Sanfilippo Syndrome, Type D: A Spectrophotometric Assay with Prenatal Diagnostic Potential

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ABSTRACT. Sanfilippo syndrome, type D (MPS IIID), is characterized by moderate physical abnormalities, progressive mental deterioration, and deficient activity of N-acetylglucosamine 6-sulfate sulfatase, a lysosomal hydrolase involved in the degradation of heparin, keratan sulfate, and heparan sulfate. To date, demonstration of the enzyme deficiency typically relies on a radiolabeled trisaccharide substrate derived from heparan sulfate. In our study, we have developed a spectrophotometric assay for the determination of N-acetylglucosamine 6-sulfate sulfatase activity using the monosaccharide, N-acetylglucosamine 6-sulfate, as substrate. The reaction mixture was incubated for 6 h at 37°C and, after Dowex chromatography, released N-acetylglucosamine was measured by a modification of the method of Reissig. Assay conditions were optimized for cultured skin fibroblasts and primary cultures of amniotic fluid cells. The pH optimum for each was 5.5. The assay was linear for 24 h and up to 0.1 absorbance units. Activities of the three known MPS IIID skin fibroblast cell lines were more than 4 SD below the skin fibroblast control mean and more than 5 SD below the control mean for amniotic fluid cells. An enzyme deficiency in cultured amniotic fluid cells of the same magnitude as the skin fibroblasts of the known patients would be detectable and, therefore, prenatal diagnosis by this method is feasible. (*Pediatr Res* 26:462-466, 1989)

Abbreviations

AFC, amniotic fluid cells
GlcNAc, N-acetylglucosamine
GlcNAc(6S), N-acetylglucosamine 6-sulfate
GlcNAc 6-sulfatase, N-acetylglucosamine 6-sulfate sulfatase
MPS, mucopolysaccharidosis
MPS IIID, Sanfilippo syndrome, type D
DMAB, *p*-dimethylaminobenzaldehyde
NMR, nuclear magnetic resonance

The Sanfilippo syndrome is a lysosomal storage disease and has been designated as MPS III. The four types of the Sanfilippo syndrome (A, B, C, and D) have a clinical presentation characterized by physical changes similar to, but milder than, other mucopolysaccharidoses, severe mental deterioration that is progressive, and increased urinary excretion of partially degraded heparan sulfate (1). Although the four types cannot be distinguished on clinical grounds alone (2) each is distinct in having deficient activity of a different lysosomal enzyme involved in the metabolism of heparan sulfate.

MPS IIID was first recognized when Kresse *et al.* (3) reported two patients with clinical manifestations of the Sanfilippo syndrome who had heparan sulfaturia and who were unable to release sulfate from GlcNAc(6S) residues in oligosaccharides derived from heparan sulfate. MPS IIID appears to be rare and, to date, there are only seven reported patients (4-7). The diagnosis was confirmed by enzyme assay for six of these patients and selected clinical and biochemical findings for them are summarized in Table 1. The diagnosis of MPS IIID is confirmed by demonstrating a deficiency of GlcNAc 6-sulfatase, EC 3.1.6.14 in cultured fibroblasts or leukocytes. The substrate used typically is the tritium labeled trisaccharide *O*-(α -D-6-sulfo-2-acetamido-2-deoxyglucosyl)-(1 $\rightarrow$ 4)-*O*-uronosyl-(1 $\rightarrow$ 4)-2,5-anhydro-D-[1-³H]mannitol (8) made from heparan sulfate through a lengthy sequence of steps (9). The use of the radiolabeled monosaccharide [1-¹⁴C]GlcNAc(6S) for diagnosis of MPS IIID has been reported but has not found general usage perhaps because the reported rate of desulfation was greater for the trisaccharide and because free sulfate, an effective inhibitor of enzyme activity, was found to be difficult to remove after the preparation of GlcNAc(6S) by direct sulfation of GlcNAc with chlorosulfonic acid (10).

GlcNAc 6-sulfatase has been purified from human urine (9) and human liver (11). Robertson *et al.* (12) localized the gene for this enzyme to chromosome region 12q14.

Our study was undertaken to develop an effective diagnostic assay for MPS IIID using an inexpensive and easily prepared monosaccharide substrate with a spectrophotometric procedure for detection of free GlcNAc, a reaction product, after desulfation by GlcNAc 6-sulfatase. By eliminating the use of radiolabeled materials and the laborious preparation of the tritium labeled trisaccharide substrate, the assay would be within the capabilities of many additional laboratories, potentially making the diagnosis more readily available. GlcNAc 6-sulfatase has not been studied in primary cultures of amniotic fluid cells and therefore, additional aspects of this study were to determine selected characteristics of the enzyme in this important tissue source and to determine the potential applicability of the spectrophotometric assay to prenatal diagnosis. Preliminary results of this study have been reported (13).

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Table 1. Selected clinical and biochemical findings reported in patients with enzymatically confirmed MPS IIID

	Patient (Reference)					
	1 (4)	2 (4)	3 (5)	4 (5)	5 (6)	6 (7)
Origin*	Italian	Italian	Italian	Italian	Italian	French-Irish
Consanguinity	No	Yes	No†	No†	No	Not reported
Sex	F	F	M	F	M	F
Age at report	4 ⁹ / ₁₂	10 ⁶ / ₁₂	9 ⁸ / ₁₂	5 ⁴ / ₁₂	9	5
Age at first sign of neurological involvement	3 ⁹ / ₁₂	3	8	2	Not reported	3
Ocular involvement	No	Pigmentary retinal degeneration	No	No	No	Optic atrophy
Enzyme activity (% of normal mean)	6.1	6.1	4.6	4.4	0‡	<3.4
Mental retardation	Mild	Severe	Mild	Severe	Mild	Severe
Coarse facies/hirsutism/mild dysostosis multiplex and limitation of elbow extension	Yes	Yes	Yes	Yes	Yes	Yes
Hepatomegaly	Yes	Yes	Yes	No	No	Yes

* One patient in literature, not described clinically, is of East Indian origin.

† Reportedly nonconsanguineous but from same small town.

‡ Affected brother with similar features at age 1³/₁₂ but enzyme deficiency not confirmed.

MATERIALS AND METHODS

Chemical reagents. Reagents were purchased as follows: Sephadex G-10, Pharmacia Fine Chemicals (Uppsala, Sweden); N-acetylglucosamine, N-acetylglucosamine 6-sulfate, 3,3-dimethylglutaric acid, *p*-dimethylaminobenzaldehyde, and bicinechonic protein assay kit, Sigma Chemical Company (St. Louis, MO); Dowex AG 1-X8, 200-400 mesh, chloride form, Bio-Rad (Richmond, CA); potassium tetraborate, Fisher Scientific (Fair Lawn, NJ); chloroform and chlorosulfonic acid, Aldrich Chemical Company (Milwaukee, WI); pyridine, Pierce Chemical Co. (Rockford, IL). All other chemicals were of reagent grade.

Method of Reissig. Reissig *et al.* (14) described a spectrophotometric assay that is both sensitive and highly specific for N-acetylated hexosamines. A modification of this method was used for the detection of GlcNAc(6S) during its preparation and for the detection of released GlcNAc in the enzyme assay described below. Two reagents were used: potassium tetraborate ($K_2B_4O_7 \cdot 4H_2O$), 0.6 M with respect to B_4O_7 , adjusted to pH 9.5 with concentrated HCl and 1 g of DMAB dissolved in 10 mL of glacial acetic acid containing 12.5% (vol/vol) of 10 N HCl. The stock DMAB reagent was diluted immediately before use with 9 vol of glacial acetic acid to 1 vol of the stock solution. A 500- μ L sample was heated in boiling water for 3 min after the addition of 100 μ L of the potassium tetraborate reagent. The sample was then cooled for 3 min in a room temperature water bath followed by the addition of 3 mL of the diluted DMAB reagent and incubation in a 37°C water bath for 20 min. It was again cooled for 3 min in a room temperature water bath and the absorbance was measured in 1.0 cm quartz cuvettes at 585 nm with a Bausch & Lomb Spectronic 2000 spectrophotometer (Fisher). For larger or smaller samples the reagents were scaled up or down proportionately. The assay was optimized for detection of GlcNAc with respect to the pH of the potassium tetraborate reagent, after which the following assay characteristics were determined for GlcNAc: absorption spectrum, change in absorbance with respect to time, and change in absorbance with respect to concentration.

Substrate preparation. GlcNAc(6S) was prepared by direct sulfation of GlcNAc with chlorosulfonic acid by modification of the methods of Lloyd (15) and Ishihara *et al.* (16). A total of 1 g of GlcNAc was dissolved in 33 mL of pyridine. This solution was cooled to 0°C followed by the dropwise addition of 680 μ L of chlorosulfonic acid in 3.3 mL of chloroform with constant stirring. The mixture was stirred continuously for 30 min at 0°C

and then for 1 h at room temperature, after which it was neutralized with 5 M NaOH (approximately 5.5 mL). After neutralization, an oily layer separated to the bottom. This layer was extracted with a glass pipette and diluted in water. Initially, free SO_4^{2-} was precipitated with $Ba(OH)_2$ and Ba^{+2} was then removed by precipitation with dry ice or CO_2 gas after which the supernatant was diluted to reduce conductivity and applied to a column (1.5×10 cm) of Dowex AG1-X8, 200-400 mesh, chloride form. The precipitation steps were subsequently eliminated by desalting over a column (2.0×125 cm) of Sephadex G-10 eluted with 5% ethanol in water. The elution of SO_4^{2-} and Cl^- were followed by $Ba(OH)_2$ and $AgNO_3$ precipitation, respectively. The elution of N-acetylhexosamine was followed by the modified Reissig method. The peak fractions having a positive reaction were pooled, concentrated by rotary evaporation in a Büchi model RE-111A rotary evaporator (Fisher), diluted to reduce conductivity and applied to a column (1.5×10 cm) of Dowex AG1-X8, 200-400 mesh, chloride form, washed with 100 mL H_2O and eluted stepwise with 100 mL each of, 0.015 M, 0.2 M, and 0.5 M NaCl. Fractions eluted with 0.2 M NaCl were combined, rotary evaporated, and desalted over a column (1.0×180 cm) of Sephadex G-10 eluted with 5% ethanol in water. Several preparations of GlcNAc(6S) were combined, desalted a second time over a column (2.0×125 cm) of Sephadex G-10, characterized for content, and used as substrate for assay of GlcNAc 6-sulfatase activity. During the course of study, GlcNAc(6S) became available as a commercial preparation; however, it was available only intermittently and varied in physical appearance. The commercial preparation and the GlcNAc(6S) made by direct sulfation were compared as described below.

Substrate characterization. Thin-layer chromatography was performed with glass or polyester backed silica gel plates (Sigma). The two solvent systems used were *n*-butanol:acetic acid: H_2O (50:25:25) and *n*-propanol: H_2O (88:12). Plates were developed for 15 h and dried, after which each lane was marked in 1-cm sections, each of which was then scraped into 500 μ L of H_2O in a 13×100 mm glass tube and assayed by the modified method of Reissig. Molecular size exclusion gel chromatography was performed with a column (1.0×180 cm) of Sephadex G-10 eluted with 5% ethanol. Anion exchange chromatography was accomplished with 1 mL of Dowex AG 1-X8, 200-400 mesh, chloride form, in a disposable polyethylene filter column (Fisher) washed with 5 mL of H_2O and eluted stepwise with 5 mL each of 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 and 1.0 M NaCl. Samples for

NMR spectroscopy were prepared as 5 mM solutions in D₂O (99.8%) and analyses were performed on a Bruker WH-400 spectrometer.

Cell culture. Five skin fibroblast cell lines, recovered from liquid nitrogen storage, and an additional five cell lines from normal individuals were used as controls along with cell line GM 302A (NIGMS Human Genetic Mutant Cell Repository, Camden, NJ). Cell lines from three patients with Sanfilippo syndrome, type D, were available for study: GM 5093 (NIGMS Human Genetic Mutant Cell Repository, Camden, NJ), WG 1004 (The Repository for Mutant Human Cell Strains, Montreal, Quebec), and MGE-SFD (graciously provided by Dr. E. Kolodny, Waltham, MA). Other sulfatase-deficient cell lines were obtained from the NIGMS Human Genetic Mutant Cell Repository, Camden, New Jersey as follows: GM 0615 (Hunter syndrome), GM 2849 (Maroteaux-Lamy syndrome), GM 1881 (Sanfilippo A Syndrome), GM 4681 (multiple sulfatase deficiency), and GM 3245 (multiple sulfatase deficiency). One additional sulfatase deficient cell line was recovered from liquid nitrogen (Laboratory of Medical Genetics, University of Alabama at Birmingham): MGE-MSD (multiple sulfatase deficiency).

Cell cultures were maintained at 37°C with 5% CO₂ in closed flasks (Corning Glass Works, Corning, NY) with McCoy's 5a medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO), 1% antibiotic combination (penicillin 10 000 U/mL, streptomycin 10 000 µg/mL) and gentamicin sulfate (Elkins-Sinn, Inc., Cherry Hill, NJ) 40 µg/mL. Cells were subcultured at confluency after trypsinization. For enzyme studies, cells were harvested 10–14 d after subculture in 0.15 M NaCl with disposable plastic scrapers (Costar, Cambridge, MA), cooled in an ice bath, sonicated five times for 5 s each time, centrifuged at 9000 × *g* for 30 min at 5°C and the supernatant retained for enzyme assay. Proteins were measured with the bicinchoninic protein assay kit using BSA as a standard.

Primary amniotic fluid cell cultures with normal karyotypes (Laboratory of Medical Genetics, University of Alabama at Birmingham) were maintained in 25 cm² flasks in the same manner described above for skin fibroblasts and were harvested after 3–4 wk in culture, without having been subcultured. They were prepared for enzyme assay in the same manner as the skin fibroblasts.

Enzyme assay. The incubation mixture consisted of 100 µL of 100 mM 3,3-dimethylglutarate, pH 5.5, 50 µL of 31 mM GlcNAc(6S) containing 100 µg of sodium azide (NaN₃), and 50 µL of fibroblast sonicate (30–60 µg protein) in 0.15 M NaCl at 37°C. After 6 h, the reaction mixture was diluted with 300 µL H₂O and added to 1 mL of Dowex AG 1-X8, 200–400 mesh, chloride form. The void volume and three 500 µL fractions, eluted with 1.5 mM NaCl, were analyzed for released GlcNAc.

Enzyme activity in samples from primary amniotic fluid cell cultures was studied for the effect of repeated freezing in liquid nitrogen, repeated sonication, heat inactivation at 55 and 70°C,

and inhibition by Na₂SO₄ and NaCl. The *K_m* and *V_{max}* were determined by the method of Wilkinson (17).

RESULTS

Reissig method. The absorbance maximum for GlcNAc in 500 µL H₂O occurred at 585 nm and the absorbance for a 10 µg sample decreased by only 2.0% over a 25-min period. Absorbance with increasing concentrations of GlcNAc was linear from 0 to 0.2 mM and maximum absorbance occurred with the potassium tetraborate solution at pH 9.5.

Substrate preparation and characterization. Sephadex G-10 chromatography effectively removed free SO₄²⁻ and Cl⁻ (Fig. 1) after direct sulfation by chlorosulfonic acid. The sulfated monosaccharide, in spite of its larger molecular size, eluted later than free SO₄²⁻ which could be attributed to ionic interaction or possibly adsorption to the gel. The GlcNAc(6S) prepared by direct sulfation and the commercial preparation co-migrated on thin-layer chromatography in both solvent systems, co-eluted from Sephadex G-10, coeluted from Dowex AG 1-X8 with 0.2 M NaCl and the NMR spectra were identical (data not shown).

Enzyme assay. The pH optimum for GlcNAc 6-sulfatase from skin fibroblasts and primary cultures of amniotic fluid cells was 5.5 (Fig. 2). The formation of GlcNAc with increasing protein concentration was essentially linear to at least 0.1 absorbance units for skin fibroblasts and at least 0.05 absorbance units for amniotic fluid cells. The formation of GlcNAc with time was linear for at least 24 h for both tissue sources.

The specific activities for GlcNAc 6-sulfatase from both cultured skin fibroblasts and primary cultures of amniotic fluid cells were not normally distributed but after a log_e transformation (Fig. 3), a normal distribution could not be rejected statistically by the Shapiro-Wilk test (18). The specific activities for these normal controls are summarized in Table 2. The sp act of GlcNAc 6-sulfatase in cultured skin fibroblasts from normal controls, patients with Sanfilippo syndrome, type D and MPS patients with other sulfatase deficiencies are shown in Table 3. Activities of the three known MPS IIID skin fibroblast cell lines were more than 4 SD below the normal control mean for fibroblasts and more than 5 SD below the control mean for amniotic fluid cells (log-transformed data). Activities of the three known MPS IIID skin fibroblast cell lines, based on the untransformed data, were 2.6, 0, and 7.8% of the normal control mean and these values compared favorably with those reported for specific activities of MPS IIID patients using other assay procedures (Table 1).

Enzyme properties in amniotic fluid cells. The *K_m* and *V_{max}* for GlcNAc 6-sulfatase from primary cultures of amniotic fluid cells, were 24.0 mM and 20.7 µg GlcNAc released/h/mg protein respectively. At 55°C, sp act was reduced 35% during the first 60 min after which enzyme activity was relatively stable up to 120 min. At 70°C, however, specific activity was reduced by 70 and

Fig. 1. Sephadex G-10 chromatography (2 × 125 cm) for desalting GlcNAc(6S) prepared by direct sulfation. Absorbance (●) determined by the modified method of Reissig as described in "Methods and Materials." Conductivity (○) reduced to scale.

Fig. 2. Effect of pH on the activity of N-acetylglucosamine 6-sulfatase. Enzyme sources were cultured skin fibroblasts from a normal control (●) and a composite of 25 primary cultures of amniotic fluid cells (○).

Fig. 3. Frequency distribution of specific activities (A) and their log-transformed values (B) for N-acetylglucosamine 6-sulfate sulfatase from 122 primary cultures of amniotic fluid cells.

Table 2. Sp act and their natural log-transformed values for N-acetylglucosamine 6-sulfate sulfatase in cultured skin fibroblasts from normal controls and primary cultures of normal amniotic fluid cells

	Skin fibroblasts (n = 11)		Amniotic fluid cells (n = 122)	
	Sp act*	ln	Sp act*	ln
Mean	7.7	1.9	6.2	1.7
SD	4.1	0.5	2.6	0.4
Minimum	3.8	1.3	1.9	0.6
Maximum	17.0	2.8	13.0	2.6
Coefficient of variation	41.7	23.9	53.8	24.2

* μg GlcNAc released/h/mg protein.

Table 3. N-acetylglucosamine 6-sulfate sulfatase activity in cultured skin fibroblasts from normals, patients with MPS IIID, and patients with other sulfatase deficiencies

Cell line		Sp act*	ln
Normals (n = 11)		7.7 ± 4.1	1.9 ± 0.5
Sanfilippo D	GM 5093	0.2	-1.6
Sanfilippo D	WG 1004	0.0	
Sanfilippo D	MGE-SFD	0.6	-0.5
Multiple sulfatase	GM 4681	0.0	
Multiple sulfatase	GM 3245	0.6	-0.5
Multiple sulfatase	MGE-MSD	0.3	-1.2
Hunter	GM 0615	3.0	1.1
Maroteaux-Lamy	GM 2849	4.1	1.4
Sanfilippo A	GM 1881	4.8	1.6

* The specific activity (μg GlcNAc released/h/mg protein) for the 11 normal controls is the group mean $\pm$ SD. The specific activities for the cell lines from MPS patients is the result of a single determination in the same experiment.

100% after 30 and 90 min, respectively. Repeated freezing, up to five times, had little effect on enzyme activity with only a 16% decrease after the first three freeze cycles. Sonication for 45 s in 5-s intervals did not affect enzyme activity adversely. Sodium sulfate was highly inhibitory to GlcNAc 6-sulfatase activity causing 95% inhibition and 100% inhibition when present in 10 and 30 mM concentrations, respectively. Sodium chloride was considerably less inhibitory. The sp act in a normal control with 8.0 mM NaCl (due to the NaCl present in the enzyme preparation) was reduced only 13 and 28% when the total NaCl concentration in the incubation mixture was raised to 40 and 160 mM, respectively.

DISCUSSION

Cell lines from patients with MPS IIID, were clearly distinguished from normal controls by the procedure presented. Cell lines from patients with multiple sulfatase deficiency were also deficient in GlcNAc 6-sulfatase activity and the differential diagnosis would require assays of additional sulfatases. The assay is effective, relatively rapid, and simple to perform. The sulfated monosaccharide substrate is easier to prepare than oligosaccharide substrates and is also available commercially. The use of a spectrophotometric procedure obviates the need to work with radioactive materials.

GlcNAc 6-sulfatase from primary cultures of amniotic fluid cells was found to be a relatively stable enzyme with respect to heat inactivation, repeated freezing and repeated sonication. This is advantageous for enzyme isolation and sample preparation, which necessitate such potentially damaging procedures. Basner *et al.* (9) found greater heat sensitivity for GlcNAc 6-sulfatase from human urine, with more than 90% of enzyme activity toward a trisaccharide substrate destroyed after only 3 min at 70°C. Inhibition by Na_2SO_4 was potent and similar in magnitude to that found by Basner *et al.* (9) who reported that a 17-mM concentration gave $\geq 95\%$ inhibition. This emphasizes the importance of using a substrate preparation free of SO_4^{2-} . The effect of NaCl was less than that suggested by Kresse *et al.* (8) who recommended that cell pellets not be homogenized in 0.15 M NaCl because this concentration was considered highly inhibitory toward GlcNAc 6-sulfatase activity. The primary cultures of amniotic fluid cells used in this study were prepared in 0.15 M NaCl although the concentration in the recommended incubation mixture was substantially less.

The deficiency of GlcNAc 6-sulfatase activity found in multiple sulfatase deficient cell lines was not consistent with the findings of Yutaka *et al.* (19). They used a disaccharide substrate and made multiple determinations of the sp act of GlcNAc 6-sulfatase in several cultures from a single multiple sulfatase deficient fibroblast cell line. The activity they reported was usually normal but in some samples a reduction of between 30–50% of the control values was found. They proposed that the normal activity levels might have resulted from the fact that cells were cultured in medium that was maintained at a pH of 7.4 and similar results were obtained by Fluharty *et al.* (20) who reported a rise in arylsulfatase A (EC 3.1.6.1) activity when multiple sulfatase-deficient cell lines were maintained at that same pH. The cell lines in the present study were maintained in a $\text{CO}_2\text{-HCO}_3^-$ buffered medium which creates a lower pH.

Many biologic functions fail to follow a truly normal distribution and the transformed data are often shown to follow a log-normal distribution (21, 22); for example, a log-normal distribution was also found by Vance *et al.* (23) for N-acetyl- α -D-glucosaminidase (EC 3.2.1.50), another heparan sulfate-degrading enzyme, the deficiency of which results in Sanfilippo syndrome, type B. The enzyme sources for their study were serum and plasma.

There has been no report of prenatal diagnosis for MPS IIID but it would be feasible using this spectrophotometric assay if it is assumed that an affected fetus would have activity levels of

the same magnitude as found in the affected patients. The validity of this assumption will, of course, await the study of at-risk pregnancies.

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