Detection of Lysosomal Storage Disorders Using Tandem Mass Spectrometry

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Abstract

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Lysosomal storage disorders is a group of diverse autosomal recessive metabolic diseases. These disorders are caused by a deficiency in an enzyme necessary for the catabolic degradation of proteins, glycolipids, and glycosaminoglycans in the lysosome. Although these disorders can be caused by different mutations of different enzymes, they all manifest themselves through the abnormal accumulation of material within the affected cells. For decades lysosomal storage disorders were considered interesting neurodegenerative fatal disorders with no possibility for treatment. A lot of progress has been made in understanding these diseases and the enzymes behind the malfunction as well as in treatments for some of these disorders. Treatments are more successful when initiated before symptoms manifest; therefore early diagnosis is desired.

Tandem mass spectrometry has become a powerful tool in clinical analysis. It is also used in newborn screening laboratories for inborn error detection. Our work focuses on developing assays to detect lysosomal storage diseases in newborns by measuring substrate concentrations and measuring enzyme activity using tandem mass spectrometry as a quantification tool.

A new direct assay for palmitoyl protein thioestarase **1** was developed for the clinical diagnosis of infantile neuronal ceroid lipofuscinosis. Dried blood spots (DBS) are incubated in an assay cocktail containing detergent, synthetic peptide substrate and internal standard at 37 ºC for 10 hours. Product and internal standard are detected by electrospray tandem mass spectrometry in positive ion mode using selected reaction monitoring.

A new method for determination of sulfatide levels in blood for the detection of metachromatic leukodystrophy (MLD) was also attempted. Measurement of urinary sulfatide levels is used as a baseline method to distinguish healthy individuals from affected MLD individuals. In an effort to easily include our assay with other newborn blood spot screening methods, sulfatides were extracted from dried urine and blood spots using organic solvent and subsequently analyzed using ultra high performance liquid chromatography tandem mass spectrometry. An acceptable separation of sulfatide levels between MLD and non-MLD dried blood spots however was not obtained; because MLD and non-MLD affected individuals had measurable levels of sulfatide. On the other hand, sulfatide levels in dried urine from MLD affected individuals were elevated when compared to non- MLD individuals.

A multiplex assay using a single DBS was developed to screen for 6 disorders: Fabry, Pompe, Niemann-Pick A/B, Krabbe, Gaucher, and mucopolysaccharidosis type I. Samples were analyzed using flow injection tandem mass spectrometry. We demonstrate substantial differences in enzyme activity between blanks and blood punches for all six disorders studied. A new cassette of substrates was introduced to improve assay performance.

New reagents for mucopolysaccharidoses type II and type VI were synthesized to improve assay performance. Results show that the new reagents have enhanced mass spectrometry sensitivity and thus, a lower detection limit.

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Dedication

This dissertation is dedicated to my mother Ana Rodriguez de Bárcenas and to the memory of my father Diomedes Bárcenas; without their unconditional love and support this work would have not been possible. Thank you for believing in me.

Chapter 1.

Introduction.

1.1 MASS SPECTROMETRY.

Mass spectrometry is an analytical technique that produces spectra of the mass to charge ratios (*m/z*) of ionized atoms or molecules in the gas phase. Mass to charge ratios are measured in a mass spectrometer; the instrument has three main components: an ion source, a mass analyzer, and an ion detector. There are different ionization methods; mass analyzers and ion detectors. The choice of each component will depend on the design of the instrument and the expected performance.

Sir J.J Thomsom and his positive rays experiments opened the doors to the development of mass spectrometry as we know it today. He observed that when different gases were subjected to a gas discharge (ionization method), positive rays with varying masses were produced.[1] He soon realized the outreach of his discovery and its implications in the chemical analysis field and published "Rays of Positive Electricity and Their Application to Chemical Analysis". However, it took many years for the chemistry community to realize the potential in Thomson's work. [2] He is now considered the "father of mass spectrometry".

Many mass analyzers have been developed: sector instruments (based on Thomson's original studies), time of flight, ion traps, and quadrupole analyzers. Equally, there are many types of ion sources: electron ionization, soft ionization methods such as electrospray ionization, ambient ionization techniques including direct analysis in real time (DART) and matrix-assisted laser desorption ionization (MALDI).

Since the instrument used for the work discussed here had only an electrospray source and a quadrupole analyzer, only those components will be discussed in more depth.

Electrospray ionization (ESI) appeared in the late 1980s and since then has transformed the mass spectrometry field. ESI is a method in which ions that are present in a solution can be transferred to the gas phase. A technique that allows the transfer from solution to the gas phase constitutes a great advantage since many of the chemical and biochemical systems are in solution.[3] ESI invention is credited to John Fenn and he was awarded the 2002 Nobel Prize in Chemistry for his work in developing electrospray. Fenn et al. introduced the first ESI-MS in 1984,[4-5] their ESI source idea was based in previous work proposed by Malcom Dole.[3] In 1985 Fenn et al. used ESI as an interface liquid chromatography-mass spectrometry[6], the most common interface nowadays. ESI allows the formation of multiply charged ions[7]. That discovery led to the analysis of large molecules like peptides and proteins, a difficult task when z=1 and the molecular mass is out of the range of most mass spectrometers.

Electrospray is a soft ionization method, meaning the ions get to the mass analyzer without the breakage of chemical bonds. Ions in electrospray are produced by applying an electric field to the liquid inside the capillary tube. Charges will start to separate due to the presence of the electric field and if the field is strong enough, droplets will brake from the tip of the capillary. A flow of warm nitrogen gas is used to help the dissolvation. The mechanism to convert solvated ions to gas phase is not well understood but there are two proposed theories: the charge residue mechanism and the ion evaporation mechanism.[8] The ions formed typically have added a proton, but they can also form sodium or potassium adducts. Both positive and negative ions can be formed; deprotonation to yield negative ions will depend on the chemical species. The ions formed then go through the mass analyzer.

The mass analyzer is where the ions get separated according to their mass to charge ratio and is the core, often called the “heart”, of the instrument. Wolfang Paul invented the quadrupole

analyzer in the 1950s, and his work on the quadrupole and the ion trap were recognized with a Nobel Prize in 1989.[9]

A quadrupole analyzer is made up of four cylindrical rods (ideally hyperbolic section) placed parallel to each other. A direct current (DC) potential is applied to a pair of rods while the other pair is connected to an alternate radio-frequency potential.[8,10]A diagram of a quadrupole analyzer is shown in Figure 1.1. An electrical field pulses the ions towards the quadrupole. Positively charged ions will be moving towards the negatively charged rod and vice versa due to the electrostatic attraction, but once the polarity is reversed, the ion will change the trajectory before it collides with the rod. As a result of these polarity changes only ions with a narrow range of *m/z* values go through the analyzer and into the detector. The rest of the ions with the unstable trajectories will collide with the rods.[10-11]

Among the advantages of quadrupoles analyzers are the robustness, low cost, and ease of maintenance. The ability to perform tandem mass spectrometry (MS/MS, coupled mass analyzers) analysis is another advantage. The most common configuration is a tandem quadrupole instrument. A tandem quadrupole or triple quadrupole instrument has three components: a quadrupole mass analyzer, a radio frequency (RF) only quadrupole, hexapole, or octapole (typical configurations use one of these three as a collision cell), and a second quadrupole mass analyzer.[8]

There are four ways to scan the ions in a tandem quadrupole. The first mode consists of choosing an *m/z* ion with the first quadrupole, the ion undergoes collision and the fragments produced are analyzed in the second analyzer. This method is known as product ion scan or daughter scan. The second mode consists of fixing the second analyzer to a set *m/z* value while scanning the masses in the first quadrupole. This method is known as precursor ion scan or parent scan. A third

alternative is to scan both masses together with a constant mass offset. This method is called neutral loss scan. The fourth alternative is to fix the masses for both of the quadrupoles so detection only occurs when the specified precursor ion forms the specified fragment ion. This method is called selected reaction monitoring (SRM).

1.2 LYSOSOMAL STORAGE DISORDERS.

The concept of lysosomal storage disorders (LSDs) was first introduced in 1965 by Henri Hers to explain how the absence of an enzyme, α-glucosidase, could cause a fatal condition, Pompe disease.[12] The undegraded substrates accumulate in the lysosome, causing damage in the organelle and eventually leading to malfunction of the organ. This concept led to the discovery of other lysosomal storage disorders.[12]

The lysosome, often called the stomach of the cell, is characterized by the presence of a membrane, a low internal pH (acidic pH 4-5), and vesicles that contain hydrolytic enzymes. The lysosome is in charge of breaking down materials into substances that can be used by the cell.[13]

LSDs like other genetic diseases are incurable but they can be treated. For many years, LSDs were considered interesting fatal degenerative disorders. Bone marrow transplantation and enzyme replacement therapy were among the first treatments attempted for these disorders and even showed promising results in some cases (e.g. enzyme replacement therapy for Gaucher disease). However, complications arise with these methods, especially with respect to neurological symptoms.[14] Gene therapy and combination therapies offer an alternative to treat LSDs.[14-18]

LSDs comprise a group of more than 50 inherited disorders, some of which are summarized in Table 1. The majority of the disorders are caused by a single enzyme deficiency but others like I cell disease result from loss of several enzymes caused by an underlying defect in a common protein.[12] LSD symptoms and severity vary among the disorders. Each disorder, however, has a spectrum of manifestation depending on the mutation and the residual enzyme activity. The difference in the clinical manifestations and symptoms often causes a delay in diagnosis.[19]

LSDs as a group have an estimated prevalence of 1:7,000 to 1:9,000 in the European populations.[20] Incidence of LSDs as a group rivals that of other disorders routinely screened for in the newborn period.[14] Advances in treatment development have also opened the debate for newborn screening of LSDs in the United States and other countries.[21]

1.3 NEWBORN SCREENING AND THE USE OF TANDEM MASS SPECTROMETRY.

Newborn screening is a public help program design to screen babies for some genetic and metabolic disorders that can impact the infants' long-term health. A card with blood from the baby's heel is collected at birth. These blood spots are used to screen for genetic disorders. The idea is that early detection and prompt intervention would improve the quality of life of the children.

Chamoles et al.[22-23] demonstrated that enzymes retain activity in dried blood spots (DBS). This discovery allows assaying enzyme activity using DBS cards collected from newborns. Our group efforts have been focused on expanding the world of newborn screening and clinical diagnosis by developing assays for lysosomal storage disorders. We use tandem mass spectrometry to

quantify enzyme activity. ESI-MS/MS is a powerful technique and has been used previously for clinical applications. Newborn screening laboratories use tandem mass spectrometry for the detection of metabolic disorders. We propose assays that are compatible with the newborn screening laboratories, so when screening for lysosomal storage disorders is mandated by the state the assays can be put to work without further delay.

In 1999, Gerber published a screening assay to simultaneously screen for GM1 gangliosidosis and MPS-III type B.[24] Assays for All MPS-III types (A-D)[25-26], Niemann Pick, and Krabbe[27] were also developed. In 2004 Li[28] developed a multiplex assay to screen for five disorders (Fabry, Gaucher, Niemann-Pick, Pompe, and Krabbe); incubating the samples separately and combining prior MS/MS analysis. Li was also the first to use DBS as the enzyme source, while the previous assays used cell lysates. Assays were also developed for MPS-I[29-30], MPS-II[31-32], MPS-IVA[33], and MPS-VI[34]. Duffey[35] created a triplex assay to screen for Fabry, Pompe, and MPS-I and a pilot study using this method was conducted in the Washington state newborn screening lab, where more than 100,000 anonymous newborn DBS were analyzed.[19]

Spacil[36] designed a multiplex assay to screen for 9 disorders that could be incubated with one or two DBS punches using UHPLC-MS/MS. The duration of the UHPLC-MS/MS analysis was two minutes, which is important because high throughput is required for the assay to be used in newborn screening laboratories.

The work described in this thesis is a contribution to the improvement of screening for lysosomal storage diseases. In the first section, an assay for infantile neuronal ceroid lipofuscinosis is described. The second section describes the efforts towards finding a screening test for metachromatic leukodystrophy[37]. The third section is a description of an alternate assay to Spacil's multiplex, using flow injection analysis and new and improved assay reagents.

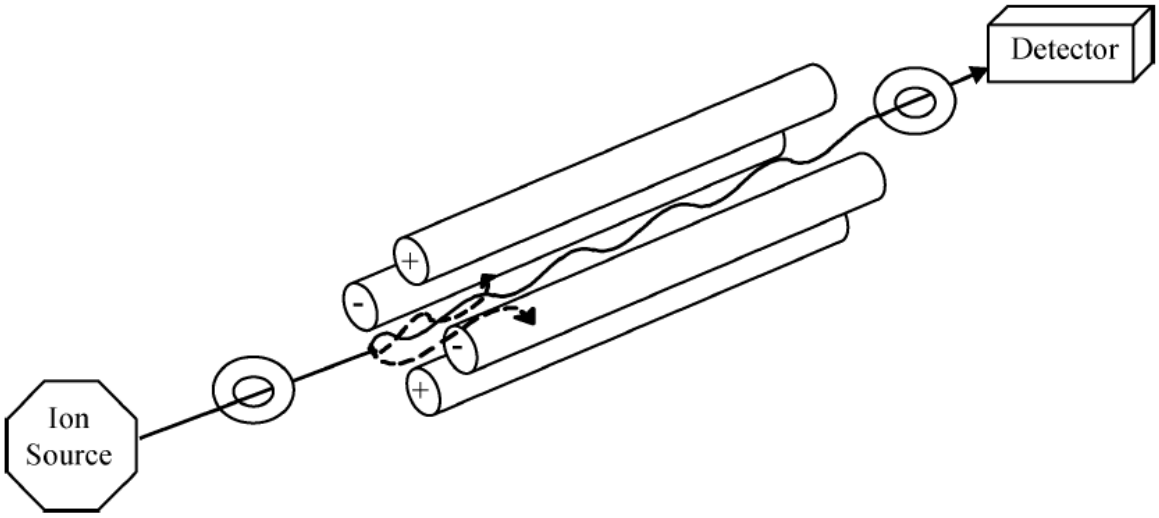

Figure 1.1. Diagram of a quadrupole analyzer. Reproduced from El-Aneed et al.[10]

Table 1. Lysosomal Storage Disorders.[14]

Mucopolysaccharidoses (MPS)	**Neuronal Ceroid Lipofuscinoses (NCL)**
MPS-I (Hurler)	Type I (Infantile, Santavuori-Haltia disease)
MPS-II (Hunter)	Type II (late infantile, Jansky-Bielchowsky disease)
MPS-III (Sanfilippo type A, B, C and D)	Type III (juvenile, Batten disease)
MPS-IV (Morquio type A and B)	Type IV (adult)
MPS-VI (Maroteaux-Lamy)	Type V (late infantile variant)
MPS-VII (Sly)	Hyaluronidase deficiency
Sphingolipidoses	Danon disease
Gaucher disease	Cystinosis
Fabry disease	Sialuria
Niemann-Pick type A, B and C	Pompe disease
Farber	Wolman's disease
Krabbe	Galactosialidosis
Metachromatic Leukodystrophy	Sialidosis
Gm1-gangliosidosis	Salla disease
Gm2-gangliosidisis (Tay-sachs, Sandhoff, activator deficiency)	α–mannosidosis
Mucolipidoses	β–mannosidosis
Type II (I cell disease)	Fucosidosis
Type III (pseudo- hurler polydystrophy)	Aspartylglucosaminuria
Type IV	Schindler disease

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Chapter 2.

Direct Assay of Palmitoyl Protein Thioesterase 1 for the Detection of Infantile Neuronal Ceroid Lipofuscinosis Using Tandem Mass Spectrometry.

Abstract

We report in a direct assay of palmitoyl protein thioesterase 1 (PPT1) enzyme, an enzyme responsible of removing thioester linked fatty acids from cysteine residues. The assay was developed for use in the clinical diagnosis of infantile neuronal lipofuscinosis (infantile CNL1). Dried blood spots (DBS) are incubated in assay cocktail containing detergent, 46 nmoles of a synthetic peptide substrate and 2.6 nmoles of the internal standard (heptapeptide AAAPFGC) at 37 ºC for 10 hours. The reaction is quenched and followed by a liquid/liquid extraction with ethyl acetate (EA) to remove Triton X-100 and C-18 solid phase extraction to desalt the sample. Product quantitation is done using electrospray ionization tandem mass spectrometry. Prior to MS/MS analysis the solution is incubated for 30 min at 37ºC with Tris (2-Carboxyethyl) Phosphine Hydrochloride (TCEP) to reduce disulfide bonds. The K_M for PPT1 was 229.6 μM and V_{max} 216.6 μmol/h*L. Enzyme activity among a random sample of 62 unaffected newborns ranged from 82.3 to 221.9 μmol/h*L and an average activity of 155.7 μmol/h*L; this result is clearly distinguished from enzyme activity in 5 infantile CNL1 patients ranging from 21.4 to 27.4 μmol/h*L with an average of 23.9 μmol/h*L. Intraassay coefficient of variation (CV) was 3.2% and the interassay CV was 15.1%. The developed assay can be multiplexed into a single mass spectrometry injection with other assays to diagnose different forms of neuronal ceroid lipofuscinoses.

2.1 INTRODUCTION.

The Neuronal Ceroid Lipofuscinoses (NCL) comprise a group of hereditary lysosomal storage disorders characterized by the accumulation of autofluorescent material rich in lipids, proteins and carbohydrates in the brain and other tissues.[1] The disorders manifest most dramatically in the brain. NCLs collectively constitute the most common hereditary neurodegenerative disorder in childhood.[2] NCL is distributed worldwide with an incidence of 1:100,000 live births and the overall incidence in the US has been estimated in 1:12,500.[2-3] NCLs are classified as lysosomal storage disorders, however unlike other lysosomal storage disorders, characterization of the accumulated material has not led to the discovery of the underlying defect. In classical LSDs enzyme deficiencies produce the accumulation of specific undegraded substrates or metabolites; meanwhile, in NCLs the main storage material is the subunit c of mitochondrial ATP synthase or sphingolipid activators proteins A and D. [2-3]

There are at least 8 different types of NCL known based on the onset of the symptoms, and the enzymes causing the deficiency have been identified in most cases. There is no universally used nomenclature for NCL but it has been suggested that names for the disorders include the affected gene and the age of onset.[4] Out of all NCL manifestations infantile and classical late infantile account for the majority of cases of NCL.

Infantile Neuronal Ceroid Lipofuscinosis also known as Santavouria-Haltia disease (INCL, NCL1, or CNL1, infantile) is the most severe form of NCL and is caused by a mutation in the *CNL1* gene, which encodes the Palmitoyl Protein Thioesterase 1 abbreviated as PPT1 (EC 3.1.2.22) an enzyme responsible for removing fatty acids attached to cysteine protein residues via thioester linkages.[5] PPT1 enzyme is structurally similar to lipases and has a peptide-binding site and a well-defined fatty acid binding pocket.[6] In contrast with most hydrolases, PPT1 has a

neutral optimum pH, and it was not considered a lysosomal enzyme until after its purification and cloning which revealed structural features consistent with an extracytoplasmic location.[7] More than 40 different disease causing mutations on *CLN1* have been reported.[3] One exception is the Finnish population, where the disorder is prevalent. The majority of the Finish children affected with the disorder are homozygous to a single point mutation that completely inactivates the enzyme. Therefore, the Finish cases constitute the most severe form of the disease.[8]

Children affected with infantile CNL1 developmental milestones are normal until the age of 8 months.[8] The disease starts manifesting in the second half of the first year and symptoms include visual deterioration leading to blindness, seizures, and mental and motor deterioration leading to vegetative stage. Most children with infantile CNL1 die around age of 10.[1, 3]

Histochemical findings in patients show that the storage material is resistant to lipid solvents. The storage material consists of 40% protein and 35% lipid by dry weight.[9] The major proteins in the storage material are saposins A and D. Saposins A and D accumulate in tissues of patients of several lysosomal storage diseases and the significance of its major accumulation in INCL is unknown. [1]

The material in the cells accumulates forming deposits that vary in shape and size. The structure of the larger deposits suggests that they are formed by fusion of small spherical globules. Such deposits are called granular osmiophilic deposits. [10]

Infantile CNL1 is an incurable disorder. No treatment is available but the existence of two completely deficient mice models has accelerated treatment development. So far neuronal stem cell transplantation has arisen as a viable treatment strategy. When normal human neuronal stem cells were transplanted into PPT1 deficient mice the cells migrated through the brain. The cells provided enough PPT1 activity to decrease autofluorescence, improve motor function and delay

host neuron lost. These data were used to support a phase one neuronal stem cell trial in children with infantile CNL1. [11] Given the complex manifestation of infantile CNL1 and the multiple organ system involvement, a combination therapy approach could be advantageous. Macauley et al. supported this hypothesis when they observed an enhancement of the therapeutic effects of enzyme replacement therapy when combined with bone marrow transplantation.[12]

Because infantile CNL1 is characterized by early onset (6 months to 1 year) and rapid progression, the success of any potential therapy strongly depends on early diagnosis.

The diagnosis of INCL is based on a combination of findings. Rapid psychomotor deterioration and retinal deterioration shown by the extinction of the electroretinogram. Symptom observation is generally too late to efficiently start treatment. Electron microscopy remains an important diagnostic method but it is tedious and not readily available.

Since the enzyme causing the disorder was identified new diagnostic tools employing enzymatic assays have been developed. Camp et al. developed an assay using S-[^{3}H]palmitoyl-H-Ras protein as a substrate.[7] In 1999, van Diggelen et al. presented an enzymatic assay using the fluorometric substrate 4-methylumbelliferyl-6-thiopalmitoyl-β-D-galactopyranoside for pre- and post-natal diagnosis of infantile CNL1.[13] In 2003 Lukacs et al. analyzed enzyme activity in dried blood spots using a fluorometric substrate.[14]

Current assays for INCL measure fluorescence but require the addition of a second enzyme to yield a fluorescent compound[13-14].

Here we report on the development of the first assay using electrospray ionization tandem mass spectrometry (ESI-MS/MS) to directly measure PPT1 activity using a synthetic substrate. In an effort to provide clinical tools to detect the two most common NCL manifestations, the assay

was multiplexed with an assay to screen for classical late infantile neuronal ceroid lipofuscinosis, an assay that was developed in our group.

2.2 EXPERIMENTAL

Materials. All water used was purified by a Millipore Milli-Q 18MΩ System. N-acetylated heptapeptides ALLPFGC and AAAPFGC were purchased from Lifetein (Hillsborough, NJ). Palmitoyl chloride 98% was acquired from Sigma-Aldrich (St Louis, MO). All solvents used were technical grade supplied by Sigma-Aldrich. Triton X-100 purchased from ACROS (NJ, USA). Tris (2-carboxyethyl) phosphine hydrochloride was purchased from Sigma-Aldrich. Solid phase extraction C-18 Omix pipette tips were supplied by Agilent (Santa Clara, CA). All experiments were conducted in compliance with Institutional Review Board guidelines. All infantile CLN1 affected patients had been diagnosed previously with established clinical and biochemical procedures. DBS were stored at -20 °C in zip-lock plastic bags (one bag sealed inside a second bag). Zip-lock bags were kept in a sealed plastic box containing desiccant (anhydrous $CaSO_4$ granules).

Substrate Synthesis. Substrate for PPT1 was prepared by S-acylation of peptide ALLPFGC following a procedure reported by Rijkers et al.[15] and summarized in scheme 2.1. N-acetylated peptide ALLPFGC (20 mg, 26.3 μmoles) was dissolved in a 3:1 mixture of dichloromethane/dimethylformamide and mixed with triethylamine (73 μL, 523 μmoles). After stirring for 5 minutes, a 0.1 M solution of palmitoyl chloride (162 μL, 534 μmoles) in dichloromethane was added slowly. The mixture was allowed to react for 15 hours at room

temperature. Upon reaction completion the solvent is removed under reduced pressure. The product was re-dissolved in acetonitrile and filtered. The filtered solution was purified using silica gel column chromatography eluted with a 5:1 dichloromethane/methanol mixture containing 0.5% acetic acid (R_f 0.76). Substrate was isolated as a white solid in 69% yield (18.1mg, 18 μmoles). Mass spectrometry data confirmed the identity of the substrate shown in Figure 2.1.

Assay Protocol. 3mm dried blood spots punches were placed in a 1.5 mL polypropylene Eppendorf tube and 100 μL of a 100 mM phosphate buffer solution (pH = 7.0) 0.08% Triton X-100 containing 46 nmoles of substrate and 2.6 nmoles of N-acetylated peptide AAAPFGC as an internal standard. The solution was then vortexed for 1 minute and incubated for 10 h at 37ºC in a thermostated air shaker at 250 rpm. After the incubation period, the samples were placed in an ice bath. Ethyl acetate (300 μL) and 100 μL of deionized water were added to the assay mixture. Samples were vortexed for 1 minute and centrifuged for 5 minutes at 3000 rpm. A liquid/liquid extraction was performed to remove Triton X-100 from the samples. Solid phase extraction C-18 pipette tips were used to capture and desalt the assay product and internal standard eluting with 200 μL of a 50:50 acetonitrile/water (1% acetic acid) mixture. Prior to injection into the mass spectrometer, TCEP was added to the solution with a final concentration of 800 μM and the mixture incubated for 30 minutes at 37ºC to reduce disulfide bonds in the peptides.

Mass Spectrometry. ESI-MS/MS was carried out on a Waters Quattro Micro tandem quadrupole instrument using a positive ionization mode and selected reaction monitoring (SRM).

Sample injection was 10 μL for each analysis and flow injection was 0.2 mL/min of an 80:20 ACN/H_2O 0.1% formic acid solution.

Mass spectrometer settings were: capillary voltage 3.5 kV; cone 35 V; extractor 2.0 V; RF lens, 0.2 V; source temperature, 120ºC; desolvation temperature, 250ºC; cone gas flow, 50L/h; desolvation gas flow, 500L/h; LM 1 resolution, 14.8; HM 1 resolution, 14.8; ion energy 1, 0.2 eV; entrance, 2 V; collision, 25 eV; exit, 15 V; LM 2 resolution, 14.8; HM 2 resolution, 14.8; ion energy 2, 1.0 eV; multiplier, 650 V; gas cell pirani pressure, 2.21 x 10^{-3} mbar; dwell time 100ms.

2.3 RESULTS AND DISCUSSION.

Assay and sample work-up conditions. In order to analyze the samples using ESI-MS/MS it is necessary for the samples to be dissolved in a compatible solvent free of non-volatile salts and buffers. The presence of a surfactant in the assay buffer further accentuates the need for buffer removal from the samples. Liquid/liquid extraction of PPT1 product (PPT1-P) and internal standard (PPT1-IS) in different organic solvents was investigated. We found that ethyl acetate, n-butanol and 8:1:1 ethyl acetate/n-butanol/1-hexanol were unsuccessful in extracting the product and internal standard. Nonionic Triton X-100 is also extracted into the organic layer and interferes with the spectrometric analysis. Liquid/liquid extraction with ethyl acetate can be used to remove Triton X-100 but is unsuccessful for the recovery of the enzymatic product and internal standard. Solid phase extraction (SPE) was then investigated. C-18 SPE pipette tips (Omix tips) were used to extract the PPT1 product and internal standard from the aqueous phase of the assay. SPE selectively retains the PPT1 product and internal standard while buffer salts can be washed away. Elution with a 50:50 mixture of acetonitrile/water was selected to elute the

peptides with recoveries of 52.3 and 49.8% for product and internal standard respectively. The acetonitrile/water mixture eliminates undesirable substrate from the analysis mixture. Prior to injecting the samples in the mass spectrometer the disulfide bonds formed under the assay conditions must be reduced. TCEP was selected as the reducing agent, and the minimum concentration required to reduce all the disulfide bonds was determined to be 800 μM. (Figure 2.2).

Reported fluorometric assays use a pH 4.0 buffer for the incubation.[13-14] However the enzyme has a neutral optimum pH when acting on the natural substrate. [7] Therefore pH dependence of the assay was investigated, and the results are illustrated in Figure 2.3. At pH values above 7.0 non-enzymatic hydrolysis of the thioester occurs with greater frequency than enzymatic hydrolysis.

Mass Spectrometry. Acetylated peptides were readily protonated in the mass spectrometer, forming singly charged positive ions. Cleavage of the peptide occurs predominantly through charge-directed pathways[16]. There is a nomenclature to designate the fragment ion types produced by the cleavage of different bonds along the peptide backbone or side chain (Scheme 2.2)[17]. Cleavage of the backbone typically occurs at the peptide amide bond to produce **b** ions, if the amino terminus retains the charge, or **y** ions if the carboxy terminus retains the charge[18]. The mass transitions monitored were selected according to the peptide fragmentation pattern shown in Figure 2.4, e.g *m/z* 762.5→ *m/z* 423.5 and *m/z* 678.45→ *m/z* 423.5 from PPT1-P and PPT1-IS respectively correspond with the formation of the y_4 ions of the peptides. The amount of product formed was calculated using the SRM intensity ratio of the product to the internal standard, the known concentration of the internal standard, and the response ratio. The response ratio was

determined to be 1.104 and showed good linearity (R^2 = 0.98891) as seen in Figure 2.5. Enzymatic activity was calculated as μmol/(h*L of blood) from the amount of product formed, the incubation time, and the volume of blood, 3.2 μL, calculated from the estimated volume of a blood spot (10 μL) and the punch/DBS area ratio.

PPT1 Enzyme Kinetics. Assay conditions were optimized for this assay by varying the incubation time, area of blood spots and concentration of substrate to determine K_m and V_{max}. The time course study of the assay showed that enzyme activity increases in a linear fashion up until 12 hours, after which the product formation reaches a maximum (Figure 2.6). An incubation time of 10 hours was selected to ensure enzyme kinetics were in the pseudo-linear stage.

The amount of enzyme was also evaluated by adding more than 1 blood spot to the assay cocktail. The results show an increase in the amount of product formed as the amount of enzyme is increased (Figure 2.7). We concluded that a single DBS provided enough enzyme to readily measure product formation using tandem mass spectrometry.

Michaelis-Menten parameters were also evaluated for the PPT1 enzyme by varying substrate concentration from 0 to 500 μM with triplicate assays at each concentration level, and the data was fitted to a non-linear least square models. Results are shown in Figure 2.8. The calculated values of K_M and V_{max} were determined to be 229.6 μM and 216.6 μmol/h*L respectively. A substrate concentration of 460 μM was used in an attempt to minimize the effect of inhibitors present in blood.

Clinical Sample Analysis. The assay was performed on 62 random newborn samples obtained from the Washington state newborn screening lab, and five diagnosed infantile CLN1 patients (Figure 2.9). Unaffected newborns showed a range of activity from 82.3 to 221.9 μmol/h*L and an average of 155.7 μmol/h*L; infantile CLN1 patients displayed a range between 21.4 to 27.4 μmol/h*L and an average of 23.9 μmol/h*L. Blanks combining all the components of the assay but replacing the DBS punch with a filter paper punch were analyzed, and the activities were in the range of 6.5 to 13.4 μmol/h*L with a mean value of 9.03 μmol/h*L.

Assay precision was calculated using a DBS from a healthy adult control sample. Intra-assay coefficient of variation (CV) was 3.2% (n = 5), calculated from five injections of the sample from the incubation of a single DBS. Inter-assay variation CV was 15.1% and involved 10 injections from different DBS punches while avoiding the blood spot perimeter.

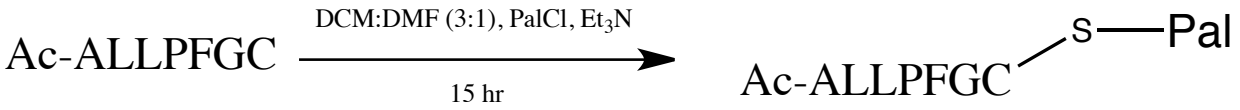
The assay was combined in a single mass spectrometry injection with an assay developed by a coworker to diagnose late infantile CLN2. Due to different pH conditions for the different enzymes the assays cannot be incubated together but are combined after the sample workup into a single mass spectrometry injection. The results of this assay are presented in Figure 2.10. Forty random newborns samples had an activity range of 95.0 to 281.8 μmol/h*L with a mean activity of 160.4 μmol/h*L; 5 infantile CNL1 affected newborns had a range of activity of 28.3-53.9 μmol/h*L and a mean activity of 42.9 μmol/h*L; PPT1activity of 5 late infantile CNL2 affected newborns ranged between 43.8 and 96.1 μmol/h*L with an average activity of 74.5 μmol/h*L. Blanks of the assay were once again evaluated and the range of activity was 13.3 to 14.9 μmol/h*L with an average activity of 13.9 μmol/h*L.

2.4 CONCLUSIONS.

Electrospray ionization tandem mass spectrometry is a powerful tool for use in clinical applications, as demonstrated by its use to measure PPT1 activity in dried blood spots. Our method is the first mass spectrometric assay for the diagnosis of infantile CNL1. It also offers the advantage of using a substrate structurally similar to the natural substrate of the enzyme as oppose to the fluorescence substrate and eliminates the need for a coupling enzyme, typical in fluorometric assays.

Assay results from previously diagnosed infantile CNL1 patients show a marked deficiency in enzyme activity, and there is a well-established separation between the healthy and affected samples. This result demonstrated a tandem mass spectrometry assay of PPT1 using dried blood spots is suitable for large scale screening of infantile CNL1 if treatment becomes available and the need for newborn screening emerges.

Due to the high specificity of tandem mass spectrometry and the orthogonality of the substrate, product, and internal standard masses, the assay can multiplexed into a single mass spectrometry injection with other assays to diagnose neuronal ceroid lipofuscinoses. This concept was demonstrated by multiplexing with an assay developed in our lab to diagnose classical late infantile CLN2.



Scheme 2.1. Synthesis of palmitoylated substrate.

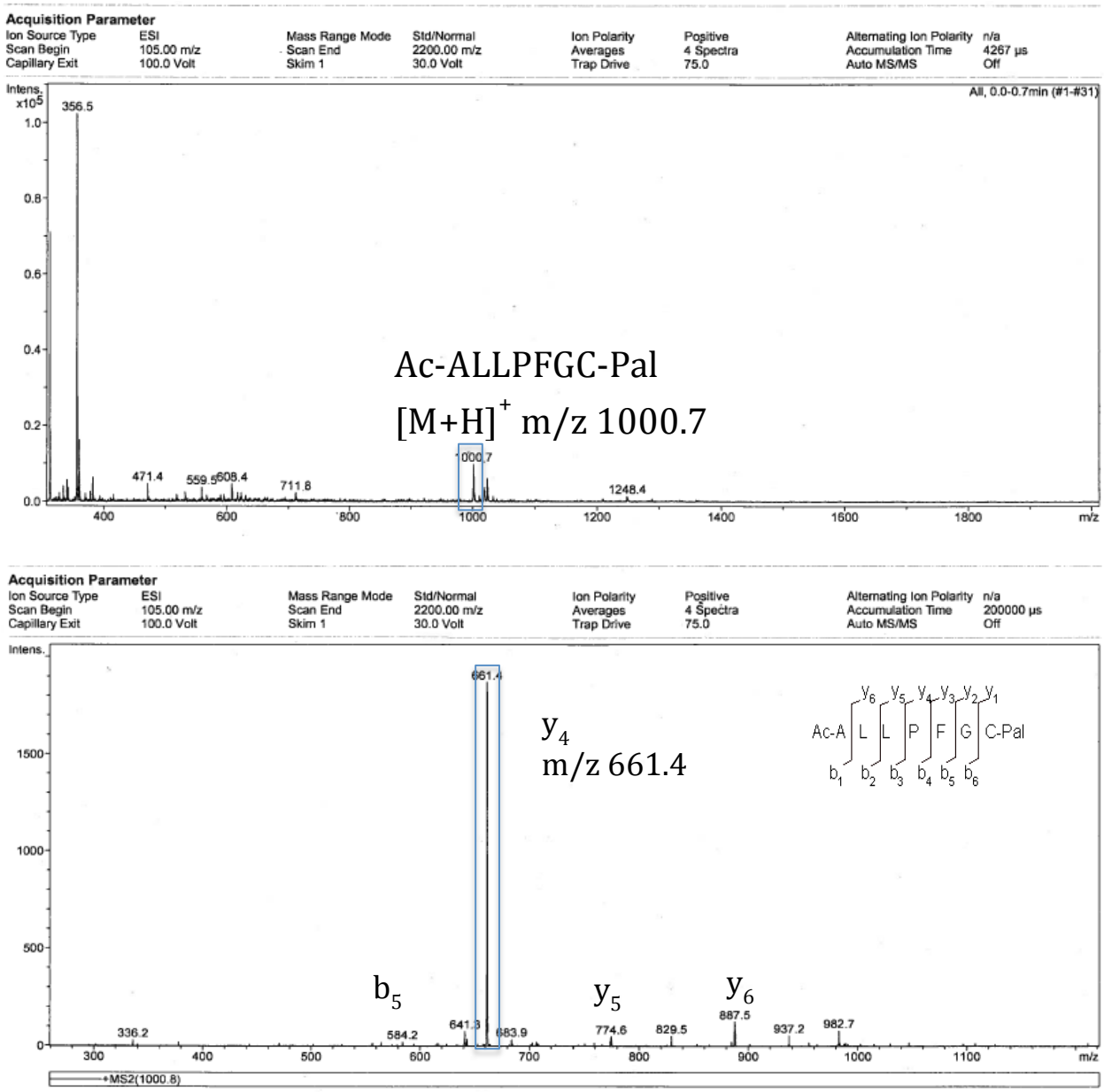


Figure 2.1. Top: MS spectrum of the synthesized palmitoylated substrate. Bottom: MS/MS spectrum of synthesized palmitoylated substrate. Predominant fragment is y_4 ion.

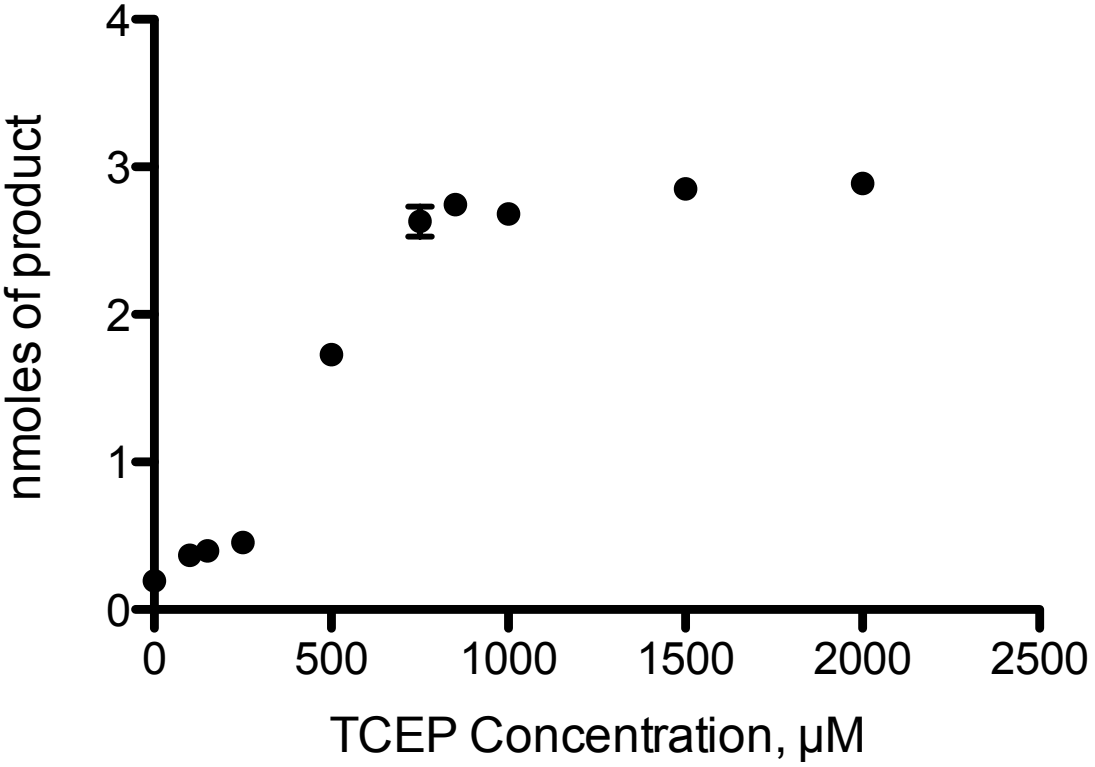


Figure 2.2. Optimization of TCEP concentration using a 3nmoles solution of PPT1 product. Error bars are one standard deviation of triplicate measurements.

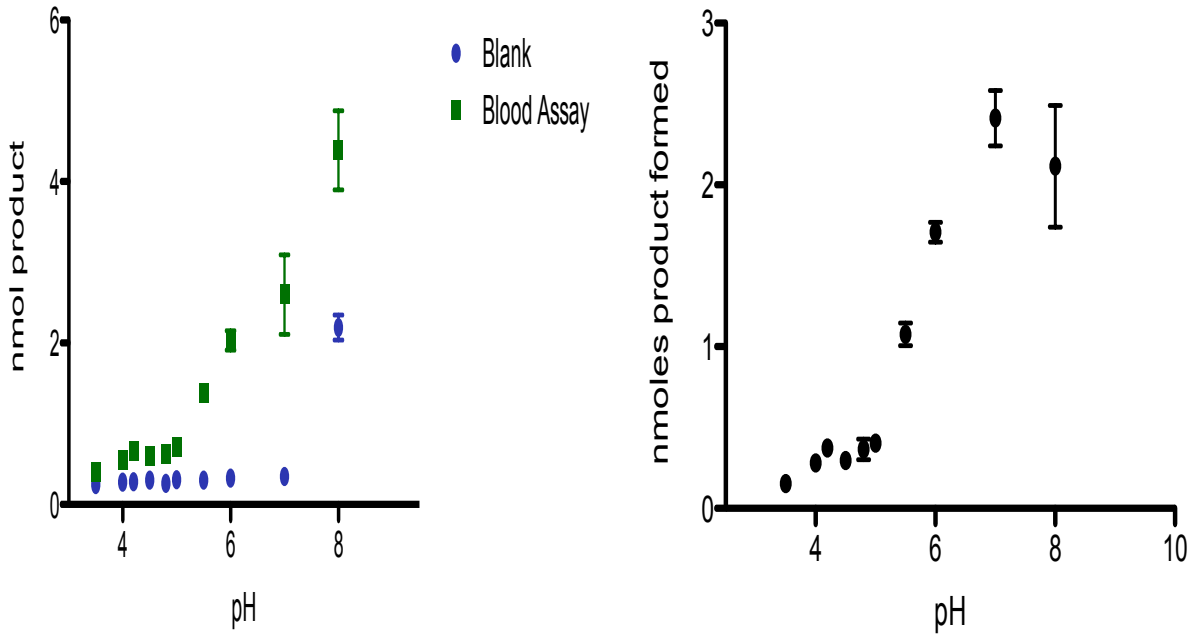
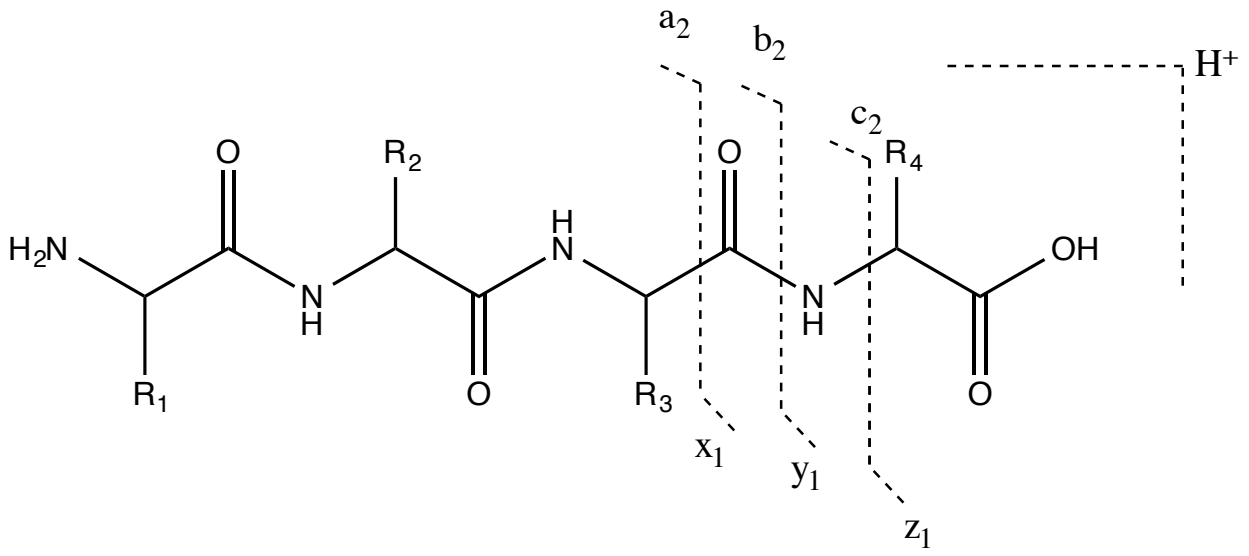


Figure 2.3. pH dependence of the PPT1 enzyme activity. Left: Product formed both enzymatically and non enzymatically. Right: Product formed by enzymatic hydrolysis blank corrected.



Scheme 2.2. Nomenclature of common peptide fragment ion types.

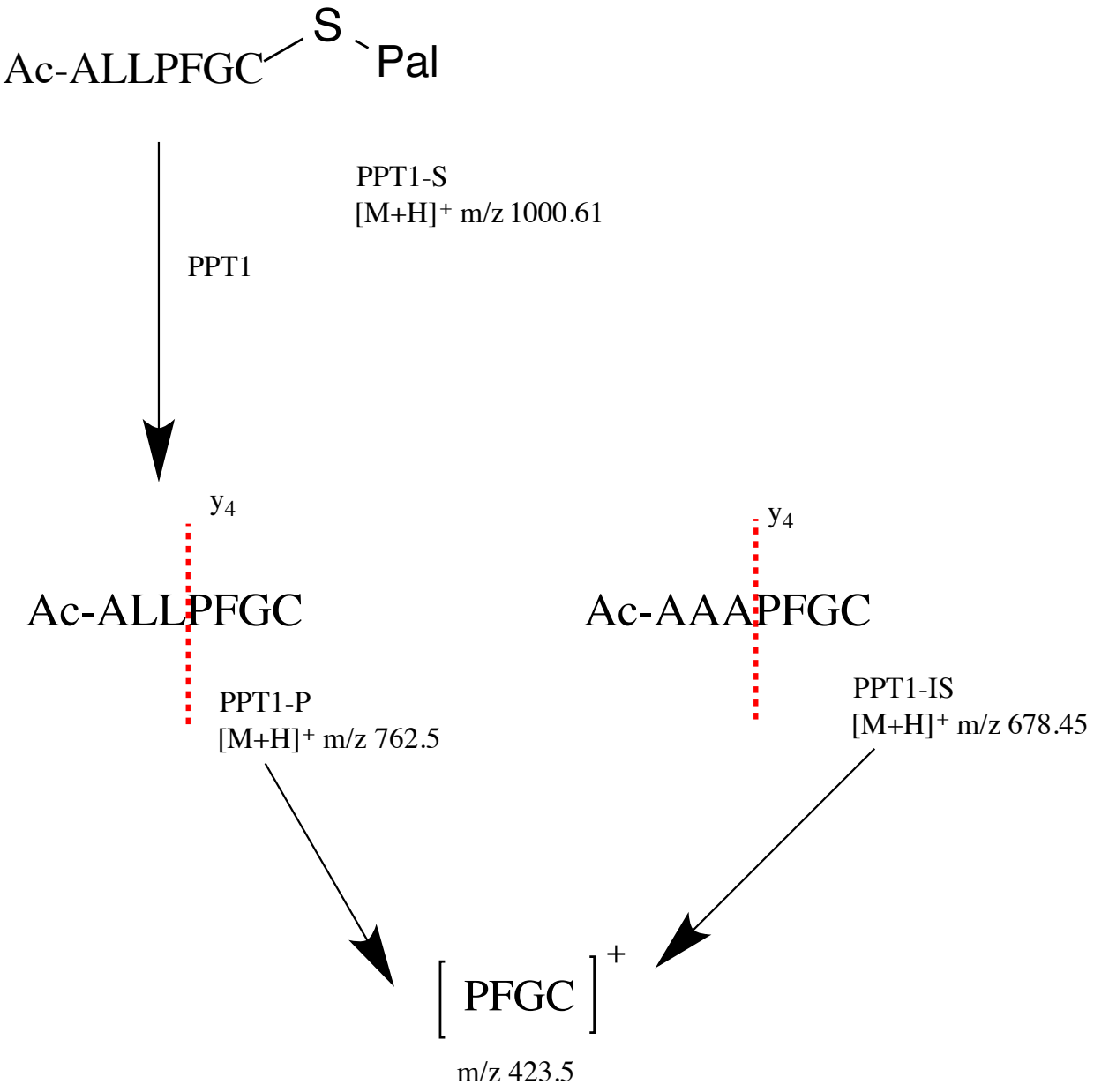


Figure 2.4. MS/MS Fragmentation of the PPT1 assay product and internal standard. Reporter ion for both species is the y_4 ion.

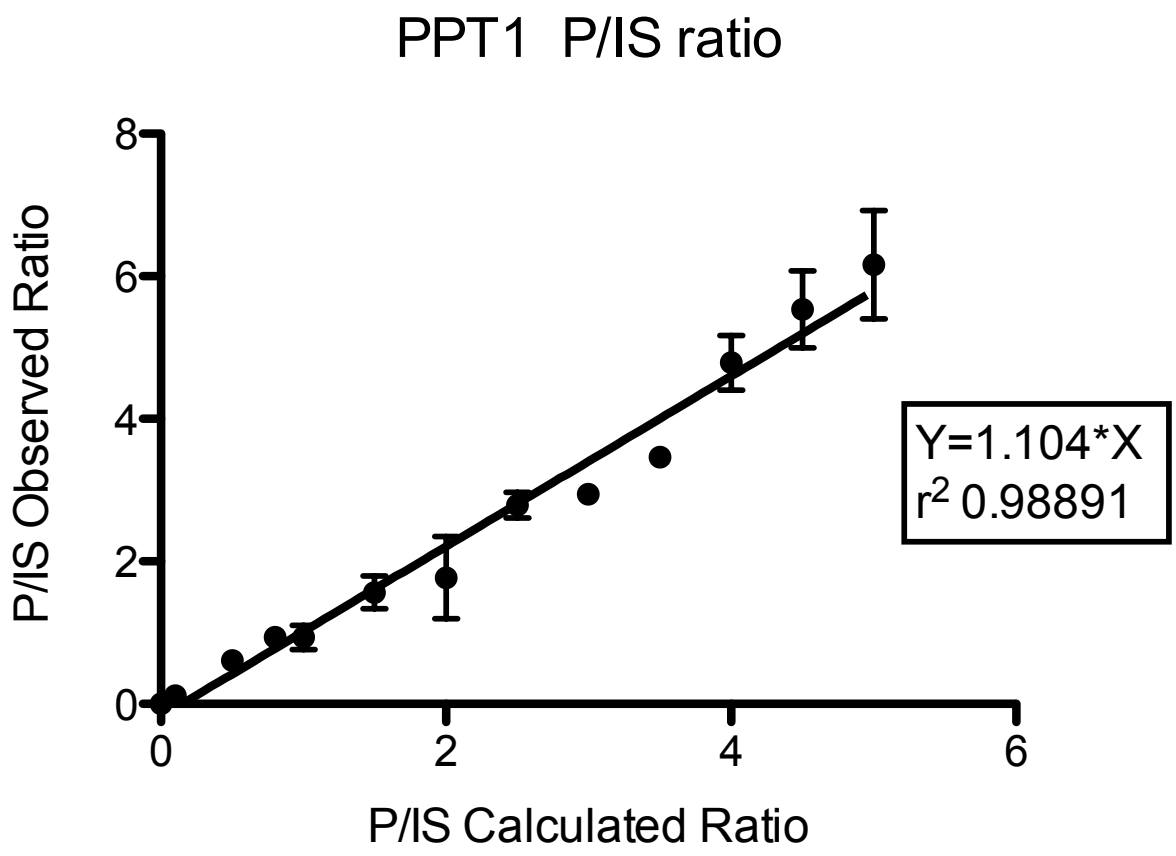


Figure 2.5. Response ratio of the PPT1-product to the PPT1- internal standard. Error bars represent one standard deviation of triplicate measurements.

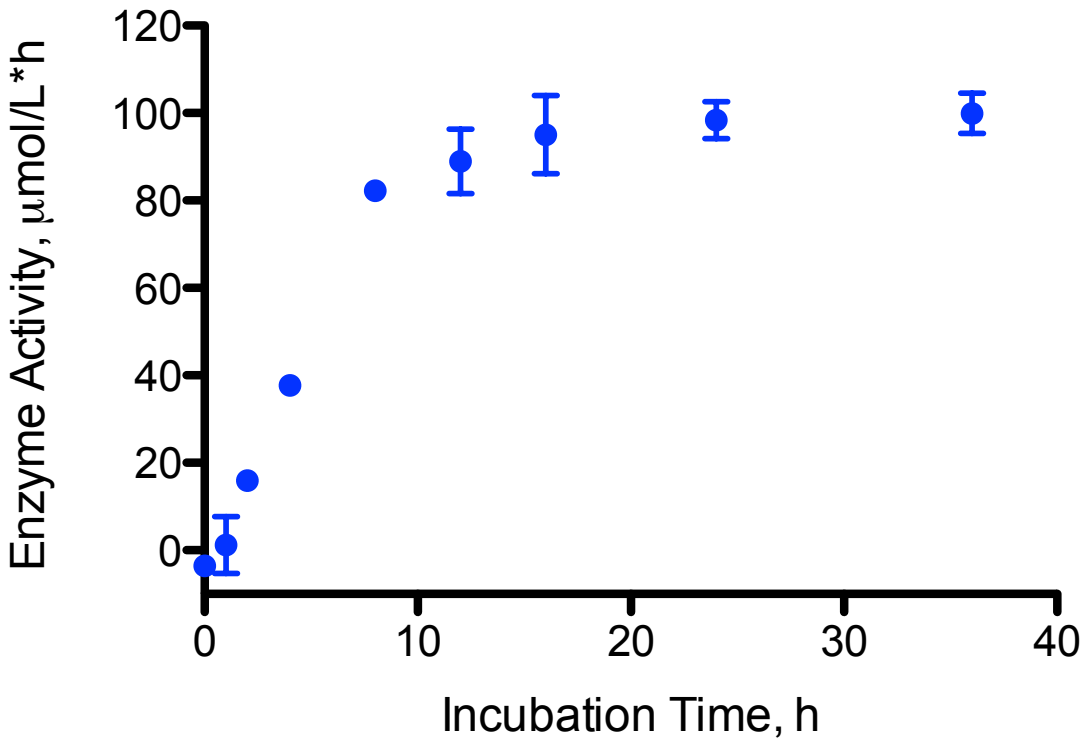


Figure 2.6. Enzyme activity as a function of the incubation time. Error bars represent one standard deviation of triplicate measurements.

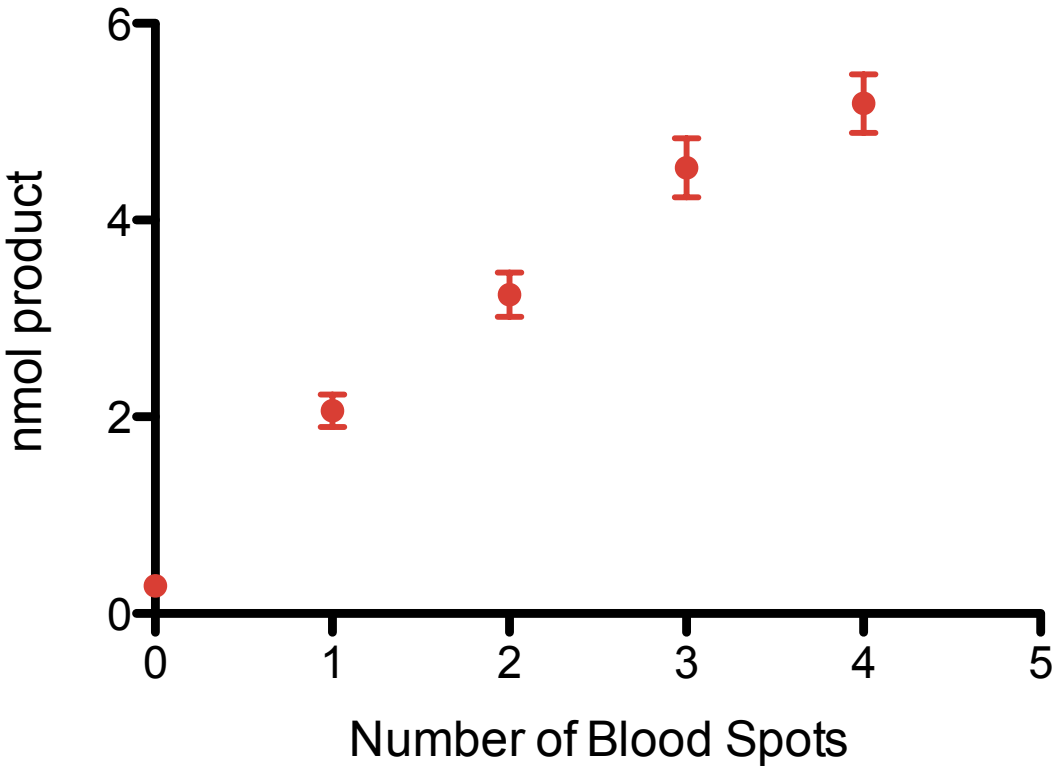


Figure 2.7. Product formed as a function of the amount of enzyme present in the assay. Error bars represent one standard deviation of triplicate measurements.

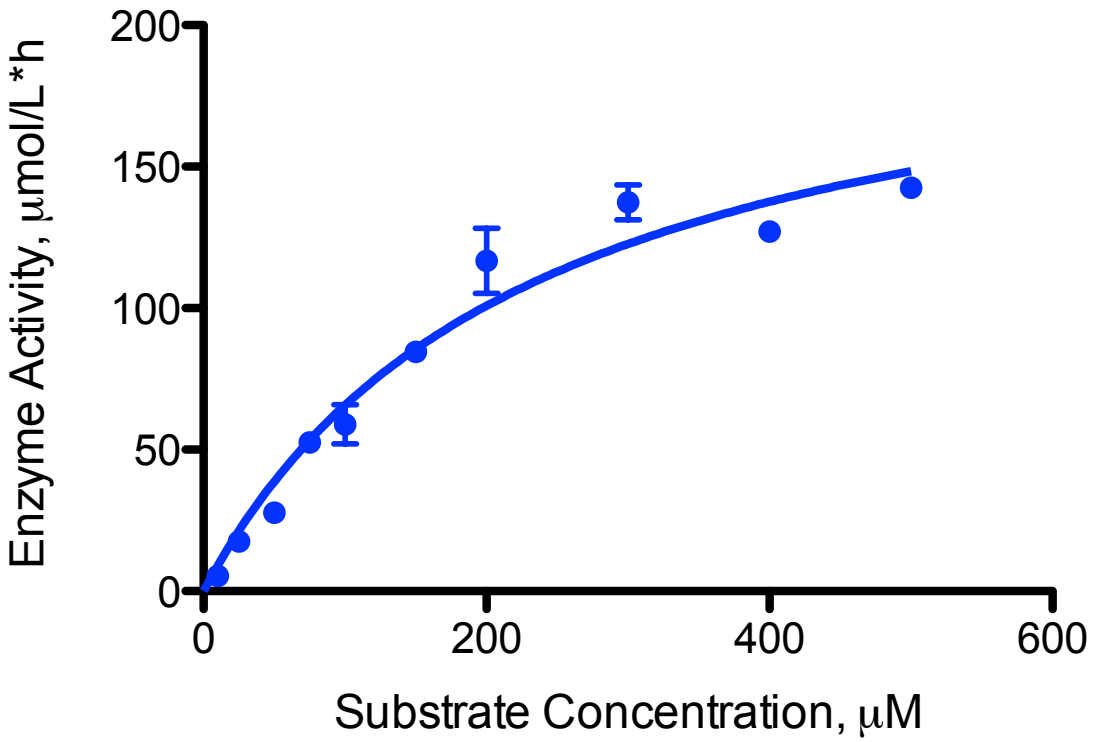


Figure 2.8. Michaelis-Menten Curve. Enzyme activity as a function of substrate concentration. Error bars represent one standard deviation of triplicate measurements.

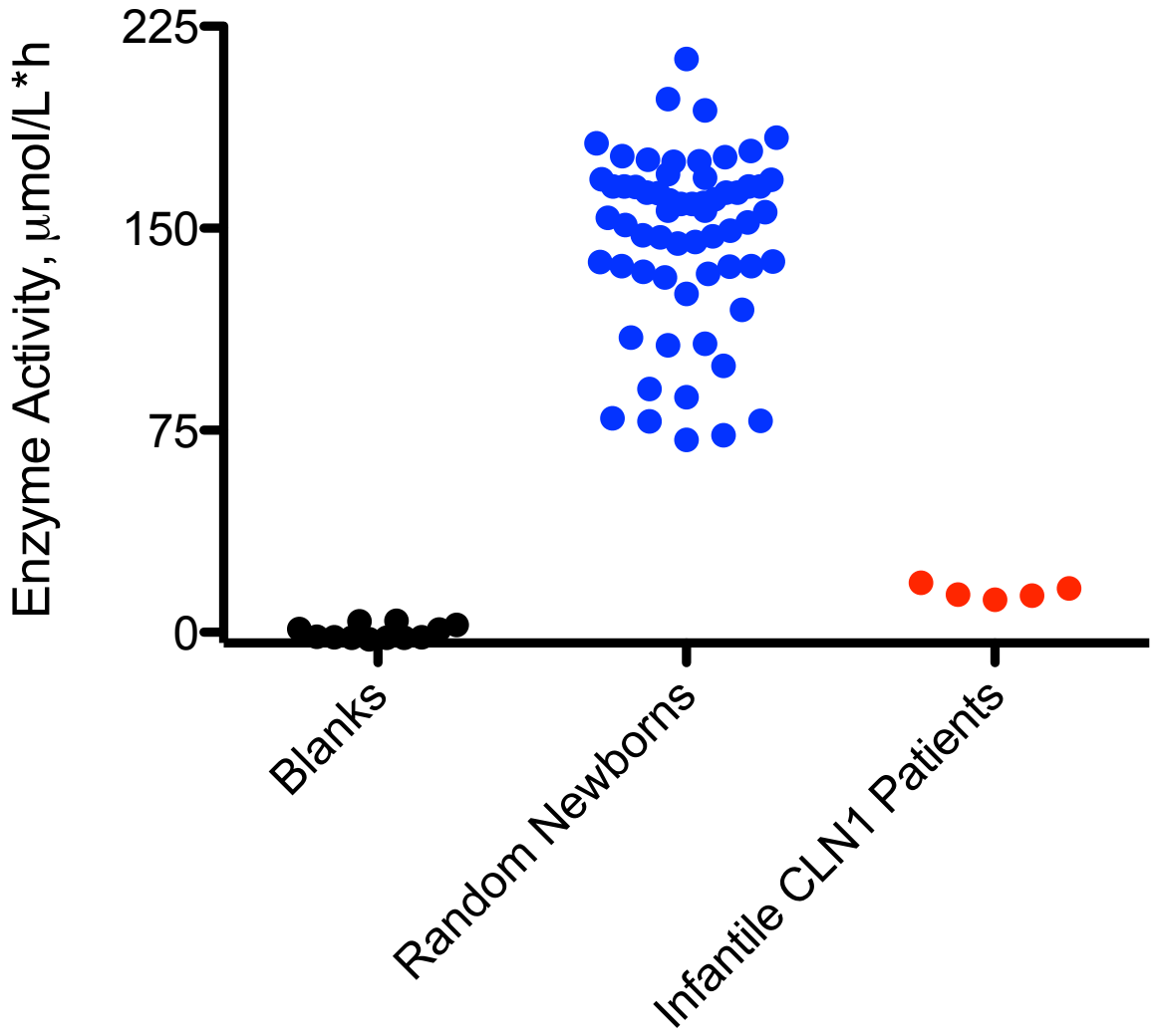


Figure 2.9. PPT1 enzyme activity of 12 blanks, 62 random newborns and 5 diagnosed infantile CNL1 patients.

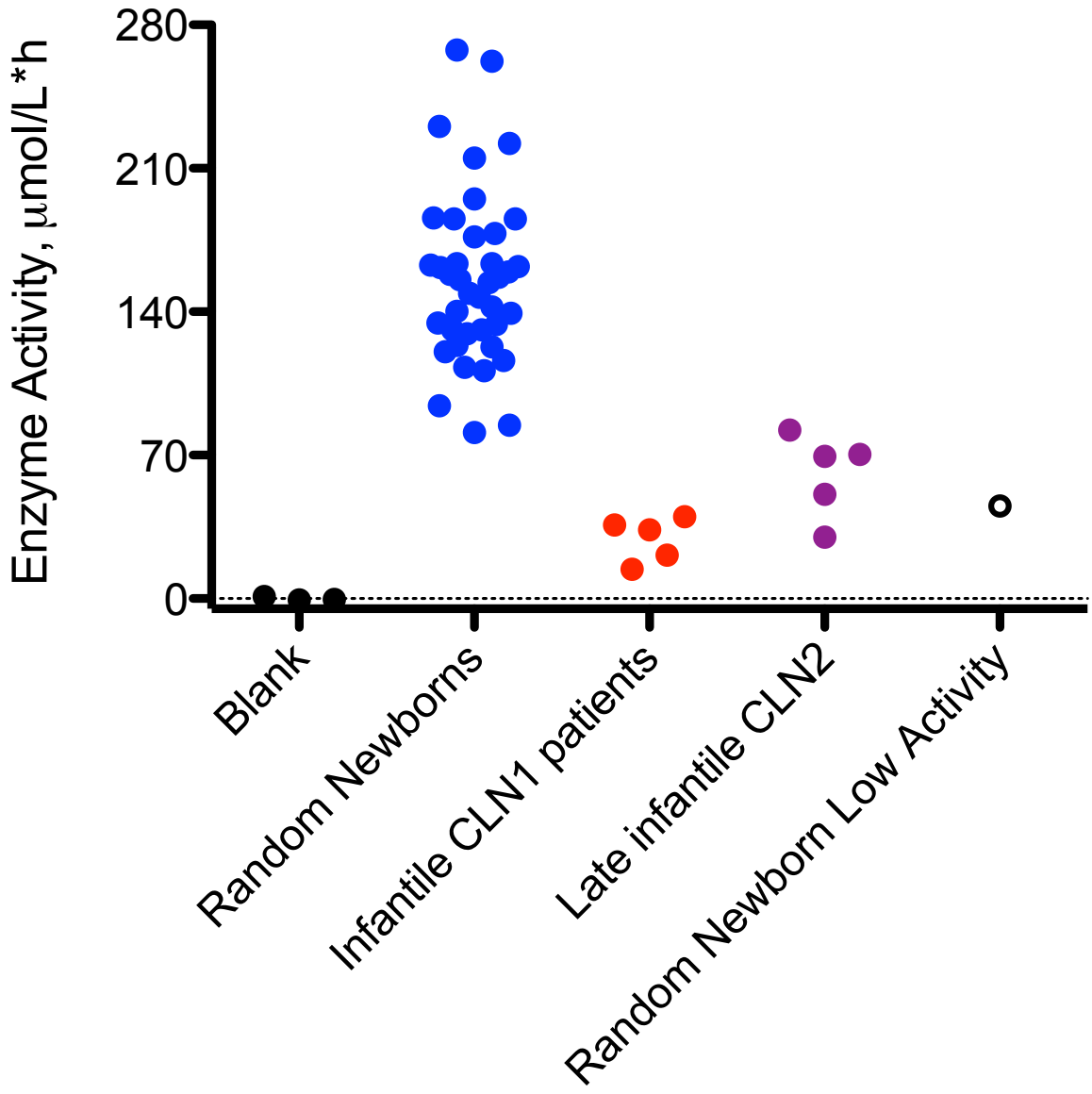


Figure 2.10. PPT1 enzyme activity of 3 blanks, 40 random newborns, 5 diagnosed infantile CNL1 patients and 5 diagnosed late infantile CLN2 patients. Duplex mass spectrometry assay.

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Chapter 3.

Sulfatide Analysis in Dried Urine and Blood Spots for the Detection of Metachromatic Leukodystrophy using Ultra High Performance Liquid Chromatrography-Tandem Mass Spectrometry.

Abstract.

Metachromatic Leukodystrophy (MLD) is a recessively inherited lysosomal storage disorder caused by a deficiency in the enzyme Arylsulfatase A (ASA) resulting in the accumulation of sulfated glycolipids in tissues. Diagnosis of MLD is complicated by the existence of an ASA pseudodeficiency with the estimated prevalence of 1-10% in the general population. Determination of urinary sulfatide concentrations is a method of choice to distinguish between healthy individuals and MLD patients, as the sulfatide levels in urine are significantly elevated in affected individuals. Presumably, an analogous correlation of the sulfatide levels in blood can be used to diagnose MLD patients. The proposed method uses dried blood spots (DBS) to determine sulfatide concentration in blood samples.

Sulfatides from dried urine and blood spots were extracted into organic solvent in the presence of internal standard added for improved precision and accuracy baseline concentration comparison. The extraction mixture was first vortexed, then centrifuged, and the supernatant was dried under a stream of nitrogen to prevent oxidation. The sample was reconstituted in methanol and injected onto the LC-MS. The sulfatides were analyzed using a UHPLC HSS T3 C18 analytical column (50×2.1 mm; 1.7 μm) as the stationary phase and a tertiary solvent system (isopropyl alcohol/

methanol/water) as the mobile phase at a flow rate of 0.4 mL/min. A Waters Xevo TQ instrument was used for tandem mass spectrometry detection.

Several sulfatide molecular species were increased in dried urine samples from all MLD samples compared to non-MLD samples. Specifically, sulfatides of low molecular weight were increased in DBS from MLD patients, but the sulfatide levels were relatively low. An acceptable separation in sulfatide levels between MLD and non-MLD samples was obtained when dried urine spots were used but not when DBS were used, because DBS from non-MLD individuals have measurable levels of sulfatides.

3.1 INTRODUCTION.

Metachromatic Leukodystrophy (MLD) is an autosomal recessively inherited lysosomal storage disorder. In 1910, German neurologist Dr. Alois Alzheimer published an article describing for the first time the metachromatic staining of the nervous system of a patient that would later be described as the suffering from the adult form of MLD.[1]

MLD has an incidence in 1 in every 100,000 live births[1-2] and is caused by a deficiency in Arylsulfatase A (ASA, EC 3.1.6.1), the enzyme generally responsible for hydrolysis of sulfate groups on glycolipids or more rarely of its activator protein saposin-B. A deficiency in this enzyme results in the accumulation of sulfated glycolipids, particularly 3-O-sulfogalactosylceramides or sulfatides (Figure 3.1). Sulfatide accumulation results in the destruction of myelin sheaths in the central and peripheral nervous systems and, to a lesser extent, it can result in accumulation in other organs such as the kidneys, gallbladder and the liver. In patients with MLD the sulfatides accumulate in the lysosomes of these tissues and are

responsible for the metachromatic staining. [1, 3]

Depending on the age of the patient at the onset of the symptoms, three different types of MLD are recognized: infantile, late infantile and adult MLD. Late-infantile MLD is the most common form and usually appears between 18 and 24 month-old infants, often shortly after a child takes his or her first steps. On the other hand, the juvenile form emerges between 4 and 16 years of age, while the adult form, which accounts for approximately 20% of all cases, is defined by the onset of the disease beyond 16 years of age.[4] In the case of late infantile MLD, death usually occurs within 5 years of the symptoms' onset. [1]

Clinical manifestations differ in each of the subtypes but they all share a common defect in the nervous system. Symptoms of late infantile MLD include gait disturbance, decreased tendon reflexes, mental regression, loss of speech, and optical atrophy and ataxia. [1]

Until recently, treatment for MLD consisted solely of supportive care, but the emergence of therapeutic alternatives like enzyme replacement therapy and hematopoietic cell transplantation bring new hopes for inherited disorders such as MLD. Currently, however, no treatment can reverse the fatal outcome of the disease and therapy is only supportive.[5] Enzyme replacement therapy has recently entered the clinical trial phase but only with still significant limitations; namely, the enzyme cannot cross the blood-brain barrier, thereby having little to no effect on the central nervous system, and it also requires lifelong administration for maximum efficiency. [5-6]

Hematopoietic stem cell transplantation proved ineffective in patients with marked neurological symptoms or with aggressive onsets of the disease, probably caused by the slow replacement of the resident tissues by the transplanted hematopoietic cell progeny compared to the rapid progression of the disease. [5-6] Gene therapy emerges as an attractive treatment option for MLD. Biffi et al. demonstrated that MLD manifestations could be prevented and corrected using a

lentiviral vector based hematopoietic stem cell gene therapy when using a mouse model. Based on this result they designed a phase I/II trial and even though their preliminary data looks promising, follow-up on the patients will be necessary to assess the full therapeutic potential of the therapy. [7] If a treatment for MLD becomes available, there is a need to develop diagnostic tests suitable for large scale screening since the success of the treatment depends on early diagnosis.

Diagnosis of MLD primarily occurs when the first symptoms start to manifest. The most useful laboratory tests are brain CT and MRI, or measurements of nerve conduction.[1] Diagnosis of MLD based on clinical manifestations requires further confirmation by biochemical assays. A widely used method is known as the sulfatide-loading test, which measures the ability of cultured cells to breakdown sulfatides.[8] This method is labor intensive and hence, not suitable for large screening.

To such end, an enzymatic assay is available and measures ASA enzyme activity in leukocytes or cultured fibroblasts using radioactively labeled sulfatide[9], synthetic non-specific substrates such as 4-methylumbelliferyl sulfate[10], or p-nitrocatechol sulfate[11]. Nonetheless, enzymatic assays face a major hurdle since a very frequent ASA allele (frequency 7.3 - 15%) is known to be associated with substantial ASA deficiency.[12]Relatedly, individuals who are homozygous for this allele have low residual ASA activity but are clinically healthy. This condition has therefore been called ASA pseudodeficiency (ASA-PD); however, the low ASA activity is sufficient to sustain a normal cerebroside sulfate catabolism.[3, 13]

Our group has attempted to develop an enzymatic assay to measure ASA activity in dried blood spots using tandem mass spectrometry. The results show that an enzymatic assay failed to work

in dried blood spots and it was concluded that some type/types of proteins or enzymes are more than likely interacting with the substrate itself.[14]

Nevertheless, another amenable method aimed at substantiating MLD diagnosis is to determine sulfatide concentration. MLD affected individuals have shown elevated concentrations of sulfatides in urine. Natowicz et al. developed a method to isolate sulfatides from urine samples and analyze them using HPLC.[15] Via this method, the sulfatides are extracted from urine and separated from glycerol-based lipids by alkaline hydrolysis, then isolated by ion-exchange chromatography, further hydrolyzed to galactosylceramide before perbenzoylation, and lastly quantified by HPLC. Whitfield [16] developed also a method to analyze urinary sulfatides, using electrospray tandem mass spectrometry. In their method, the Whitfield group extracted the sulfatide from urine using (liquid-liquid extraction) chloroform/methanol and then analyzed the samples using tandem mass spectrometry.

Furthermore, Kuchar et al.[17] developed their own method to profile sulfatide in urine samples using tandem mass spectrometry. The procedure allows isolation of urinary sulfatides by solid-phase extraction on DEAE-cellulose membranes and transportation on a dry membrane, followed by elution and tandem mass spectrometry (MS/MS) analysis in the clinical laboratory.

To our knowledge, there is no published data available on sulfatide accumulation in the blood. We therefore attempted to develop a protocol to detect and quantify sulfatide accumulation in dried blood spots, with the most common sample being available in newborn screening laboratories. To establish our method we also collected dry urine samples from affected MLD patients to compare sulfatide levels in the different biological fluids.

3.2 EXPERIMENTAL.

Materials. All water used was purified by a Millipore Milli-Q 18MΩ System. All the solvents used were technical grade. Hexane was supplied by Sigma-Aldrich and Macron Fine Chemicals provided ethyl acetate. Methanol, chloroform and all UHPLC solvents used were LC-MS grade solvents from Fisher Scientific. The C17:0 sulfatide (sulfatide with a heptadecanoyl acyl chain on the amino group of the sphingosine backbone) internal standard was obtained from Avanti Polar Lipids. Dried urine and blood samples were obtained with the help of the MLD Foundation. All experiments were conducted according to Institutional Review Board guidelines. DBS were collected by puncturing the patients' fingertips with lancet and by letting the blood drip onto filter paper, which was air dried for ~2 hrs and then mailed to the University of Washington. DBS from random, anonymous newborns were obtained from the Washington State Newborn Screening Lab after being stored at 18 °C for 8-10 mths. Urine was collected on Whatman 5 paper disks (70 mm), which were allowed to air dry for ~2 hrs at room temperature, then placed in a zip-lock plastic bag for shipment at ambient temperature. After arrival, all urine and DBS samples were stored at -10 to -20 °C in a closed jar with dessicant.

Sulfatide Synthesis. Synthesis of C24:0 sulfatide was carried out according to previously published procedures.[18-19] The synthetic procedure is summarized in scheme 3.1.

Briefly, lignoceric acid (53.7 mg; 0.146 mmoles) and N-hydroxysuccinimide (23.5 mg; 0.20 moles) were dissolved in 4 mL of a 2:1 acetonitrile: dimethyl formamide (DMF) mixture, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. EDC (28 mg; 0.146 mmoles) was later added. The mixture was allowed to react for 24 hrs at room temperature. The n-hydroxysuccinimide ester of lignoceric acid was obtained with a 54.7% yield.

For the second step, the reaction products of step one, (1), (8.0 mg; 17.2 μmoles) were combined

with psychosine (8.2 mg; 17.8 μmoles) and dissolved in 500 μL of anhydrous DMF, to which 5 μL of diisopropylethylamine were added. The reaction was allowed to proceed for 60 hrs. The product was purified by HPLC (protein & peptide C18, VYDAC, Cat nº 218TP1010), using a binary water/methanol solvent system. A gradient of 30% to 100% methanol over 30 minutes and hold at 100% for 25 minutes. Sulfation of the galactoceramide was carried out by dissolving the product of (2) (6.8 mg; 8.4 μmoles) in 4 mL of anhydrous methanol and dibutyltin (IV) oxide (3.1 mg; 12.5 μmoles) was added. The reaction mixture was heated under reflux for 2 hrs. The reaction was allowed to cool and the solvent was evaporated under vacuum. The residue was redissolved in tetrahydrofuran. Sulfur trioxide trimethylamine complex (1.74 mg; 12.5 μmoles) was added and the solution was stirred for 30 hrs at room temperature. The product was purified by HPLC using the conditions described above. The mass spectrum and NMR data of the product are shown in Figure 3.2.

Sulfatide Extraction. To evaluate the extraction yield of the sulfatides from the filter paper, a 3mm punch was spiked with C17:0 and C24:0 sulfatides. The paper punch was spiked with 100 pmoles of each sulfatide and allowed to dry for 2 hrs. The punch was then placed in a 1.5 mL polypropylene tube, and 1 mL of ethyl acetate was added to the sample. The sample was then mixed in a vortex mixer and centrifuged at 3000 rpm for 3 minutes. 0.8 mL of the supernatant was collected and dried under a nitrogen stream. The samples were reconstituted in 100 μL of methanol and subjected to analysis by the tandem mass spectrometer.

Processing of DBS and urine spots samples for analysis. 10 mm diameter punch of the DBS or urine spot was placed in a glass tube. Sulfatides were extracted using the procedure described

above. The ethyl acetate used for extraction contained 2 pmoles of C17:0 sulfatide as an internal standard.

Mass Spectrometry. Samples were analyzed using Ultra High Performance Liquid Chromatography (UHPLC) tandem mass spectrometry. UHPLC was carried out with a Waters ACQUITY system with a HSS T3 C18 analytical column (50 x 2.1 mm, 1.8 μm) with a HSS T3 (5 x 2.1 mm, 1.8 μm) VanGuard guard column (Waters Corp.). The elution solvent was water with 0.1% formic acid (solvent A) and 2- propanol/methanol (80/20) with 0.1% formic acid (solvent B). The solvent program was 82% solvent B to 92% solvent B over 1.3 min, hold at 92% solvent B for 1.7 min, all at a flow rate of 0.4 ml/min. The total run time was 3 min. Tandem mass spectrometry was carried out on a Waters Xevo TQ instrument used in negative ion mode. The injection volume was 10 μl. All mass spectrometer settings are given in Table 3.1.

3.3 RESULTS AND DISCUSSION.

Sulfatide Extraction. To determine the extraction yield of sulfatides from urine and blood spots different solvent systems were evaluated. The systems explored were 3:2 hexane/2-propanol, 2:1 chloroform/methanol and ethyl acetate. Placing the paper punch in a buffer solution to dissolve the sulfatides was also assessed for efficiency. Three mm filter paper punch spiked with sulfatides was placed in 50 μL of 100 mM Tris-HCl (pH 7.4) buffer and sonicated for 5 minutes. One hundred and fifty μL of water were then added and liquid/liquid extraction was performed using the method described above.

Results are summarized in Table 3.2. Our results show that a buffer solution doesn't aid the extraction. The chosen mix of 3:2 Hexane/2-propanol proved unsuitable for sulfatide extraction due to its low recovery (or low recoveries). Conversely the chloroform/methanol (2:1) mixture and ethyl acetate solvent were both independently effective in the sulfatide extraction from filter punches, but due to the toxicity of chloroform, ethyl acetate was selected as the extraction solvent.

Likewise, extraction yields from a paper punch spotted with 100 pmoles of C17:0 and C24:0 sulfatides and each subjected to ethyl acetate extraction were determined to be 74.1% and 76.4% respectively.

Extraction yields from urine and blood spots were also determined by spiking the punches with various amounts of C17:0 sulfatide (from 0 to 6 pmoles), and by measuring the response using tandem mass spectrometry. Figure 3.3 shows a linear curve when the response of the extracted sulfatides is plotted against the response of the sulfatides directly injected into the instrument without extraction, thereby ensuring a direct correlation between the two (or explain why it is important to know that). The slopes of the curves for urine and DBS were 0.49 and 0.56 respectively, indicating that about 50% of the sulfatides were extracted from the filter paper containing the biological fluid.

Mass Spectrometry. Sulfatides are analyzed in the negative mode using tandem mass spectrometry. All sulfatide precursor ions $[M-H]^-$ produce a common fragment ion at *m/z* 96.9 corresponding to $[HSO_4]^-$, selected as the reporter ion. Ionization of sulfatide in the positive mode was also explored and yielded a common fragment ion at *m/z* 264.2 corresponding to the same protonated dienylideneimine derived from the ceramide (Figure 3.4).[20] Different ion

polarities produce fragment ions that are independent of the acyl chain length but with different sensitivities and selectivities. We found that the negative ion mode provided better sensitivity for our measurements. Positive ion mode can be used as a confirmatory fragment since *m/z* 97 is not specific to $[HSO_4]^-$, but can also be due to $[H_2PO_4]^-$; the two cannot be distinguished from one another without performing high-resolution mass spectrometry experiments. Precursor ion and fragment masses for all the sulfatide isoforms analyzed are given in Table 3.3.

Sulfatide levels in dried urine spots. Altogether, we analyzed 14 MLD patients and 8 non-MLD individuals. All 14 MLD patients showed elevated concentrations of sulfatide in urine samples. Sulfatide levels for all molecular species detected in all 14 MLD patients are shown in Figure 3.5. Sulfatide levels for non- MLD individuals were not included in the graphs because we did not observe any elevation of sulfatide in healthy individuals. A lack of detectable urinary sulfatide species in non-MLD individuals has been previously reported.[15-17] An example for the sulfatide species C24:1-OH is shown in Figure 3.6 (third panel). A well-defined peak can be observed in the chromatogram; by contrast, no discernable peak was seen for this species when urine from a non-MLD individual was analyzed (Fig. 3.6, bottom panel). The most abundant sulfatides in the MLD patients were C22:0, C22:0-OH, C24:0-OH, and C24:1. The concentrations of these were well increased in all 14 MLD patients. The next most abundant group was C16:0, C16:0-OH, C20:0, C20:0-OH, C22:1-OH, C23:0-OH, C24:0, and C24:1-OH. Their concentrations were also well increased in all 14 MLD patients. The least abundant sulfatide species were 18:0, 18:0-OH, 26:0, 26:0-H, 26:1, and 26:1-OH. These were increased in most but not all of the 14 MLD patients. Some of the MLD patients showed no detectable MS/MS signal for a subset of these low abundant sulfatides. Sulfatide levels from all urine

samples are provided in Table 3.4. These trends are similar to those reported previously for urinary sulfatides in MLD patients.[16-17]

Sulfatide in dried blood spots. Sulfatide levels from 50 non- MLD individuals and 11 MLD patients were determined. Only 11 out of the 14 patients provided (large) enough blood spots to perform the analysis. DBS results showed a different behavior than the one observed for urine samples. Figure 3.6 (top panel) shows a well-defined peak in the ion trace for C16:0 sulfatide in DBS for a MLD patient. The second panel of the figure shows that the same sulfatide species can be detected with confidence in a non-MLD individual. In contrast with urine samples, where no discernable peaks are seen in the ion chromatogram, sulfatide species can be detected in DBS samples from unaffected individuals. Sulfatide levels from all DBS samples are summarized in Table 3.5. Another interesting result was that only low molecular weight species C16:0 and C16:0-OH were elevated in concentrations while higher molecular weight species were not. Figure 3.7 shows the levels of C16:0 and C16:0-OH sulfatides in MLD patients and non-MLD individuals. The levels of sulfatides in MLD patients are higher than in non-MLD patients but there is an apparent overlap in the sulfatide levels for some of the MLD patients and the non-MLD individuals.

3.4 CONCLUSIONS.

In this study we have confirmed earlier reports that sulfatide molecular species are significantly increased in urine from MLD patients versus non-MLD individuals. In our studies, we used adult urine samples from non-MLD individuals because we have no ready access to urine samples from newborns. We also used urine samples from MLD children rather than newborns, because

these were available with the help of the MLD foundation.

As mentioned before, because urine is not routinely collected in newborn screening laboratories and since there are no reported levels of sulfatide in blood, we analyzed the sulfatide molecular species in DBS using UHPLC/MS/MS. For these studies we used DBS from random newborns (non-MLD) obtained from the Washington newborn screening laboratory and DBS from young children with MLD (obtained with the help of the MLD Foundation). The results clearly showed that sulfatide levels are increased in some but not all of the MLD patient DBS samples compared to non-MLD individuals. However, there is significant overlap between the sulfatide levels in a subset of MLD patients and those in the non-MLD samples, so much so that the analysis of sulfatide levels in DBS seems problematic for screening of MLD. Sulfatides were detectable by UHPLC/MS/MS analysis in persons without MLD. This stands in contrast with urine samples, for which our results showed essentially no detectable levels of sulfatide molecular species, consistent with the results reported in the literature. The level of sulfatides detected in DBS from non-MLD individuals leads to a significant overlap in sulfatide levels between MLD and non-MLD samples. This overlap is expected to become problematic in large scale screening results when trying to identify a small number of individuals who are at risk to develop MLD. Another problem that arises from our findings is that sulfatide levels in DBS are low enough to make it difficult to use of positive ion mode tandem mass spectrometry to confirm the identity of the species, where the *m/z* of interest can originate from $[HSO_4]^-$ or $[H_2PO_4]^-$. Our results continue to support the use of urinary sulfatides for the diagnosis of MLD.

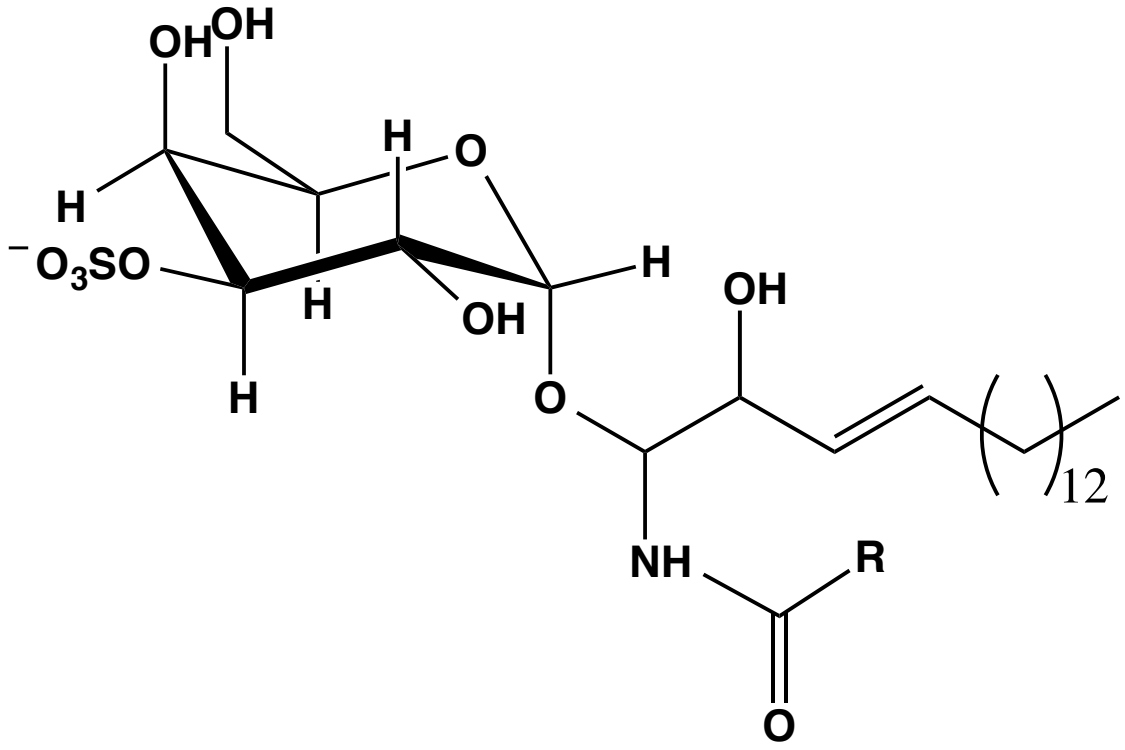
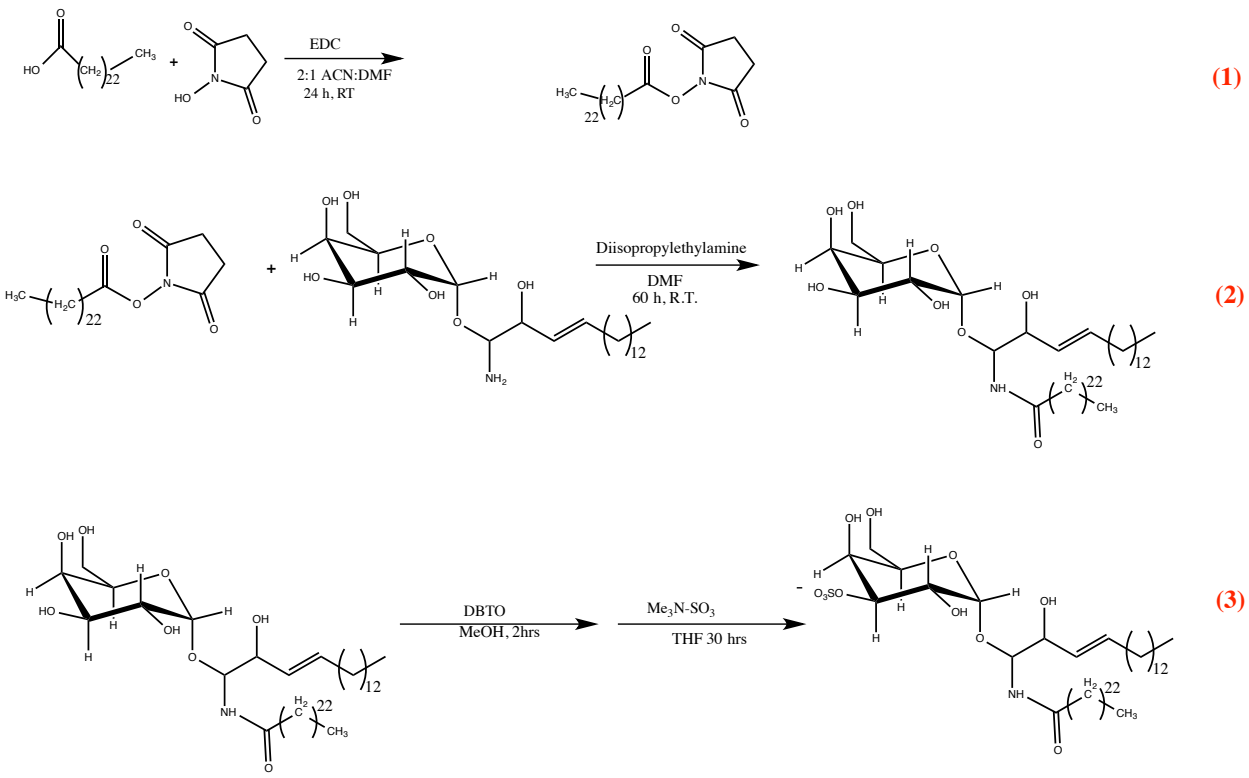


Figure 3.1. General structure of sulfatides.



Scheme 3.1. C24:0 sulfatide synthesis.

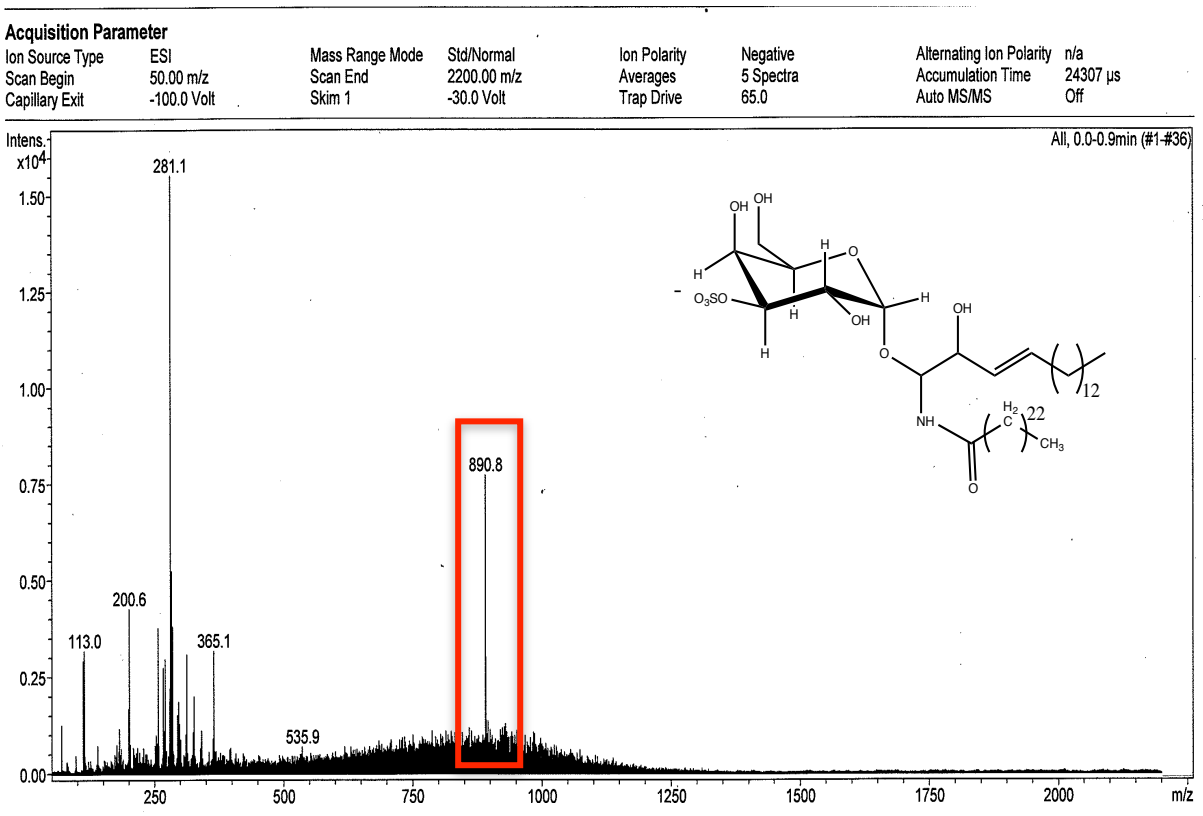


Figure 3.2. Mass Spectrum of C24:0 sulfatide in the negative mode. *m/z* 890.8 is the desired product.

Product (3): ^{1}H NMR (300 MHz, CD3OD): δ5.66 (m, 1H CH=C**H**), δ5.41 (m, 1H, C**H**=CH), δ4.32 (d, 1H, H-l), δ4.26 (m, 2H, H-3, H4), δ4.11 (dd, 1H, OCH$_a$**H$_b$**CNH), δ4.0 (dd, 1H, C**H**OHCNH), δ3.92 (m, 1H, CHNH), δ3.72 (m, 3H, H$_2$-6, H-2), δ3.59 (dd, 1H, OC**H$_a$**H$_b$CNH), δ3.56 (dd, 1H, H-5), δ2.15 (t, 2H, NHCOC**H$_2$**), δ2.0 (m, 2H, CH=CHC**H$_2$**), δ1.6 (br s, 2H, NHCOCH$_2$C**H$_2$**), δ1.2 (br s, 62H, C**H$_2$**), δ0.9 (t, 6H, C**H$_3$**)

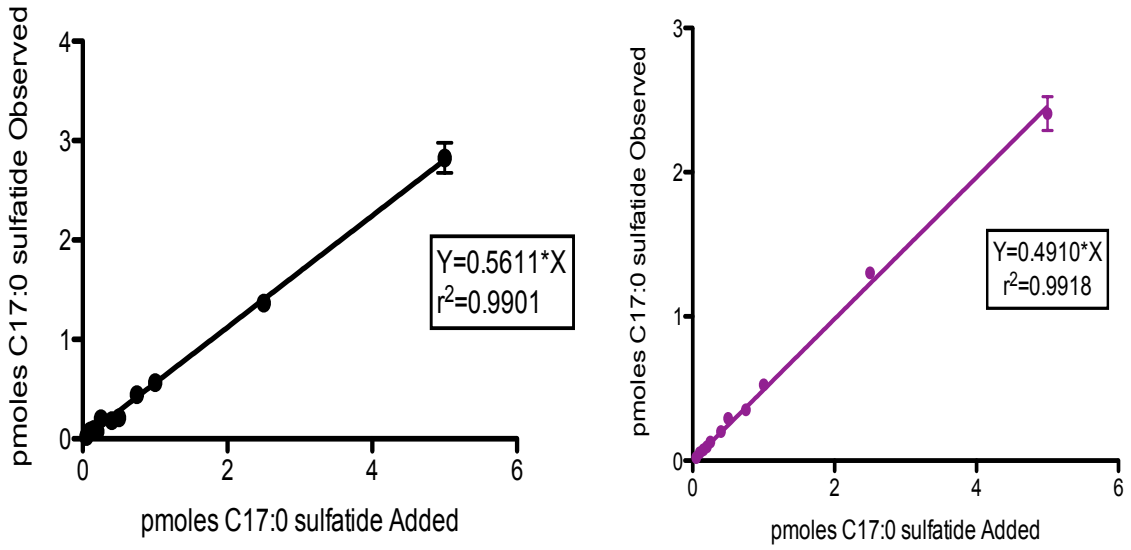


Figure 3.3. Sulfatide recovery in biological samples. DBS (left panel) and urine samples (right panel). Error bars represent one standard deviation of triplicate measurements.

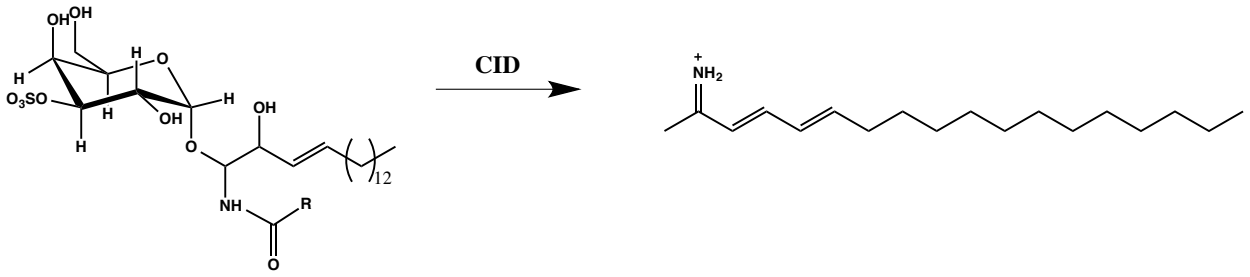


Figure 3.4. Collision induced dissociation of ceramides in positive ion mode. Common fragment ion formed at *m/z* 264.2.

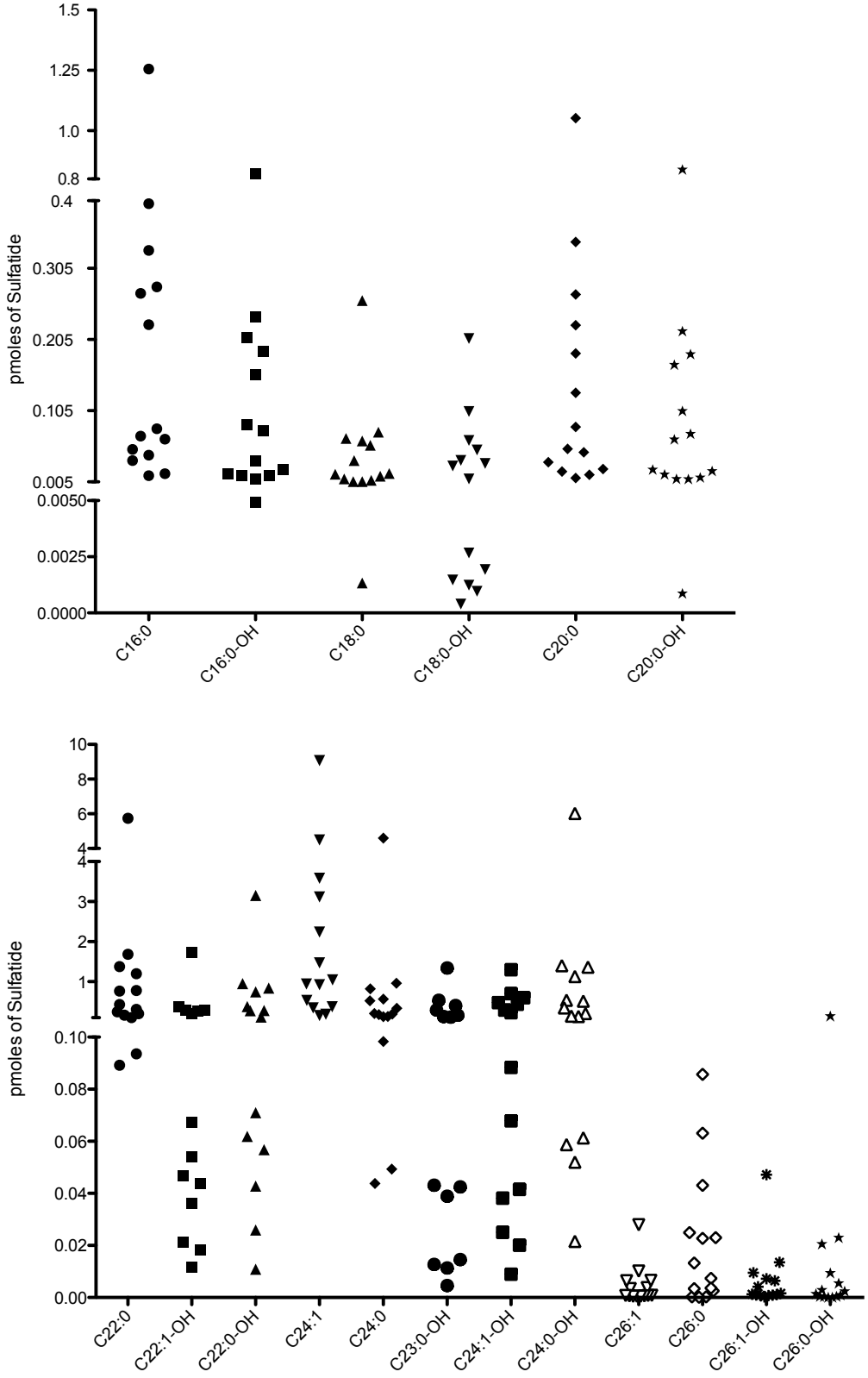


Figure 3.5. Levels of sulfatide molecular species in 14 MLD affected individuals.

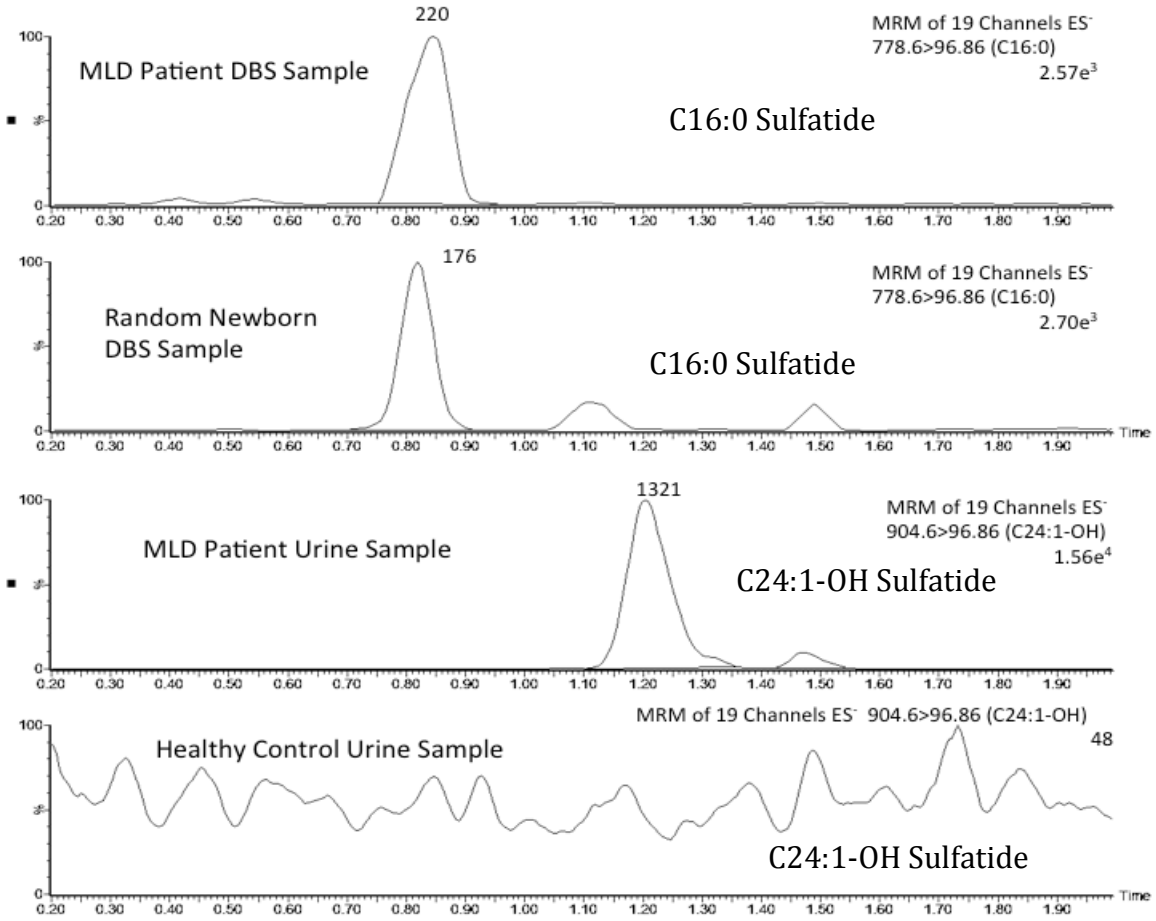


Figure 3.6. Selected ion chromatogram of the indicated species. DBS from an MLD patient (top), DBS from a non-MLD affected individual (second panel), urine from an MLD affected individual (third panel) and urine from a non-MLD individual (bottom panel). Y-axis is the relative intensity.

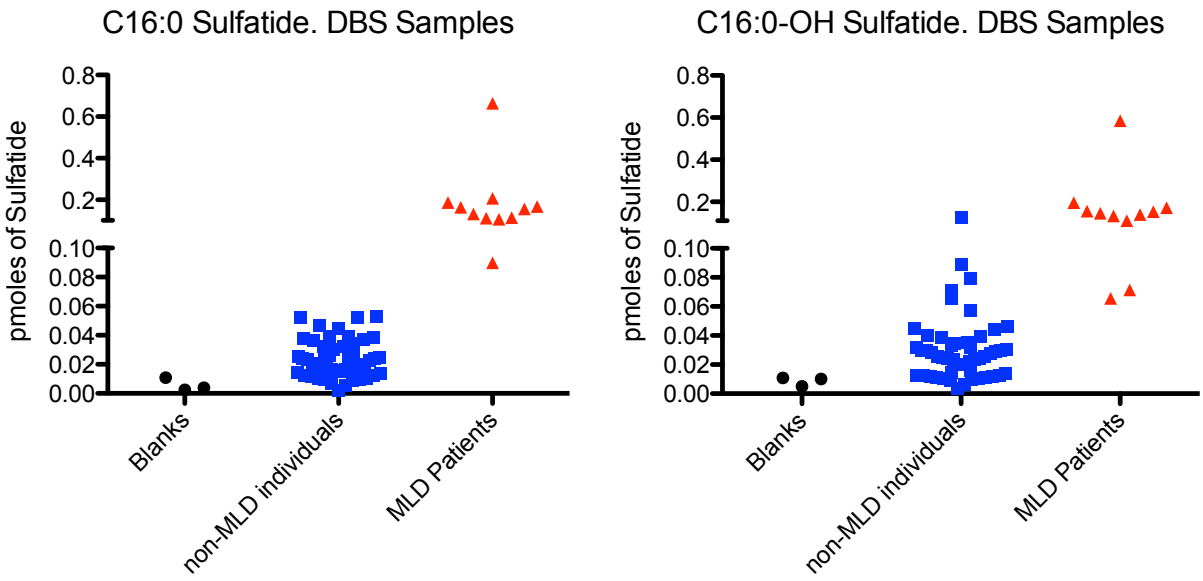


Figure 3.7. Sulfatide levels of C16:0 and C16:0-OH in DBS from 11 MLD patients and 50 DBS from non-MLD individuals. Blanks are 3 blood-free filter paper punches.

Table 3.1. Xevo-TQ MS/MS experimental parameters.

Parameter (units)	
Capillary voltage (V)	3500
Extractor (V)	3.00
Source temperature (°C)	150
Desolvation temperature (°C)	450
Cone Gas Flow (L/h)	25
Desolvation Gas Flow (L/h)	800
LM 1 Resolution	2.8
HM 1 Resolution	14.9
Ion Energy	0.8
Collision Cell Entrance Potential (V)	0.50
Collision Cell Exit Potential (V)	0.50
LM 2 Resolution	2.9
HM 2 Resolution	14.7
Ion Energy 2	1.1
Multiplier (V)	512.8
Collision Gas	Argon

Table 3.2. Sulfatide extraction yield using different organic solvents for the extraction.

Organic Solvent System	Extraction Yield (%)	RSD (%)	Buffer Addition
3:2 Hexane: 2-propanol	9.4	10.2	Yes
2:1 Chloroform: Methanol	24.4	12.2	
Ethyl Acetate	34.5	9.7	
3:2 Hexane: 2-propanol	36.5	7.4	No
2:1 Chloroform: Methanol	68.8	4.2	
Ethyl Acetate	72.9	5.4	

Table 3.3. SRM parameters for all sulfatide species.

Analyte	**SRM transition (*m/z*)**	**Cone Voltage (V)**	**Collision Energy (eV)**
C16:0 Sulfatide	778.60 → 96.86	100	62
C17:0 Sulfatide	792.477→ 96.86	100	62
C16:0-OH Sulfatide	794.60 → 96.86	100	62
C18:0 Sulfatide	806.50 → 96.86	100	62
C18:0-OH Sulfatide	822.40 → 96.86	100	62
C20:0 Sulfatide	834.70 → 96.86	100	62
C20:0-OH Sulfatide	850.60 → 96.86	100	62
C22:0 Sulfatide	862.60 → 96.86	100	62
C22:1-OH Sulfatide	876.70 → 96.86	100	62
C22:0-OH Sulfatide	878.60 → 96.86	100	62
C24:1 Sulfatide	888.60 → 96.86	100	62
C24:0 Sulfatide	890.60 → 96.86	100	65
C23:0-OH Sulfatide	892.60 → 96.86	100	62
C24:1-OH Sulfatide	904.60 → 96.86	100	62
C24:0-OH Sulfatide	906.60 → 96.86	100	62
C26:1 Sulfatide	916.70 → 96.86	100	62
C26:0 Sulfatide	918.70 → 96.86	100	62
C26:1-OH Sulfatide	932.70→ 96.86	100	62
C26:0-OH Sulfatide	934.70 → 96.86	100	62

Table 3.4. Sulfatide levels (pmoles) in urine spots. Each sample was injected in triplicates to obtain the indicated mean activity and standard deviation.

	C16:0	C16:0-OH	C18:0	C18:0-OH	C20:0	C20:0-OH
Blank_1	0.0027±0.03	0.000±0.000	0.0021±0.0022	0.0012±0.0011	0.0012 ±0.001	0.0000±0.0000
Blank_2	0.0004±0.0004	0.0004±0.0000	0.0004±0.0004	0.0001±0.0002	0.0006 ±0.0002	0.0008±0.0002
Blank_3	0.0006±0.0004	0.0000±0.0000	0.0007±0.0000	0.0004±0.0004	0.0030 ±0.002	0.0011±0.0004
Control_1	0.0003±0.0002	0.0003±0.0001	0.0002±0.0000	0.0004±0.0002	0.0005 ±0.0003	0.0003±0.0002
Control_2	0.0004±0.0004	0.0002±0.00006	0.0002±0.0000	0.0005±0.0004	0.0003 ±0.0001	0.0004±0.0005
Control_3	0.0002±0.00006	0.0002±0.0002	0.0001±0.0001	0.0003±0.0002	0.0007 ±0.0006	0.0003±0.0001
Control_4	0.0004±0.0002	0.0006±0.0002	0.0002±0.0002	0.0006±0.0005	0.0002 ±0.0001	0.0007±0.0008
Control_5	0.0002±0.0002	0.0005±0.0005	0.0004±0.0003	0.0003±0.0002	0.0002 ±0.0002	0.0004±0.0001
Control_6	0.0009±0.0006	0.0003±0.0001	0.0002±0.0001	0.0001±0.0001	0.0003 ±0.0003	0.0002±0.0002
Control_7	0.0005±0.0002	0.0003±0.0002	0.0001±0.0001	0.0005±0.0004	0.0004 ±0.0003	0.0007±0.0006
Control_8	0.0003±0.0001	0.0002±0.0001	0.0004±0.0003	0.0009±0.0004	0.0004 ±0.0003	0.0005±0.0006
MLD_Patient_1	0.3957±0.006	0.2364±0.002	0.0749±0.02	0.0631±0.01	0.3421 ±0.02	0.2169±0.03
MLD_Patient_2	0.0351±0.009	0.0134±0.003	0.0054±0.0000	0.0019±0.001	0.0105 ±0.003	0.0089±0.0002
MLD_Patient_3	1.2551±0.02	0.8221±0.06	0.2597±0.04	0.2065±0.02	1.0522 ±0.02	0.8389±0.07
MLD_Patient_4	0.2788±0.03	0.2072±0.02	0.0351±0.006	0.1040±0.02	0.1301 ±0.02	0.1695±0.04
MLD_Patient_5	0.3302±0.04	0.1883±0.006	0.0563±0.02	0.0497±0.02	0.2682 ±0.008	0.1844±0.02
MLD_Patient_6	0.0166±0.004	0.0050±0.001	0.0013±0.0008	0.0004±0.0000	0.0195 ±0.006	0.0008±0.0005
MLD_Patient_7	0.0507±0.004	0.0218±0.004	0.0071±0.001	0.0010±0.0014	0.0230 ±0.005	0.0222±0.002
MLD_Patient_8	0.0796±0.01	0.0343±0.004	0.0168±0.001	0.0091±0.003	0.0822 ±0.01	0.0202±0.002
MLD_Patient_9	0.0650±0.004	0.0767±0.006	0.0158±0.003	0.0308±0.0022	0.0513 ±0.006	0.0647±0.006
MLD_Patient_10	0.0138±0.003	0.0157±0.001	0.0091±0.002	0.0015±0.001	0.0149 ±0.002	0.0112±0.0006
MLD_Patient_11	0.0693±0.007	0.0092±0.007	0.0128±0.0068	0.0027±0.0003	0.0467 ±0.01	0.0091±0.002
MLD_Patient_12	0.0426±0.007	0.0141±0.002	0.0054±0.0007	0.0012±0.0008	0.0328 ±0.0008	0.0155±0.001
MLD_Patient_13	0.2697±0.005	0.1553±0.006	0.0625±0.02	0.0275±0.006	0.2250 ±0.07	0.1045±0.02
MLD_Patient_14	0.2257±0.02	0.0851±0.02	0.0660±0.02	0.0353±0.008	0.1855 ±0.02	0.0724±0.003

	C22:0	C22:1-OH	C22:0-OH	C24:1	C24:0	C23:0-OH
Blank_1	0.0000±0.0000	0.0000±0.0000	0.0000±0.0000	0.0110±0.02	0.0024±0.004	0.0002±0.0004
Blank_2	0.0001±0.0002	0.0007±0.001	0.00001±0.0002	0.0027±0.002	0.0008±0.0006	0.0005±0.0002
Blank_3	0.0002±0.0002	0.0005±0.0006	0.0006±0.0004	0.0233±0.02	0.0004±0.0006	0.0008±0.0006
Control_1	0.0001±0.0001	0.0003±0.0001	0.0003±0.00004	0.0095±0.003	0.0008±0.0002	0.0003±0.0003
Control_2	0.0001±0.0001	0.0002±0.0002	0.0003±0.0002	0.0054±0.006	0.0007±0.0007	0.0004±0.0002
Control_3	0.0002±0.00006	0.0003±0.0000	0.0005±0.0003	0.0047±0.001	0.0005±0.0006	0.0005±0.0001
Control_4	0.0001±0.00006	0.0002±0.0001	0.0004±0.0003	0.0029±0.002	0.0005±0.0005	0.0005±0.0003
Control_5	0.0002±0.0001	0.0002±0.0002	0.0003±0.0002	0.0048±0.002	0.0009±0.0008	0.0003±0.0002
Control_6	0.0004±0.0003	0.0005±0.0002	0.0003±0.0002	0.0065±0.003	0.0007±0.0002	0.0005±0.0004
Control_7	0.0001±0.00004	0.0006±0.0003	0.0003±0.0002	0.0037±0.003	0.0007±0.0008	0.0003±0.0003

Control_8	0.0003±0.0002	0.0010±0.0002	0.009±0.0003	0.0025±0.001	0.0004±0.0003	0.0003±0.0002
MLD_Patient_1	1.683±0.06	0.3774±0.03	0.9497±0.06	4.4894±0.09	0.9589±0.01	0.4063±0.02
MLD_Patient_2	0.0892±0.006	0.0183±0.004	0.0259±0.002	0.1853±0.008	0.0493±0.005	0.0146±0.003
MLD_Patient_3	5.7361±0.03	1.7224±0.2	3.1528±0.06	9.0666±0.1	4.5966±0.1	1.3411±0.1
MLD_Patient_4	0.7767±0.08	0.2774±0.0422	0.8405±0.1	1.4690±0.5	0.5201±0.02	0.5383±0.03
MLD_Patient_5	1.3772±0.02	0.2942±0.02	0.7479±0.01	3.5840±0.2	0.8186±0.06	0.2914±0.02
MLD_Patient_6	0.0936±0.009	0.0117±0.005	0.0109±0.002	0.1538±0.04	0.0438±0.006	0.0046±0.002
MLD_Patient_7	0.1988±0.005	0.0467±0.004	0.0710±0.004	0.3734±0.03	0.2072±0.006	0.0432±0.004
MLD_Patient_8	0.4302±0.03	0.0672±0.007	0.1186±0.01	0.9226±0.09	0.1890±0.02	0.0389±0.002
MLD_Patient_9	0.3054±0.02	0.0542±0.007	0.2740±0.01	1.0461±0.2	0.1789±0.004	0.1178±0.01
MLD_Patient_10	0.1018±0.02	0.0213±0.003	0.0619±0.0006	0.3446±0.04	0.0983±0.01	0.0426±0.003
MLD_Patient_11	0.2486±0.01	0.0439±0.007	0.0568±0.01	0.9418±0.06	0.1239±0.009	0.0127±0.003
MLD_Patient_12	0.1618±0.001	0.0362±0.003	0.0428±0.005	0.5299±0.05	0.1305±0.002	0.0114±0.002
MLD_Patient_13	1.1974±0.05	0.2739±0.007	0.3798±0.02	3.1138±0.1	0.5623±0.06	0.1608±0.05
MLD_Patient_14	0.7642±0.07	0.1892±0.02	0.2806±0.02	2.2378±0.09	0.3364±0.005	0.1312±0.02

	C24:1-OH	C24:0-OH	C26:1	C26:0	C26:1-OH	C26:0-OH
Blank_1	0.0002±0.0004	0.0000±0.0000	0.0000±0.0000	0.0000±0.0000	0.0010±0.001	0.0000±0.0000
Blank_2	0.0002±0.0002	0.0005±0.0002	0.0008±0.001	0.0005±0.0006	0.0005±0.0002	0.0010±0.0006
Blank_3	0.0010±0.0008	0.0005±0.0006	0.0005±0.0004	0.0005±0.0004	0.0006±0.0008	0.0006±0.0002
Control_1	0.0004±0.0004	0.0004±0.0003	0.0004±0.0003	0.0004±0.0003	0.0002±0.0001	0.0004±0.0003
Control_2	0.0002±0.0001	0.0002±0.0001	0.0010±0.0004	0.0009±0.0006	0.0006±0.0004	0.0002±0.0001
Control_3	0.0002±0.0001	0.0001±0.0001	0.0007±0.0007	0.0002±0.0002	0.0005±0.0004	0.0007±0.0004
Control_4	0.0004±0.0001	0.0004±0.0003	0.0007±0.0005	0.0003±0.0002	0.0005±0.0005	0.0001±0.0001
Control_5	0.0008±0.0005	0.0007±0.001	0.0007±0.0004	0.0005±0.0004	0.0010±0.0002	0.0007±0.0006
Control_6	0.0004±0.0003	0.0006±0.0006	0.0004±0.0002	0.0003±0.0002	0.0005±0.0002	0.0004±0.0001
Control_7	0.0006±0.0006	00005±0.0003	0.0008±0.0005	0.0006±0.0003	0.0006±0.0005	0.0005±0.0001
Control_8	0.0003±0.0002	0.0006±0.0007	0.0007±0.0006	0.0003±0.0002	0.0004±0.0001	0.0007±0.0005
MLD_Patient_1	0.5985±0.06	1.3625±0.06	0.0066±0.005	0.0431±0.02	0.0135±0.01	0.0205±0.01
MLD_Patient_2	0.0201±0.002	0.0613±0.01	0.0007±0.0004	0.0002±0.0002	0.0008±0.0002	0.0005±0.0006
MLD_Patient_3	1.2962±0.02	6.0179±0.1	0.0279±0.0063	0.0857±0.009	0.0472±0.006	0.1383±0.03
MLD_Patient_4	0.7015±0.1	1.3996±0.2	0.0102±0.003	0.0631±0.03	0.0073±0.002	0.0094±0.003
MLD_Patient_5	0.4322±0.08	1.1297±0.03	0.0038±0.0024	0.0249±0.009	0.0095±0.008	0.0230±0.007
MLD_Patient_6	0.0089±0.0006	0.0215±0.0009	0.0005±0.0002	0.0002±0.0002	0.0002±0.0002	0.0004±0.0000
MLD_Patient_7	0.0415±0.004	0.2046±0.01	0.0006±0.0004	0.0034±0.0006	0.0008±0.0006	0.0029±0.004
MLD_Patient_8	0.0884±0.006	0.1282±0.01	0.0005±0.0002	0.0025±0.002	0.0007±0.0004	0.0013±0.0002
MLD_Patient_9	0.4752±0.006	0.5118±0.007	0.0006±0.0006	0.0230±0.004	0.0064±0.0043	0.0010±0.0008
MLD_Patient_10	0.0678±0.005	0.1401±0.02	0.0034±0.002	0.0074±0.002	0.0012±.00009	0.0005±0.0004
MLD_Patient_11	0.0250±0.003	0.0520±0.01	0.0005±0.0002	0.0036±0.003	0.0011±0.0006	0.0001±0.0002
MLD_Patient_12	0.0381±0.003	0.0588±0.007	0.0007±0.0004	0.0001±0.0002	0.0016±0.001	0.0001±0.0002
MLD_Patient_13	0.2892±0.03	0.5352±0.06	0.0064±0.009	0.0227±0.008	0.0041±0.006	0.0055±0.003
MLD_Patient_14	0.2231±0.01	0.3448±0.02	0.0008±0.0002	0.0133±0.003	0.0010±0.0002	0.0024±0.001

Table 3.5. Sulfatide levels (pmole) in DBS. Each sample was injected in triplicates to obtain the indicated mean activity and standard deviation.

	C16:0	C16:0-OH	C18:0	C18:0-OH	C20:0	C20:0-OH
Blank_1	0.0109 ± 0.01	0.0101 ± 0.004	0.0059 ± 0.003	0.0020 ± 0.001	0.0017 ± 0.001	0.0138 ± 0.01
Blank_2	0.0039 ± 0.002	0.0109 ± 0.02	0.0032 ± 0.001	0.0012 ± 0.001	0.0054 ± 0.005	0.0094 ± 0.005
Blank_3	0.0026 ± 0.002	0.0050 ± 0.005	0.0015 ± 0.001	0.0026 ± 0.001	0.0017 ± 0.001	0.0031 ± 0.001
Control_1	0.0520 ± 0.01	0.0888 ± 0.08	0.0066 ± 0.005	0.0150 ± 0.02	0.0021 ± 0.001	0.0117 ± 0.01
Control_2	0.0525 ± 0.008	0.0396 ± 0.03	0.0242 ± 0.02	0.0056 ± 0.006	0.0200 ± 0.01	0.0079 ± 0.007
Control_3	0.0259 ± 0.02	0.0399 ± 0.01	0.0098 ± 0.01	0.0031 ± 0.002	0.0090 ± 0.006	0.0032 ± 0.002
Control_4	0.0343 ± 0.03	0.0320 ± 0.01	0.0059 ± 0.003	0.0013 ± 0.001	0.0266 ± 0.02	0.0192 ± 0.01
Control_5	0.0320 ± 0.007	0.0247 ± 0.01	0.0202 ± 0.009	0.0036 ± 0.004	0.0089 ± 0.005	0.0048 ± 0.005
Control_6	0.0168 ± 0.01	0.0341 ± 0.01	0.0587 ± 0.08	0.0016 ± 0.001	0.0114 ± 0.01	0.0051 ± 0.008
Control_7	0.0379 ± 0.004	0.0114 ± 0.003	0.0017 ± 0.003	0.0047 ± 0.002	0.0056 ± 0.001	0.0093 ± 0.004
Control_8	0.0160 ± 0.01	0.0062 ± 0.006	0.0030 ± 0.002	0.0016 ± 0.002	0.0074 ± 0.002	0.0021 ± 0.001
Control_9	0.0163 ± 0.007	0.0145 ± 0.02	0.0098 ± 0.006	0.0056 ± 0.004	0.0462 ± 0.06	0.0009 ± 0.001
Control_10	0.0103 ± 0.009	0.0203 ± 0.009	0.0144 ± 0.005	0.0058 ± 0.006	0.0034 ± 0.002	0.0112 ± 0.007
Control_11	0.0105 ± 0.01	0.0125 ± 0.01	0.0215 ± 0.02	0.0075 ± 0.01	0.0015 ± 0.001	0.0023 ± 0.002
Control_12	0.0227 ± 0.009	0.0242 ± 0.02	0.0027 ± 0.001	0.0048 ± 0.004	0.0083 ± 0.005	0.0079 ± 0.002
Control_13	0.0242 ± 0.02	0.0160 ± 0.005	0.0097 ± 0.008	0.0121 ± 0.02	0.0059 ± 0.006	0.0071 ± 0.004
Control_14	0.0463 ± 0.02	0.0124 ± 0.008	0.0079 ± 0.006	0.0050 ± 0.006	0.0146 ± 0.009	0.0058 ± 0.001
Control_15	0.0089 ± 0.004	0.0116 ± 0.007	0.0309 ± 0.03	0.0085 ± 0.006	0.0069 ± 0.006	0.0051 ± 0.002
Control_16	0.0052 ± 0.008	0.0140 ± 0.01	0.0192 ± 0.02	0.0134 ± 0.01	0.0023 ± 0.002	0.0098 ± 0.003
Control_17	0.0202 ± 0.02	0.0107 ± 0.009	0.0137 ± 0.01	0.0015 ± 0.002	0.0030 ± 0.002	0.0062 ± 0.008
Control_18	0.0179 ± 0.02	0.0098 ± 0.01	0.0160 ± 0.02	0.0102 ± 0.009	0.0054 ± 0.004	0.0036 ± 0.002
Control_19	0.0070 ± 0.010	0.0110 ± 0.01	0.0019 ± 0.002	0.0066 ± 0.003	0.0056 ± 0.006	0.0048 ± 0.001
Control_20	0.0390 ± 0.03	0.0038 ± 0.004	0.0090 ± 0.007	0.0011 ± 0.001	0.0048 ± 0.003	0.0149 ± 0.004
Control_21	0.0124 ± 0.009	0.0306 ± 0.03	0.0052 ± 0.006	0.0024 ± 0.001	0.0058 ± 0.005	0.0039 ± 0.002
Control_22	0.0095 ± 0.007	0.0117 ± 0.007	0.0019 ± 0.001	0.0052 ± 0.002	0.0141 ± 0.02	0.0032 ± 0.003
Control_23	0.0273 ± 0.02	0.0351 ± 0.008	0.0222 ± 0.01	0.0055 ± 0.003	0.0159 ± 0.02	0.0427 ± 0.05
Control_24	0.0450 ± 0.02	0.0296 ± 0.01	0.0055 ± 0.002	0.0060 ± 0.003	0.0031 ± 0.002	0.0056 ± 0.005
Control_25	0.0184 ± 0.009	0.0300 ± 0.008	0.0106 ± 0.007	0.0129 ± 0.01	0.0097 ± 0.007	0.0019 ± 0.001
Control_26	0.0373 ± 0.02	0.0244 ± 0.006	0.0046 ± 0.003	0.0044 ± 0.003	0.0153 ± 0.02	0.0247 ± 0.032
Control_27	0.0238 ± 0.02	0.0709 ± 0.02	0.0120 ± 0.008	0.0034 ± 0.003	0.0078 ± 0.006	0.0089 ± 0.008
Control_28	0.0386 ± 0.02	0.0793 ± 0.05	0.0059 ± 0.003	0.0026 ± 0.001	0.0015 ± 0.001	0.0026 ± 0.002
Control_29	0.0253 ± 0.02	0.0653 ± 0.04	0.0035 ± 0.002	0.0051 ± 0.004	0.0247 ± 0.04	0.0222 ± 0.02
Control_30	0.0527 ± 0.02	0.1244 ± 0.09	0.0028 ± 0.001	0.0086 ± 0.005	0.0017 ± 0.001	0.0026 ± 0.002
Control_31	0.0095 ± 0.007	0.0204 ± 0.03	0.0019 ± 0.001	0.0030 ± 0.001	0.0028 ± 0.004	0.0097 ± 0.006
Control_32	0.0125 ± 0.02	0.0254 ± 0.01	0.0375 ± 0.05	0.0023 ± 0.002	0.0039 ± 0.003	0.0071 ± 0.01
Control_33	0.0140 ± 0.02	0.0387 ± 0.04	0.0277 ± 0.02	0.0011 ± 0.001	0.0089 ± 0.006	0.0019 ± 0.001
Control_34	0.0114 ± 0.01	0.0285 ± 0.03	0.0196 ± 0.01	0.0035 ± 0.0002	0.0062 ± 0.003	0.0078 ± 0.004
Control_35	0.0271 ± 0.02	0.0333 ± 0.02	0.0193 ± 0.02	0.0026 ± 0.003	0.0038 ± 0.002	0.0102 ± 0.01
Control_36	0.0196 ± 0.03	0.0107 ± 0.005	0.0129 ± 0.01	0.0023 ± 0.001	0.0109 ± 0.007	0.0050 ± 0.004

Control_37	0.0167 ± 0.02	0.0287 ± 0.008	0.0077 ± 0.004	0.0019 ± 0.0002	0.0118 ± 0.007	0.0098 ± 0.005
Control_38	0.0171 ± 0.02	0.0091 ± 0.001	0.0118 ± 0.02	0.0009 ± 0.001	0.0020 ± 0.002	0.0251 ± 0.01
Control_39	0.0367 ± 0.03	0.0571 ± 0.03	0.0052 ± 0.002	0.0021 ± 0.001	0.0024 ± 0.001	0.0028 ± 0.003
Control_40	0.0391 ± 0.01	0.0274 ± 0.005	0.0138 ± 0.004	0.0019 ± 0.002	0.0193 ± 0.02	0.0028 ± 0.001
Control_41	0.0253 ± 0.02	0.0126 ± 0.01	0.0149 ± 0.004	0.0159 ± 0.02	0.0046 ± 0.004	0.0060 ± 0.003
Control_42	0.0322 ± 0.02	0.0297 ± 0.01	0.0187 ± 0.009	0.0219 ± 0.03	0.0120 ± 0.02	0.0032 ± 0.003
Control_43	0.0179 ± 0.02	0.0316 ± 0.03	0.0074 ± 0.008	0.0028 ± 0.002	0.0038 ± 0.002	0.0066 ± 0.01
Control_44	0.0250 ± 0.03	0.0236 ± 0.02	0.0040 ± 0.005	0.0012 ± 0.0004	0.0019 ± 0.001	0.0013 ± 0.001
Control_45	0.0232 ± 0.02	0.0223 ± 0.02	0.0074 ± 0.006	0.0019 ± 0.002	0.0021 ± 0.001	0.0030 ± 0.003
Control_46	0.0017 ± 0.002	0.0451 ± 0.02	0.0052 ± 0.005	0.0011 ± 0.001	0.0040 ± 0.003	0.0043 ± 0.002
Control_47	0.0144 ± 0.01	0.0238 ± 0.005	0.0056 ± 0.006	0.0005 ± 0.001	0.0016 ± 0.002	0.0109 ± 0.007
Control_48	0.0150 ± 0.02	0.0258 ± 0.03	0.0168 ± 0.01	0.0097 ± 0.01	0.0197 ± 0.02	0.0173 ± 0.02
Control_49	0.0208 ± 0.02	0.0462 ± 0.02	0.0402 ± 0.05	0.0105 ± 0.009	0.0044 ± 0.005	0.0232 ± 0.02
Control_50	0.0306 ± 0.03	0.0443 ± 0.03	0.0243 ± 0.02	0.0091 ± 0.004	0.0050 ± 0.001	0.0021 ± 0.002
MLD_Patient_1	0.0278±0.01	0.1100±0.02	0.0040±0.002	0.0001±0.0000	0.0041±0.004	0.0025±0.003
MLD_Patient_2	ND[1]	ND	ND	ND	ND	ND
MLD_Patient_3	0.6667±0.02	0.5858±0.03	0.0424±0.005	0.0025±0.001	0.0096±0.008	0.0024±0.002
MLD_Patient_4	ND	ND	ND	ND	ND	ND
MLD_Patient_5	0.1323±0.02	0.1728±0.05	0.0066±0.002	0.0017±0.002	0.0012±0.0008	0.0006±0.0006
MLD_Patient_6	ND	ND	ND	ND	ND	ND
MLD_Patient_7	0.1117±0.09	0.1528±0.1	0.0100±0.009	0.0005±0.0004	0.0021±0.003	0.0002±0.0002
MLD_Patient_8	0.1065±0.04	0.1453±0.03	0.0091±0.0055	0.0007±0.0006	0.0024±0.0023	0.0002±0.0002
MLD_Patient_9	0.1150±0.05	0.0655±0.02	0.0007±0.0004	0.0005±0.0002	0.0002±0.0002	0.0000±0.0000
MLD_Patient_10	0.0900±0.03	0.0715±0.009	0.0057±0.006	0.0004±0.0004	0.0005±0.0002	0.0013±0.001
MLD_Patient_11	0.1639±0.02	0.1394±0.006	0.0048±0.001	0.0002±0.0002	0.0053±0.004	0.0012±0.002
MLD_Patient_12	0.1567±0.01	0.1963±0.06	0.0053±0.003	0.0000±0.0000	0.0018±0.002	0.0006±0.0002
MLD_Patient_13	0.1868±0.003	0.1566±0.02	0.0148±0.006	0.0016±0.002	0.0028±0.003	0.0007±0.0004
MLD_Patient_14	0.1678±0.03	0.1330±0.01	0.0076±0.002	0.0008±0.0004	0.0012±0.001	0.0012±0.0006

	C22:0	C22:1-OH	C22:0-OH	C24:1	C24:0	C23:0-OH
Blank_1	0.0026 ± 0.002	0.0063 ± 0.007	0.0024 ± 0.002	0.0021 ± 0.002	0.0126 ± 0.007	0.0107 ± 0.009
Blank_2	0.0024 ± 0.001	0.0016 ± 0.001	0.0044 ± 0.005	0.0094 ± 0.006	0.0270 ± 0.03	0.0048 ± 0.002
Blank_3	0.0069 ± 0.002	0.0012 ± 0.001	0.0046 ± 0.002	0.0093 ± 0.008	0.0062 ± 0.005	0.0048 ± 0.003
Control_1	0.0129 ± 0.01	0.0055 ± 0.005	0.0099 ± 0.01	0.0128 ± 0.009	0.0367 ± 0.02	0.0077 ± 0.005
Control_2	0.0077 ± 0.006	0.0040 ± 0.004	0.0094 ± 0.006	0.0103 ± 0.01	0.0110 ± 0.01	0.0030 ± 0.002
Control_3	0.0064 ± 0.005	0.0095 ± 0.003	0.0173 ± 0.02	0.0290 ± 0.04	0.0246 ± 0.01	0.0184 ± 0.02
Control_4	0.0071 ± 0.005	0.0157 ± 0.02	0.0505 ± 0.08	0.0223 ± 0.02	0.0408 ± 0.02	0.0079 ± 0.001
Control_5	0.0082 ± 0.01	0.0040 ± 0.001	0.0243 ± 0.03	0.0176 ± 0.02	0.0175 ± 0.02	0.0021 ± 0.001
Control_6	0.0235 ± 0.02	0.0019 ± 0.002	0.0044 ± 0.004	0.0169 ± 0.02	0.0717 ± 0.02	0.0103 ± 0.004
Control_7	0.0050 ± 0.003	0.0074 ± 0.003	0.0395 ± 0.03	0.0036 ± 0.002	0.0188 ± 0.01	0.0060 ± 0.004
Control_8	0.0050 ± 0.006	0.0019 ± 0.001	0.0031 ± 0.002	0.0064 ± 0.004	0.0124 ± 0.01	0.0030 ± 0.001
Control_9	0.0028 ± 0.002	0.0130 ± 0.001	0.0005 ± 0.000	0.0021 ± 0.002	0.0365 ± 0.03	0.0164 ± 0.01
Control_10	0.0137 ± 0.009	0.0082 ± 0.005	0.0150 ± 0.01	0.0239 ± 0.02	0.0160 ± 0.01	0.0040 ± 0.001

Control_11	0.0074 ± 0.01	0.0075 ± 0.007	0.0060 ± 0.003	0.0030 ± 0.001	0.0262 ± 0.007	0.0101 ± 0.002
Control_12	0.0085 ± 0.01	0.0027 ± 0.001	0.0066 ± 0.005	0.0157 ± 0.01	0.0265 ± 0.04	0.0140 ± 0.003
Control_13	0.0079 ± 0.007	0.0051 ± 0.003	0.0019 ± 0.001	0.0116 ± 0.007	0.0398 ± 0.04	0.0060 ± 0.003
Control_14	0.0047 ± 0.005	0.0181 ± 0.01	0.0030 ± 0.001	0.0027 ± 0.003	0.0352 ± 0.03	0.0039 ± 0.002
Control_15	0.0027 ± 0.003	0.0130 ± 0.02	0.0032 ± 0.002	0.0157 ± 0.02	0.0621 ± 0.06	0.0062 ± 0.003
Control_16	0.0024 ± 0.002	0.0133 ± 0.005	0.0296 ± 0.03	0.0090 ± 0.003	0.0136 ± 0.02	0.0074 ± 0.008
Control_17	0.0023 ± 0.001	0.0023 ± 0.001	0.0188 ± 0.02	0.0073 ± 0.006	0.0251 ± 0.008	0.0043 ± 0.006
Control_18	0.0036 ± 0.002	0.0048 ± 0.004	0.0246 ± 0.02	0.0224 ± 0.01	0.0094 ± 0.004	0.0035 ± 0.002
Control_19	0.0050 ± 0.002	0.0154 ± 0.01	0.0058 ± 0.005	0.0034 ± 0.004	0.0074 ± 0.003	0.0044 ± 0.002
Control_20	0.0026 ± 0.004	0.0039 ± 0.004	0.0101 ± 0.002	0.0250 ± 0.03	0.0175 ± 0.02	0.0078 ± 0.006
Control_21	0.0034 ± 0.003	0.0023 ± 0.001	0.0298 ± 0.04	0.0183 ± 0.02	0.0371 ± 0.04	0.0056 ± 0.006
Control_22	0.0062 ± 0.009	0.0124 ± 0.004	0.0297 ± 0.03	0.0121 ± 0.01	0.0015 ± 0.002	0.0099 ± 0.003
Control_23	0.0071 ± 0.008	0.0016 ± 0.001	0.0134 ± 0.02	0.0171 ± 0.008	0.0085 ± 0.005	0.0064 ± 0.002
Control_24	0.0011 ± 0.002	0.0077 ± 0.008	0.0439 ± 0.05	0.0386 ± 0.03	0.0922 ± 0.08	0.0031 ± 0.001
Control_25	0.0128 ± 0.01	0.0078 ± 0.001	0.0257 ± 0.03	0.0239 ± 0.02	0.1247 ± 0.1	0.0071 ± 0.006
Control_26	0.0074 ± 0.006	0.0020 ± 0.003	0.0404 ± 0.05	0.0674 ± 0.08	0.0412 ± 0.06	0.0102 ± 0.006
Control_27	0.0059 ± 0.005	0.0048 ± 0.003	0.0117 ± 0.01	0.0038 ± 0.004	0.0528 ± 0.05	0.0106 ± 0.004
Control_28	0.0069 ± 0.006	0.0090 ± 0.006	0.0121 ± 0.01	0.0132 ± 0.006	0.0396 ± 0.05	0.0034 ± 0.001
Control_29	0.0052 ± 0.005	0.0062 ± 0.002	0.0085 ± 0.003	0.0337 ± 0.04	0.0662 ± 0.03	0.0101 ± 0.007
Control_30	0.0040 ± 0.002	0.0212 ± 0.01	0.0099 ± 0.01	0.0146 ± 0.006	0.0857 ± 0.03	0.0075 ± 0.002
Control_31	0.0058 ± 0.003	0.0059 ± 0.006	0.0023 ± 0.002	0.0024 ± 0.002	0.0017 ± 0.001	0.0013 ± 0.001
Control_32	0.0043 ± 0.003	0.0058 ± 0.002	0.0051 ± 0.004	0.0112 ± 0.008	0.0275 ± 0.04	0.0019 ± 0.001
Control_33	0.0050 ± 0.004	0.0024 ± 0.002	0.0028 ± 0.004	0.0040 ± 0.003	0.0529 ± 0.06	0.0026 ± 0.002
Control_34	0.0055 ± 0.003	0.0008 ± 0.000	0.0126 ± 0.006	0.0047 ± 0.001	0.0026 ± 0.001	0.0036 ± 0.003
Control_35	0.0042 ± 0.001	0.0199 ± 0.02	0.0118 ± 0.01	0.0434 ± 0.03	0.0222 ± 0.007	0.0157 ± 0.004
Control_36	0.0060 ± 0.006	0.0086 ± 0.006	0.0349 ± 0.04	0.0015 ± 0.001	0.0040 ± 0.002	0.0043 ± 0.003
Control_37	0.0032 ± 0.002	0.0017 ± 0.001	0.0069 ± 0.006	0.0152 ± 0.007	0.0361 ± 0.04	0.0165 ± 0.01
Control_38	0.0054 ± 0.002	0.0085 ± 0.005	0.0226 ± 0.02	0.0078 ± 0.007	0.1229 ± 0.08	0.0099 ± 0.004
Control_39	0.0020 ± 0.001	0.0085 ± 0.006	0.0031 ± 0.002	0.0079 ± 0.005	0.0408 ± 0.04	0.0046 ± 0.002
Control_40	0.0090 ± 0.004	0.0054 ± 0.002	0.0116 ± 0.004	0.0180 ± 0.007	0.0425 ± 0.05	0.0048 ± 0.004
Control_41	0.0152 ± 0.01	0.0024 ± 0.002	0.0141 ± 0.008	0.0089 ± 0.008	0.0443 ± 0.04	0.0102 ± 0.005
Control_42	0.0234 ± 0.02	0.0044 ± 0.004	0.0459 ± 0.06	0.0304 ± 0.01	0.0801 ± 0.08	0.0048 ± 0.002
Control_43	0.0044 ± 0.001	0.0060 ± 0.005	0.0019 ± 0.001	0.0008 ± 0.0004	0.0087 ± 0.008	0.0110 ± 0.007
Control_44	0.0083 ± 0.002	0.0055 ± 0.002	0.0044 ± 0.002	0.0157 ± 0.02	0.0042 ± 0.001	0.0031 ± 0.001
Control_45	0.0027 ± 0.003	0.0019 ± 0.001	0.0034 ± 0.002	0.0064 ± 0.01	0.0090 ± 0.009	0.0032 ± 0.004
Control_46	0.0028 ± 0.002	0.0016 ± 0.002	0.0056 ± 0.004	0.0120 ± 0.007	0.0048 ± 0.002	0.0062 ± 0.005
Control_47	0.0047 ± 0.001	0.0048 ± 0.001	0.0286 ± 0.03	0.0051 ± 0.005	0.0153 ± 0.01	0.0093 ± 0.010
Control_48	0.0030 ± 0.001	0.0106 ± 0.009	0.0279 ± 0.03	0.0058 ± 0.003	0.0396 ± 0.03	0.0031 ± 0.003
Control_49	0.0040 ± 0.003	0.0079 ± 0.005	0.0314 ± 0.03	0.0192 ± 0.02	0.0395 ± 0.03	0.0124 ± 0.01
Control_50	0.0206 ± 0.02	0.0099 ± 0.008	0.0825 ± 0.1	0.0165 ± 0.01	0.0171 ± 0.01	0.0181 ± 0.01
MLD_Patient_1	0.0019±0.0009	0.0035±0.002	0.0420±0.01	0.0028±0.001	0.0011±0.001	0.0023±0.002
MLD_Patient_2	ND	ND	ND	ND	ND	ND
MLD_Patient_3	0.0041±0.002	0.0085±0.002	0.0096±0.002	0.1285±0.006	0.0169±0.0009	0.0021±0.002

MLD_Patient_4	ND	ND	ND	ND	ND	ND
MLD_Patient_5	0.0012±0.001	0.0012±0.001	0.0008±0.0004	0.0073±0.0007	0.0015±0.001	0.0008±0.0008
MLD_Patient_6	ND	ND	ND	ND	ND	ND
MLD_Patient_7	0.0006±0.0006	0.0005±0.0002	0.0027±0.003	0.0494±0.004	0.0013±0.001	0.0010±0.001
MLD_Patient_8	0.0030±0.003	0.0041±0.006	0.0031±0.004	0.0154±0.007	0.0027±0.002	0.0002±0.0002
MLD_Patient_9	0.0005±0.0002	0.0005±0.0002	0.0005±0.0002	0.0021±0.002	0.0008±0.0006	0.0004±0.0000
MLD_Patient_10	0.0012±0.001	0.0007±0.0006	0.0010±0.001	0.0030±0.001	0.0006±0.0006	0.0005±0.0006
MLD_Patient_11	0.0353±0.004	0.0008±0.0002	0.0145±0.003	0.0248±0.009	0.0042±0.001	0.0036±0.005
MLD_Patient_12	0.0018±0.001	0.0017±0.002	0.0005±0.0002	0.0082±0.003	0.0006±0.0002	0.0010±0.0008
MLD_Patient_13	0.0047±0.004	0.0027±0.0006	0.0062±0.004	0.0444±0.01	0.0012±0.0004	0.0010±0.0004
MLD_Patient_14	0.0111±0.004	0.0042±0.003	0.0034±0.003	0.0285±0.01	0.0031±0.0012	0.0013±0.0006

	C24:1-OH	C24:0-OH	C26:1	C26:0	C26:1-OH	C26:0-OH
Blank_1	0.0028 ± 0.002	0.0034 ± 0.003	0.0026 ± 0.001	0.0032 ± 0.002	0.0052 ± 0.007	0.0028 ± 0.001
Blank_2	0.0051 ± 0.002	0.0058 ± 0.003	0.0019 ± 0.001	0.0031 ± 0.002	0.0019 ± 0.0002	0.0066 ± 0.006
Blank_3	0.0024 ± 0.002	0.0011 ± 0.001	0.0013 ± 0.0002	0.0036 ± 0.003	0.0095 ± 0.009	0.0030 ± 0.001
Control_1	0.0091 ± 0.003	0.0046 ± 0.001	0.0026 ± 0.002	0.0067 ± 0.002	0.0062 ± 0.001	0.0058 ± 0.001
Control_2	0.0054 ± 0.004	0.0398 ± 0.05	0.0062 ± 0.005	0.0052 ± 0.004	0.0038 ± 0.004	0.0036 ± 0.002
Control_3	0.0243 ± 0.02	0.0091 ± 0.007	0.0039 ± 0.004	0.0026 ± 0.001	0.0063 ± 0.003	0.0079 ± 0.007
Control_4	0.0101 ± 0.004	0.0044 ± 0.003	0.0425 ± 0.05	0.0056 ± 0.006	0.0073 ± 0.005	0.0032 ± 0.002
Control_5	0.0207 ± 0.03	0.0273 ± 0.035	0.0035 ± 0.003	0.0075 ± 0.006	0.0028 ± 0.002	0.0013 ± 0.002
Control_6	0.0090 ± 0.003	0.0177 ± 0.02	0.0149 ± 0.02	0.0089 ± 0.004	0.0083 ± 0.008	0.0040 ± 0.003
Control_7	0.0075 ± 0.001	0.0172 ± 0.02	0.0034 ± 0.003	0.0042 ± 0.004	0.0017 ± 0.001	0.0047 ± 0.002
Control_8	0.0046 ± 0.004	0.0102 ± 0.008	0.0007 ± 0.001	0.0021 ± 0.001	0.0021 ± 0.001	0.0047 ± 0.004
Control_9	0.0021 ± 0.001	0.0153 ± 0.01	0.0054 ± 0.007	0.0044 ± 0.001	0.0012 ± 0.001	0.0197 ± 0.01
Control_10	0.0103 ± 0.01	0.0058 ± 0.004	0.0039 ± 0.003	0.0035 ± 0.002	0.0042 ± 0.004	0.0064 ± 0.003
Control_11	0.0044 ± 0.004	0.0030 ± 0.001	0.0056 ± 0.004	0.0047 ± 0.002	0.0101 ± 0.005	0.0176 ± 0.02
Control_12	0.0026 ± 0.001	0.0031 ± 0.001	0.0039 ± 0.003	0.0028 ± 0.002	0.0099 ± 0.001	0.0021 ± 0.001
Control_13	0.0118 ± 0.01	0.0048 ± 0.001	0.0094 ± 0.006	0.0047 ± 0.003	0.0050 ± 0.003	0.0056 ± 0.004
Control_14	0.0052 ± 0.003	0.0073 ± 0.006	0.0253 ± 0.03	0.0063 ± 0.004	0.0048 ± 0.004	0.0020 ± 0.001
Control_15	0.0054 ± 0.002	0.0126 ± 0.02	0.0026 ± 0.002	0.0077 ± 0.004	0.0148 ± 0.004	0.0089 ± 0.009
Control_16	0.0017 ± 0.002	0.0019 ± 0.001	0.0074 ± 0.003	0.0017 ± 0.002	0.0090 ± 0.005	0.0040 ± 0.005
Control_17	0.0187 ± 0.01	0.0035 ± 0.003	0.0161 ± 0.02	0.0059 ± 0.002	0.0050 ± 0.003	0.0098 ± 0.003
Control_18	0.0191 ± 0.02	0.0066 ± 0.002	0.0145 ± 0.003	0.0021 ± 0.002	0.0067 ± 0.005	0.0112 ± 0.009
Control_19	0.0038 ± 0.002	0.0130 ± 0.01	0.0044 ± 0.005	0.0159 ± 0.008	0.0019 ± 0.002	0.0046 ± 0.004
Control_20	0.0048 ± 0.002	0.0085 ± 0.005	0.0099 ± 0.003	0.0020 ± 0.002	0.0101 ± 0.003	0.0048 ± 0.004
Control_21	0.0090 ± 0.009	0.0191 ± 0.006	0.0051 ± 0.004	0.0179 ± 0.02	0.0056 ± 0.003	0.0020 ± 0.001
Control_22	0.0136 ± 0.004	0.0031 ± 0.002	0.0028 ± 0.004	0.0122 ± 0.01	0.0066 ± 0.004	0.0032 ± 0.002
Control_23	0.0120 ± 0.01	0.0098 ± 0.003	0.0043 ± 0.001	0.0202 ± 0.03	0.0191 ± 0.02	0.0087 ± 0.008
Control_24	0.0075 ± 0.007	0.0282 ± 0.02	0.0046 ± 0.005	0.0129 ± 0.01	0.0113 ± 0.006	0.0109 ± 0.01
Control_25	0.0023 ± 0.001	0.0180 ± 0.02	0.0236 ± 0.02	0.0110 ± 0.006	0.0052 ± 0.003	0.0017 ± 0.001
Control_26	0.0051 ± 0.004	0.0083 ± 0.008	0.0040 ± 0.002	0.0077 ± 0.002	0.0071 ± 0.006	0.0086 ± 0.01
Control_27	0.0077 ± 0.003	0.0112 ± 0.007	0.0188 ± 0.03	0.0064 ± 0.006	0.0102 ± 0.003	0.0050 ± 0.003

Control_28	0.0234 ± 0.01	0.0044 ± 0.003	0.0023 ± 0.002	0.0024 ± 0.002	0.0024 ± 0.002	0.0114 ± 0.006
Control_29	0.0040 ± 0.003	0.0474 ± 0.05	0.0051 ± 0.003	0.0202 ± 0.01	0.0058 ± 0.002	0.0021 ± 0.001
Control_30	0.0113 ± 0.009	0.0292 ± 0.02	0.0016 ± 0.001	0.0040 ± 0.002	0.0047 ± 0.003	0.0051 ± 0.003
Control_31	0.0024 ± 0.001	0.0089 ± 0.008	0.0132 ± 0.004	0.0059 ± 0.008	0.0030 ± 0.002	0.0086 ± 0.006
Control_32	0.0062 ± 0.006	0.0098 ± 0.003	0.0016 ± 0.001	0.0083 ± 0.007	0.0067 ± 0.007	0.0028 ± 0.002
Control_33	0.0069 ± 0.004	0.0095 ± 0.001	0.0005 ± 0.001	0.0058 ± 0.003	0.0071 ± 0.002	0.0019 ± 0.002
Control_34	0.0071 ± 0.005	0.0039 ± 0.004	0.0060 ± 0.005	0.0070 ± 0.005	0.0019 ± 0.001	0.0058 ± 0.004
Control_35	0.0094 ± 0.01	0.0226 ± 0.03	0.0042 ± 0.002	0.0074 ± 0.006	0.0042 ± 0.001	0.0036 ± 0.004
Control_36	0.0109 ± 0.003	0.0146 ± 0.02	0.0038 ± 0.001	0.0083 ± 0.001	0.0024 ± 0.001	0.0120 ± 0.009
Control_37	0.0043 ± 0.003	0.0089 ± 0.01	0.0024 ± 0.001	0.0058 ± 0.005	0.0046 ± 0.003	0.0066 ± 0.002
Control_38	0.0128 ± 0.01	0.0066 ± 0.004	0.0052 ± 0.001	0.0054 ± 0.001	0.0034 ± 0.003	0.0019 ± 0.001
Control_39	0.0142 ± 0.01	0.0181 ± 0.02	0.0063 ± 0.004	0.0048 ± 0.003	0.0082 ± 0.006	0.0030 ± 0.001
Control_40	0.0020 ± 0.001	0.0513 ± 0.07	0.0058 ± 0.002	0.0093 ± 0.007	0.0066 ± 0.005	0.0036 ± 0.003
Control_41	0.0090 ± 0.005	0.0083 ± 0.009	0.0038 ± 0.002	0.0026 ± 0.001	0.0114 ± 0.005	0.0048 ± 0.002
Control_42	0.0106 ± 0.004	0.0038 ± 0.002	0.0181 ± 0.02	0.0445 ± 0.06	0.0017 ± 0.001	0.0116 ± 0.01
Control_43	0.0085 ± 0.003	0.0058 ± 0.002	0.0024 ± 0.001	0.0063 ± 0.007	0.0015 ± 0.001	0.0034 ± 0.002
Control_44	0.0085 ± 0.006	0.0027 ± 0.002	0.0011 ± 0.001	0.0019 ± 0.002	0.0050 ± 0.002	0.0064 ± 0.008
Control_45	0.0032 ± 0.003	0.0083 ± 0.005	0.0055 ± 0.004	0.0024 ± 0.002	0.0008 ± 0.001	0.0027 ± 0.001
Control_46	0.0188 ± 0.02	0.0059 ± 0.003	0.0028 ± 0.002	0.0030 ± 0.001	0.0050 ± 0.002	0.0091 ± 0.007
Control_47	0.0021 ± 0.001	0.0110 ± 0.009	0.0012 ± 0.0004	0.0153 ± 0.01	0.0071 ± 0.003	0.0016 ± 0.002
Control_48	0.0064 ± 0.004	0.0050 ± 0.004	0.0038 ± 0.001	0.0030 ± 0.002	0.0026 ± 0.001	0.0043 ± 0.007
Control_49	0.0430 ± 0.06	0.0105 ± 0.002	0.0062 ± 0.008	0.0062 ± 0.002	0.0044 ± 0.003	0.0046 ± 0.002
Control_50	0.0308 ± 0.02	0.0579 ± 0.08	0.0095 ± 0.004	0.0070 ± 0.004	0.0069 ± 0.004	0.0039 ± 0.003
MLD_Patient_1	0.0089±0.002	0.0412±0.002	0.0021±0.0008	0.0012±0.002	0.0017±0.0004	0.0006±0.0004
MLD_Patient_2	ND	ND	ND	ND	ND	ND
MLD_Patient_3	0.0121±0.003	0.0022±0.0004	0.0044±0.003	0.0016±0.002	0.0019±0.002	0.0018±0.001
MLD_Patient_4	ND	ND	ND	ND	ND	ND
MLD_Patient_5	0.0024±0.003	0.0031±0.002	0.0019±0.002	0.0006±0.0002	0.0004±0.0000	0.0005±0.0002
MLD_Patient_6	ND	ND	ND	ND	ND	ND
MLD_Patient_7	0.0025±0.003	0.0030±0.002	0.0017±0.002	0.0010±0.001	0.0006±0.0004	0.0007±0.0004
MLD_Patient_8	0.0010±0.001	0.0024±0.002	0.0008±0.0006	0.0007±0.0000	0.0007±0.001	0.0008±0.0004
MLD_Patient_9	0.0005±0.001	0.0008±0.0006	0.0005±0.0002	0.0004±0.0000	0.0000±0.0000	0.0004±0.0004
MLD_Patient_10	0.0015±0.001	0.0011±0.001	0.0004±0.0000	0.0005±0.0002	0.0002±0.0002	0.0002±0.0004
MLD_Patient_11	0.0057±0.001	0.0103±0.0002	0.0008±0.0006	0.0004±0.0000	0.0006±0.0004	0.0006±0.0002
MLD_Patient_12	0.0027±0.004	0.0004±0.0000	0.0001±0.0002	0.0008±0.0008	0.0002±0.0004	0.0004±0.0000
MLD_Patient_13	0.0028±0.002	0.0025±0.001	0.0013±0.001	0.0008±0.0006	0.0022±0.001	0.0006±0.0002
MLD_Patient_14	0.0019±0.001	0.0017±0.001	0.0006±0.0004	0.0006±0.0004	0.0004±0.0000	0.0005±0.0002

[1]ND, not determined since there was insufficient blood on the card to obtain a 1 cm punch.

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Chapter 4.

Multiplex Newborn Screening of Lysosomal Storage Diseases using Flow Injection Tandem Mass Spectrometry.

Abstract.

Lysosomal Storage Disorders (LSD) comprise a group of over 50 degenerative diseases with a combined incidence of 1 in every 5,000 to 7,000 individuals. As a group, LSDs incidence rivals that of disorders commonly screened for in the newborn screening period. Chamoles discovered that lysosomal enzymes retain activity in dried blood spots (DBS) and can be used to detect enzyme deficiencies for patients suffering from LSDs. For over a decade, our group has been developing enzyme assays based on tandem mass spectrometry for the detection of lysosomal storage disorders using DBS. Our recent efforts have been focused on developing multiplex assays.

We have developed a multiplex assay to screen for six disorders: Krabbe, Pompe, Niemann-Pick A/B, Fabry and mucopolysaccharidosis type I using a single DBS. A 3-mm punch of DBS is incubated in a common buffer cocktail containing synthetic enzyme-specific substrates and internal standards for 16 hours at 37ºC. After incubation, the assays are quenched, and products are extracted into ethyl acetate in the presence of internal standards. Our results demonstrate that solid phase extraction is not required. The organic solvent is dried down and reconstituted in 100 μL of infusion solvent (80:20 methanol/water) and analyzed on a Waters Xevo tandem quadrupole instrument using electrospray ionization and selected reaction monitoring (SRM) in positive ion mode.

Assay protocols were tested using quality control DBS obtained from the center for disease control. Our results show substantial differences for blood and assay blanks. Blood to no blood ratios ranged from 10.89 for Gaucher disease to 46.11 for Fabry disease.

The use of new reagents for Krabbe, Gaucher and mucopolysaccharidosis I assays was also studied. Our results showed an improvement of the assay performance. Blood to no blood ratios ranged from 20.6 for Gaucher disease to 244 for mucopolysaccharidosis I.

The developed 6-plex assay can be further expanded to screen for more disorders.

4.1 INTRODUCTION.

Lysosomal storage disorders (LSDs) comprises a group of autosomal recessively inherited disorders.[1] LSDs are caused by a deficiency in a lysosomal enzyme. Different disorders have different onsets and symptoms but they all share the abnormal accumulation of material in the cells.[2-4] Therapy for a group of lysosomal storage disorders has become available in the recent years. Enzyme replacement therapy has been successfully used in Gaucher (acid ß-glucocerebrosidase deficiency, ABG) disease type 1 and Fabry disease (acid α-galactosidase deficiency, GLA) to alleviate clinical symptoms.[5]

Enzyme replacement therapy (ERT) has been registered for Gaucher disease, Fabry, Pompe (α-glucosidase deficiency, GAA), and Mucopolysaccharidoses (MPS) type I (α-L-iduronidase deficiency, IDUA), II (iduronate-2-sulfatase deficiency, ID2S) and VI (N-acetylgalactosamine 4-sulfatase deficiency, GAL4S). ERT for MPS-VIA (N-acetylgalactosamine 6-sulfatase, GAL6S), metachromatic leukodystrophy (arylsulfatase A deficiency, ASA), Niemann-Pick A/B (acid sphingomyelinase deficiency, ASM), and Wolman (lysosomal acid lipase, LAL) disease are in different stages of clinical evaluations. [4, 6]

Hematopoietic stem cell transplantation (HSCT) has been proposed as a therapy for Krabbe (ß-galactocerobrosidase deficiency, GALC), but it has only been beneficial to patients diagnosed at birth.[5] HSCT is only used in a subset of patients with MPS-I, metachromatic leukodystrophy and Krabbe and is in clinical trials for Niemann-Pick A/B (acid sphingomyelinase deficiency, ASM).[4]

Additionally, gene therapy and chaperone therapies have been suggested and investigated for other disorders, leading way to several clinical trials. [1-2, 4, 6-7]

It is worth noting that LSDs treatments are more successful when initiated before any symptom manifestation; therefore, early diagnosis is desired. The existence of therapeutic alternatives has made LSDs candidates for newborn screening programs.[5, 8] New York State currently offers newborn screening for Krabbe.[9]

Nowadays, the use of tandem mass spectrometry as a tool in newborn screening for inborn errors has become routine practice.[10-11]

Chamoles et al.[12-13] were the first to report that lysososmal enzymes retain activity in dried blood spots and hence used them to detect enzyme deficiencies using fluorometric methods. Our group has been developing enzymatic assays to detect LSDs using dried blood spots as the enzyme source using tandem mass spectrometry. The method consists in designing a synthetic substrate for each of the enzymes of interest along with a respective internal standard, and monitoring product formation using tandem mass spectrometry. Each compound would have a unique mass, which would allow us to combine several assays in a single mass spectrometry injection (multiplexing).

Zhou et al. developed an assay to screen for Niemann-Pick A/B.[14] In 2004 Li et al. developed an assay to screen for Krabbe disease using dried blood spots. [15]

Li et al.[16] later expanded their assay to screen for five disorders, Krabbe, Niemann-Pick A/B, Gaucher, Fabry and Pompe. This first multiplex assay employed five different incubation buffers, one for each disorder; samples were combined after sample workup that included solid phase extraction. Further work on multiplexing from our group include a triplex assay to detect Fabry, Pompe and MPS-I[17] using flow injection analysis that eliminated the use of solid phase extraction. This triplex assay was used to carry out a pilot study in the Washington State newborn screening lab where more than 110,000 anonymous newborn samples were screened, yielding results that showed that the technique had a positive predictive value equal to those of the current methods used to detect lysosomal storage disorders.[3]

The triplex assay development triggered an effort to expand our work and include LC-MS/MS platforms. The above-mentioned triplex was performed using LC-MS[18] and was later expanded to screen for nine disorders (Fabry, Pompe, Krabbe, Niemann-Pick A/B, Gaucher, MPS-I, MPS-II, MPS-IVA, and MPS-VI) using a dual column UHPLC-MS/MS system.[19]

In a continuous effort to develop assays that are fully compatible with the newborn screening laboratories, and to address their concerns, we decided to develop an alternative flow injection analysis method to screen for the nine disorders included in our previous UHPLC-MS/MS method. Here we report on both the development of a 6-plex FIA-MS/MS assay (Fabry, Pompe, Gaucher, Krabbe, Niemann-Pick and MPS-I), a simplified procedure that eliminates solid phase extraction, and on the development of new reagents that improve the sensitivity of the assay.

4.2 EXPERIMENTAL.

Materials. Ammonium formate, sodium taurocholate hydrate and acarbose were purchased form Sigma-Aldrich. Ethyl acetate was purchased from Macron Fine Chemicals. Methanol and all other solvents Optima LC-MS grade were purchased from Fisher Scientific. Substrates (S) and internal standards (IS) for the enzymatic assays of α-glucosidase (GAA), α-galactosidase (GLA), α-L-iduronidase (IDUA), acid sphingomyelinase (ASM), β-glucocerebrosidase (ABG), and β-galactocerebrosidase (GALC) were obtained from the center for disease control (CDC, Atlanta). A second generation of substrates and internal standards for ABG (ABG-C5-S; ABG-C5-d3-IS), GALC (GALC-C7-S; GALC-C7-d5-IS), IDUA (IDUA-acetyl-C6; IDUA-acetyl-C6-d5-IS), and one internal standard for ASM (ASM-d5-IS) were all synthesized in the Gelb laboratory. [14]
Quality control dried blood spots (DBS) were obtained from the CDC. DBS were stored at -20 °C in zip-lock plastic bags (one bag sealed inside a second bag). Zip-lock bags were kept in a sealed plastic box with desiccant.

Stock solutions. Ammonium formate buffer (100 mM, pH 4.4) was prepared in deionized water and the pH was adjusted using formic acid (using a pH meter and two calibration buffers, pH 4.0 and 7.0).

Buffer additives were prepared as follows: sodium taurocholate hydrate stock solution was prepared in ammonium formate buffer, final concentration 100g/L. Acarbose stock solution was prepared in deionized water (0.8 mM). All buffer and additives solutions are stored at 4ºC.

The S/IS vials received from the CDC were dissolved in methanol and subsequently kept at -20ºC, GAA (6 mL), GLA (10 mL), IDUA (6mL), GALC (4mL), ABG (4mL), ASM (4mL). The

Gelb laboratory's substrates and internal standards were accurately weighted using an analytical balance and stock solutions were prepared by dissolving in methanol and stored at -20ºC.
New reagents for GALC, ABG, ASM, and IDUA were carefully weighted and stock solutions were prepared in methanol and stored at -20ºC.

6-plex assay cocktail preparation. The assay buffer is prepared by adding 1/10 of sodium taurocholate hydrate stock solution, 1/100 of acarbose stock solution and ammonium formate buffer, pH 4.4. Appropriate amounts of the S/IS stock solutions for all six enzyme assays were combined in a glass capped vial, dried down under a stream of nitrogen, and reconstituted in the buffer cocktail. The assay mixture contains 200 µM GAA-S, 2.0 µM GAA-IS; 600 µM GLA-S, 1.2 µM GLA-IS; 500 µM IDUA-S, 3.5 µM IDUA-IS; 150 µM ASM-S; 2.7 µM ASM-IS; 450 µM GALC-S, 2.8 µM GALC-IS; 300 µM ABG-S, 5.9 µM ABG-IS; 8.0 µM acarbose, and 10 g/L sodium taurocholate in ammonium formate, pH 4.4.

Assay Protocol. A 3 mm DBS punch is placed in each well of a 96-well microtiter plate (1 mL deep well plate from Corning Costar, Fisher Scientific), and 30 µL of 6-plex assay cocktail is added. The plate is sealed with sealing film (AxySeal, VWR International). The sealed plate is placed in the incubator at 37 °C with orbital shaking at 250 rpm for 16 hrs.
After incubation, 100 µL of a 1:1 mixture of ethyl acetate/methanol was added to each well. Additional 400 µL ethyl acetate and 200 µL water portions were added to each well and mixed. Plates were covered with a sealing film and centrifuged at 3000 rpm for 5 min at room temperature. Aliquots (200 µL) of the organic phase were removed from the plate and transferred to a new 96-well plate (0.5 mL, Axygen Scientific, VWR International), the solvent was

evaporated under a nitrogen stream, and the samples were reconstituted in 100 µL of the infusion solvent. The plates were covered with aluminum foil and subjected to analysis.

Mass Spectrometry. Samples were analyzed using flow injection tandem mass spectrometry. The elution solvent was an 80:20 methanol/water mixture with 5mM ammonium formate. The flow stream from the autosampler at the time of injection is 0.3 mL/min. After sample injection, the flow rate is dropped to 0.2 ml/min for 0.1 min, then dropped to 0.03 ml/min for 0.15 min, held at 0.03 mL/min for 0.65 min, then raised to 0.4 mL/min at 0.9 min, and finally raised to 0.3 mL/min at 1 min. The total analysis time is 1.5 minutes. Tandem mass spectrometry was carried out on a Waters Xevo TQ instrument in positive ion mode and selected reaction monitoring (SRM). Injection volume was 10 µl. Mass spectrometer settings are given in Table 4.1.

4.3 RESULTS AND DISCUSSION.

Signal suppression. Single buffer incubation for six disorders was first tried in our lab by Spacil[19]. Because the assays were analyzed by LC-MS/MS, signal suppression is not a problem. Nonetheless, using flow injection analysis arises concern on the effects that the presence of substrates, products and internal standards might have on one another. Orsini et al.[20] performed a 4+1-plex (GAA, GLA, GALC, and ABG in one buffer, and ASM incubated separately) and reported the interference of ABG substrate in the quantitation of ABG and GALC product to internal standard ratios; consequently, solid phase extraction was used to remove the substrate.

We prepared a series of solutions of products, internal standards and substrates in order to investigate mutual signal suppression. Firstly, solutions of the product in estimated assay

concentration were prepared and injected individually; secondly, a mixture of all products was analyzed. Additionally, a third mixture including all internal standards was analyzed, followed by one last mixture containing substrates, products and internal standards. Our results show that there is no suppression of product signal due to the presence of other products and/or internal standards and that the absolute signal intensity for the products decrease when substrates are present in the mixture (Figure 4.1a). Loss of signal intensity for the products is tolerated because the internal standards are expected to all exhibit the same behavior, thereby providing an acceptable baseline. In fact, products to internal standards ratios used to calculate enzyme activity remain within 10% of their value when comparing mixtures with and without substrates (Figure 4.1b). We found no evidence of complete depletion of product signal due to the presence of substrates in the mixture.

Assay and sample work-up. Buffer pH values for the individual assays (GAA, GLA, GALC, ABG, ASM, IDUA)[14, 16, 21-22] range from 3.6 to 5.5. A common buffer for all enzymes, ammonium formate, pH 4.4, was used to incubate our assays based on previous work by Duffey[17] and Spacil[19]. Sodium taurocholate is required to help dissolve the lipophilic substrates (GALC, ABG and ASM)[16, 21]. Acarbose is used to selectively inhibit maltase glucoamylase, an enzyme that interferes with our Pompe assay.[16, 23]

In an effort to improve solubility of the substrates in assay buffer GALC-C7-S and ABG-C5-S substrates and their respective internal standards were designed and synthesized.

A new internal standard for ASM (ASM-d5-IS), a deuterated analog of the enzymatic product was introduced to the assay to replace the CDC ASM internal standard.

New IDUA substrate was designed to include a bis-amide unit similar to the Pompe (GAA) substrate, a compound that has higher protonation efficiency and, as a result, lower detection limits.[18, 24] Therefore, by redesigning the substrates we hoped to improve the sensitivity of the assay. Structures for all substrates, products and internal standards are compiled in Figures 4.2 and 4.3.

Furthermore, previously reported multiplex methods[8, 20] employ solid phase extraction (SPE) to ensure all salts and other buffer components are not injected into the mass spectrometer.

We compared our liquid/liquid extraction protocol described above with the SPE method proposed by Orsini et al.,[20] where the samples are redissolved and passed through a filter plate pre-packed with silica following ethyl acetate extraction. Our results show no improvement of the calculated enzyme activity when using SPE versus liquid/liquid extraction (Figure 4.4). Consequently, a simplified workup method using liquid/liquid extraction was chosen to carry out our experiments.

Mass Spectrometry. All the designed products and internal standards were protonated in the mass spectrometer, forming singly charged positive ions. Common structural features in the reagents allow separating the fragmentation pattern into two groups: t-butyl containing carbamates (GLA, GAA, IDUA) readily loose isobutylene and carbon dioxide[24] while ceramides (ASM, GALC, ABG, GALC-C7-S and ABG-C5-S) form an iminium ion containing the sphingosine moeity[16]. The major fragmentation for IDUA-acetyl-C6-S is shown in Figure 4.3. Fragmentation of IDUA-Acetyl-C6-Product and its internal standard is analogous to that of the substrate. The monitored mass transitions are summarized in Table 4.2. Enzymatic activity was

calculated as μmol/(h*L of blood) from the ratio of product to internal standard, using the following formula:

$A_e = (P/IS)*[IS]*V_a/(3.2*t_i)$

where P/IS is the observed ratio of product to internal standard from the ESI-MS/MS, [IS] is the concentration of internal standard in micromoles, V_a is the assay volume in microliters, t_i is the incubation time, and the volume of blood, 3.2 μL, was calculated from the estimated volume of a blood spot (10 μL) and from the punch/DBS area ratio.

In-source substrate fragmentation of substrate to yield the enzymatic product in the mass spectrometer was evaluated. In an effort to minimize substrate decomposition, we focused on four parameters of interest: capillary voltage, desolvation gas flow, desolvation and source temperature Solutions of assay-relevant concentrations substrate and internal standard were used while monitoring product channels in an attempt to minimize the background generated by substrate decomposition. Capillary voltage of 2.5, 3.0 and 3.5 kV were evaluated. The effect on substrate decomposition was less then 5 % for all the 6-plex substrates. A capillary voltage of 3.0 kV was selected. Desolvation gas flow also proved to have a minimal effect, and a gas flow of 450L/h was selected for further use.

Desolvation temperatures of 150, 250 and 500ºC were considered. Lowering the temperature to 150ºC reduced in-source fragmentation by 60% or more for all analytes, with the exception of IDUA, which remained roughly constant across all temperatures considered (Figure 4.6). Given these results, 150ºC was chosen as the desolvation temperature.

Source temperature had a major impact on substrate decomposition; the temperatures considered were 90ºC and 150ºC. Our results demonstrated that decreasing the temperature to 90ºC reduces

in-source fragmentation by 10% on ASM, 40% for IDUA, 54% for GAA, 78% for GLA, 90% for ABG, and 95% for GALC (Figure 4.7).

Sample analysis. The assay was tested on quality control DBS obtained from the CDC. Blanks combining all the components of the assay but replacing the DBS punch with a filter paper punch were also analyzed. Results are summarized in Table 4.3. The assays showed a clear distinction between assay samples and blanks for the six disorders studied, where the lowest blood to no blood ratios belonged to ABG and GALC, with ratio values 10.89 and 11.61 respectively; this may be due to the poor solubility of the substrates in the assay buffer. We hypothesized that improving the solubility of the substrates for these two enzymes will improve the performance of the assay.

Second-generation substrates assay. Consequently, a new set of substrates and internal standards were designed for GALC and ABG and synthesized with the goals of improving the solubility of the reagents and the overall assay performance. As previously mentioned, a new substrate and internal standard was also designed for IDUA. Results of the 6-plex assay using the new reagents are presented in Table 4.4. Blood to no blood ratios for the new substrates showed some improvement when compared to the previous results discussed above. Shorter acyls chains helped deliver more substrate into solution and thus the enzyme activity improved. IDUA's new substrate not only provided enhanced mass spectrometry sensitivity, but also ease of synthesis and purification, where the background interferences are therefore lower and the blood to no blood ratio is greatly increased.[25]

4.4 CONCLUSIONS.

We have developed a multiplex assay to screen for six disorders (Pompe, Fabry, Niemann-Pick A/B, Krabbe, Gaucher and MPS-I), which include a simple liquid-liquid extraction, a single incubation and flow injection analysis, all from a single 3-mm DBS punch. First and foremost, our multiplex 6-plex assay protocol is fully compatible with settings commonly used in newborn screening laboratories and our results showed a substantial difference in blood to no blood ratios for all the disorders analyzed.

Moreover, we have introduced a new cassette of substrates to improve the assay performance, the results from which showed that blood to no blood ratios were improved when compared to the original set of substrates provided by the CDC. The second-generation substrates have an improved solubility, a difficulty resolved from the previous substrate, and enhanced mass spectrometry sensitivity for IDUA. A pilot study using our improved 6-plex assay is currently being planned in the Washington State newborn screening laboratory.

The use of mass-orthogonal enzymatic products and internal standards for selected reaction monitoring and quantitation of enzyme activities and the high specificity of mass spectrometry offers the opportunity of further expanding our multiplex assay to detect more disorders.

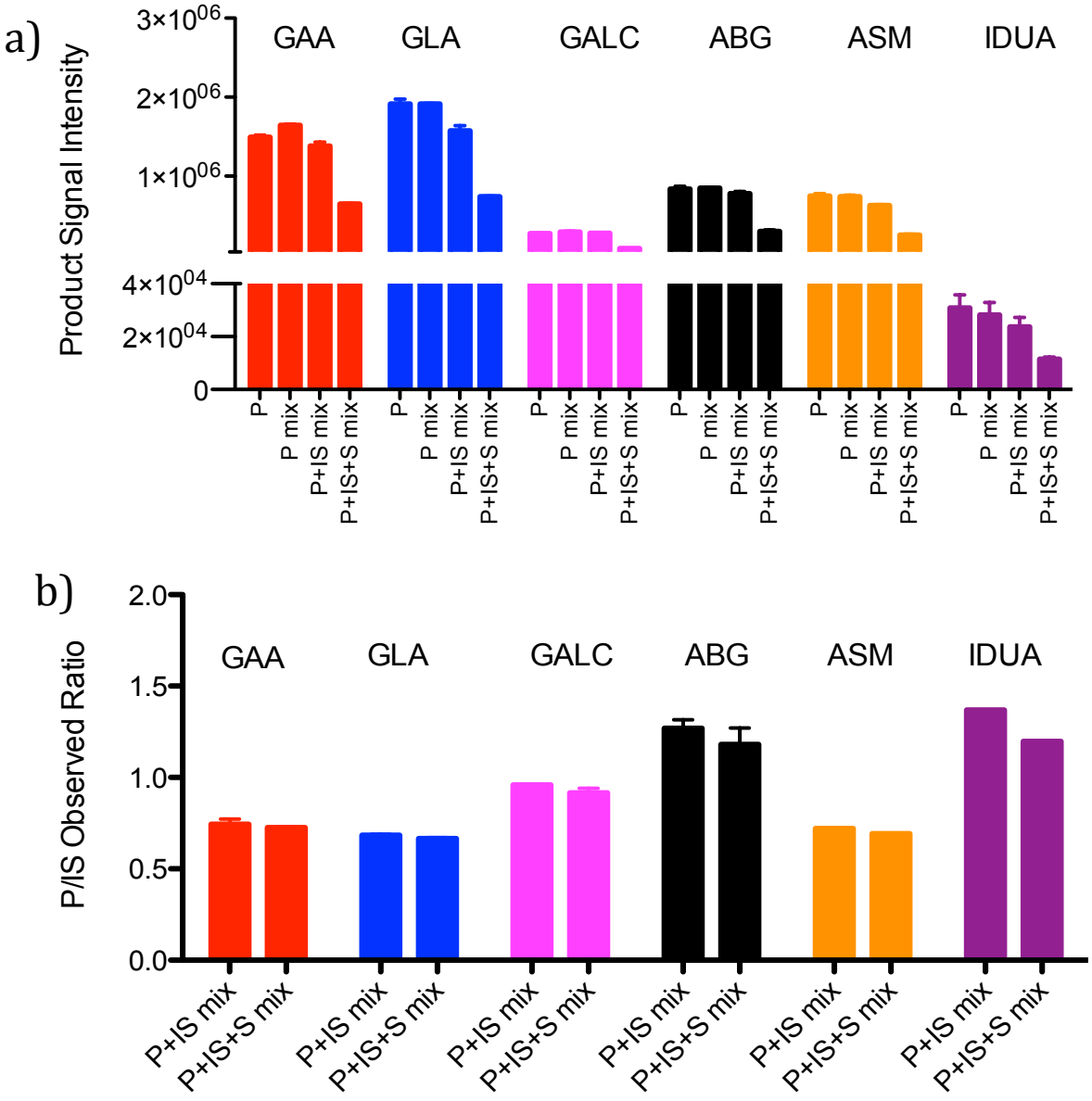

Figure 4.1. Signal suppression on the 6-plex assays. a) Absolute counts for each of the products when injected alone or as a component of the mixture. b) Product to internal ratio observed in the mixtures. Error bars represent one standard deviation of triplicate measurements.

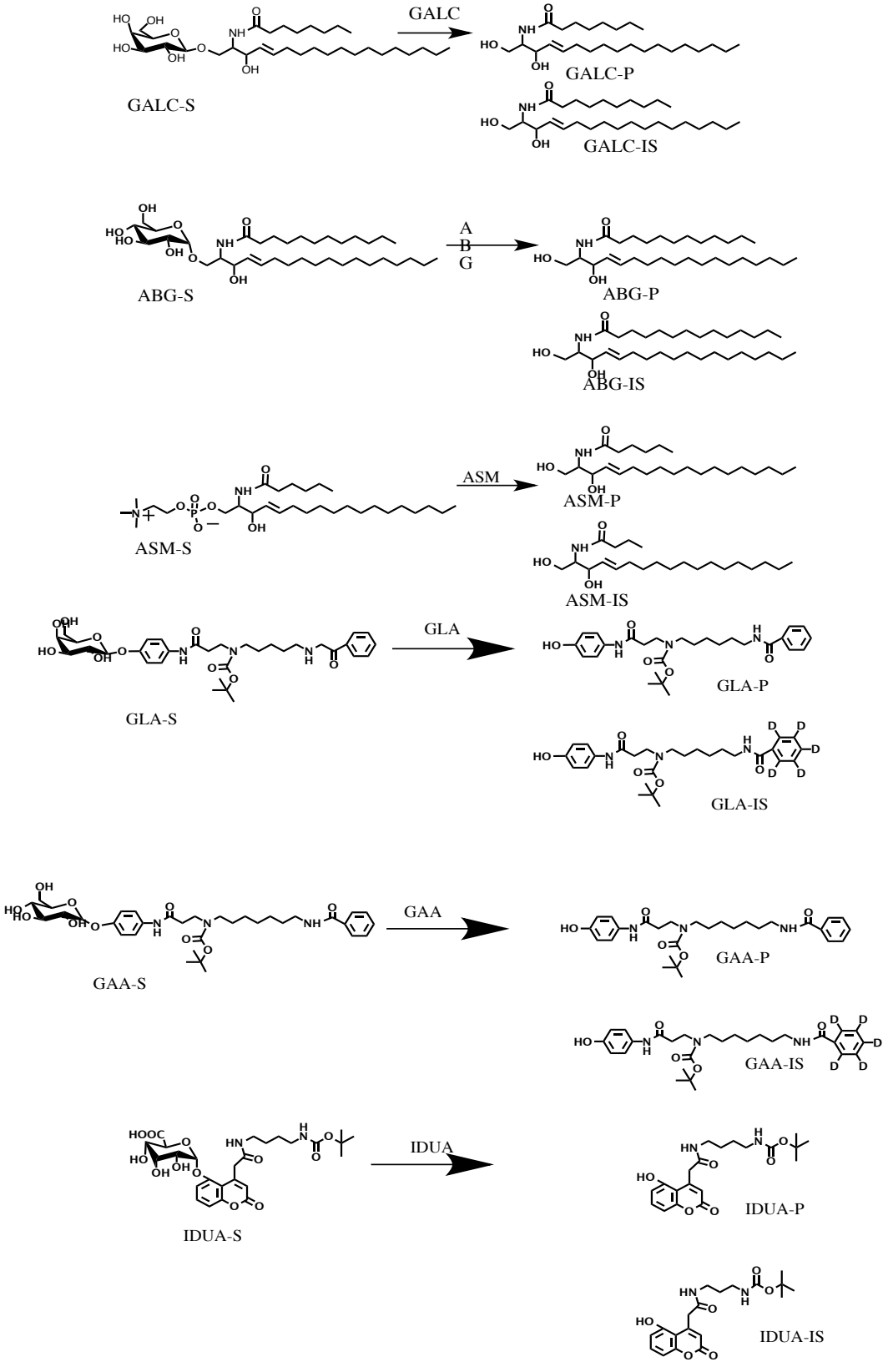

Figure 4.2. Structure of 6-plex substrates, products and internal standards.

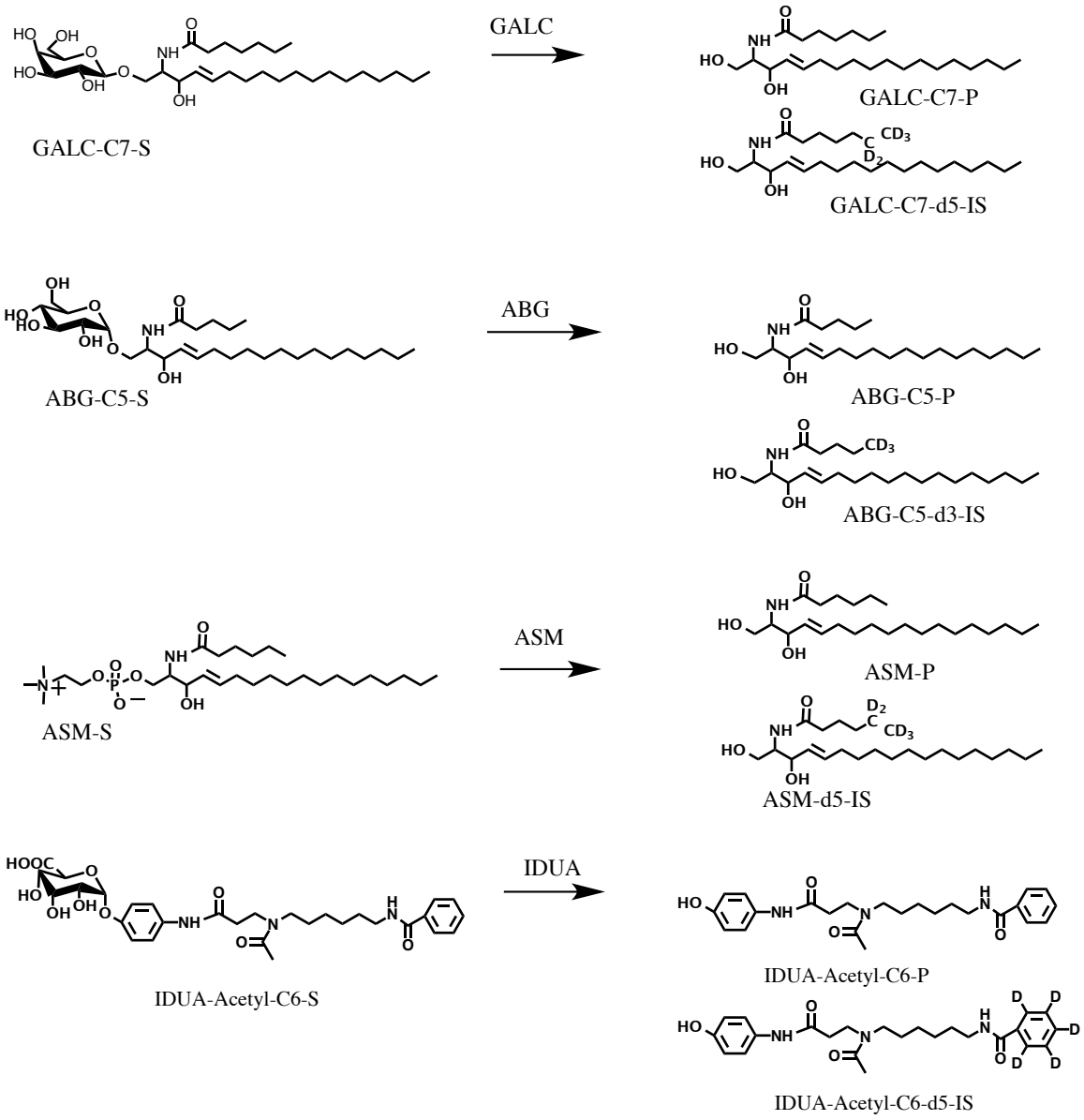

Figure 4.3. Structures of the second-generation reagents for ABG, GALC, ASM, and IDUA.

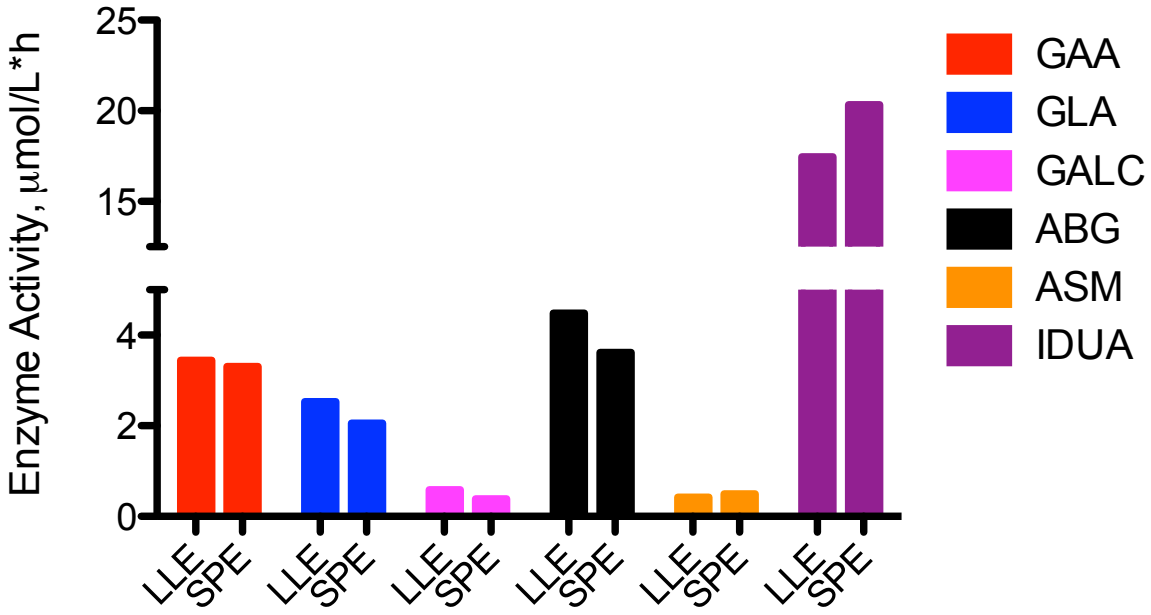

Figure 4.4. Comparison between liquid/liquid extraction (LLE) and silica gel solid phase extraction (SPE). Samples incubated in parallel. Results shown are the average of triplicate injections.

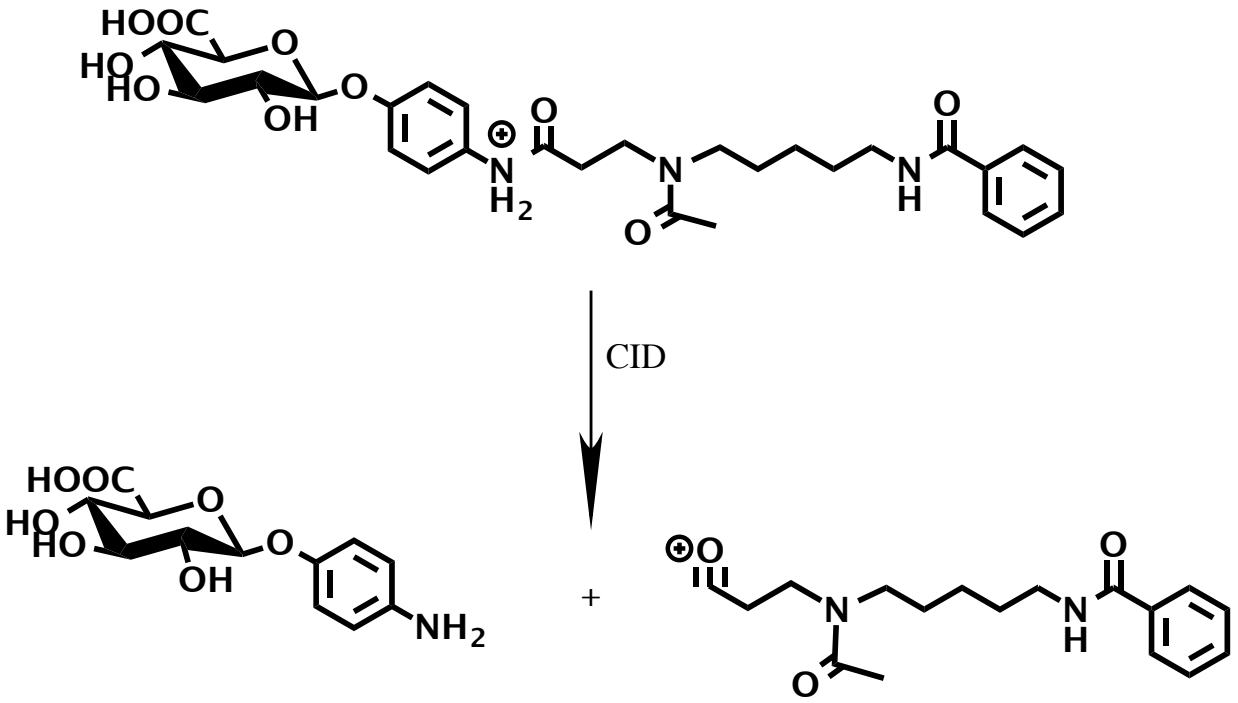

Figure 4.5. Fragmentation pathway for IDUA-acetyl-C6-S. Product and internal standard undergo the same pathway.

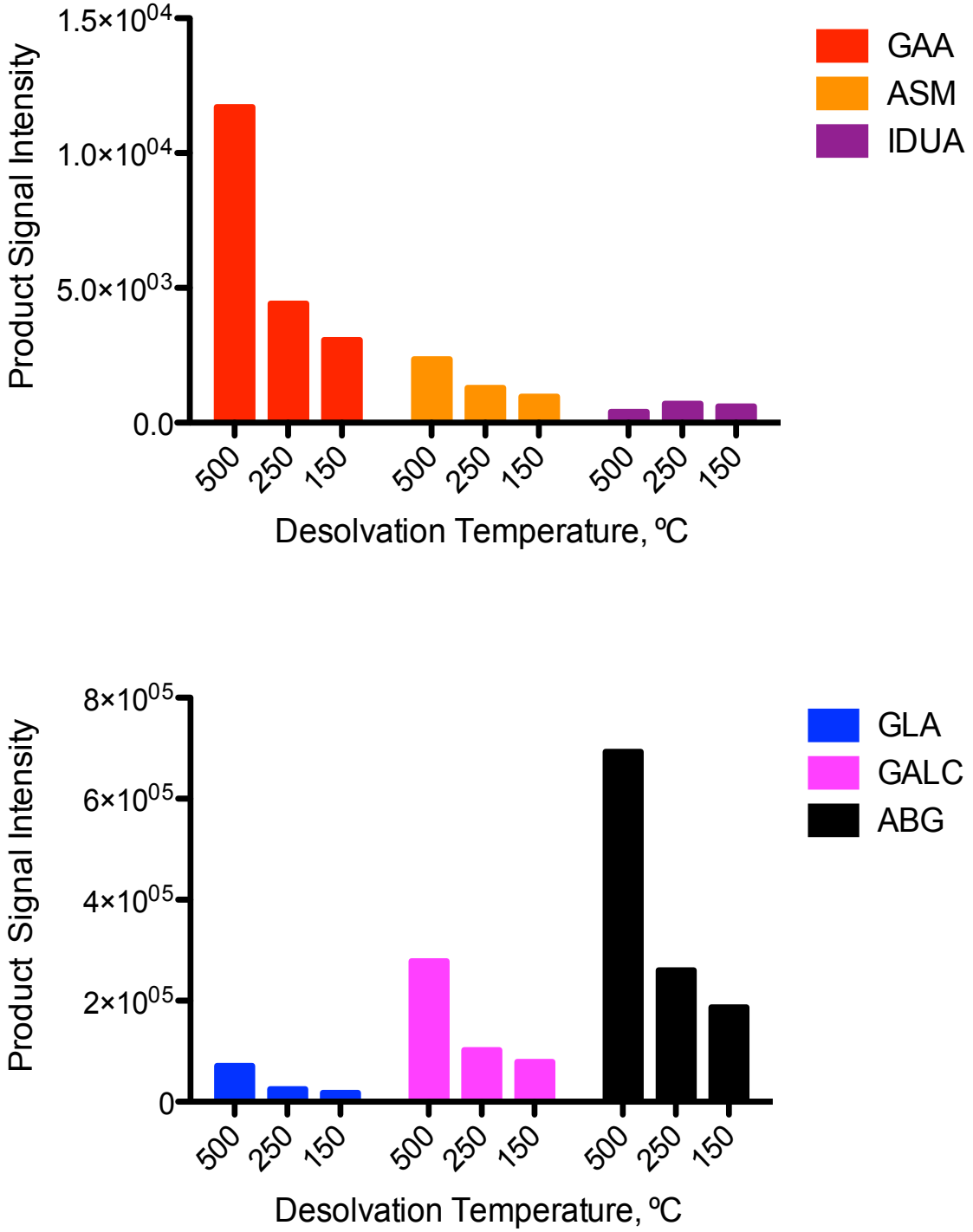

Figure 4.6. In-source decomposition of the substrate as a function of the desolvation temperature.

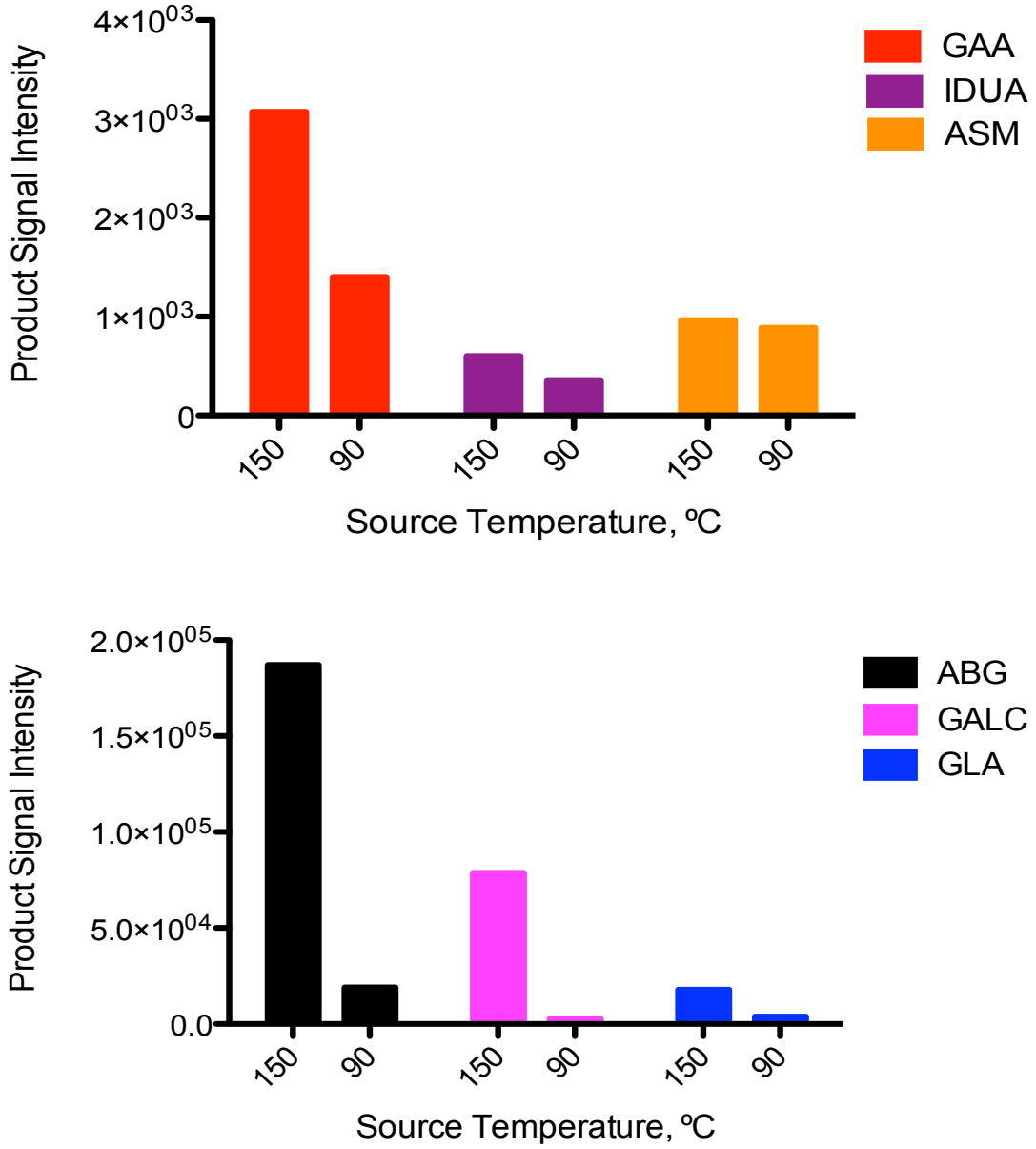

Figure 4.7. In-source decomposition of the substrate as a function of the source temperature.

Table 4.1. XEVO-TQ mass spectrometer settings.

Parameter (units)	
Capillary voltage (V)	3000
Extractor (V)	3.00
Source temperature (°C)	90
Desolvation temperature (°C)	150
Cone Gas Flow (L/h)	50
Desolvation Gas Flow (L/h)	450
LM 1 Resolution	2.6
HM 1 Resolution	15.0
Ion Energy	0.5
Collision Cell Entrance Potential (V)	0.50
Collision Cell Exit Potential (V)	0.50
LM 2 Resolution	2.8
HM 2 Resolution	14.7
Ion Energy 2	0.6
Multiplier (V)	493.04
Collision Gas	Argon

Table 4.2. Selected reaction monitoring transitions for all substrates (S), products (P) and internal standards (IS) of the 6-plex assay.

Analyte	SRM transition (*m/z*)	Cone Voltage (V)	Collision Energy (eV)
GAA-S	660.35 → 560.30	18	15
GAA-P	498.27 → 398.23	18	15
GAA-IS	503.33 → 403.28	18	15
GLA-S	646.33 → 546.28	18	15
GLA-P	484.25 → 384.20	18	15
GLA-IS	489.31 → 389.26	18	15
IDUA-S	567.26 → 467.20	7	11
IDUA-P	391.19 → 291.13	7	11
IDUA-IS	377.17 → 277.12	7	11
ABG-S	644.50 → 264.20	22	21
ABG-P	482.40 → 264.20	22	21
ABG-IS	510.50 → 264.20	22	21
ASM-S	563.40 → 184.00	15	22
ASM-P	398.28 → 264.20	15	22
ASM-IS	370.30 → 264.20	15	22
GALC-S	588.50 → 264.20	16	20
GALC-P	426.33 → 264.20	16	20
GALC-IS	454.40 → 264.20	16	20
ABG-C5-S	546.34 → 264.20	14	20
ABG-C5-P	384.25 → 264.20	16	20
ABG-C5-d3-IS	387.36→ 264.20	16	20
GALC-C7-S	574.34→ 264.20	16	18
GALC-C7-P	412.17 → 264.20	16	18
GALC-C7-d5-IS	417.42 → 264.20	16	18
ASM-d5-IS	403.43 → 264.20	15	22
IDUA-acetyl-C6-S	602.23→ 317.16	18	16
IDUA-acetyl-C6-P	426.20→ 317.17	24	16
IDUA-acetyl-C6-d5-IS	431.23→ 322.20	24	16

Table 4.3. Enzyme activity for all six disorders in CDC quality control DBS. Values reported are mean (CV) of triplicate injections.

	Enzyme Activity (µmol/h*L of blood)				
	Blank	**QC Low**	**QC Med**	**QC High**	**QC High/Blank**
GAA	0.15 (5.6)	0.69 (4.5)	3.08 (0.8)	5.12 (1.4)	34.42
GLA	0.10 (3.1)	0.66 (5.5)	2.38 (2.6)	4.57 (2.1)	46.11
GALC	0.11 (7.3)	0.18 (7.6)	0.68 (3.3)	1.28 (2.9)	11.61
ABG	0.45 (3.2)	1.22 (2.3)	4.02 (2.2)	4.93 (1.4)	10.89
ASM	0.025 (16.6)	0.05 (7.0)	0.24 (4.9)	0.53 (2.5)	20.88
IDUA	0.13 (11.8)	0.67 (3.1)	2.14 (2.2)	3.09 (2.8)	23.8

Table 4.4. Enzyme activity for all six disorders in CDC quality control DBS using the second generation substrates. Values reported are mean (CV) of triplicate injections.

	Enzyme Activity (μmol/h*L of blood)				
	Blank	**QC Low**	**QC Med**	**QC High**	**QC High/Blank**
GAA	0.052 (5.8)	0.27 (3.5)	1.90 (4.1)	2.73 (0.99)	65.4
GLA	0.072 (5.7)	0.671 (1.1)	2.86 (2.0)	5.37 (1.4)	74.6
GALC-C7-S	0.023 (8.7)	0.103 (11.5)	0.511 (4.9)	1.06 (5.3)	46.1
ABG-C5-S	0.36 (3.2)	0.97 (2.3)	4.07 (2.1)	7.42 (1.8)	20.6
ASM (d5 IS)	0.026 (11.1)	0.08 (7.7)	0.5 (6.0)	1.3 (1.5)	50
IDUA-acetyl-C6-S	0.01 (3.7)	0.23 (7.3)	1.49 (1.8)	2.44 (5.1)	244

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Chapter 5.

Advances on the assays for mucopolysaccharidoses type II, IVA and VI.

Abstract.

Mucopolysaccharidoses are a group of lysosomal storage disorders that encompasses a collection of enzymes responsible for the degradation of glycosaminoglycans. Therapeutic alternatives have been introduced for some of these disorders (MPS-I, MPS-II and MPS-VI) while clinical trials have been initiated for others. There is an interest in early detection of the disorders. Our group has developed assays to detect MPS-I, II, IIIA-D, IVA, and VI.

In addition, we have developed a second-generation of substrates for MPS-II and MPS-VI enzymes. Our results that the new reagents provide a more sensitive assay than those previously reported in literature. Our assay protocols are simple, do not require solid phase extraction, and can be multiplexed with assays to detect other lysososmal storage disorders. These assays are fully compatible with newborn screening laboratory settings.

We demonstrate substantial differences in enzyme activity between blanks and blood punches, a 97-fold difference for our new MPS-VI substrate and 74-fold difference for MPS-II.

5.1 INTRODUCTION.

The mucopolysaccharidoses (MPS) are a group of disorders caused by deficiency in an enzyme responsible for degrading glycosaminoglycans, heparan, dermatan, keratan, or chondroitin sulfate.[1] The group of enzymes responsible for the breakdown of these substrates includes five sulfatases, four exoglycosidases and one acetyl-N-transferase.

MPS type II or Hunter disease is caused by a deficiency of the enzyme iduronate-2-sulfatase

(ID2S), encoded by a gene located in the X chromosome, and, therefore, affects mainly males.[1-2] In 2011 Wolfe et al.[2] developed an assay to detect MPS-II using tandem mass spectrometry. Khaliq et al.[3] developed an assay for MPS-IVA or Morquio syndrome type A (N-acetylgalactosamine-6-sulfate sulfatase deficiency, GAL6S), and Duffey et al.[4] developed an assay to detect MPS-VI (N-acetylgalactosamine-4-sulfate sulfatase deficiency, GAL4S).

In 2013, Spacil et al.[5] published a multiplex assay to screen for nine disorders using UHPLC-MS/MS, as a part of the continuous efforts from our group to expand multiplexing capabilities. Our group has since then decided to develop a flow injection analysis alternative to screen for the same nine disorders included in our previous UHPLC-MS/MS protocol.

Here we report on the development of new substrates for MPS-II and MPS-VI to improve assay sensitivity.

5.2 EXPERIMENTAL.

Materials. Ammonium formate, sodium taurocholate hydrate, acarbose, barium acetate, and cerium (III) acetate hydrate were purchased form Sigma-Aldrich. 2-Acetamido-2-deoxy-D-glucono-1,5-lactone was obtained from Santa Cruz Biotechnology. Ethyl acetate was purchased from Macron Fine Chemicals. Optima LC-MS grade methanol and all other solvents were purchased from Fisher Scientific.

Substrates for iduronate-2-sulfatase (ID2S), N-acetylgalactosamine 6-sulfatase (GAL6S) and N-acetylgalactosamine 4-sulfatase (GAL4S) were synthesized in the Gelb laboratory according with previously reported procedures,[2, 6] except for the GAL6S-S in which the BOC group was replaced with a d_9-BOC group. A second-generation of substrates has been designed and

synthesized in the Gelb laboratory for ID2S and GAL4S[7]. Ongoing efforts are focused on a new substrate for GAL6S.

All experiments were conducted with Internal Review Board approval from the University of Washington. Dried blood spots (DBS) from healthy adults were used to develop our assays. DBS were stored at -20ºC in sealed plastic bags inside a glass jar with dessicant.

Stock solutions. Ammonium formate buffer (100 mM, pH 4.4) was prepared in deionized water and the pH was adjusted using formic acid (using a pH meter and two calibration buffers, pH 4.0 and 7.0).

A second ammonium buffer (100 mM, pH 4.0) used for GAL6S and GAL4S assays was prepared following the protocol described above.

Buffer additives were prepared as follows: sodium taurocholate hydrate stock solution was prepared in ammonium formate buffer, final concentration 100g/L. Acarbose stock solution was prepared in deionized water (0.8 mM). Barium acetate (150 mM) and cerium (III) hydrate (100 mM) stock solutions were prepared in deionized water. All buffer and additives solutions are stored at 4ºC. Stock solution of 2-acetamido-2-deoxy-D-glucono-1,5-lactone, 15 mM was prepared in deionized water and stored at -20°C in a glass vial.

ID2S, GAL4S, GAL6S, substrates and internal standards were accurately weighted using an analytical balance and dissolved in methanol. The solutions were stored at -20ºC.

ID2S assay buffer. ID2S assay buffer is prepared by mixing 1/10 of sodium taurocholate solution, 1/100 of acarbose solution, 1/20 of barium acetate and 1/20 cerium (III) acetate hydrate stock solutions. An aliquot of ID2S-S and ID2S-IS stock solutions in methanol is dried down

using a stream of nitrogen. Add the appropriate volume of ID2S buffer to give a final concentration of ID2S-S of 1 mM and ID2S-IS of 5 μM.

GAL4S and GAL6S assay buffer. GAL4S and GAL6S assay buffer is prepared by mixing 1/20 of barium acetate and 1/20 cerium (III) acetate hydrate stock solutions and 1/100 of 2-acetamido-2-deoxy-D-glucono-1,5-lactone stock solution and ammonium formate buffer, 100 mM, pH 4.0. An aliquot of GAL6S-S, GAL6S-IS, GAL4S-S and GAL4S-IS stock solutions in methanol is dried down using a stream of nitrogen. Add the appropriate volume of GAL4S and GAL6S buffer to give a final concentration of 1mM GAL4S-S and 5 μM GAL4S-IS and 2 mM GAL6S-S and 5 μM GAL6S-IS.

Assay Protocol. A 3mm DBS punch was placed in a 96 well plate and 30 μL of assay buffer were added to each well. Plates were sealed with sealing film and incubated for 16 hours at 37ºC with orbital shaking. After incubation, 200 μL of a 44 mM citric acid in water was added to each well. Ethyl acetate (400 μL) and water (100 μL) portions were added to each well, and samples were mixed. The plates were sealed and centrifuged for 5 minutes at 3000 rpm. Aliquots of 200 μL of ethyl acetate were collected and transferred to a shallow well plate.

GAL4S assays were carried out as previously described for the ID2S assay. After incubation, two different workup protocols were used. In the first workup the reaction was quenched with 100 μL of 1:1 ethyl acetate/methanol. Additional 400 μL of ethyl acetate and 100 μL of water were added to each well, and plates were processed as for the ID2S assay. In the second workup protocol, quenching of incubated samples was done by adding 100 μL of a suspension of DEAE cellulose (Whatman DE52 pre-swollen, 16 mg resin per 100 mL water) followed by 400 μL of

ethyl acetate.[4] The samples were processed as above. GAL6S assay can be incubated in the same buffer with GAL4S with the same two quench/workup procedures.

Extraction studies. The various enzyme products (300 pmoles) were dissolved in 30 μL of assay buffer to give a 10 mM analyte solution. The sample was processed by liquid-liquid extraction as above and submitted to flow-injection tandem mass spectrometry. The amount of product detected by mass spectrometry was compared to that measured when 300 pmoles of enzymatic product was directly injected into the instrument.

Mass Spectrometry. Samples were analyzed using flow injection tandem mass spectrometry. The elution solvent was an 80:20 methanol/water mixture with 5mM ammonium formate. The flow stream from the auto sampler at injection is 0.3 mL/min. After sample injection, the flow rate is dropped to 0.2 ml/min for 0.1 min, then dropped to 0.03 ml/min for 0.15 min, and held at 0.03 mL/min for 0.65 min, then raised to 0.4 mL/min at 0.9 min, then to 0.3 mL/min at 1 min. Total analysis time is 1.5 minutes. Tandem mass spectrometry was carried out on a Waters Xevo TQ instrument in positive ion mode and selected reaction monitoring (SRM). Injection volume was 10 μl. Mass spectrometer settings are given in Table 5.1. Mass spectrometer settings are identical to those described in chapter 4 for the multiplex assay.

5.3 RESULTS AND DISCUSSION.

Assay and sample workup. Assay buffer for the different enzymes have different pH values. GAL4S and GAL6S were incubated at pH 4.0 according to previously published methods from our group[3-4]. On the other hand, ID2S assay was developed in a buffer pH 5.5[2] but our

experiments were carried out at pH 4.4. ID2S assay can then be incubated with the 6-plex assay described in chapter 4. Sulfatases are partially inhibited by the presence of free sulfate and phosphate in blood; this interference was eliminated by the addition of barium and cerium solutions to the buffer. 2-acetamido-2-deoxy-D-glucono-1,5-lactone was included in the buffer to selectively inhibit β-hexosaminidase, which interferes with the GAL4S assay.

Substrate depletion is critical for the sulfatases assays. Sulfated substrates undergo fragmentation in the transfer between the electrospray source and the first quadrupole and forms product ions in-source. Even though in-source fragmentation is a minor process, the large concentrations of substrate present in the assay compared to product formed it becomes of importance and creates a large background. Post incubation purification is therefore a critical step for the assay. An anion exchange resin is used in the GAL4S and GAL6S to capture the substrate, while the respective products will be extracted into ethyl acetate in the presence of internal standards. ID2S assay on the contrary, cannot be purified using an anion exchange resin due to the fact that the assay product contains iduronic acid, deprotonated at assay pH. Lowering the pH by adding citric acid protonates the iduronic acid making it easily extractable into the ethyl acetate layer while the substrate remains in the aqueous phase.

New ID2S and GAL4S substrates were designed to include a bis-amide unit similar to the Pompe (GAA) substrate, a compound with higher protonation efficiency.[8-9] The introduction of this new set of substrates will improve the sensitivity of the mass spectrometry detection. Structures for all substrates, products and internal standards are compiled in Figure 5.1. Second-generation substrates for ID2S and GAL4S are shown in Figure 5.2.

Extraction of the products into ethyl acetate for the newly designed reagents was evaluated using the protocol described above. Extraction yields were 60.5% for ID2S-P and 28.5% for GAL4S-P. Internal standards are expected to extract to the same extent as their respective product.

Mass Spectrometry. All the substrate, products and internal standards easily protonate in the mass spectrometer to form positive singly charged ions. Substrates, products and internal standards for ID2S, GAL6S and GAL4S have a common structural feature: a t-butyl carbamate. Major fragmentation pathway is the loss of isobutylene and carbon dioxide.[9] Second-generation substrates and internal standards were designed and tested for each assay in an attempt to improve the sensitivity of our method. Cleavage pathway for major fragment formation is illustrated in Figure 5.3 (e.g. ID2S-S). This new set of reagents exhibits the same fragmentation pattern. The mass transitions monitored are summarized in Table 5.3. Enzymatic activity was calculated as μmol/(h*L of blood) using the formula described in chapter 4 (A_e = (P/IS)*[IS]*V_a/(3.2*t_i)).

ID2S sample analysis. ID2S substrate and newly designed ID2S-pentanoyl-C6 substrate assays were incubated in parallel. Our results show improvements in the blood to no blood ratio, 37.9 for the old substrate and 75 for our new reagent (Table 5.3). Additionally, mass spectrometry signal was increased by a factor of 5.8. Our ID2S was developed in the 6-plex-assay buffer with the goal of expanding the assay to a 7-plex. We tested this hypothesis and incubated an assay using CDC 6-plex reagents (the new reagents discussed in chapter 4 were in preparation) and ID2S-pentanoyl-C6-S. Sample workup was carried out as described for the individual ID2S-assay and enzyme activity results are summarized in Table 5.4. Enzyme activities for ID2S-

pentanoyl-C6-S individual assay and the multiplex assay are comparable. This result demonstrates that sulfatase ID2S can be added to the 6-plex-assay previously described to convert it into a 7-plex assay if desired.

GAL6S and GAL4S assay. Incubation of DBS in a cocktail buffer with substrates and internal for these two enzymes was investigated. Preliminary results show these two assays can be multiplexed (Table 5.5). Unfortunately, the product signal in the mass spectrometer is too low and complicates the applicability of the assay in newborn screening laboratories. A new set of substrates for these enzymes are needed. Current efforts are focused on developing new reagents for GAL6S. Results on a new substrate for GAL4S are discussed below.

GAL4S assay. Second-generation substrate for GAL4S was designed and synthesized. Assays using both old and new substrates were incubated in parallel (Table 5.6). Sample workup using anion exchange followed by ethyl acetate extraction and ethyl acetate liquid/liquid extractions only were used. Our results show a 8-fold enhancement in sensitivity for the enzymatic product. The overall assay performance with anion exchange quench is also improved, as shown by the increase of the blood to no blood ratio from 4.9 to 97.4 when the new reagent is used instead of the old reagent. Ethyl acetate extraction results also show improved assay performance of the new substrate, however, the blood to no blood ratio is smaller (17.2). The results suggest that removing the substrate using anion exchange decreases the background. Our results also suggest that the largest portion of the background is produced by in-source fragmentation of the substrate to form product ions.

5.4 CONCLUSIONS.

We have introduced a new set of substrates to improve ID2S and GAL4S assay performance, the results from which showed that the second-generation substrates have enhanced mass spectrometry sensitivity. In addition, blood to no blood ratios were improved when compared to the original substrates. Moreover, we have demonstrated that the new ID2S assay can be multiplexed with the 6-plex-assay developed to detect six other lysosomal disorders (Krabbe, Pompe, Niemann-Pick A/B, Fabry and MPS-I) described in chapter 4.

We are in the process of designing a new and improved reagent for the GAL6S assay.

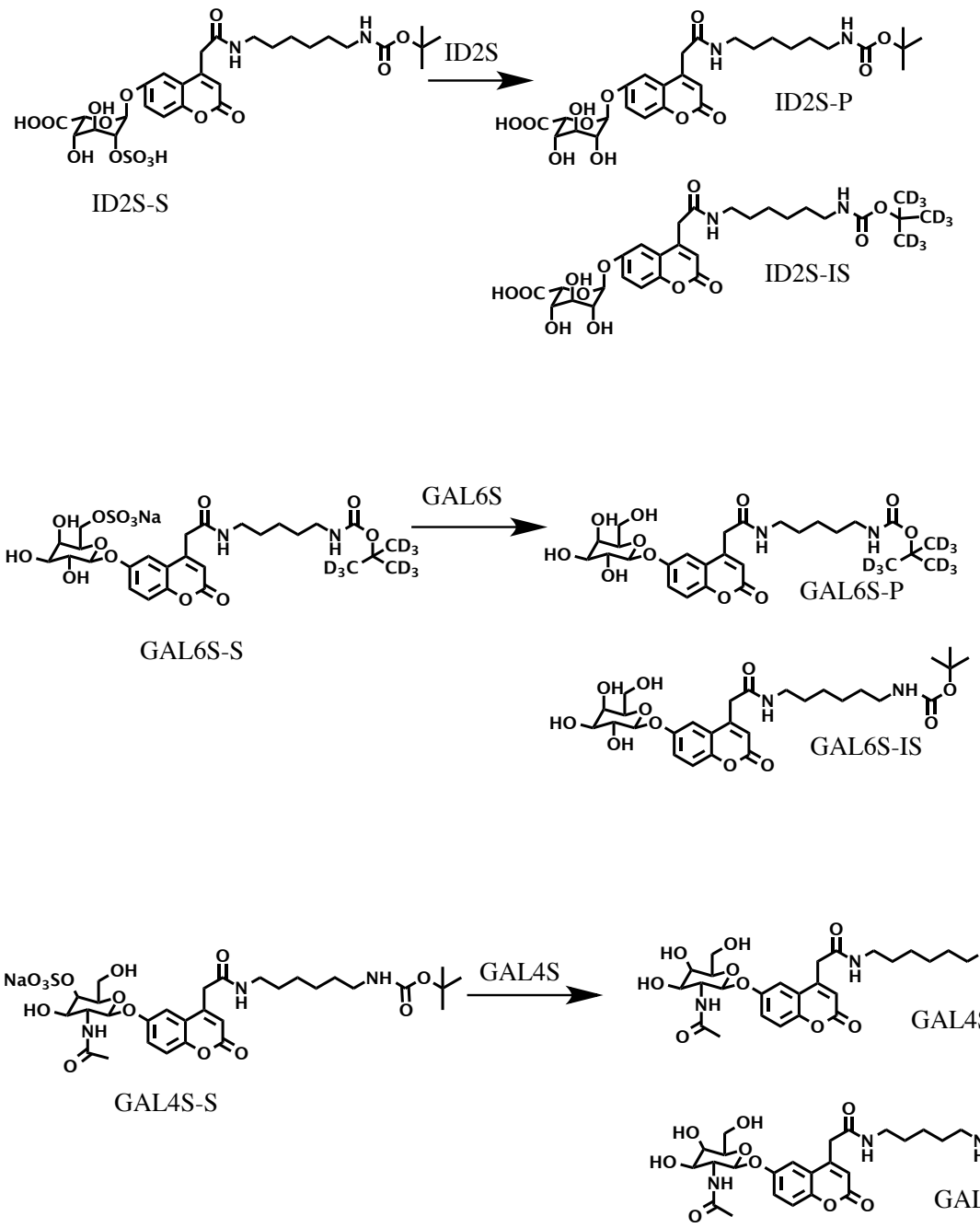

Figure 5.1. Substrates, products, and internal standards structures for ID2S, GAL6S and GAL4S according to the previously published methods.[2, 6]

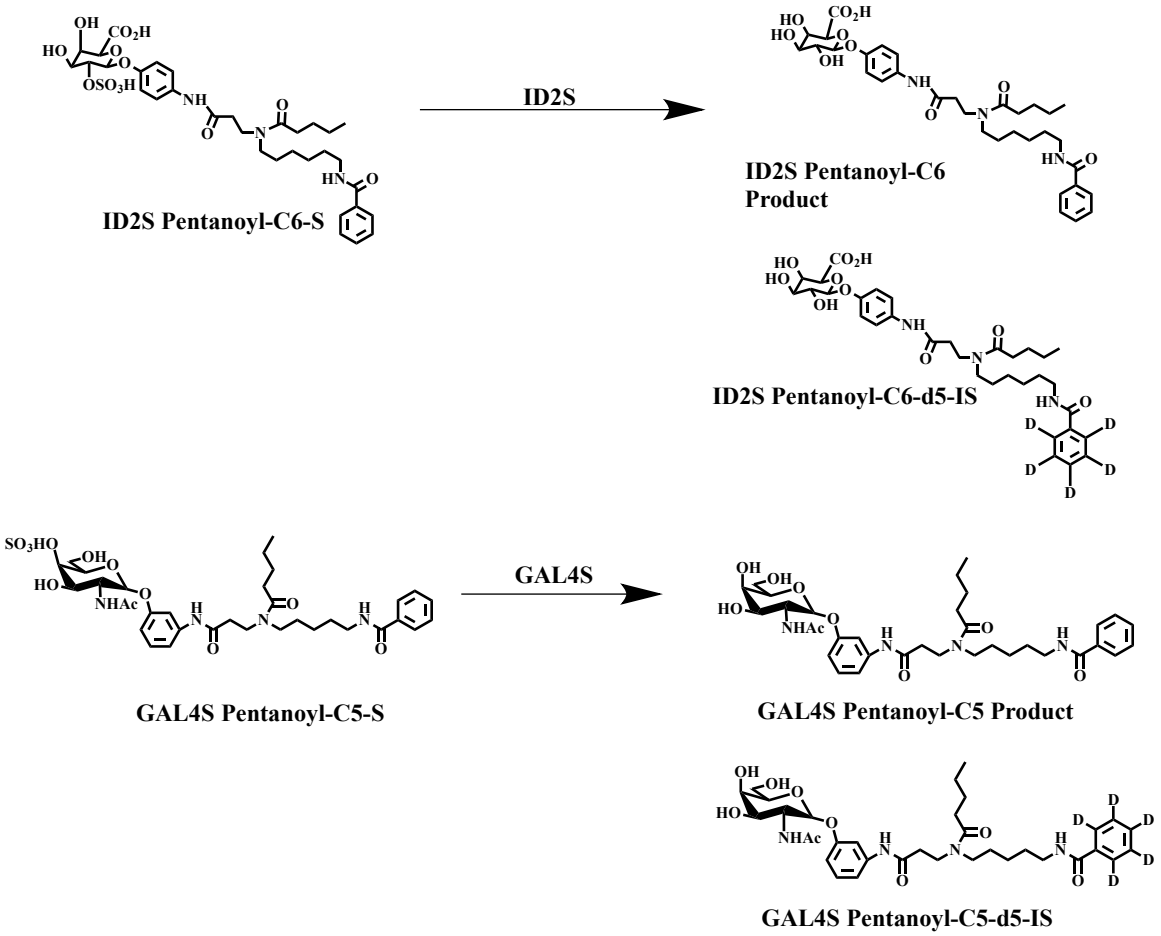

Figure 5.2. Structures of the second-generation reagents for ID2S and GAL4S.

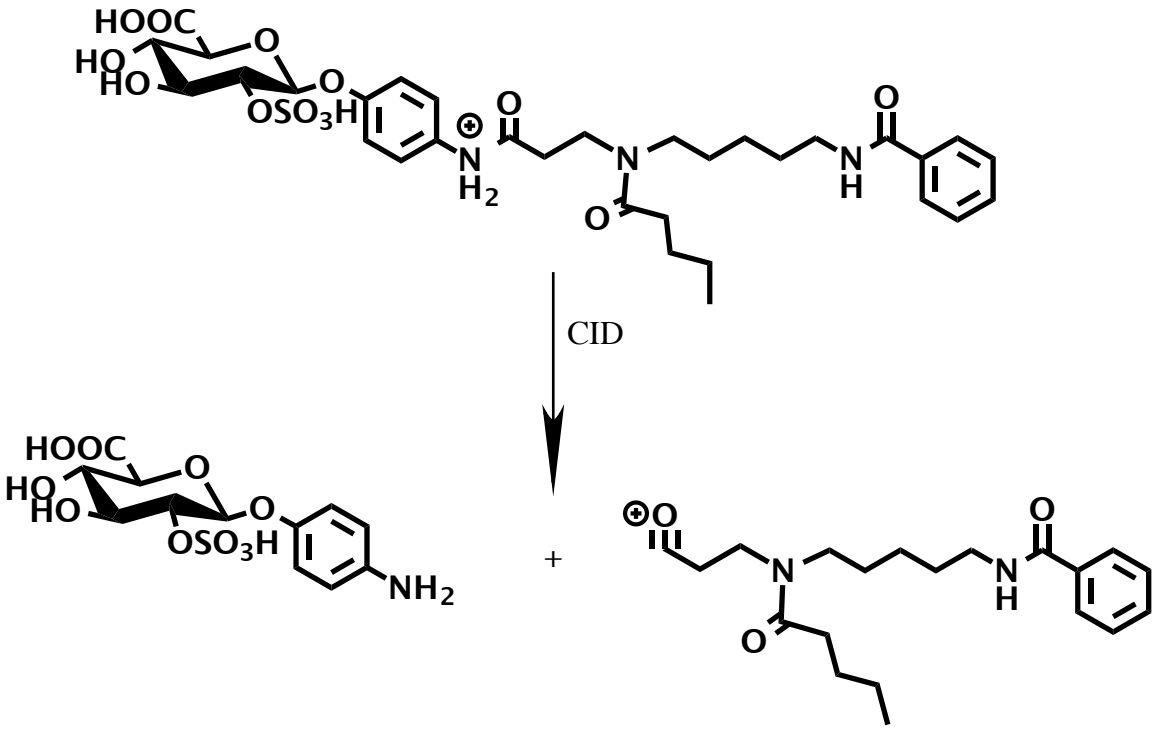

Figure 5.3. Fragmentation pathway for ID2S-pentanoyl-C6-S. All other synthesized compounds undergo an identical fragmentation pathway.

Table 5.1. XEVO-TQ mass spectrometer settings.

Parameter (units)	
Capillary voltage (V)	3000
Extractor (V)	3.00
Source temperature (°C)	90
Desolvation temperature (°C)	150
Cone Gas Flow (L/h)	50
Desolvation Gas Flow (L/h)	450
LM 1 Resolution	2.6
HM 1 Resolution	15.0
Ion Energy	0.5
Collision Cell Entrance Potential (V)	0.50
Collision Cell Exit Potential (V)	0.50
LM 2 Resolution	2.8
HM 2 Resolution	14.7
Ion Energy 2	0.6
Multiplier (V)	493.04
Collision Gas	Argon

Table 5.2. Selected reaction monitoring transitions for all substrates (S), products (P) and internal standards (IS) for the sulfatases.

Analyte	**SRM transition (m/z)**	**Cone Voltage (V)**	**Collision Energy (eV)**
ID2S Substrate	697.20 → 597.20	10	11
ID2S Product	595.25 → 495.20	10	11
ID2S Int. Std.	604.31 → 496.20	10	11
ID2S-pentanoyl-C6-S	724.60→ 359.17	15	26
ID2S-pentanoyl-C6-P	644.26→ 359.16	15	20
ID2S-pentanoyl-C6-d5-IS	649.36 → 364.26	16	20
GAL4S-S	724.24 → 624.24	12	11
GAL4S-P	622.30 → 522.24	12	11
GAL4S-IS	608.28 → 508.23	12	11
GAL4S-pentanoyl-C5-S	737.39→ 345.24	15	16
GAL4S-pentanoyl-C5-P	657.45→ 345.19	18	20
GAL4S-pentanoyl-C5-IS	662.50→ 350.25	18	20
GAL6S-S	656.33 → 548.28	10	19
GAL6S-P	576.26 → 468.20	10	19
GAL6S-IS	581.27 → 481.22	10	19

Table 5.3. Product ion counts and enzyme activity for ID2S using both old and second-generation substrate in healthy adult DBS. Values reported are mean (CV) of triplicate injections.

	Product Ion Counts		Enzyme Activity (μmol/h*L)		
	Blank	**DBS**	**Blank**	**DBS**	**DBS/Blank**
ID2S	3.10E+01	1.24E+03	0.076 (8.3)	2.88 (1.90)	37.93
ID2S-pentanoyl-C6-S	3.62E+02	7.24E+03	0.060 (4.3)	4.40 (8.5)	75

Table 5.4. Enzyme activity for new ID2S-Pentanoyl-C6-S and 6-plex disorders in healthy adult DBS. Single DBS incubation for all 7 disorders. Values reported are mean (CV) of triplicate injections.

	Enzyme Activity (μmol/h*L)		
	Blank	**DBS**	**DBS/blank**
GAA	0.1 (6.1)	3.81 (1.5)	38.1
GLA	0.09 (8.5)	2.65 (2.9)	29.4
GALC	0.13 (8.2)	1.33 (6.6)	10.2
ABG	0.36 (3.3)	3.8 (1.9)	10.6
ASM	0.016 (5.4)	0.62 (2.3)	38.8
IDUA	0.11 (9.2)	2.32 (3.0)	21.1
ID2S-pentanoyl-C6-S	0.066 (10.8)	4.93 (6.3)	74.7

Table 5.5. Product ion counts and enzyme activity for GAL6S and GAL4S in healthy adult DBS. Values reported are mean (CV) of triplicate injections.

	Ion Counts Product		Enzyme activity (μmol/h*L)		
	Blank	**DBS**	**Blank**	**DBS**	**DBS/Blank**
GAL6S	3.12E+02	1.80E+03	0.074 (24)	0.84 (10.9)	11.42
GAL4S	2.99E+01	1.13E+03	0.03 (33)	1.24 (10.2)	39.97

Table 5.6. Product ion counts and enzyme activity for GAL4S in healthy adult DBS. Values reported are mean (CV) of triplicate injections.

	Product ion count		Enzyme activity (μmol/h*L)			
	Blank	**DBS**	**Blank**	**DBS**	**DBS/Blank**	
GAL4S	2.91E+02	1.26E+03	0.29 (5.4)	1.41 (4.7)	4.9	DEAE sample workup
GAL4S-pentanoyl-C5-S	1.21E+02	1.12E+04	0.125 (7.9)	12.17 (2.4)	97.4	
GAL4S	3.06E+02	1.30E+03	0.23 (12.3)	1.14 (4.9)	4.9	Ethyl acetate only
GAL4S-pentanoyl-C5-S	8.93E+02	1.03E+04	0.74 (4.4)	12.73 (3.3)	17.2	

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