Urinary Glycosaminoglycan Electrophoresis With Optimized Keratan Sulfate Separation Using Peltier System for the Screening of Mucopolysaccharidoses

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Abstract
The purpose of this communication is to indicate a simple and rapid method with a small volume of urine sample to detect urine glycosaminoglycan (GAG) and serve as a screening procedure for mucopolysaccharidoses (MPSs). Total GAG measurement for patients with MPS disorders is considered to be the first step in diagnosis of those heterogeneous group of lysosomal storage disorders presenting clinical phenotype. In this study, modified 9-dimethylmethylen blue method is used for total GAG measurement. Following GAG quantitation, the procedure described here allows GAG isolation from a very a small volume of urine sample and subjected to high-resolution GAG electrophoresis, which can be easily performed in routine clinical diagnostic laboratories. Glycosaminoglycan precipitation is a modified method based on total GAG concentration in the urine. For optimized isolation of total GAG for electrophoresis, instead of considering the urine creatinine concentration, 300 μg/mL GAG containing urine is considered to be the target concentration for the best precipitation with 1000 μL cetylpyridinium chloride (CPC)/citrate buffer. Glycosaminoglycan concentration-based precipitation of urine with CPC allows the laboratory to be able to work with a small volume of urine sample by keeping the precipitating ratio with CPC constant for samples that contain GAG less than 300 μg/mL. Based on the effect of cold buffer using low voltage, GAGs high-resolution electrophoresis banding patterns described here enable a clear separation of keratan sulfate from chondroitin sulfate as well as dermatan sulfate (DS1 and DS2) and heparan sulfate. By this procedure, GAG patterns are more clear, easily identified, and provide a guide for the enzyme analysis deficient in the MPS disorders.

Keywords
keratan sulfate, glycosaminoglycans, mucopolysaccharidoses, glycosaminoglycan electrophoresis, cellulose acetate

Introduction
Cellulose acetate (CA) membrane has been used for many years as the supporting medium for glycosaminoglycans (GAGs). Almost all the experimental techniques in urine GAG electrophoresis (GAGE) involve the use of buffers with different pH and voltage to optimize GAG separation and to make an accurate interpretation in order to facilitate further enzymatic or molecular analysis.1-3 The major GAG excreted by the healthy individuals is chondroitin-6-sulfate, whereas heparan sulfate (HS), dermatan sulfate (DS), and keratan sulfate (KS) are excreted in excess in patients with mucopolysaccharidosis (MPS). In well-optimized electrophoretic assay condition, chondroitin-4-sulfate separation also appears to be justified. The GAGs can migrate as compact, clear bands without tailing. The method outlined here is simple, requires less volume of urine sample, less time consumption, and can be easily performed in any hospital or clinic diagnostic laboratory.

Method
Measurement of Total GAG
In this study, direct spectrophotometric determination of GAGs in centrifuged urine samples with 1,9-dimethylmethylen blue (DMB) color reagent at basic pH (8.0) is used for total GAG measurement. This method is the most reliable in terms of false-negative results,4-6 although it is not totally free of them.

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Measurements are carried out on microtiter plates with double wavelength 580/690 nm using a regression equation polynomial second order. Microplate method facilitates to measure urine GAGs quantitatively in many urine samples simultaneously. The value observed for total GAG as mg/L is corrected with regard to the amount of urine creatinine value obtained by the automated dry chemistry analyzer Vitros 350 (Ortho-Clinical Diagnostic NY, USA). The final result is expressed as mg GAGs/mmol creatinine in an individual to compare the result with the established age-dependent reference values by this method. Quantitation obtained using this method is compared with the results reported by an interlaboratory assay from European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism (ERNDIM) External Quality Assessment Scheme for quantitative urine total GAG values. The results in this program correspond to the mean value for results submitted by 70 to 79 laboratories in which 24 laboratories use the same DMB method. The quantitative results agree with those obtained by the ERNDIM scheme: intraclass correlation coefficient 0.931 with a 95% confidence interval 0.837 to 0.971, thus proving the accuracy of the method. The method described here is presented as a poster at Society for the Study of Inborn Errors of Metabolism (SSIEM) meeting, Birmingham, United Kingdom, 2012. Detailed information can be obtained from the link https://meeting.tfigroup.com/TFI/media/uploaded/EVTFI/event_109/P-208.pdf.

Precipitation of GAGs

For precipitation of GAGs, primarily, mg/L GAG-containing urine concentration is taken into account as follows: 1000 μL cetylpyridinium chloride (CPC)/citrate buffer pH 4.8 was added to 300 μg GAG-containing urine samples. Following incubation at cold temperature (4-10°C) for overnight, the urine is centrifuged at 10 000 rpm for 5 minutes. The supernatant is decanted, and the pellet is dissolved in 150 μL 2 mol/L lithium chloride (Sigma, St. Louis, MO, USA) by vortexing vigorously for 60 seconds and mixed with 800 μL absolute ethanol (Sigma, St. Louis, MO, USA) until the pellet becomes an homogenous mixture which is left to stand for at least 1 hour at 4°C degree. The mixture is then centrifuged at 12 200 rpm for 5 minutes, supernatant is decanted, and the pellet that contains GAGs is dried under nitrogen gas. The final pellet is mixed with 20 μL phenol red (0.5 g/L) and vortexed vigorously to dissolve the GAGs for further GAGE.

High-Resolution Electrophoresis of Isolated Urinary GAGs

For the cases where the excretion of GAGs is less distinctly increased as seen in some MPS III (Sanfilippo), MPS IV (Morquio), and MPS IS (Scheie) disorders, GAGE technique is very useful and commonly used to detect an abnormal excretion pattern in order not to miss a case. Random urine samples can be used for GAGE. The modified method that will be described here is a discontinuous electrophoresis, based upon both charge differences and different solubility in ethanolic solution.

The CA plate (TITAN III 60 × 76 mm; Helena Biosciences Europe) is prewet in buffer I 0.025 mol/L barium acetate (BaAc, pH 7.0) in the bufferizer for at least half an hour prior to electrophoresis. After blotting the plate, it is subjected to prerun to condition the wicks prior to running sample plate for 10 minutes at 145 V. Then, minimum 8 μL of prepared specimens are loaded onto the sample wells, and Z applicator is used to spot the samples on the plate through cathode application. Electrophoresis is performed at constant 145 V, 3 mA, 0 to 1 W for 12 minutes, then the CA plate is removed and soaked in cold buffer II (BaAc 0.025 mol/L, pH 7.0, 15% ethanol) for 2 minutes, and then it is blotted and re-electrophoresed at constant 145 V, 3 mA, and 0 to 1 W for 25 minutes. Cellulose acetate is once more removed and soaked in cold buffer III (BaAc 0.025 mol/L, pH 7.0, 50% ethanol) for 2 minutes. After it is blotted, the CA plate is electrophoresed this time at 145 V, 3 mA, 0 to 1 W for 36 minutes. The main purpose in this GAGE method is to provide a cool medium with low concentration of BaAc at pH 7.0. The lower the concentration of buffer used and the cooler the buffer in the chamber is, the less heat production will be caused during electrophoresis. This will facilitate the GAGs to migrate as compact, straight bands without tailing. For an optimized complete separation of GAGs from the mixture, constant pH and constant cool medium are needed. In order to maintain a cool medium, Peltier is used. Peltier, according to its structure, is a kind of a substance that when the voltage is applied, one side cools, whereas the other side warms. Cooling sides of the Peltiers are in contact with aluminum plate 20 × 30 cm where the electrophoresis tank is placed on. The system is installed with 6 Peltiers/30 W supported by 380 W power supply. The temperature of the buffer in the electrophoresis tank can be cooled down to 8°C to 11°C easily and can be maintained at constant temperature throughout the electrophoresis. Coolant sponges obtained from Helena Laboratories (Cat. No. 5045, Helena Biosciences Europe) can also be used supportively for cooling the electrophoresis chamber in addition to Peltier system for better separation of GAG bands without denaturation. After the last run, the plate is soaked in DMB dye (0.04%) for 6 minutes and destained with 1% vol/vol acetic acid for 10 minutes, and the CA can be left immersed overnight in distilled water for evaluation by densitometry.

Results

There is a clear separation of keratan sulfate in Morquio case (MPS IV A) which is from ERNDIM MPS QA Scheme sample (No:15; Figure 1). Mucopolysaccharidosis II case is also from ERNDIM QA Scheme sample (No: 16). 38104 is a normal control sample with a marked chondroitin sulfate and a faint HS band, CS 200 μg/mL) is the chondroitin sulfate standard included in that
analytical run. Mucopolysaccharidosis I and MPS VI are the confirmed cases and informed consent is obtained from each patient. The most remarkable result obtained by this method is to be able to see that DS2 (fast moving DS) is clearly separated from chondroitin sulfate in MPS I, MPS II, and MPS VI cases (Figure 1). During interpretation of GAGE, the GAG pattern differentiates whether it is MPS I, MPS II, or MPS VI. Mucopolysaccharidosis I cases excrete larger amounts of DS1 (slow moving DS) and DS2 as well as CS; CS band is wider and denser in MPS I cases when compared to MPS VI cases. Mucopolysaccharidosis II cases excrete larger amounts of DS1 (slow moving DS) and DS2 as well as CS; CS band is wider and denser in MPS I cases when compared to MPS VI cases. Mucopolysaccharidosis II cases display a very distinctive heparan sulfate band, which is thick, broad, with well-defined borders in between DS1 and DS2. Dermatan sulfate bands are also very clear to see in MPS II cases (Figure 2).

P indicates an individual “patient sample,” sent for GAGE analysis. In Figure 2: patient 1 (P1) pattern shows a normal profile with faint CS band.

As seen in the MPS III case (ERNDIM MPS no. 22), there is a pronounced HS band that is thicker and heavier when compared to chondroitin sulfate band excreted in its related urine (Figure 3). However, the electrophoretic pattern does not allow MPS III subtypes to be distinguished.

In Figure 4, quality control (QC) standard mixture is used to show the bands DS, HS, CS, and KS which does not contain DS2. Dermatan sulfate 2 band can be seen as typical excretion pattern in both MPS II case of ERNDIM QA scheme sample 01-2015 and MPS VI confirmed case. In the same figure, marked-broad HS band can be seen in MPS III patient sample.
of ERNDIM QA scheme sample 03-2015, which is denser when compared to CS band in the same patient. QC sample and MPS IV case both contain thick broad marked KS band.

The factors that affect the reproducibility of the results are listed subsequently: Buffer temperature 8°C to 11°C; voltage used: 145 V; sample volume loaded on the CA plate: 10 µL from test specimen are placed on sample well plate and super Z applicator is used to load on the CA plate; buffer pH: 7.0; and buffer concentration: 0.025 mol/L, and standard ratio of CPC–µg GAG: 1000 µL CPC + 300 µg GAG containing urine: as long as those conditions are maintained in the laboratory, one can observe a proper separation and achieve similar appearances of the bands when the test is repeated, making the method reproducible.

Discussion

This study based on qualitative analysis of GAGs to detect the abnormal distribution of sulfated GAGs in urine can be optimized by the CPC–GAG complex using 1000 µL CPC for 300 µg GAG containing urine. This ratio-based precipitation regarding the total GAG content of certain amount of urine rather than the urine creatinine eliminates overloading the CA plate with excessive amounts of samples in order to detect keratan sulfate excreted by MPS IV cases. The length of time required to achieve an effective separation of GAGs depends on the cooling efficiency of the apparatus by the Peltier system used in this method. The cooler the system along with increasing the time of electrophoresis, the better the separation is. BaAc molarity as 0.025 mol/L is the optimized molarity that causes less heat production but optimal migration for every type of GAG based on their charge differences and differential solubility in ethanol-containing solutions. Ensuring the coolness of the plate gives fairly good band patterns appearing to be straight enough to differentiate the GAGs for your every analytical run, preventing the middle section to be squashed where the heparan, dermatan, and chondroitin sulfate species migrate properly (Figure 2). Most of the laboratories that perform GAGE analysis use a system of circulating cold water (cooled to 15°C). However, using Peltiers, there is no longer a need for a cold water generating system. The buffer in the chamber can be cooled down to 8°C to 11°C effectively by the Peltiers and can be adapted to various electrophoresis set-up systems.

Conclusion

This study aims to briefly explain some details for the novice laboratories on MPS screening in order to easily perform GAGE from a small volume of urine samples to obtain reproducible results. This practical first-line screening test provides an important guide for further lysosomal enzyme activities based on fluorometric, radiometric, immunochemical protein filing, and electrospray ionization tandem mass spectrometric assays. Glycosaminoglycan electrophoresis is a very valuable and a cost-effective test to screen and rule out MPS disorders if the test can be accurately adjusted in the laboratory. In this short original article, it is stated that an effective and efficient buffer pH, molarity of the BaAc together with optimized time of electrophoresis under steady low temperature 8°C to 11°C, supported by Peltiers will generally give you reproducible, clear-cut results in every analytical run.

Straight bands obtained with this electrophoresis can be quantitatively interpreted by densitometry and confidently reported on the laboratory report of the patient as well. I believe that these simple modifications indicated in this method will ease the work of laboratory technicians and facilitate the interpretation of the results by the laboratory professionals, simply eliminate the fear for the test due to inherent widespread difficulty of the technique silently shared between laboratories.

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