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Fluorophore-assisted electrophoresis of urinary carbohydrates for the identification of patients with oligosaccharidosis- and mucopolysaccharidosis-type lysosomal storage diseases

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Lysosomal storage diseases result from defects in the activity of the lysosomal enzymes that break down macromolecules in the cell. These enzyme defects contribute to over 30 separate storage diseases that result in neuromuscular and intellectual impairment and, in some cases, early childhood death. This report describes a new method for identifying defects in the lysosomal enzymes and in the metabolic pathway that functions in the degradation of complex carbohydrates. The method involves identifying 'abnormal' carbohydrates in the urine of affected patients using fluorescent carbohydrate tags and polyacrylamide gel electrophoresis. Currently, the method can be used as a simple screen for the identification of at least 12 different lysosomal storage diseases using a single electrophoretic procedure. Both oligosaccharidoses and mucopolysaccharidoses (MPS) can be identified, and in many cases the MPS subtype can be determined. In addition, the method can be used to confirm enzymatically the results of the initial screening test. We believe that this method will become extremely useful not only in the diagnosis of these diseases but in the management of patients on therapy.

Keywords: carbohydrate analysis, electrophoresis, glycosidases, imaging systems, lysosomal storage disease, scientific software

Introduction

The family of lysosomal storage diseases (LSDs) is composed of over 30 different inherited metabolic diseases [1, 2]. The published incidence and prevalence rates for this large group of diseases vary widely, from 1:10000 for the mucopolysaccharidoses [3] to 5:10000 for all LSDs [4], but it is generally appreciated that these conditions are underdiagnosed. The clinical indications of disease, which include mental retardation, skeletal defects and early childhood death, are a result of the accumulation of incompletely degraded complex carbohydrates and glycolipids in the cell. This accumulation is caused by the deficient activity of one or more lysosomal enzymes or enzyme activator proteins, or by disruption of the lysosomal enzyme transport system. In the disease state, the incompletely degraded macromolecules are stored in the lysosome, resulting in lysosomal swelling and an overall disruption of normal cellular physiology.

Disease diagnosis is usually based on either the measurement of defective enzyme activities by plasma or fibroblast enzyme assay [5], genotypic

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analysis using nucleic acid probes [6] or the identification of the accumulated carbohydrates in tissues or body fluids [7]. Because it is not practical to assay directly for all known enzyme defects (see Discussion), semiquantitative methods for measuring accumulated materials in storage disease patients have been developed. Most of the LSDs result in the excretion of the storage products into body fluids, plasma and urine, and it is the latter that is generally used for analysis. Methods that have been used to measure storage products in urine include spectrophotometry [8], paper chromatography [9], high-performance liquid chromatography (HPLC) [10], thin-layer chromatography [11, 12] and cellulose acetate electrophoresis [13]. The last two are most commonly used in clinical reference laboratories. Owing to the wide variety of complex carbohydrates associated with each of the diseases, no single analytical method has been found that can be used as a primary screening test for all of the LSDs.

This report describes a new electrophoresis-based method for screening children with storage diseases that result in the accumulation of complex carbohydrates. The method involves identifying and measuring urinary carbohydrates using a new technique called fluorophore-assisted carbohydrate electrophoresis (FACE). FACE involves labeling the urinary carbohydrates with a fluorescent tag, ANTS (8-aminonaphthalene 1,3,6-trisulfonic acid, disodium salt), under reducing conditions followed by high-resolution polyacrylamide gel electrophoresis [14, 15]. The carbohydrate banding patterns on the gel are imaged using UV light with a charge-coupled device (CCD)-based imaging system (Glyko), and image analysis is performed using a MS Windows-based analytic software package (Glyko).

FACE analysis is useful for the identification of at least two major types of LSD, the oligosaccharidoses and the mucopolysaccharidoses (MPS). For the purpose of disease identification, we define an oligosaccharidosis as any storage disease that results in the excretion of neutral and sialic acid-containing complex oligosaccharides into the urine (Table 1). This includes the general class of glycoproteinoses (fucosidosis, mannosidosis and sialidosis types I and II) and also GM1 and GM2

Table 1. Lysosomal storage diseases that result in excretion of complex carbohydrates into the urine

Storage disease	Defective enzyme	Excreted storage product
Oligosaccharidoses		
GM1 gangliosidosis	β-Galactosidase	Oligosaccharides with β-linked galactose
GM2, type I Tay–Sachs	β-Hexosaminidase	Oligosaccharides with β-linked GlcNAc
GM2, type II Sandhoff	β-Hexosaminidase	Oligosaccharides with β-linked GlcNAc
Fucosidosis	α-Fucosidase	Oligosaccharides with α-linked fucose
Mannosidosis	α-Mannosidase	Oligosaccharides with α-linked mannose
ML-I, sialidosis	Neuraminidase	Oligosaccharides with terminal NANA
ML-II, I-cell	Transport defect	Mixed oligosaccharides
ML-III, pseudo-Hurler	Transport defect	Mixed oligosaccharides
Mucopolysaccharidoses		
MPS I, Hurler, Scheie	α-Iduronidase	DS/HS
MPS II, Hunter	Iduronate sulfatase	DS/HS
MPS IIIA, Sanfilippo A	Heparin sulfaminidase	HS/CS
MPS IIIB, Sanfilippo B	α-*N*-aetylglucosaminidase	HS/CS
MPS IIIC, Sanfilippo C	*N*-acetyl CoA: GluNc transferase	HS/CS
MPS IIID, Sanfilippo D	*N*-acetyl GlcNAc C-6 sulfatase	HS/CS
MPS IVA, Morquio A	*N*-acetyl GalNAc C-6 sulfatase	KS/CS
MPS IVB, Morquio B	Keratan sulfate β-galactosidase	KS/CS
MPS VI, Maroteaux–Lamy	Arylsulfatase B	DS/HS
MPS VII, Sly	β-Glucuronidase	CS

The oligosaccharidoses result in the excretion of complex oligosaccharides with monosaccharides either α or β linked to the non-reducing end of the oligosaccharide. The non-reducing end-terminal sugar is indicated for each disease. The MPS diseases result in the excretion of the glycosaminoglycans indicated.
DS = dermatan sulfate, HS = heparan sulfate, CS = chondroitin sulfate, KS = keratan sulfate.

gangliosidoses, generally classified as sphingolipidoses, and a number of the mucolipidoses [ML-I sialidosis, ML-II (I-cell) and ML-III] [16]. For identifying the different oligosaccharidoses, FACE of the urinary carbohydrates results in a banding pattern that is unique and characteristic of each disease. This band pattern information is used to determine the amount and distribution of complex oligosaccharides in patients and to determine a probability for each oligosaccharidosis. The characteristic banding patterns seen for each oligosaccharidosis can be used to train a neural network database within the software. Disease identification is confirmed following the initial screen by a shift of the banding pattern resulting from exoenzyme digestion of the labeled oligosaccharides.

To identify patients with the mucopolysaccharidoses, the sulfated carbohydrates excreted into the urine are first precipitated with cetylpyridinium chloride (CPC) and the precipitated carbohydrates are then labeled and separated by electrophoresis. Characteristic carbohydrate banding patterns are identified for each major MPS disease class, MPS I to MPS VII. Confirmation of the disease class is accomplished by analyzing the monosaccharide content of the carbohydrates in the CPC precipitate.

Materials and methods

The initial FACE screening test for identifying LSDs involves profiling the urinary oligosaccharides directly (oligosaccharidoses) and profiling the urinary carbohydrates following precipitation with CPC (mucopolysaccharidoses). This initial profiling will define the general class of disease (oligosaccharidoses or MPS), and in some cases the subclass (Table 1). Subsequent experiments are described for confirming the disease or defining the subtype. An outline of the general strategy is shown in Figure 1.

Collection of patient samples

Urine samples were obtained from patients with different LSDs. The samples were obtained from certified clinical reference laboratories including Dr Reuben Matalon, Miami Children's Hospital, Dr Larry Sweetman, Children's Hospital in Los Angeles, and one of us (T.G.), as well as others listed in the acknowledgements. The samples were received by Glyko, in dry ice, along with the definitive diagnosis, which was based on 'state-of-the-art' standard, thin-layer chromatography (TLC), electrophoresis and enzyme assays that were being routinely performed by the laboratory

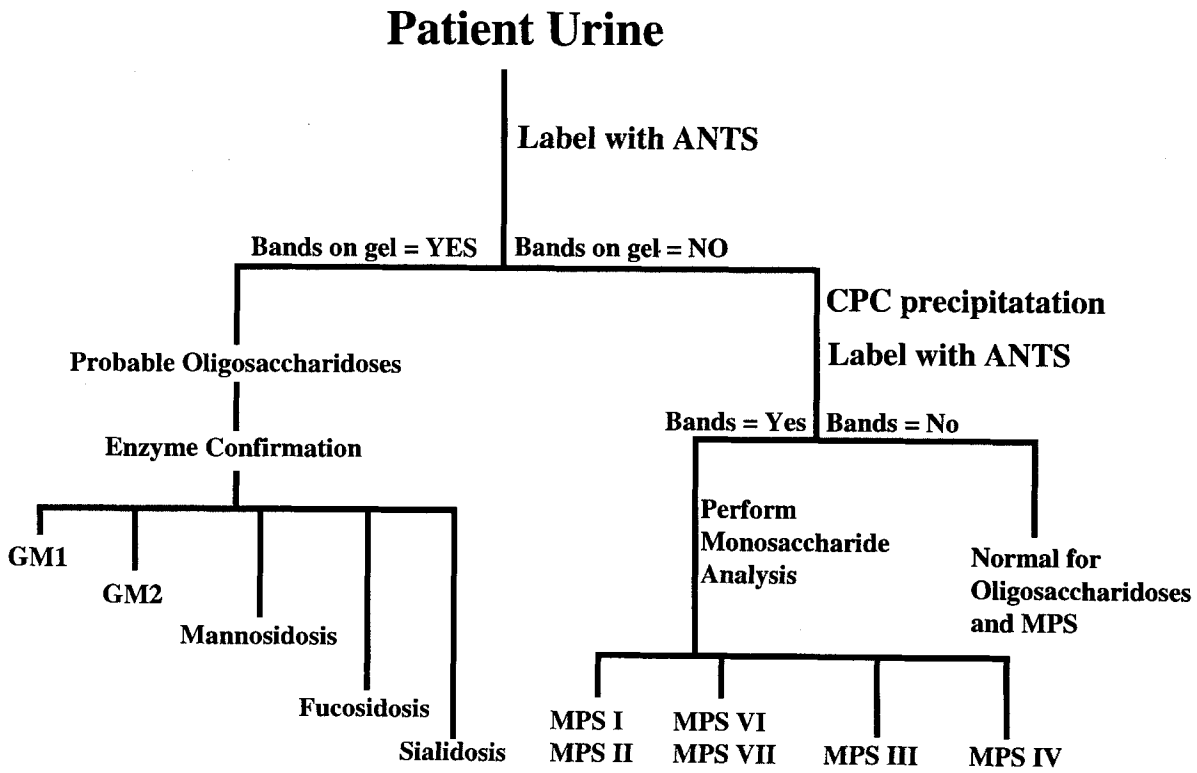

Figure 1. FACE strategy for identifying patients with oligosaccharidosis and mucopolysaccharidosis-type lysosomal storage disease.

for patient diagnoses [11–13]. The samples were obtained either by collecting the urine directly from the urine stream or by placing a disk of Whatman glass microfiber filter (7.0 cm) (Whatman, Maidstone, UK) in the diaper at bedtime. Urine carbohydrates collected on filter paper were recovered by cutting one 7.0-cm filter into small squares and soaking the squares in water overnight. The eluted carbohydrates were dried in a centrifugal vacuum evaporator, and the dried residue was resuspended in 50 μl of Milli-Q DI water. Fresh urine was frozen and stored at −70°C. Urine that appeared cloudy upon thawing was filtered through an Ultrafree-MC, 30000 NMWL Filter (Millipore, Bedford, MA, USA) prior to analysis.

Urine oligosaccharide profiling

Urinary carbohydrates were profiled using a FACE urinary carbohydrate analysis kit developed by Glyko (Novato, CA, USA). Fifty microliters of urine, filtered urine or resuspended carbohydrates recovered from filter paper was dried in a 1.5-ml microfuge tube using a centrifugal vacuum evaporator. The dried residue was resuspended in 5 μl of the fluorophore ANTS, 0.15 M in 15% acetic acid, and 5 μl of 1 M $NaCNBH_4$ in DMSO as supplied in the kit. Although the labeling reaction was complete in 2 h at 45°C, for convenience some of the samples were labeled overnight at 37°C. Both incubation conditions resulted in the same banding pattern (data not shown). The samples were dried and resuspended in 20 μl of water. Two microliters of the resuspended material was combined with 2 μl of 2× loading buffer (25% glycerol containing 0.05% bromophenol blue). The entire 4 μl sample was loaded into a single lane of a precast 35% polyacrylamide slab gel supplied in the kit. An electrophoresis box (Glyko) was assembled and the chiller was set for 0°C. The gel was run at 20 mA constant current for 1–2 h. A migration and quantification standard prepared from a partial digest of wheat starch (oligo ladder standard, Glyko) was loaded into the outside lanes of the gel. This standard migrates as a ladder of glucose polymers on the gel and the position of maltotetraose (indicated on figures as G4) was used for quantification. Following electrophoresis, the gel profiles of fluorescently labeled carbohydrates were imaged and individual oligosaccharide bands were quantified using the FACE imaging system (Glyko) as described below. For the purposes of band quantification, the amount of urinary carbohydrates was normalized against urine creatinine levels as determined on a Beckman Creatinine Analyzer 2 Model no. 6641 (Beckman Diagnostic Systems, Brea, CA, USA) using reagents supplied by Beckman.

Enzymatic confirmation of oligosaccharidoses

To confirm the identity of the defective enzyme activity, 2 μl of the labeled oligosaccharide mixture remaining from the urinary oligosaccharide profile above was aliquoted into each of six tubes. To each tube was added 14 μl of 200 mM $NaPO_4$ buffer pH 5.5. Each tube then received 2 μl of one of the following enzymes: α-fucosidase (Sigma, bovine epididymis fucosidase), α-mannosidase (Glyko, MANase II), neuraminidase (Glyko, NANase III), β-galactosidase (Glyko, GALase I) and β-hexosaminidase (Glyko, HEXase II). Prior to setting up the digestions each enzyme lot was assayed to determine enzyme activity and specificity using the appropriate *p*-nitrophenol-glycosyl substrates (Sigma) under the assay conditions (buffer, pH) recommended by the supplier. The last of the six tubes served as an undigested control and received 2 μl of water. The reactions were incubated for 2 h at 37°C, dried in a centrifugal vacuum evaporator and resuspended in 4 μl of 12% glycerol. The entire 4 μl of each reaction was loaded into separate lanes of a 35% polyacrylamide gel along with the undigested control and the wheat starch standard. Electrophoresis was performed as described above for oligosaccharidoses profiling.

Urine glycosaminoglycan (GAG) profiling

For MPS determinations, 0.5 ml of urine was aliquoted into a tube containing chondroitinase AC (Sigma C-2780, 20 μl, 100 mU). The mixture was incubated for 1 h at 37°C, and the remaining high molecular weight GAGs were precipitated from the urine by adding 0.5 ml of CPC (cetylpyridinium chloride) reagent (0.2 M sodium citrate, 0.1% CPC, pH 4.8). The mixture was incubated for 30 min at 37°C. The CPC precipitate was recovered by centrifugation for 5 min at 14000 ***g***, and the supernatant was discarded. The CPC pellet was washed by dissolving the pellet in 67 μl of 2 M LiCl, adding 267 μl of cold ethanol and reprecipitating the CPC for 2 h at 4°C. The washed CPC pellet was recovered by centrifugation for 5 min at 14000 ***g*** and resuspended in 50 μl of water. Fifteen microliters was removed for monosaccharide compositional analysis as described below and 20 μl was labeled with ANTS as described above for oligosaccharide profiling. The labeled carbohydrates were dried in a centrifugal vacuum evaporator for 15 min, 8 μl of water and 8 μl of

25% glycerol was added, a 4 μl aliquot from each sample was loaded into separate wells of a 35% polyacrylamide gel and electrophoresis was performed as described for oligosaccharide profiling.

Classification of mucopolysaccharidoses by monosaccharide analysis

The 15 μl aliquot of the urine CPC precipitate from above was dried and resuspended in 50 μl of 4 N HCl and incubated for 2 h at 100°C. The hydrolysis reactions were frozen at −70°C and lyophilized to dryness. The samples were resuspended in 10 μl of re-*N*-acetylation buffer (100 mM ammonium carbonate buffer, pH 9.4) and 1 μl of analytical-grade acetic anhydride (Sigma). The samples were incubated for 20 min on ice and dried in a centrifugal vacuum evaporator. The free monosaccharides were labeled by adding 2.5 μl of AMAC (2-aminoacridone), 0.3 M in DMSO, 2.5 μl of 30% acetic acid, and 5 μl of 1 M $NaCNBH_4$ in DMSO [17]. The labeling reaction was incubated overnight at 37°C and dried in a centrifugal vacuum evaporator for approximately 15 min. Each sample was resuspended in 10 μl of DMSO, 10 μl of water and 20 μl of 25% glycerol. Four microliters of each sample was loaded into a lane of a monosaccharide composition gel (Glyko) and 4 μl of monosaccharide standard containing 50 pmol of each of the AMAC-labeled GlcNAc, GalNAc, GlcUA, GalUA and galactose was loaded into both outside lanes (unlabeled standards obtained from Sigma). Because IdUA is not available commercially because of chemical instability, IdUA is not included in the mix of standards and the location of IdUA on the gel was determined from the hydrolysis pattern of purified dermatan sulfate (Sigma). An electrophoresis box (Glyko) was assembled and the chiller was set for 5°C. The power supply was set for a constant current of 30 mA per gel and electrophoresis was complete in approximately 45 min.

Gel imaging and carbohydrate analysis

FACE imaging was performed using a Glyko FACE imaging system. Following electrophoresis, the gel was inserted into the imager, under long-wave UV excitation, and an electronic image of the fluorescent carbohydrate banding pattern of the gel was acquired by a CCD as a digital image [17, 18]. The image was displayed on a computer screen using FACE imaging software through an MS-Windows interface. This imaging system allowed for detection of individual carbohydrate bands into the low picomolar range. Determination of the position of the carbohydrate bands in each lane was made relative to the ladder of glucose polymers in the wheat starch standard that was included in a single lane of each gel as a quantification standard.

Design of the FACE software database and neural net

Identification of the FACE carbohydrate pattern of each oligosaccharidosis was performed by visual inspection and by comparing the carbohydrate band positions and intensities of the sample with a database of band patterns of known disease samples. The current database for the oligosaccharidoses contains a total of 73 patient samples including patients with fucosidosis ($n = 12$), GM1 gangliosidosis ($n = 18$), GM2 gangliosidosis ($n = 17$, of which nine are Sandhoff and eight are Tay–Sachs), mannosidosis ($n = 10$) and sialidosis ($n = 16$). Each sample was from a different patient and, to the best of our knowledge, the patients were not from the same immediate family.

In the database, the band patterns are stored numerically as individual records, consisting of the banding patterns with individual band quantity and location on the gel. Additional patient data were also entered into the software, including symptoms, medications, age and sex as well as the urine creatinine level and the amount of each urine sample loaded on the gel. A neural net was trained using this accumulated data to recognize over 30 different features of each sample lane that uniquely characterize each storage disease and using these features to discriminate between the various diseases. The trained neural net is invoked by selecting a lane from the image of a gel and the software then presents the sample lane band data for analysis by the neural net. The results of this analysis are then presented as percentage probabilities for each disease. The essential features considered in the development of the neural net are described in references 18 & 19. In addition, the database may be accessed directly by Borland International's database manager Paradox. For further analysis or graphics the data or gel image was exported to spreadsheet and graphical programs [20].

Results

Quantification of urinary oligosaccharides

The oligosaccharidosis detection method is performed by measuring the amount and electrophoresis pattern of the labeled carbohydrates from

5 μl of urine. Using the FACE imaging system to quantify the carbohydrate bands above the location of G4, normal children contain an average of 69 ± 42 (mean ± SD) pmol of carbohydrate per μg of creatinine ($n = 35$) above the G4 band located in the standard (Figure 2). Patients with oligosaccharidoses contain between 304 and 3151 pmol of carbohydrate per μg of creatinine depending on the type of disease [15]. Of the eight oligosaccharidoses listed in Table 1, the least total amount of carbohydrate above G4 was found in fucosidosis patients with 304 ± 125 (mean ± SD) pmol of carbohydrate per μg of creatinine ($n = 12$), and the greatest amount was found in GM1 gangliosidosis patients with 3151 ± 1045 (mean ± SD) pmol of carbohydrate per μg of creatinine ($n = 18$). Normal urine has been reported to contain a number of small glucose polymers resulting from normal glycogen catabolism [21] and various blood group-specific oligosaccharides in ABO secretors [22, 23]. Using the FACE analysis shown in Figure 2, 5 μl of normal urine does not contain a significant number of carbohydrate bands that migrate above maltotetraose (G4) when labeled and profiled on a gel, although the reported glucose tetrasaccharide migrating at G4 is observed [21]. The level of urinary carbohydrates increases with the creatinine level of the urine, but we have found that loading the labeled carbohydrates from 5 μl of urine on the gel results in acceptably low carbohydrate background levels but is an optimal sample volume for detecting the oligosaccharidoses. Quantification of the amount of carbohydrates and normalization of the carbohydrate band data to the creatinine level of the urine is performed by the software after the electrophoresis and imaging is complete.

Screening for oligosaccharidoses

In general, the glycosaminoglycans present in MPS patients are not in high enough concentration in raw urine to be seen without prior CPC concentration. Therefore, the presence of carbohydrate bands above G4 in raw urine indicates the existence of 'abnormal' oligosaccharides and a high probability of one of the oligosaccharidoses. As shown in Figure 3, each oligosaccharidosis displays a characteristic carbohydrate banding pattern which can be thought of as a 'fingerprint' for that disease, although the relative intensities of the bands may vary from patient to patient. Because this pattern is essentially identical in different patients with the same disease, an initial identification of the type of oligosaccharidosis can be made based on the urinary carbohydrate banding pattern. As with many tests performed on biological fluids, the presence of microbial contamination of the urine may result in spurious results, in this case altered carbohydrate banding patterns, either because of the contribution of bacterial

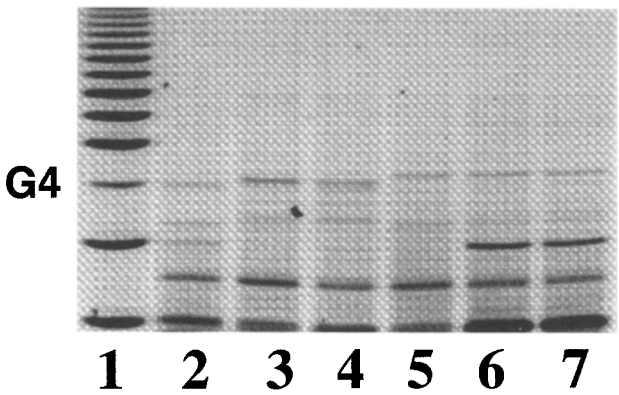

Figure 2. FACE profile of urine from normal children. Lane 1 contains a ladder of glucose polymers prepared from wheat starch. The location of the linear tetrasaccharide, maltotetraose, in the standard is indicated by G4. Lanes 2–7 each contain the carbohydrates from 5 μl of urine obtained from six normal children, age 3–4 years. Normal urine generally does not contain a significant amount of carbohydrate higher than the position of G4 on the gel.

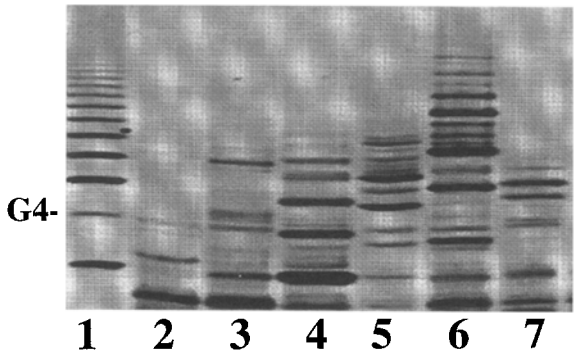

Figure 3. FACE analysis of the urinary carbohydrates excreted from children with various oligosaccharidoses. Lane 1 contains a ladder of glucose polymers prepared from wheat starch and the location of maltotetraose is indicated by G4. The remaining lanes contain the oligosaccharides in 5 μl of urine from a normal child (lane 2), and from patients with fucosidosis (lane 3), mannosidosis (lane 4), ML-I sialidosis (lane 5), GM1 gangliosidosis (lane 6) and GM2, type II, gangliosidosis (Sandhoff) (lane 7). Note the presence of carbohydrate bands above G4 in these diseases which are not present in normal urine.

polysaccharides to the pattern or as a result of the presence of bacterial glycosidases, which might result in the degradation of the excreted oligosaccharides. Procedures designed to minimize bacterial as well as yeast and fungal contamination should be employed when collecting and handling samples, and the possibility of contamination should always be considered when evaluating the data.

Confirmation of oligosaccharidoses

Following the initial urine profiling, final confirmation of the oligosaccharidoses is made enzymatically. The carbohydrate banding pattern obtained in patients with each oligosaccharidosis is unique for each disease because the pattern is due to the presence of partially degraded oligosaccharides in the urine. These oligosaccharides terminate at their non-reducing end with the monosaccharide that corresponds to the defective lysosomal enzyme activity. For example, the urine of patients with α-mannosidosis contains oligosaccharides that terminate in α-linked mannose (Figure 4) [24]. These abnormal oligosaccharides are present in the urine because of the lack of normal levels of the lysosomal enzyme α-mannosidase in this patient [25]. Confirmation of the identity of the defective enzyme is based on the susceptibility of these oligosaccharides to digestion with exoglycosidases (Figure 5). Digestion of the labeled oligosaccharides from a mannosidosis patient results in complete degradation of the urinary carbohydrates and thereby confirms that these oligosaccharides contain mannose at the non-reducing end (Figure 5, lane 3). In more complex structures where the penultimate sugar at the non-reducing end is not the same as the terminal sugar, a positive test for enzymatic digestion will appear as a shift in the overall banding pattern. For example, if the carbohydrates from a patient with GM1 gangliosidosis (β-galactosidase deficiency) are digested with β-galactosidase, a downward shift of the banding pattern results, thus confirming the susceptibility of the oligosaccharides to the exoenzyme (Figure 6, lane 4). A complete collapse of the banding pattern does not occur because the next sugar in the chain is probably GlcNAc. Digestion of these oligosaccharides with a test series of glycosidases, each corresponding to a different oligosaccharidosis, confirms that only the enzyme corresponding to the defective activity will generate a band pattern shift (Figures 5 and 6).

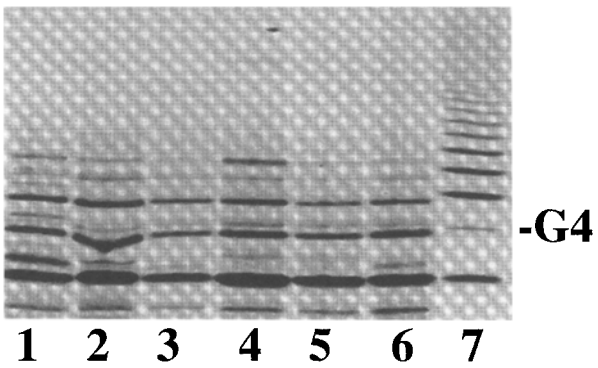

Figure 4. Urinary oligosaccharide patterns in patients with mannosidosis. Lanes 1–7 contain the oligosaccharides in 5 μl of urine from six mannosidosis patients. Mannosidosis patients excrete complex oligosaccharides that contain mannose residues at the non-reducing terminus. The ladder pattern shown in these patients is due to the presence of undegraded high-mannose type oligosaccharides, Man_4-GlcNAc to Man_9-GlcNAc. Lane 7 contains a ladder of glucose polymers prepared from wheat starch, and the location of maltotetraose is indicated by G4.

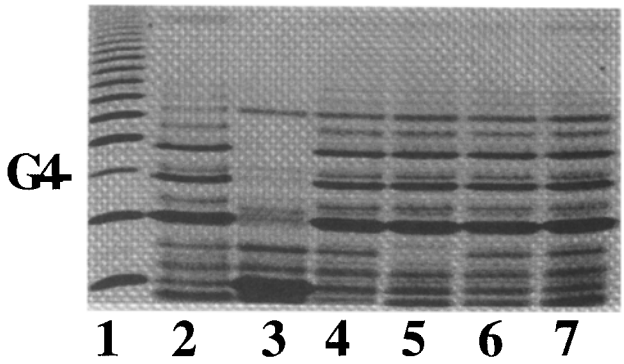

Figure 5. Enzymatic confirmation of mannosidosis. The labeled oligosaccharides from the patient sample shown in lane 4 were digested with a variety of exoglycosidases to determine the nature of the non-reducing terminal sugar residues. Lanes 2–7 show the digestion products using α-fucosidase (lane 2), α-mannosidase (lane 3), β-galactosidase (lane 4), neuraminidase (lane 5), β-hexosaminidase (lane 6) and an undigested control (lane 7). α-Mannosidase is the only enzyme of this series that causes a collapse of the banding pattern (lane 3), thus confirming α-mannosidase as the defective enzyme in this patient. Lane 1 contains a ladder of glucose polymers prepared from wheat starch, and the location of maltotetraose is indicated by G4.

Screen for mucopolysaccharidoses

Owing to their size and charge heterogeneity, the glycosaminoglycans (GAGs) excreted by patients with mucopolysaccharidoses are difficult to profile

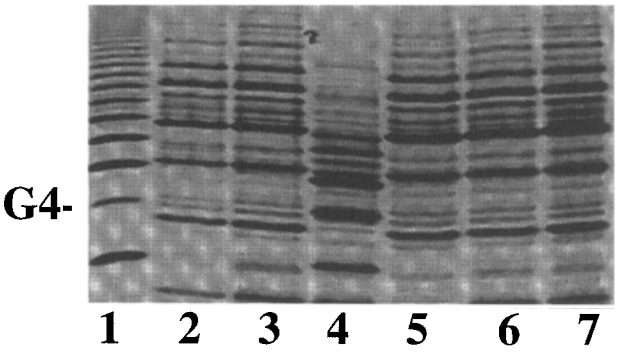

Figure 6. Enzymatic confirmation of GM1 gangliosidosis. The labeled oligosaccharides from a patient suspected of having GM1 gangliosidosis, based on an initial profiling test, were digested with a variety of exoglycosidases to determine the nature of the non-reducing terminal sugar residues. Lanes 2–7 show the digestion products using α-fucosidase (lane 2), α-mannosidase (lane 3), β-galactosidase (lane 4), neuraminidase (lane 5), β-hexosaminidase (lane 6) and an undigested control (lane 7). When compared with lane 7 (undigested), β-galactosidase is the only enzyme of this series that causes a shift of the banding pattern (lane 4). This confirms β-galactosidase as the defective enzyme and that this patient suffers from GM1 gangliosidosis. Lane 1 contains a ladder of glucose polymers prepared from wheat starch, and the location of maltotetraose is indicated by G4.

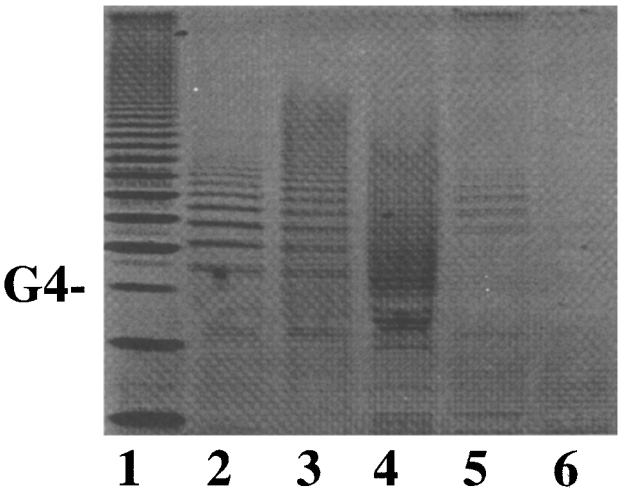

Figure 7. FACE analysis of glycosaminoglycans in patients with different MPS diseases. The GAGs were recovered from 0.5 ml of urine using CPC precipitation following chondroitinase AC digestion, and the equivalent of 50 μl of urine was loaded into each lane of the gel. Lanes 2–6 show the GAGs isolated from patients with MPS I (lane 2), MPS II (lane 3), MPS III (lane 4) and MPS IV (lane 5) and from a normal child (lane 6). Each MPS disease reveals a different carbohydrate banding pattern owing to the presence of different GAGs, and normal urine does not contain significant amounts of GAGs following chondroitinase AC treatment. Lane 1 contains a ladder of glucose polymers prepared from wheat starch, and the location of maltotetraose is indicated by G4.

on gels directly from raw urine. Therefore, the absence of a banding pattern above G4 in raw urine does not necessarily eliminate the possibility of the patient having one of the MPS storage diseases. MPS patients excrete large charged glycosaminoglycans into the urine which can be concentrated from a larger amount of urine (0.5 ml is usually adequate) using CPC precipitation. CPC is a quaternary ammonium salt that acts as a cationic detergent which complexes with the negatively charged GAGs present in MPS urine and causes the GAGs to precipitate [26]. The GAGs in the CPC precipitate are then labeled with a fluorescent tag (ANTS) and visualized on the gel. Figure 7 shows the fluorophore-labeled CPC precipitate isolated from a number of different MPS diseases. Each MPS disease generates a characteristic banding pattern, although the pattern differences are generally not as clear and recognizable as those observed with the oligosaccharidoses. The different patterns result from different enzyme defects that cause each MPS disease (Table 1) and the type of GAG that accumulates in each disease, whether it be heparan sulfate, dermatan sulfate or keratan sulfate (Table 2).

MPS monosaccharide composition

Glycosaminoglycans are polymers of repeating disaccharides and the monosaccharide composition of the disaccharides is different and characteristic of each type of GAG excreted (Table 2). Therefore, further differentiation of the MPS diseases can be made based on the monosaccharide composition of the glycosaminoglycans recovered from the urine. Young children normally excrete relatively large amounts of chondroitin sulfate A and C into the urine [27], as will patients with certain types of metabolic bone diseases [28]. This creates a significant 'background' of GalNAc which complicates the monosaccharide analysis. Although increased levels of chondroitin sulfate in the urine are characteristic of MPS III, MPS IV and MPS VII, identification and differentiation of these diseases can still be made following the removal of chondroitin sulfate A and C based on the monosaccharide composition of the other GAGs excreted. In the case of MPS VII, which is caused by a deficiency in β-glucuronidase activity, the diagnosis is based on the accumulation of chondroitin

Table 2. Monosaccharide analysis of urinary carbohydrates from patients with mucopolysaccharidoses

MPS disease	Urinary GAGs	GAG monosaccharide composition			
		GalNAc	GlcNAc	IdUA	Gal
MPS I, Hurler, Scheie	DS/HS	+	+	+	−
MPS II, Hunter	DS/HS	+	+	+	−
MPS III, Sanfilippo	HS	−	+	+	−
MPS IV, Morquio	KS	−	+	−	+
MPS VI, Maroteaux–Lamy	DS	+	−	+	−
MPS VII, Sly	HS	+	−	+	−

Urinary glycosaminoglycans were precipitated using CPC following digestion with chondroitinase AC to remove chondrotin sulfate.
GAG = glycosaminoglycan, DS = dermatan sulfate, HS = heparan sulfate, KS = keratan sulfate, GalNAc = *N*-acetylgalactosamine, GlcNAc = *N*-acetylglucosamine, IdUA = iduronic acid, Gal = galactose.

sulfate A and C. A determination of the amount of CPC precipitable material obtained before and after chondroitinase digestion is used to identify patients with MPS VII.

In order to eliminate the non-charged monosaccharides in the raw urine, which would also interfer with analysis, the MPS monosaccharide analysis is performed on the urine CPC precipitate. For example, MPS I and MPS II result in the excretion of both heparan sulfate and dermatan sulfate into the urine. Based on the monosaccharide composition of these glycosaminoglycans, the urine from patients with MPS I or MPS II will contain GalNAc, GlcUA and IdUA (Figure 8, lanes 2 and 3). In MPS I and MPS II keratan sulfate will not be excreted into the urine, so the urine monosaccharide composition will not contain significant amounts of galactose. Galactose will, however, be present in the urine of patients with MPS IV, in whom keratan sulfate is the major excreted product (Figure 8, lane 5). Patients with MPS III excrete only heparan sulfate, so the urine will contain GlcNAc, GlcUA and iduronic acid but will not contain GalNAc (Figure 8, lane 4). This type of analysis is only reliable when the normal 'background' of chondroitin sulfate A and C is removed prior to CPC precipitation as described above and in the Materials and methods section.

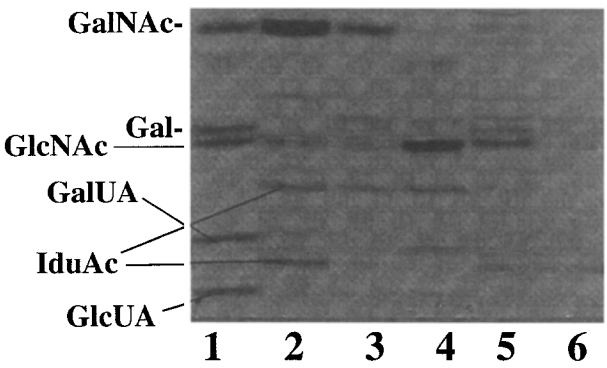

Figure 8. Monosaccharide analysis of urinary glycosaminoglycans. The CPC-precipitated GAGs from MPS patients were hydrolyzed to monosaccharides. The monosaccharides were labeled and analyzed by FACE. Lanes 2–6 show the GAGs isolated from patients with MPS I (lane 2), MPS II (lane 3), MPS III (lane 4) and MPS IV (lane 5) and from a normal child (lane 6). The monosaccharide composition of each disease can be used to differentiate and identify the type of MPS. Lane 1 shows the location of key monosaccharides in a mixture indicated along the left side of the gel (iduronic acid is not included in the standard mix). The identity of the monosaccharides in a test sample is determined by co-migration with each of the monosaccharides in the standard mix.

Discussion

Lysosomal storage diseases cause neuromuscular and intellectual impairment in children. Although treatment through bone marrow transplantation [29–31] and enzyme replacement therapy [32] is available for a few, in general most of these diseases are still considered incurable. As these diseases are generally thought to be underreported, part of the effort to deal with these diseases has been to develop more reliable and

routine diagnostic screening tests so that the parents of afflicted children can be advised of their child's disorder and receive appropriate counseling and management.

In this report we describe a novel method for identifying and differentiating two major types of LSDs, the oligosaccharidoses and the mucopolysaccharidoses. These two groups represent over half of the known LSDs. Both of these types of storage diseases result in excretion of accumulated carbohydrates into the urine, and the method described here is based on the profiling and monosaccharide analysis of the 'abnormal' carbohydrates. We believe that the accurate determination of urinary carbohydrates provides the most reliable method for routine screening for LSDs. Indeed, the only reported method for identifying patients with sialic acid storage diseases (e.g. Salla's disease) is through the determination of increased levels of sialic acid in tissues or urine [33].

Although detection of carbohydrates excreted into the urine is one method that is currently being used for diagnosing these diseases, other methods have been developed [34]. The most well-developed alternative method for the diagnosis of these diseases is based on measuring enzyme levels in plasma, fibroblasts and tissue [35]. These enzyme assay tests rely on the availability of a wide variety of high-quality fluorescent or radiochemically tagged substrates, each with the correct sugars, in the correct anomeric configurations. In order for the tests to be reliable, these synthetic substrates must be essentially free of excess sugar, nonconjugated fluorescent dye or other contaminants that, if present, may inadvertently inhibit enzyme activity in the assay. In addition, even with a complete picture of the clinical symptoms and history of the patient to help limit the possibilities, most unknown patients need to receive a complete range of enzyme tests in the hunt for the defective activity. Therefore, the FACE system offers a fast, economical initial screening method that can be used to reduce the list of possible diseases prior to enzyme tests, which are used for confirmation.

In the future, genetic testing may find a place in the diagnosis of LSD, although to our knowledge no routine LSD diagnosis is currently being made using DNA-based technology. This is in spite of the fact that the genetic defects responsible for a number of these diseases, including Tay–Sachs [36] and Gaucher disease [37], are known. Another important consideration in the eventual development of both DNA-based genetic testing and immunochemical-based tests is that many of these storage diseases are not caused by defective enzymes *per se*, but by defective intralysosomal enzyme activity. In addition to defective enzymes, a reduction in intralysosomal enzyme activity may result from defective enzyme transport systems [38], defective activator proteins [39, 40] or problems of enzyme instability in the acid environment of the lysosome [41]. Patients with these diseases show accumulation of incompletely degraded carbohydrates in tissues and urine, but will probably show normal enzyme genotypes and normal enzyme protein levels [42] and may show normal catalytic activity against synthetic substrates [43].

Clearly with the complexities that this group of LSDs present, confirmed disease diagnosis will inevitably be based on a combination of different types of assays. Confirmation of disease by enzyme assay is currently being used to support conclusions based on urinary carbohydrate analysis and will probably remain essential for differentiating subtypes and for the sphingolipidoses, in which products other than reducing carbohydrates accumulate. The FACE method offers a simple system of identifying two major types of storage diseases using a single electrophoretic method. The method described here offers both qualitative as well as quantitative information about the nature and level of complex carbohydrates excreted into the urine and the opportunity for an independent enzymatic confirmation.

The sensitivity of the FACE method means that early detection of disease may be possible. This would improve the possibilities of treatment before the disease becomes disabling and encourage the development of the appropriate therapeutics. There are a number of exciting new strategies for the treatment of these diseases. As many LSDs are caused by genetic lesions in the DNA encoding each associated lysosomal enzyme, these diseases are excellent candidates for therapeutic drug development based on enzyme replacement, enzyme supplementation or genetic therapy. One such drug is currently being used in the clinic to treat patients with Gaucher disease, a storage disease involving glycosphingolipid catabolism [32]. Other drugs are in development worldwide. Once the appropriate drugs are developed, we believe that the FACE method of urinary carbohydrate analysis will be extremely useful for following patients on therapy. In following enzyme replacement therapy, for example, measuring plasma or tissue enzyme levels may not be a reliable measurement of therapeutic efficacy owing to the requirement and problems encountered in the

targeting of replacement enzymes to the lysosomes of appropriate cells. A more accurate clinical picture may be obtained by measuring the reduction in the level of excreted storage products, as this would reflect the rate of accumulation in the tissue. Our hope is that these technologies will eventually merge into effective diagnostic and therapeutic programs that will modify the natural course of these diseases.

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