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Neonatal Screening for Mucopolysaccharidoses by Determination of Glycosaminoglycans in the Eluate of Urine-Impregnated Paper: Preliminary Results of an Improved DMB-Based Procedure

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Background: The fact that mucopolysaccharidoses (MPSes) are now treatable, and that the earlier treatment is initiated the better, is an indication for neonatal screening. The most efficient approach seems likely to be a multi-tier procedure in which screening for urinary glycosaminoglycan (GAG) is followed by enzyme determinations in heelprick blood of newborns screening positive. Hitherto the method of choice for the determination of GAG has been the measurement of absorbance by a complex of GAG and 1,9-dimethylmethylene blue (DMB). *Method*: We evaluated a DMB method in which absorbance by DMB is measured following its addition to the eluate obtained from paper-borne newborn urine samples and is normalized relative to urinary creatinine. Calibration is performed with chondroitin-6-sulfate (Ch-6-S). *Results*: The limits of detection and quantification of GAG were 1.98 and 5.94 mg/dl, respectively. The within-run coefficients of variation (CVs) of the GAG/creatinine ratio for 25, 31, and 70 mg/dl solutions of Ch-6-S in urine were 21.8, 16.4, and 10.5%, respectively, and the corresponding between-run CVs were 25.0, 13.5, and 10.1%. Recovery from the urine spiked with 31 mg Ch-6-S/dl was 94.8%. Accuracy was also acceptable for all other GAGs except hyaluronic acid. For neonatal screening, the diagnostic threshold was tentatively established as 800 mg GAG/g creatinine, the 95th centile of samples from 903 infants aged 3–28 days, but the value of the GAG/creatinine ratio was negatively correlated with age. Application of the new method to samples from older individuals with and without MPS achieved 100% sensitivity and specificity when used with an age-dependent threshold taken from the literature on the original DMB method. *Conclusion*: If used in the first tier of a multi-tier screening protocol, the proposed method would allow the detection of abnormal levels of all GAGs except hyaluronic acid. J. Clin. Lab. Anal. 24:149–153, 2010.

Key words: newborn screening; lysosomal disorders; mucopolysaccharidoses; DMB reagent; urine in paper specimen

INTRODUCTION

Over 50 lysosomal storage disorders caused by deficient or defective lysosomal enzymes are now recognized. Although their joint prevalence is estimated as 1/8,000 or greater, their estimated individual prevalences range from 1/50,000 to as little as 1/4,000,000. Their efficient detection therefore requires a stepwise procedure based on their classification in a number of diagnostic groups. The mucopolysaccharidoses (MPSes), one such group, can be jointly identified as such by quantification of glycosaminoglycan (GAG) in urine, after which more specific tests can be carried out to identify which of the various MPSes is responsible for the positive GAG result.

There are now a number of therapeutic approaches to MPSes and other lysosomal storage diseases, including

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enzyme replacement, bone marrow transplantation, umbilical cord blood transplantation, and gene therapy, and the earlier the treatment is initiated, the better. This invites the establishment of comprehensive neonatal screening for these conditions. In anticipation of such programmes, neonatal screening methods of greater or lesser efficiency based on the detection or quantitation of urinary GAG with toluidine blue (1–3) or 1,9-dimethylmethylene blue (DMB) (4–6) have in fact been available for many years, and there continue to appear other, more costly methods, such as those based on direct determination of relevant enzymes or enzyme activities in neonatal bloodspots (7).

Here we describe a modification of the DMB method in which, instead of measuring absorbance by the DMB-GAG complex, we measure absorbance by uncomplexed DMB; this parameter is more sensitive because, due to the different absorptivities of DMB and DMB-GAG, it decreases more rapidly with increasing DMB-GAG concentration than absorbance by DMB-GAG increases. An analogous procedure was followed by Thuy and Nyhan (8) using a mixture of azure A and azure B as reagent. Its advantage over the original DMB method, in addition to its greater sensitivity, is that it eliminates both the possibility of excessive DMB concentration (which can result in severe interference from absorbance by DMB at the absorbance peak wavelength of the DMB-GAG complex) and the need for repeat measurements of diluted samples from high-GAG individuals (as high DMB concentrations can be used in the initial measurements, we use a reagent/analyte ratio 27.3 times that used by Whitley et al. (4)). As in the original Whitley DMB method (4,6), measured GAG is normalized relative to urinary creatinine (determined by a variant of Jaffé's method) in order to compensate for variation in urine concentration, paper saturation, and elution efficiency. The new method requires no alteration of sample collection logistics in laboratories that, like ours (9) (Alonso-Fernández and Colón, unpublished) continue to use paper-borne urine samples for neonatal screening in the tradition of Berry (1) and Woolf (10).

MATERIALS AND METHODS

Materials

Reagents and solvents

For the standard assay: 1,9-DMB (Aldrich 34108-8); chondroitin-6-sulfate type C (Ch-6-S; Sigma C-4384; Sigma-Aldrich Corporation, St. Louis, MO); creatinine (Merck 5208; Merck, Darmstadt, Germany); analytical grade formic acid, sodium formiate, saturated picric acid, and sodium hydroxide; Milli Q Gradient A10 system water and 0.2 M sodium formiate buffer (formic acid+sodium formiate, pH 3.5). For the evaluation of performance with GAGs other than Ch-6-S: chondroitin-4-sulfate (Ch-4-S; Sigma C-4134); dermatan sulfate (Sigma C-3788); heparan sulfate (Sigma H-7640); heparin sulfate (Sigma H-7005) and hyaluronic acid (Sigma H-7630).

Other fungibles

Whatman 903 paper; Greiner Bio-One 44.1 mm-high 96-well microtitration plates and Greiner Bio-One flat-bottomed 96-well measurement plates.

Stock and working solutions

DMB stock solution (0.35 mM in a 1:99 mixture of 95% ethanol and 0.2 M sodium formiate buffer) is prepared in 100 ml batches (sufficient for 20 measurement plates) as per Whitley et al. (4), and is stable in the dark for 30 days at room temperature. DMB working solution is prepared daily as a 1:4 dilution of stock solution with 0.2 M sodium formiate buffer.

Calibration standards

By dilution of an aqueous solution containing 100 mg/dl of Ch-6-S and 400 mg/dl of creatinine, three other standard solutions are made up that contain 70 mg/dl Ch-6-S and 280 mg/dl creatinine, 25 mg/dl Ch-6-S and 100 mg/dl creatinine, and 10 mg/dl Ch-6-S and 40 mg/dl creatinine. Separate sheets of Whatman 903 paper are impregnated with each mixture.

Procedure

From the sample-bearing paper are cut four discs 6 mm in diameter, using a Wallac dried blood spot punch (Perkin Elmer 1296–071; Perkin Elmer, Turku, Finland), and all four are inserted in a single microtitration plate well and stirred for 40 min with 300 µl of water; as each disc absorbed approximately 10 µl of urine, the resulting eluate is an approximately 7.5-fold dilution of the urine with which the discs were impregnated. Concurrently, two sets of four discs are cut from each of the calibration standard papers, and each set is treated in the same way as the sample discs. Samples of each eluate (2×20 µl) are then transferred to two flat-bottomed measurement plate wells, where 200 µl of DMB working solution is added to one, and to the other 35 µl of 20% saturated picric acid solution followed by 35 µl of 7.5 g/l sodium hydroxide solution. Absorbance by DMB in the DMB-treated wells is measured at 584 nm 5 min after addition of the reagent using a BMG Labtech Fluostar Optima plate reader, and the calibration line constructed from the corresponding measurements of the standards is used to convert the

absorbance of the urine sample to mg Ch-6-S/dl. Absorbance by the Janowski complex in the picric acid-treated wells is measured at 492 nm 20 min after the addition of sodium hydroxide, and the calibration line constructed from the corresponding measurements of the standards is used to convert the absorbance of the urine sample to mg creatinine/dl. Finally, the ratio of the Ch-6-S measurement to the creatinine measurement is calculated.

Note that absorbance by DMB is read at 584 nm, instead of at the wavelength of the absorption peak of DMB, 597 nm, merely because we have no filter closer to the latter wavelength. Also, the wavelength at which absorbance by the Janowski complex is read, 492 nm, is closer to the 500 nm of Jaffé's original method than to the 535 nm of Whitley et al. (4).

Evaluation

Analytical quality parameters

Limits of detection and quantification were determined from measurements of ten replicate blank Whatman 903 papers. Between-run variation in calibration was evaluated in terms of the variation of the absorbances of the calibration standard eluates in seven runs with two replicates per run. Within- and between-run coefficients of variation (CVs) for the GAG/creatinine ratio were determined by measurements of Whatman 903 papers impregnated with urine spiked with 25, 31, and 70 mg/dl of Ch-6-S (the 31 mg/dl sample was prepared using a different urine from the other two); 12 replicates were run together for within-run CVs, and two in each of six runs for between-run CVs. Recovery was determined using 21 replicate papers impregnated with the urine sample spiked with 31 mg/dl of Ch-6-S. Performance with GAGs other than Ch-6-S, using Ch-6-S for calibration, was evaluated by determination, in two separate runs, of an aqueous solution containing a known concentration of the GAG in question.

Screening parameters

The diagnostic threshold and reference ranges for our neonatal population were determined on the basis of measurements of 903 paper-borne newborn urine samples obtained in the first month of life. Performance with samples from MPS patients was evaluated using samples from three local MPS I patients aged 1, 4, and 17 years and their families; and also samples kindly supplied by ERNDIM (from a 19-year-old patient with MPS VI) and by the Dr. N.A. Chamoles Neurochemistry Laboratory, Buenos Aires (samples from patients aged 1–26 years with MPS I, II, III, IV, VI, VII, and I—S, in this last case before, during and after treatment). The Buenos Aires laboratory also supplied a sample from a healthy 6-month-old infant.

Ethics

The study was approved by the Galician Biomedical Research Ethics Committee.

RESULTS AND DISCUSSION

The detection and quantification limits for GAG were determined as 1.98 and 5.94 mg/dl, respectively, and the limits for creatinine were both immeasurably small. The GAG limits are lower, and therefore less liable to give rise to false negative assays, than those reported for a method using liquid urine samples (11).

Figures 1 and 2 show, for Ch-6-S and creatinine, respectively, the average of the calibration lines constructed in seven different runs, together with the standard deviations of the corresponding calibration standard absorbances. These standard deviations imply, for example, CVs of 13 and 18% for the 10 and 70 mg/dl Ch-6-S calibration standard absorbances, respectively, and 11 and 18% for the 40 and 100 mg/dl creatinine standards, respectively. These figures are within the limit

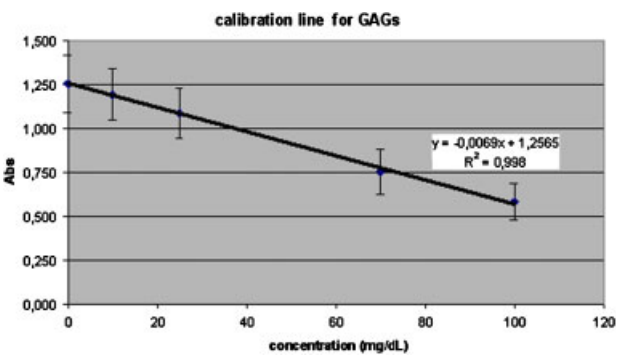

Fig. 1. Mean of seven calibration lines constructed in independent runs for the determination of urinary GAG. Error bars show standard deviations of calibration standard absorbances.

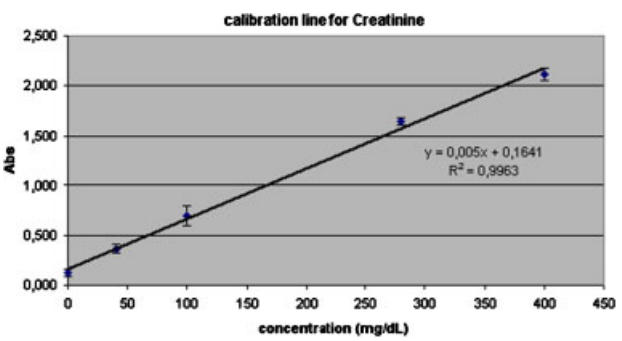

Fig. 2. Mean of seven calibration lines constructed in independent runs for the determination of urinary creatinine. Error bars show standard deviations of calibration standard absorbances.

of 20% generally considered acceptable for neonatal screening analytes. Colomé Mallolas et al. reported within- and between-assay CVs of 4.9 and 6.9%, respectively, for a GAG standard of 22 mg/dl (11), and Molinelli et al. reported values of 8.0 and 5.4% for the between-assay CVs of GAG solutions of 25 and 76 mg/l, respectively, and values of 2.7 and 0.9% for the corresponding within-assay CVs (12); however, it should be borne in mind that in both these studies it was bulk solutions that were analyzed, without any intermediate passage through absorbent paper (11,12).

The within-run CVs of the GAG/creatinine ratio were 21.8, 16.4 and 10.5 for the urine samples spiked with 25, 31, and 70 mg Ch-6-S/dl, respectively, and the corresponding between-run CVs were 25.0, 13.5 and 10.1. The between-run CV at 25 mg/dl is at the limit of what is acceptable for analysis of paper-borne neonatal screening samples (13), but all these CVs are likely to improve when the pipetting of eluate and reagent is automated and the reaction time optimized. Whitley et al. reported CVs of 20.1 and 10.2% for a normal urine and the same urine spiked with a high Ch-6-S concentration, respectively (4).

Recovery from the urine sample supplemented with 31 mg/dl of Ch-6-S was 94.8%, showing acceptable accuracy.

Table 1 lists the results of determining other GAGs using calibration lines constructed with Ch-6-S. For screening purposes the results are acceptable for all the GAGs tested except hyaluronic acid, the concentrations of which were greatly underestimated. This implies that the present method, like that of Molinelli et al. (12), should not be expected to detect MPS IX. By contrast, the present method is even more sensitive to dermatan sulfate than to Ch-6-S, which favors the detection of MPS I, II, and VI.

The 95 and 90%-coverage reference ranges of the GAG/creatinine ratio in our neonatal population, as determined from 903 paper-borne neonatal urine samples, are 0–260 and 0–800 mg GAG/g creatinine, respectively. The diagnostic threshold for newborn screening has provisionally been established as the 95th centile, 800 mg GAG/g creatinine. This is considerably higher than the cutoff proposed by Molinelli et al. (12) for infants aged up to 2 months, 330 mg/g (which is close to the 95th centile of the distribution observed by Whitley et al. (4) in samples from 435 3-week-old newborns), but it should be borne in mind that most of our newborns were much younger than 3 weeks (Fig. 3), and that urinary GAG excretion drops sharply during the first month of life (4,6,14). This negative correlation was confirmed in this study ($r = -0.1784$, 95% confidence interval [−0.2056, −0.1510]; $P < 0.0001$), and in the light of Iwata et al.'s observations (GAG/creatinine ratios of 978, 940, and 1177 mg/g in MPS II patients aged 15 days, 26 days, and 1 month, respectively, and of 1180 and 1205 mg/g in two 1-month-old MPS III patients (14)) suggests that a screening threshold of 800 mg/g should be sensitive enough for samples collected at the age of 3days, the age at which heelprick blood (and in our laboratory urine samples) are currently collected to screen for hypothyroidism, phenylketonuria, galactosaemias, cystinuria, and other disorders (15). By the same token, although this threshold makes it necessary to examine 5% of samples with a more specific method such as thin layer chromatography (we intend to reserve measurement of enzyme activities in heelprick blood for third tier screening), it seems unlikely that it can be raised much to improve its specificity if it is to be used for children aged up to 1 month. It appears that significantly greater operational efficiency can be achieved without loss of sensitivity only by using an age-dependent diagnostic threshold such as is shown in Figure 1 of (6).

The proposed method and screening threshold were tested in the first place using the samples of

TABLE 1. Performance of the Method With GAGs Other Than Ch-6-S (See *Methods* for Sources)

GAG	True concentration (mg/dl)	Assay results (mg/dl) Run 1	Run 2	Mean
Ch-4-S	25.0	27.0	21.1	24.1
	70.0	50.0	84.6	67.3
Dermatan sulfate	25.0	48.2	21.0	34.6
	70.0	99.8	80.0	89.9
Heparan sulfate	100.0	81.0	72.8	76.9
Heparin	25.0	26.7	40.6	33.7
	70.0	64.6	78.9	71.8
Hyaluronic acid	25.0	4.6	0	2.3
	70.0	15.7	24.4	20.0

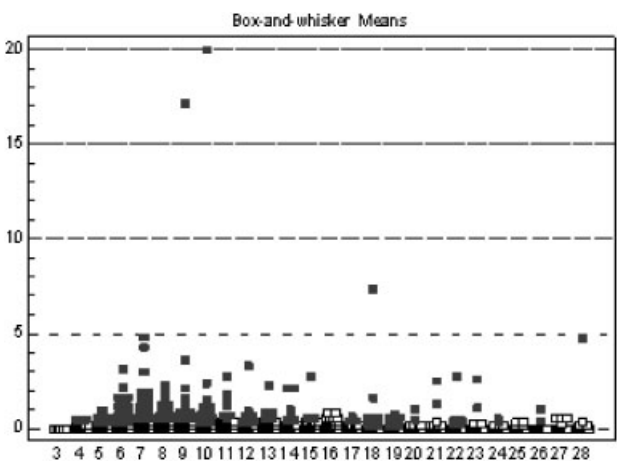

Fig. 3. GAG/creatinine ratios (mg/g) among newborns aged 3–28 days. A box-and-whisker plot is shown for the values obtained at each age.

urine-impregnated paper provided by the Dr. N.A. Chamoles Neurochemistry Laboratory. In all cases, the results were consistent with the clinically and biochemically based diagnoses made in Argentina: no GAG was detected in the sample from the healthy infant, and the GAG/creatinine ratios of the samples from MPS patients were all above the above-mentioned age-dependent threshold of Whitley et al. (6). Similarly, duplicate determinations of the ERNDIM MPS VI sample gave exactly the same value, which was likewise above Whitley et al.'s age-dependent threshold, as were the results obtained for the three local MPS I patients. In one of the three families examined, the father had a GAG/creatinine level higher than that of the proband himself, and Chamoles' fluorimetric assay (16) showed α-L-iduronidase activity to be approximately half the lower reference limit.

The samples taken before, during and after treatment from the Argentine MPS I-S patient had progressively smaller GAG/creatinine ratios, but all were above threshold. By contrast, samples taken from one of the local MPS I patients before and 24 hr after a therapeutic infusion had ratios of 300 and 40 mg/g, and no GAG was detected in a sample taken from this patient 48 hr after infusion. In spite of their different post-treatment results, both these cases suggest that the proposed procedure may be useful for monitoring the effects of treatment.

In conclusion, we have developed a more sensitive, more convenient variant of the DMB method for GAG determination, and our experience so far supports its usefulness for neonatal screening for MPS disorders. It has been included on an experimental basis in our routine screening practice and is the object of on-going evaluation.

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