clearly able to distinguish between healthy patients and patients suffering from MPS ($P < 0.001$).

TABLE 1. Urinary GAG Concentration Values of Controls and Untreated Patients

Samples	N	Age (years) (range)	GAG/Crn ^a mean $\pm$ SD (range)	GAG ^b mean $\pm$ SD (range)
Controls	26	0–1	26.1 $\pm$ 8.1 (11.3–41.5)	31.9 $\pm$ 23.0 (8.8–99.6)
Controls	20	1–2	17.1 $\pm$ 7.7 (5.7–35.5)	45.8 $\pm$ 29.4 (11.2–103.8)
Controls	17	2–4	14.8 $\pm$ 5.2 (9.4–30.3)	57.1 $\pm$ 31.5 (7.6–124.6)
Controls	31	4–6	12.0 $\pm$ 4.2 (4.9–25.9)	72.7 $\pm$ 26.3 (25.6–131.2)
Controls	79	6–10	10.3 $\pm$ 3.7 (4.6–33.2)	73.0 $\pm$ 27.8 (9.7–145.6)
Controls	97	10–15	7.2 $\pm$ 2.7 (0.6–13.8)	68.1 $\pm$ 28.3 (4.8–118.6)
Controls	28	15–20	4.6 $\pm$ 2.6 (0.8–12.9)	54.5 $\pm$ 24.4 (4.4–94.1)
Controls	14	20–50	3.2 $\pm$ 1.6 (0.5–6.7)	26.07 $\pm$ 14.67 (3.4–46.8)
Controls	8	50–90	4.3 $\pm$ 1.8 (2.2–8.3)	29.80 $\pm$ 12.98 (14.2–52.4)
MPS (not treated)	4	4–6	45.3 $\pm$ 13.0 (36.1–54.5)	196.3 $\pm$ 18.6 (183.1–209.4)
MPS (not treated)	7	6–10	63.3 $\pm$ 6.7 (54.4–68.9)	349.6 $\pm$ 70.1 (262.6–427.2)
MPS (not treated)	7	10–15	58.7 $\pm$ 22.5 (34.4–80.7)	322.1 $\pm$ 90.9 (198.0–400.6)
MPS (not treated)	1	15–20	54.0	1,025.9
MPS (not treated)	4	20–50	46.7 $\pm$ 6.8 (41.9–51.5)	416.9 $\pm$ 33.6 (393.1–440.6)

^aGAG concentration expressed as mg GAG/mmol Crn.

^bGAG concentration expressed as mg GAG/l.

TABLE 2. Reliability of the GAG-Test[®] as a Diagnostic Tool

	MPS +	MPS –
GAG-Test [®] + (positive and ambiguous)	23 (TP)	15 (FP)
GAG-Test [®] –	0 (FN)	304 (TN)

Sensitivity = TP/(TP+FN) = 1; PLR = sensitivity/(1-specificity) = 20; Specificity = TN/(FP+TN) = 0.95; NLR = (1-sensitivity)/specificity = 0.

Reliability of the GAG-Test[®]

The reliability of the GAG-test[®] as a diagnostic tool was analyzed through its specificity, sensitivity, and likelihood ratios (both positive and negative) (Table 2). The ambiguous category was considered a positive result, so that the GAG-test[®] parameter becomes a categorical parameter. Thus, the GAG-test[®] could detect 100% of the MPS cases (IH, II, III, and VI) with a resulting specificity of 95% (Table 2). It has been suggested that a test is only clinically useful when its positive likelihood ratio (PLR) is higher than 10 and its negative likelihood ratio (NLR) is lower than 0.1 (22). In our casuistic, the PRL value was 20 and the NLR was 0.

DISCUSSION

Nowadays, an increasing number of researchers and clinicians are focused on the study of lysosomal diseases, specifically MPS, owing to the wide range of specialties involved because of their symptoms and the recent availability of treatments involving enzymatic replacement therapy or substrate inhibition (23–25). Sometimes, there can be a delay in the diagnosis of MPS owing to the late onset of less progressive phenotypes. This has prompted the publication of guidelines for their diagnosis and management (26) and the possibility of neonatal screening is being developed (27). It should be borne in mind that during the first periods of the disease, physicians can detect few signs or no specific symptoms within a wide range of presentations (neurological, rheumatological, cardiorespiratory, dermatological, ophthalmological, etc.) (2). It is for this reason that patients are sometimes sent to different medical departments, causing diagnosis to be delayed.

Dye-binding techniques have been considered attractive, particularly owing to their sensitivity and potential for quantitative results. The DMB reagent used in this study was first described as a histochemical stain by Taylor and Jeffree (28). It was subsequently applied to a colorimetric assay for sulfated GAGs, and it was suggested that DMB could be used in a simple urine screening test for MPS diseases by exploiting the visual color change. By this method, the color of the DMB reagent added to urine specimens changed from dark blue to purple in the case of affected children.

The reliability of the DMB reaction as a screening method for MPS was previously tested by de Jong and Whitley (10,29,30). According to these authors, the sensitivity of the determinations using DMB as a dye ranged between 96 and 100% and the specificity from 94 to 100%. In our casuistic, the sensitivity reached 100% of the samples analyzed and the specificity 95%, which is in agreement with the results described in the literature. Some of the urine samples tested within the control group gave rise to an ambiguous result with the GAG-test[®], because our test is concentration sensitive. The test can produce false-positive results in urines with very high creatinine clearance, which is more likely to happen in older patients than in infants. As far as the likelihood ratios are concerned (PLR and NLR), the values provided by the GAG-test[®] (PLR = 20; NLR = 0) are in accordance with those which have been proposed as reference values (PLR > 10; NLR < 0.1) (22) to consider a test as clinically useful. The GAG-test[®] is clearly able to distinguish between patients suffering from MPS and healthy controls. For this reason, it has been distributed to departments of rheumatology of some collaborating hospitals and primary care centers of the area as a

screening test to detect a MPS among pediatric patients with compatible rheumatological affections.

We also examined the reliability of the GAG-test[®] as progress evaluation test, using urine samples coming from patients undergoing enzyme replacement therapy (data not shown). In this case, the test also allowed us to distinguish between patients who continued to have high values of urinary GAGs and those who had reached the normal range of the control group. Therefore, the GAG-test[®] can also be used as a method to follow up the evolution of patients, in addition to its use as a diagnostic tool for MPS.

This study has demonstrated the potential usefulness of the GAG-test[®] as a tool for detecting patients suffering from MPS. We have, therefore, developed a simple, rapid, and low-cost colorimetric test based on the color changes of DMB dye, which has shown very good results as far as sensitivity, specificity, PLR, and NLR values are concerned. The GAG-test[®] was designed for the diagnosis of MPS IH, II, III, and VI, given that MPS IS, ISH, IV, and VII might produce false-negative results, owing to their GAG excretion being likely to fall within the reference range. It should be borne in mind that if a patient was suspected of suffering from one of these attenuated types of MPS on the basis of their clinical symptoms, it would be of no use to perform the GAG-test[®], and a definite diagnosis would have to be conducted by means of enzymatic or genetic analyses.

This test would be useful for a quick screening of MPS among those patients under suspicion of suffering from MPS (IH, II, III, or VI) or among patients who present symptoms that might be compatible with MPS. The test would be able to make a quick detection, confirm, or discard a suspicion or reduce the number of individuals with whom investigations should be continued.

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