Development of Mass Spectrometry-Based Assays for Newborn Screening: Novel Approaches to Lysosomal Acid Lipase and the Mucopolysaccharidoses

Sophia Masi

A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2018

Reading Committee:

Michael Gelb, Chair

Marshall Horwitz

Dustin Maly

Jesse Zalatan

Program Authorized to Offer Degree:

Chemistry

University of Washington

Abstract

Development of Mass Spectrometry-Based Assays for Newborn Screening:

Novel Approaches to Lysosomal Acid Lipase and the Mucopolysaccharidoses

Sophia Masi

Chair of the Supervisory Committee:
Michael Gelb, Professor and Barbara L. Weinstein Endowed Chair
Department of Chemistry

Newborn screening has been an important public health tool for over fifty years. As technology improves, new testing has developed for rare diseases including lysosomal storage disorders (LSDs) in parallel with novel treatment advances. With the discovery of a specific substrate, we have developed the first mass spectrometry-based assay for lysosomal acid lipase (LAL) and a concurrent fluorescent assay. We also have shown that mass spectrometry provides a higher analytical range for three mucopolysaccharidoses (MPS): MPS Types II, IVA, and VI. With increased analytical range via mass spectrometry, we are able to further newborn screening and diagnostic testing with the newly supported option of multiplexing for all four of these LSDs.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to thank my hard-working labmates, my P.I., Mike, and the other professors on my thesis committee. I would also like to thank my eternally patient husband, Scott – I could not have done this without him. I also would like to thank my family for inspiring my love of science that has brought me to the University of Washington.

DEDICATION

This thesis is dedicated to all of the children and families who have suffered with lysosomal storage disorders.

Chapter 1. INTRODUCTION

This following text will introduce the concept of newborn screening (NBS), focusing on the development of assays for lysosomal storage disorders (LSDs).

1.1 History of Newborn Screening

Newborn screening (NBS) began in the 1960s as a public health program. The goal for this first-tier screening was to detect a wide assortment of conditions that can be drastically improved via early intervention. [1][2][3] These interventions or treatments normalize a newborn's physical and neurological development, but also can drastically increase lifespan.[1][2][3] One of the governing tenets of NBS is that assay development and research should focus on diseases for which intervention is possible, thus conditions that have known approved therapies and treatment.[1][4] With the expansion of recombinant enzyme replacement therapy, many new diseases are being evaluated for their inclusion in newborn screening.[4] In the United States, the Recommend Uniform Screening Panel (RUSP) includes all conditions for which newborn screening has been validated and in active use in the US.[4][5]

In order to be accessible to as many individuals as possible, NBS must maximize its efficiency, both in terms of cost and source of patient samples. Historically, the first newborn screening assays relied on bacterial growth in response to interactions of patient serum with treated agar.[5] Over time slow, biological-based assays were replaced by newer analytical technology, namely fluorimetry and mass spectrometry, greatly increasing the scalability of testing.[5] Currently, NBS is offered in all 50 states as well as around the world.[4][6] It is possible to use a variety of

biological materials for NBS, including whole blood, secretions, and specific tissue biopsies.[4][5] Dried blood spots (DBS) are a common and popular source for NBS samples due to the ease of obtaining such a sample and the relative longevity of enzymes and biomarkers in this form.[3][5][6][7] The use of high quality DBS and improved technology has enabled testing of all the diseases seen in Table 1.1 below, a small subset of known heritable diseases are recommended for newborn screening panels.[6][8] As technology improves, so has the desire and ability to screening for new disorders.[3][5]

Table 1.1. List of NBS Screened Diseases using Fluorimetry and Mass-Spectrometry in the USA based on the 2016 RUSP list.[6][8]

Type of Disease	Diseases	
Fatty Acid Disorders	Carnitine uptake/transport defect (CUD)	Long-chain L-3-hydroxylacyl-CoA dehydrogenase (LCHAD)
	Medium-chain-acyl-CoA dehydrogenase (MCAD)	Very long-chain acyl-CoA dehydrogenase (VLCAD)
	Trifunctional protein deficiency (TFP)	
Organic Acid Disorders	Glutaric acidemia type 1 (GA-I)	3-hydroxy 3-methylglutaric aciduria (HMG)
	Isovaleric acidemia (IVA)	3-methylcrotonyl-CoA carboxylase (3-MCC)
	Methylmalonic Acidemia cobalamin disorders (Cbl-A,B)	Beta ketothiolase deficiency (BKT)
	Methylmalonic Acidemia- Methylmalonyl-CoA mutase (MUT)	Propionic acidemia (PROP)
	Multiple carboxylase deficiency (MCD)	
Amino Acid Disorders	Argininosuccinate aciduria (ASA)	Citrulinemia type I (CIT I)
	Homocystinuria (HCY)	Maple syrup urine disease (MSUD)
	Phenylketonuria (PKU)	Tyrosinemia Type 1 (TYR-I)
Lysosomal Storage Disorders	Hurler Syndrome/Mucopolysaccharidosis type I (MPS-I)	Hunter Syndrome/ Mucopolysaccharidosis type II (MPS-II)*
	Pompe or Glycogen Storage Disease Type II (GAA)	

*Refers to disease not yet on the primary RUSP panel but are still screened for in select states.[8]

1.2 Mass Spectrometry in Newborn Screening

Although the use of DBS remains very similar to the original serum filter paper discs used in the nineteen-sixties, the means to analyze data have changed greatly in the ensuing decades.[5]

The two most common analytical detection methods used in NBS are fluorescence detection and mass spectrometry (MS).[3] With MS, it is possible to detect products of multiple functional enzyme assays (referred to as assays being "multiplexable") as well as biomarkers simultaneously.[1][3][8][9][11][12] The use of consecutive fragmentation or tandem mass spectrometry (MS/MS) further increases the high sensitivity and specificity of mass spectrometry.[1][3][8][9][11][12] However, mass spectrometry is not inherently quantitative, requiring the use of chemically identical internal standards that are isotopically-labeled and present in the sample in known amounts.[1][3][8][9][11][12] Compared to the universally-used 4MU fluorophore, mass spectrometry is able to greatly vary its substrate structure often more closely matching the natural substrate instead of a fluorometric analog. The high specificity of the mass spectrometry comes partly from the use of specific fragmentation of the desired highly ionizable analyte, further favored via tandem, or consecutive, transitions (MS/MS).[1][2][3] The Gelb lab has spent the last decade developing designer specific substrates that include both an ionizable proton-trap and unique secondary (daughter) ion fragmentation pathway in pursuit of a higher analytical range.[9][11][12] The analytical differences between fluorescence assays and mass spectrometry-based will be discussed in further detail in Chapter 3.

1.3 Lysosomal Storage Disorders

To understand lysosomal storage disorders, first the function of the lysosome must be understood. Further detailed explanation of the key enzymes of interest for this article will follow, namely information on lysosomal acid lipase and the three mucopolysaccharidoses (MPS): MPS types II, IVA, and VI.

1.3.1 *The Lysosome and Diseases of the Lysosome*

The lysosome is one of the primary cellular catabolic sites, an acidic organelle critical to cellular metabolism found in almost every cell of the body, as shown in Figure 1.1 below. [10][13][14] A large variety of substrates including proteins, lipids, and polysaccharides are broken down into monomers or useable fragments by lysosomal enzymes. [10][13][14]

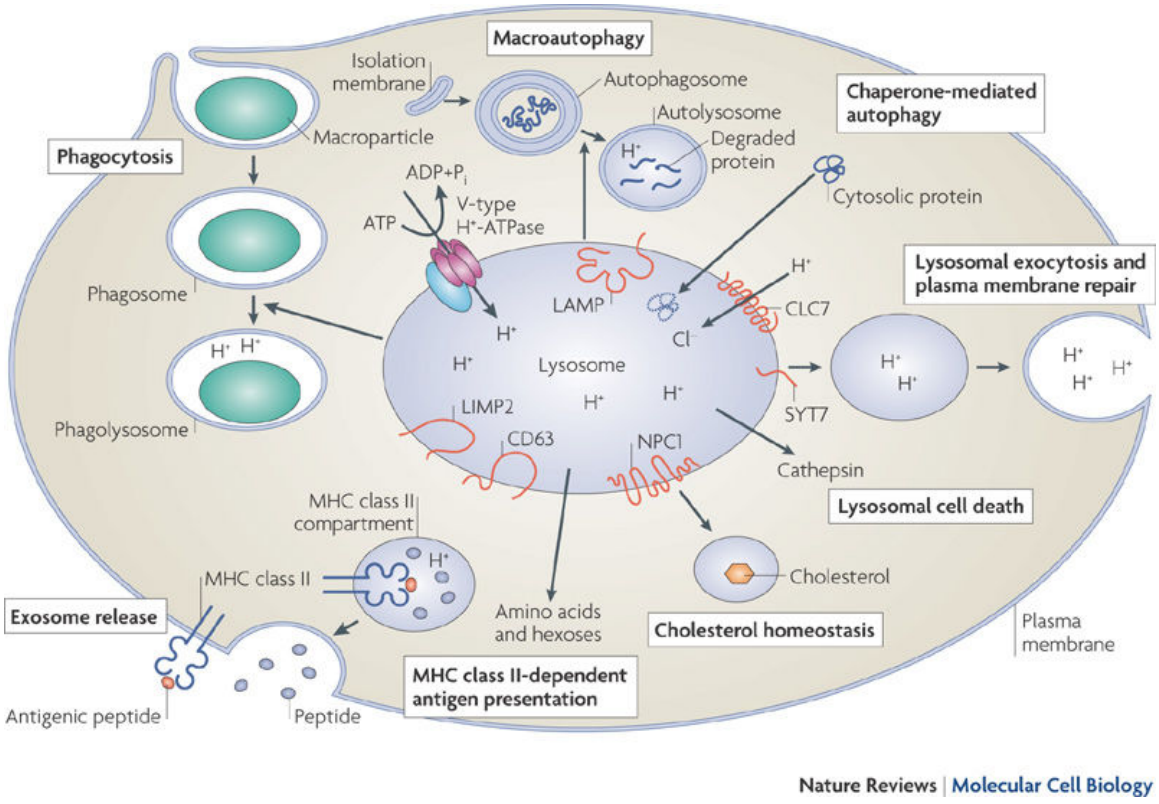


Figure 1.1. The lysosome with all of its functions in bold text. Common lysosomal proteins are also listed as follows: V-type H^+-ATPase, lysosomal-associated membrane proteins (LAMPs), lysosomal integral membrane protein 2 (LIMP2), chloride channel protein 7 (CLC7), Major-histocompatibility complex (MHC) class II antigen presentation proteins and compartments, synaptogamin 7 (SYT7), CD63, and Niemann-Pick C1 (NPC1) protein.[14]

Lysosomal storage disorders (LSDs) are a set of over 50 inborn metabolic disorders caused by a deficiency of lysosomal enzymes responsible for the breakdown of complex macromolecules in the cell, the accumulation of which leads to cellular stress and eventually cell death.[4][13][14] LSDs are rare with a combined prevalence of only 1 in 8000 live births.[4] However, each

individual disease rate is much less common, usually less than 1 in 100,000 births.[1] Detection of these diseases becomes more pressing as treatment options become available.[4][10][13][15] Depending on which exact catabolic pathway is affected, symptoms of LSDs can similarly affect nearly every tissue in the body, the extent of which is extremely variable, though death is common when the disease is untreated. [4][10][13][15][16] Examples of the diversity of LSD symptoms are shown below in Figure 1.2.

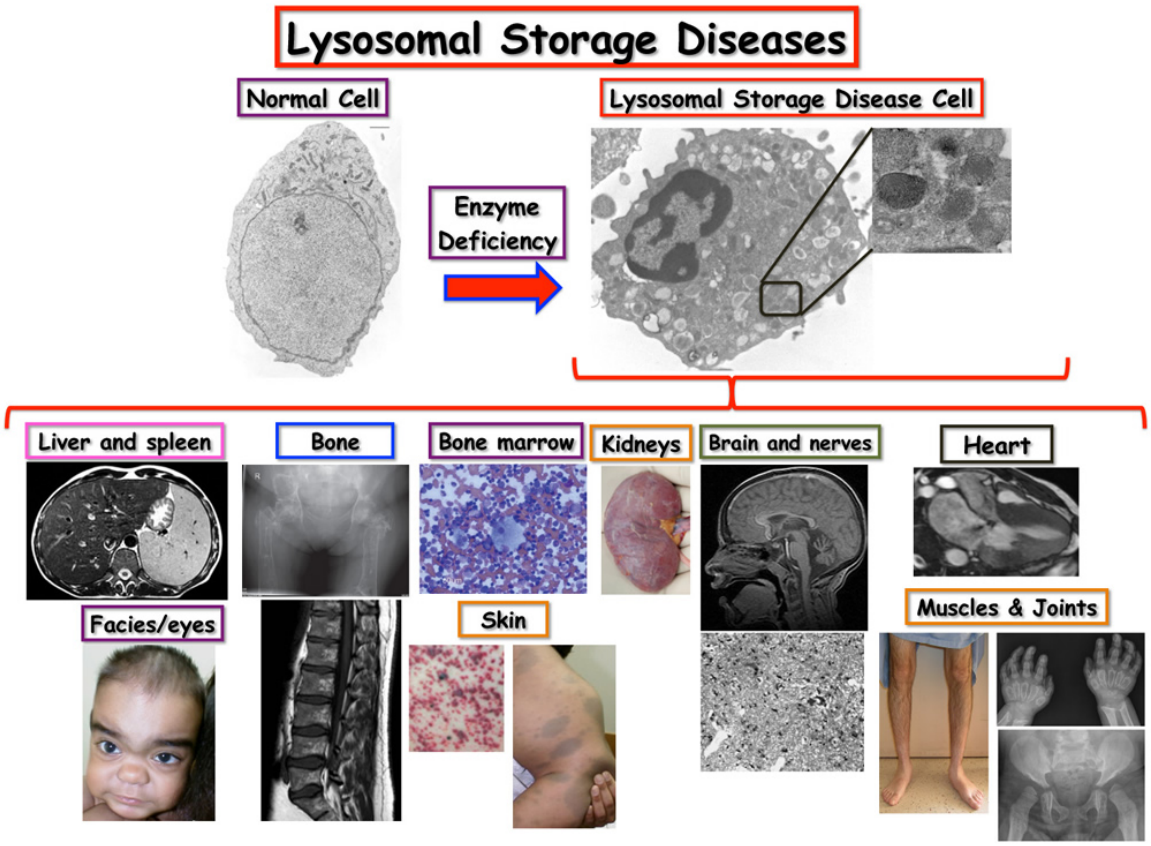


Figure 1.2. Lysosomal storage disease diagram showing the variety of organs and tissues affected by lysosomal dysfunction.[16]

Treatments for LSDs are equally varied as the symptoms and range from only symptomatic management to a complete bone marrow transplant.[4][10][13][15][16] As LSD symptoms are progressive with increased accumulation of metabolic byproducts, treatments are most effective

when administered as soon as symptoms are noted, if not sooner.[4][13] In this regard, NBS provides an excellent pathway towards early detection, and eventually better outcomes for LSDs.

1.3.2 *Lysosomal Acid Lipase*

One of the many single enzyme lysosomal storage disorders is caused via alterations in lysosomal acid lipase. Lysosomal Acid Lipase (LAL) deficiency or insufficiency is seen in two recessive disease forms: Wolman's Disease (WD) and Cholesterol Ester Storage Disease (CESD).[17][18] Wolman's disease is the more severe of the two disorders, presenting typically within the first week of life with severe hepatosplenomegaly (enlarged liver and spleen), adrenal calcification, and failure to thrive, culminating in death typically within the first year of life for affected infants.[17][18] CESD is milder in its course with affected individuals living into adulthood.[17] However, CESD patients typically suffer from resistant hypercholesterolemia (high serum cholesterol) and early-onset atherosclerosis, usually succumbing to fatal cardiac events by their thirties.[19][20][21][22][23] The difference between the two diseases of LALD is due to different levels of residual activity in lysosomal acid lipase, from the variability in the over 40 different unique mutations of the LAL gene LIPA.[24][25] The two forms of LALD are compared in Table 1.2 below.

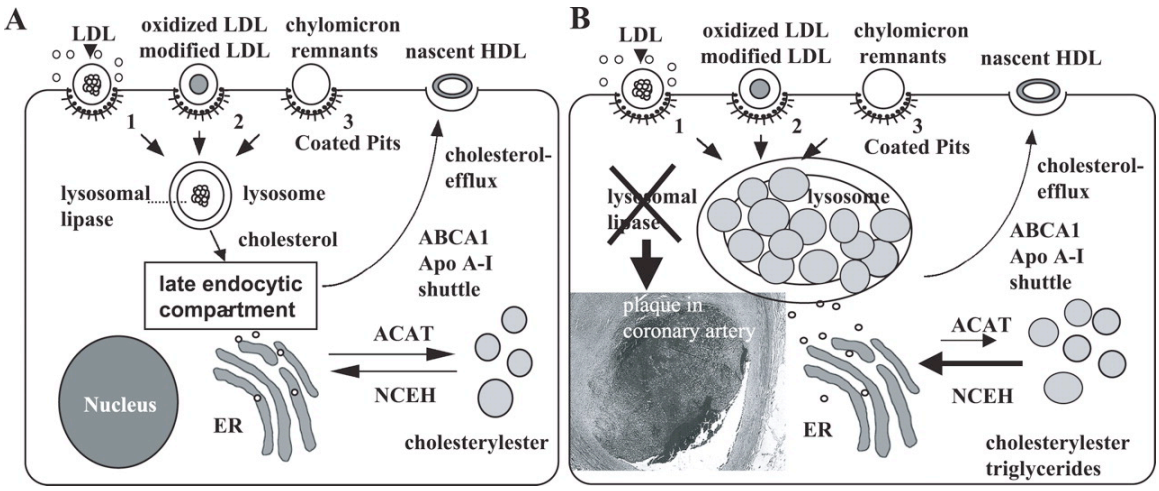


Figure 1.3. LAL involvement in lipid metabolism where (**A** left) is a healthy cell with functional LAL and (**B** right) is a cell with deficient LAL activity. In the absence of active LAL, the cholesterol esters from LDL particles accumulate in the lyso-endosome, further activating NCEH activity while suppressing ACAT-based cholesterol ester synthesis. Abbreviations shown are as follows: high-density lipoprotein (HDL), low-density lipoprotein (LDL), Acyl-coenzyme A–cholesteryl-acyl-transferase (ACAT), endoplasmic reticulum (ER), and neutral cholesteryl ester hydrolase (NCEH).[26]

Table 1.2. Comparison of the two types of Lysosomal Acid Lipase Deficiencies (LALD).[17][27]

Enzyme	Gene	Disease	Treatment Options
Lysosomal Acid Lipase (LAL)	LIPA	Wolman Disease (WD)	• Bone Marrow Transplant • Recombinant Enzyme Replacement Therapy (Kanuma-sebelipase alfa) • Supportive therapies
		Cholesterol Ester Storage Disorder (CESD)	• Recombinant Enzyme Replacement Therapy (Kanuma-sebelipase alfa) • Statins • Diet modification • Supportive therapies

The enzyme involved in both diseases, LAL, is active in the cellular cholesterol utilization pathway as shown in Figure 1.3 above.[26][27][28][29][30] LAL is able to cleave both cholesterol esters and triglycerides through a low-pH serine-catalyzed hydrolysis, giving rise to the dyslipidemias seen in LAL deficient patients. [31][32][33][34][35] Both these diseases are considered rare although in the Western population, roughly 1 in 40,000 are considered to be suffering from CESD and its heterozygote carrier rate is estimated as high as 1 in 200.[19]

Treatments for WD include bone marrow transplant, enzyme replacement therapy (sebelipase alfa), and drug therapies designed to reduce cholesterol ester accumulation in cells.[17][20][27][35]

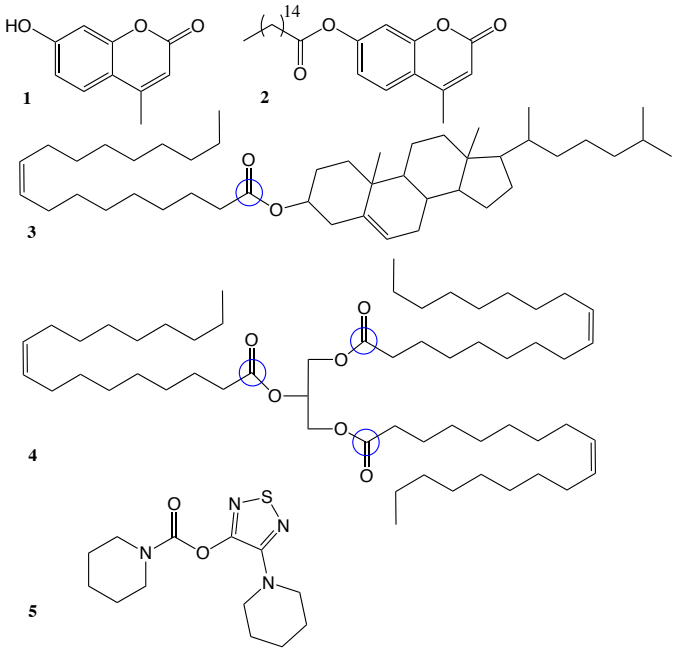


Figure 1.4. Compounds relevant to LAL. 1, 4-methylumbelliferone (4MU) is the fluorophore used for LAL fluorescent assays. 2, 4-methylumbelliferone palmitate is the fluorescent substrate used by Hamilton *et.al.*[36] In both 3, cholesterol oleate, and 4, triolein or glycerol tri-oleate, the blue circle indicates the site of a14C radiolabeled nucleus for radiometric LAL assays used by Negré *et. al.*[37], 5, Lalistat2 (LS2) is a specific LAL inhibitor (IC_{50} is 152 nM) that carbamoylates the active-site serine.[38]

Currently, there are a handful of functional enzymatic tests for LAL reported in the literature, either relying on fluorescence spectroscopy or radiometric counting, using substrates like those listed in Figure 1.4.[36][37][38][39][40] Only one newborn screening assay has been proposed recently for LAL activity using DBS as the source for samples.[36] It is based on the detection of UV fluorescence of 4-methylumbelliferone (4MU) and is dependent upon the use of specific covalent inhibitor of LAL, Lalistat2 (shown in Figure 1.4).[36][38] In the original assay from Hamilton *et. al.*, two samples (inhibited and uninhibited) are run in parallel of the same individual.[36] The original substrate, 4MU palmitate is a general lipase substrate, cleaved by multiple lipases, making the uninhibited sample measure all lipase activity in the sample while

the inhibited sample measures all lipase activity not belonging to LAL. [36] Thus determination of LAL activity is based upon a difference in fluorescent counts amongst two samples (inhibited and uninhibited). [36]

To expand screening options for LAL, we have developed a novel way of detecting LAL deficiencies that employs a tandem mass spectrometry-based (MS/MS-based) enzyme assay, using a lipophilic substrate that is specific for LAL. Eventually, such an assay would be then be multiplexed with other lysosomal storage disorders. In this report, we describe the discovery of a new substrate that is selective for LAL in DBS, and thus the activity of this enzyme can be measured in a single incubation. This new assay and an adapted form for fluorimetric analysis will be discussed further in Chapter 2.

1.3.3 *Mucopolysacharidoses*

Another large group of lysosomal storage disorders fall under the category of Mucopolysaccharidoses (MPS). This group of diseases refers to disorders with accumulation of glycosaminoglycans, a variety of branching carbohydrate peptide polymers.[41] Three such mucopolysaccharidoses are Hunter Syndrome (MPS Type II), Morquio A Syndrome (MPS Type IV), and Mauriteaux-Lamy Syndrome (MPS Type VI) caused by the defects in the following enzymes iduronide-2-sulfatase (I2S), N-acetylgalactosamine-6-sulfatase (GALNS), and N-acetylgalactosamine-4-sulfatase or Arylsulfatase B (ARSB) respectively.[41][42][43][44][45] The three mucopolysaccharidoses that we are interested in currently are detailed in Table 1.3 below. Each of the enzymes affect different GAGs although some are shared, likewise some of the symptoms are also shared.[41]

Table 1.3. Comparison chart of Mucopolysaccharidoses Types II, IVA, and VI.[41][46][47][48]

Enzyme	Gene	Disease	Treatment Options
Iduronide-2-sulfatase (I2S)	IDS	Hurler Disease or MPS type II **Affected GAG** Heparan sulfate Dermatan sulfate	• Bone Marrow Transplant • Recombinant Enzyme Replacement Therapy (idursulfase) • Supportive therapies
N-acetylgalactosamine-6-sulfatase (GALNS)	GALNS	Morquio A Syndrome or MPS type IV A **Affected GAG** Keratan sulfate Chondroitin sulfate	• Bone Marrow Transplant • Enzyme Replacement Therapy (elosulfase alfa) • Supportive therapies
N-acetylgalactosamine-4-sulfatase or Arylsulfatase B (ARSB)	K	Maroteaux-Lamy Syndrome or MPS type VI **Affected GAG** Dermatan sulfate Chondroitin sulfate	• Bone Marrow Transplant • Enzyme Replacement Therapy (galsulfase) • Supportive therapies

All three diseases now have recombinant replacement enzyme treatments and are now being piloted for use in NBS as well as diagnostic testing for these diseases.[41][42][43][44][45] These three diseases originally were assayed using a variety of fluorometric assays using 4-methylumbelliferone-hexose sugar conjugates.[41][44][49][50][51] As all three of the above enzymes remove an ionic sulfate group from the respective base sugar, a secondary enzyme is needed to remove appropriate carbohydrate monomer.[44][49][51] The Gelb lab has now developed novel synthetic substrates that are analyzed via mass spectrometer, removing the need for glycosidic cleavage.[42][43][45][50] However, the use of fluorimetric detection is still common in standard newborn screening facilities and research labs. In order to accommodate fluorometric detection, the Gelb lab has expressed a low-pH active galactosaminidase from

Paenibacillus in collaboration with Dr. Ito from Kyushu University.[52] The enzyme specifically hydrolyzes galactosamine from a small reporter fluorophore, in this case 4-methylumbelliferone, enabling fluorometric detection of both MPS types IV A and VI.[52] The study of these MPS assays will be further discussed in Chapter 3.

Chapter 2. ASSAYS OF LYSOSOMAL ACID LIPASE

The following text is an adaptation of the Clinical chemistry article (doi:10.1373/clinchem.2017.282251), which has been edited to include greater detail than initially published. [53] This article has reproduced with permission from the American Association for Clinical Chemistry.

2.1 INTRODUCTION

To expand screening options for LAL, we have proposed a novel way of detecting LAL deficiencies that employs a tandem mass spectrometry-based (MS/MS-based) enzyme assay, using a lipophilic substrate that is specific for LAL. Such an assay would be then be multiplexed with other lysosomal storage disorders. Recently, enzyme replacement therapy with recombinant LAL (sebelipase alfa) has been approved by the Food and Drug Administration for management of LAL deficiency.[54] As previously discussed, lysosomal storage disorders often benefit from early intervention, making a case for the inclusion of LAL as part of newborn screening.[55] However, given the rarity of LAL deficiency (LALD), newborn screening for this enzyme would be most feasible only the following conditions were met: if it could be added in a multiplex-fashion to an existing LSD NBS panel, if it could be done at low cost, and if the number of samples needed for analysis was reduced. Below we describe the discovery of a substrate that is selective for LAL in DBS, thereby allowing the activity of this enzyme to be measured in a single incubation for the first time via both fluorescence and tandem mass spectrometry.[36]

2.2 METHODS AND MATERIALS

The following has been taken directly from the Clinical Chemistry article (doi:10.1373/clinchem.2017.282251).[53]

2.2.1 *Materials*

All patient samples were obtained with Institutional Review Board approval from the University of Washington. DBS from anonymized samples from LAL-deficient patients were obtained from Dr. Rhona Jack (Seattle Children's Hospital). All patient samples were stored at 4°C in sealed plastic bags. Other DBS samples were stored in plastic bags at -20°C in sealed containers with desiccant. Recombinant human LAL (rhLAL) was obtained as a gift from Alexion Corp. Lalistat-2 was provided as a gift from Dr. J. Hamilton (Yorkhill Hospital, UK) or synthesized as described.[56]

2.2.2 *Synthesis*

The synthesis of all new reagents is detailed as follows from the Mast *et. al.* paper and earlier Appendix A material.[53]

1. Synthesis of Compound 7:

The synthesis of Compound 7 was accomplished via an acyl chloride esterification shown below in Figure 2.1.

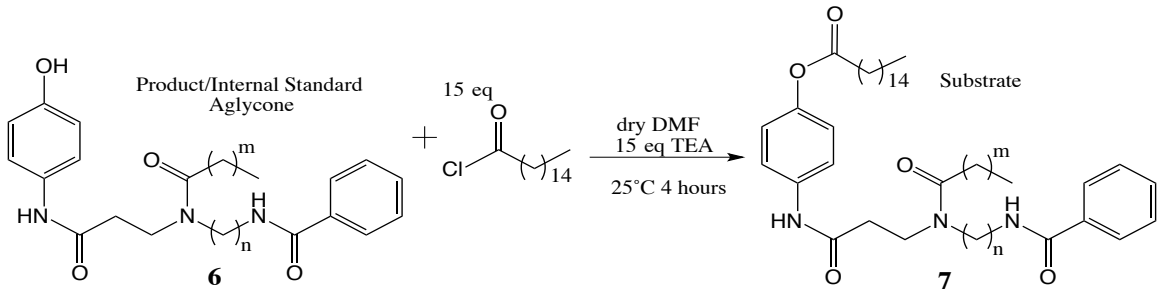


Figure 2.1. Synthesis of Compound 7, the amino-phenol with linkers n= 5 and m=3. An internal standard was obtained with one extra methylene group as compared to the product *p*-amino phenol (m = 4 instead of 3).

2. Synthesis of Compound 12.

The synthesis of Compound 12 was accomplished via Steglich esterification (Figure 2.2) to produce a cholesterol ester with a bis-amide tail.

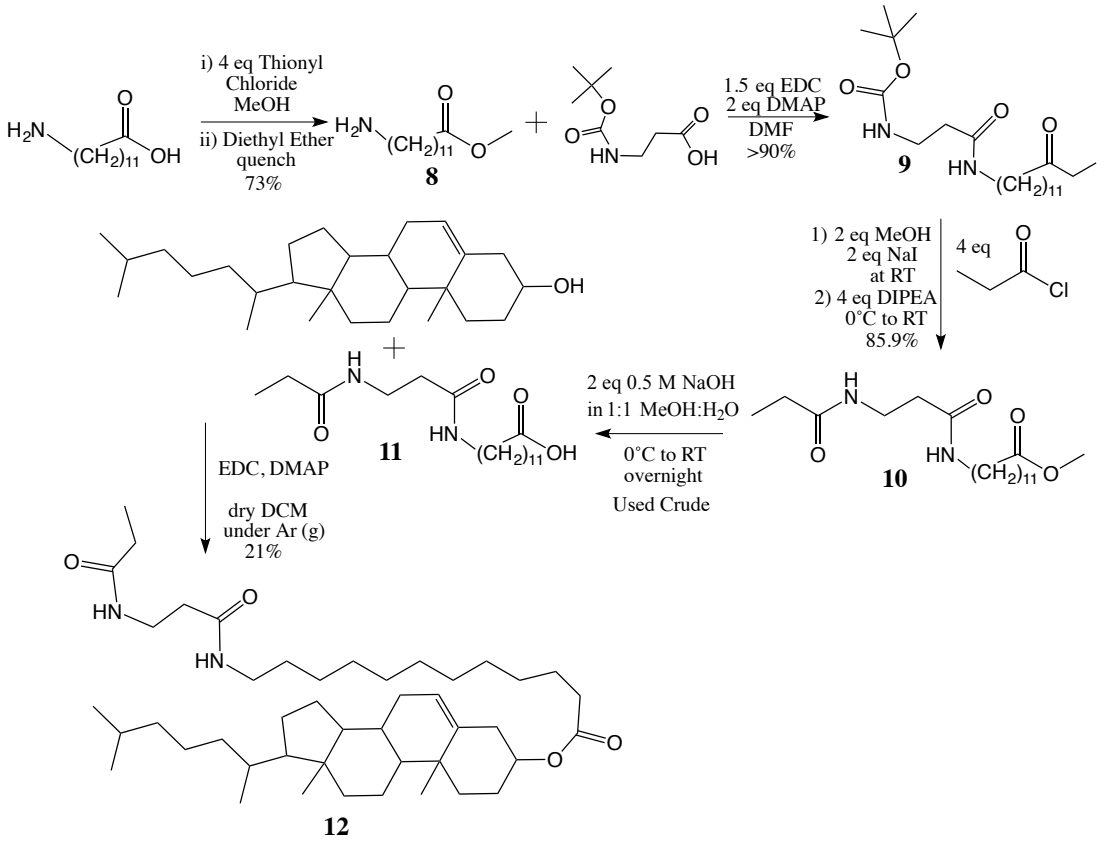


Figure 2.2. Synthesis of Compound 12. Following esterification of ω -amino-dodecanoic acid, Compound 8 was subjected to an amide condensation reaction to give Compound 9 that was then subjected to a one-pot, 2-step de-BOC-ing and acylation. The resulting Compound 10 was de-esterified and then coupled to free cholesterol to give Compound 12.

3. Synthesis of fatty acid esters.

The general synthesis of fatty acid esters involving substituted coumarins is shown in Fig. 2.3 and is described below using the following as an example.

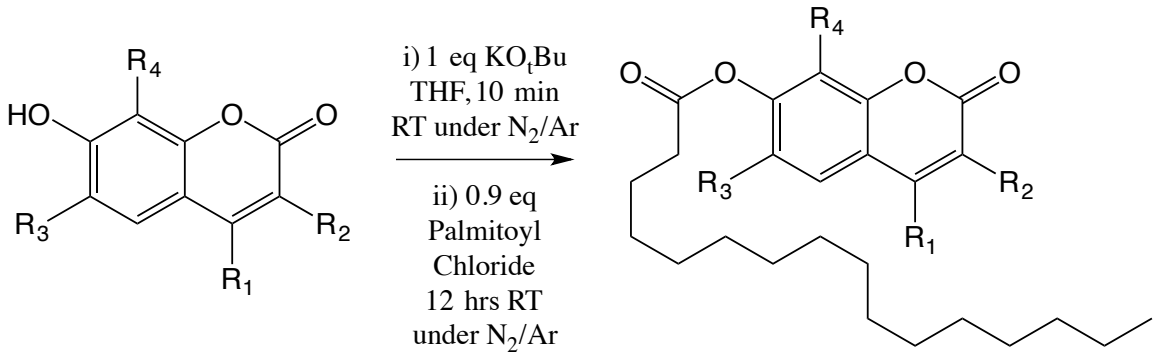


Figure 2.3. General synthetic scheme for the synthesis of substituted coumarin esters.

7-Hydroxy-8-methyl-4-propyl-2H-chromen-2-one (8 mg, 0.04 mmole, Chembridge 6367113) and potassium tert-butoxide (0.04 mmole) were placed in an oven-dried flask under Ar. Dry tetrahydrofuran (2 mL) was added, and the mixture was stirred for 10 min at ambient temperature. Palmitoyl chloride (0.03 mmole) was added via a Hamilton glass syringe, and the mixture was stirred overnight at ambient temperature under Ar. The reaction was quenched by addition of water, and the solution was extracted with methylene chloride. The organic extract was dried over anhydrous sodium sulfate, filtered and concentrated. Product was purified by reverse phase HPLC on a C18 column (YMC-Pack ODS-A, 100 x 20 mm, 5 μm, Waters, AA12S05-1020WT) with the following solvent gradient: 80% water/20 % acetonitrile for 5 min, then to 100% acetonitrile over 35 min, then hold at 100% acetonitrile for 10 min. The product eluted at approximately 45 min. Solvent was removed with a vacuum centrifuge at ambient

temperature. The structure of the product was confirmed by ^{1}H-NMR and electrospray ionization mass spectrometry.

4. Synthesis of internal standard.

Fig. 2.4 shows the synthetic route for the internal standard and it is described in detail below.

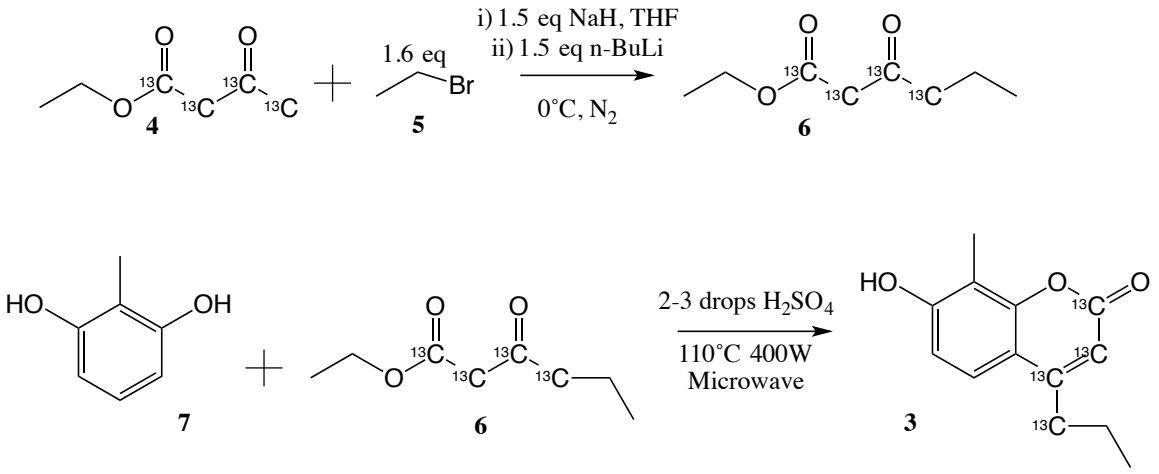


Figure 2.4. The two step synthesis of heavy-labeled internal standard.

To a flame-dried flask was added solid sodium hydride (3.8 mmole) and 12 mL of dry tetrahydrofuran at 0 °C under N_2. The mixture was stirred for 15 min. Then ethyl acetoacetate-1,2,3,4-$^{13}C_4$ (0.3 mL, 2.5 mmole, Sigma Cat. 489263) was added dropwise. Stirring was continued for 15 min. *n*-Butyl lithium (1.6 M in hexane, 3.8 mmole) was added dropwise via syringe, and stirring was continued at 0 °C. Ethyl bromide (4 mmole) was added dropwise, and then the reaction was allowed to warm to room temperature with stirring for 30 min. The reaction was quenched with water then extracted with ethyl acetate. The organic layer was washed with brine then dried over anhydrous sodium sulfate. After filtration and solvent removal, the crude product was purified using a silica column with hexane/ethyl acetate. Ethyl 3-oxohexanoate (0.3 mmole) was mixed with 2-methyl-resorcinol (0.3 mmole) and 2 drops of concentrated sulfuric acid. The mixture was heated in a sealed tube in a microwave reactor (10 min, 100 °C, 400 Watts). The reaction was quenched with water and extracted with

dichloromethane. After drying the organic layer with anhydrous sodium sulfate, filtration, and solvent removal, the crude product was purified by HPLC (same column and gradient as above). The coumarin eluted at ~70% B.

A stock solution of internal standard was prepared by weighing > 5 mg of labeled coumarin and adding absolute ethanol (stored at -20 °C) in a Teflon-septum, screw cap vial.

2.2.3 *Assay Protocols*

LC-MS/MS LAL ASSAY

Assay mixture was prepared by combining 9 volumes of 0.1 mol/L sodium acetate buffer (made by adding reagent-grade NaOH to 0.1 mol/L reagent-grade acetic acid in water), pH 4.5, containing 2.5 mmol/L sodium taurodeoxycholate hydrate (Sigma, 287245) with 1 volume of 0.5% (w/v) bovine heart cardiolipin in ethanol (Sigma, C1649) containing 3 mmol/L substrate 4-propyl-8-methyl-7-hydroxycoumarin (P-PMHC) and 50 µmol/L carbon-13-labeled 4-propyl-8-methyl-7-hydroxycoumarin (internal standard). The latter was prepared by mixing solid substrate P-PMHC with internal standard solution in absolute ethanol, removing solvent in a centrifugal concentrator under vacuum (or with a jet of nitrogen), and then mixing with commercial cardiolipin/ethanol solution. This ethanol stock could be stored at -20 °C for several months (avoid ~2–3 freeze–thaw cycles), and the aqueous buffer could be stored at 4 °C for several months. This was based on no change in assay results when new and stored solutions were used (not shown). Assay mixture was made fresh before each use.

To a 3-mm punch from a DBS in a 1.5-mL polypropylene Eppendorf tube was added 200 µL of purified water (Milli-Q, EMD Millipore). The tubes were shaken on an orbital platform shaker for1hat ambient temperature. The tube of DBS extract was mixed briefly on a vortex-type mixer, and a 10 µL aliquot was added to the well of a polypropylene, deep-well, 96-well plate (Costar,

3959). Assay mixture (30 µL, ambient temperature) was added to each well. The plate was centrifuged at 3000g for 5 min at ambient temperature to ensure that all liquid was at the well bottom. The plate was sealed with a film cover (Advantage 96-well round clear Silicone/PTFE cap mat 962611). The plate was shaken at 37 °C in an incubator with orbital mixing at 400 rpm for 3 h. The reactions were quenched by addition of 80 µL of purified water (Milli-Q, EMD Millipore) followed by 400 µL of HPLC-grade ethyl acetate. The well contents were mixed by pipetting up and down approximately 10 times. The plate was centrifuged at 3000g for 5 min at ambient temperature, and then 120 µL of the upper ethyl acetate layer was transferred to a shallow-well, 96-well polypropylene plate (Greigner, 651201). Solvent was removed at ambient temperature with a jet of N_2. To each well was added 200 µL of water/methanol (1:1, Fisher Optima Grade), and samples were mixed by pipetting up and down a few times. The plate was wrapped with aluminum foil and placed in the autosampler chamber at 8 °C in preparation for LC-MS/MS analysis.

Liquid chromatography was carried out using a Waters Acquity binary solvent pump system with a CSH, C18, 1.7-µm, 2.1 × 50-mm column (Waters, 186005296) and a CSH, C18, 1.7-µm guard column (Vanguard, 186005303). The solvent program was 99% A (70:30, water/acetonitrile, 0.1% formic acid, Fisher Optima grade)/1% B (50:50, acetonitrile/isopropanol, 0.1% formic acid) to 30% A/70% B over 1 min, then jump to 100% B and hold for 0.5 min, then to 99% A over 0.5 min (flow rate 0.8 mL/min). The total run time was 2.5 min. Mass spectrometry was carried out with a Waters Xevo TQ tandem-quadrupole instrument. Additional liquid chromatography and tandem mass spectrometry parameters are provided in Appendix 1.

FLUOROMETRIC LAL ASSAY

The fluorometric assay up to the end of the incubation was identical to the LC-MS/MS assay. After incubation, the fluorometric assay was continued as follows: Reactions were quenched by addition of 200 μL of water/methanol (1:1, Fisher Optima Grade). The well contents were mixed by pipetting up and down approximately 10 times. A portion (150 μL) of the well contents was transferred to a black, flat-bottomed 96-well microtiter plate (NUNC 437112). Samples were immediately read on a fluorometer (PerkinElmer Victor3V 1420) with an excitation wavelength of 355 nm, an emission wavelength of 460 nm, and an excitation time of 0.1 s. The fluorometer reading was converted to micromoles of product by generating a standard curve. DBS extract (60 μL) and assay mixture (450 μL) were incubated separately. To a well was added 200 μL of water/methanol solution (1:1), 10 μL of DBS extract, and 30 μL of assay mixture. To each well was added 2 μL of LAL product (4-propyl-8-methyl-7-hydroxycoumarin) (0, 0.2, 1, or 2 nmol) from an aqueous stock solution. After mixing by pipetting up and down approximately 10 times, a 150 μL aliquot was transferred to the fluorometer plate and submitted to fluorometry as above. The well in which no product 2 was added serves as the blank. Note that because DBS extract and assay mixture were incubated separately, this blank reflected (a) the fluorescence of the blood extract and substrate; (b) any fluorescence owing to non-enzymatic breakdown of substrate; and (c) any quenching of the fluorescence by components of the blood.

2.3 RESULTS

2.3.1 *Initial Assays*

The first assay for LAL performed in the Gelb laboratory was the MS/MS assay using Compound 7, an aminophenol based on the most recent MS/MS scaffold used currently for other NBS projects.[9] At the time of these initial experiments, no MS/MS protocol for LAL activity existed. Thus for a preliminary pilot test, the first assay MS/MS conditions (Appendix A) were

loosely based on the existing LAL fluorimetric work of Hamilton *et. al,* at that time, the only DBS-compatible assay in the literature.[36] We chose to mimic assay work-up in a detergent-containing buffer similar to those used in other Gelb LSD assays.[9] The reaction used the inhibitor Lalistat2 in order to detect LAL specific lipase activity from the difference in signal between the uninhibited and inhibited assays.[36] As stated previously, Lalistat2 was found to be a specific inhibitor for LAL compared to other lipases.[36][38][54] To determine enzymatic activity, the Gelb laboratory uses the Product to Internal Standard ratio (P/IS ratio) to normalize MS/MS responses. The internal standard initially used was a carbon-chain length variant, instead of an exact heavy-labeled match, for efficiency (see Figure 2.1).

This initial MS/MS assay of LAL showed increased P/IS ratio above a filter paper control for all DBS samples (see Table 2.4). Thus, some product was being formed in the presence of blood alone.

Table 2.4. Original Substrate DBS Mass Spectrometry Assay of Compound 7.

Compound	**Average Ion Counts Uninhibited DBS wells**	**Average Ion Counts Inhibited DBS wells**
LAL Substrate	220624	214994
LAL Product	23364	11274
LAL IS	25714	12133
P/IS ratio	1.06	1.01
Well Type	**Blood/No Blood Ratio**	**Resulting Lipase Activity (µmol/hr/L blood)**
Uninhibited	2.04	3.09E-06
Inhibited	2.12	2.96E-06

The data does show an interesting discrepancy between the inhibited and uninhibited DBS samples (the inhibited wells have half the MS/MS response compared to the uninhibited). It is not clear why this is but it was reproducible in multiple runs. However, there was no difference between the inhibited and uninhibited P/IS ratios as seen in Table 2.4. This can be interpreted as no detectable specific LAL activity in the assay. Two main reasons for this non-specific result

come to mind. Firstly, the enzyme does not cleave the substrate or, secondly, the assay conditions are not conducive for detecting activity. To test these aspects, a second more natural-like substrate was prepared to test LAL substrate preferences. Additionally, the assay conditions were adjusted to duplicate the incubation conditions used in the Hamilton *et. al.* assay.[36]

The cholesterol ester was suggested because in acidic pH, LAL is thought to be the dominant, if not only, esterase active towards cholesterol esters.[24][57] These highly hydrophobic esters are less likely to have non-specific esterase activity at low pH due to their dissimilarity to preferred substrates of the protein and carbohydrate esterases present in the blood. Likewise it is known that relatively few acid lipases are present in the blood.[24][57] Thus highly hydrophobic esters should theoretically provide an opportunity to report specific LAL activity, independent of the inhibitor Lalistat2. The initial hypothesis is that such specificity would eliminate the need for the inhibitor Lalistat2 because the only activity on highly hydrophobic esters should be LAL. While testing Compound 12, the above modified cholesterol ester in Figure 2.2, we only monitored total enzyme activity above a no-enzyme control i.e. a filter paper blank, to determine whether or not there was any lipase activity using the hypothesized-specific Compound 12. This modification simplified the assay protocol.

Using the assay conditions specified for Variation 2 of the MS/MS assay (see Appendix Table A), the resulting ion counts from the MS/MS assays displayed no differences in activity across the different blood samples (data not shown). The blood samples used included Quality Control (QC) samples, both Low and High, and a healthy control DBS spot.[7] Typically, as the Low QC spot has only 5% whole blood and thus only 5% of the total leukocytes possible in the High QC spot, the low should have fewer counts of product formed.[7] No change in the P/IS ratio across

all three blood samples reflects the possibility that LAL does not act on the substrate or that assay conditions remained flawed. Thus neither question was answer at this point.

2.3.2 *rhLAL Assays*

Concurrently, we wished to test isolated LAL activity in the form of recombinant human LAL (rhLAL) obtained from Synageva.[54] Using rhLAL, the final incubated assay components would be greatly simplified as the complex ionic, lipid, and protein mixture of a DBS would be eliminated. This simplification would minimize the presence of both unknown agonists and antagonists of LAL in the DBS matrix. With rhLAL, we gained a reliable positive control for enzymatic activity to replicate existing assays in the literature (specifically radiometric and fluorimetric assays). With the ability to validate our findings to literature values, we should be able to directly transfer assays using rhLAL to a DBS source for LAL.

The first rhLAL assays were run in parallel with Compound 12 MS/MS assays for the DBS samples using the same assay volumes, substrate concentrations, incubation, and work-up conditions (see Appendix Table A). These rhLAL assays showed no evidence of product formation across the three protein concentrations (4 μg, 2 μg, and 0.4 μg), which suggests that the cholesterol ester was likely a poor substrate, if one at all, for the enzyme, under the given MS/MS conditions.

Going forward, the activity of LAL against natural highly hydrophobic esters (like Compound 12) was further evaluated to understand the low activity of LAL in known literature assays.[30][31][33][36][37][39][40] To evaluate the hydrophobic esters, radiometric assays for 14C were performed using cholesterol oleate and triolein (Fig 1.4). We hoped to discover how robust rhLAL activity was for cholesterol esters and glycerol esters to translate that into a functional specific LAL assay in the absence of inhibitors. The assay conditions used duplicated

the procedure and extraction from Negré *et. al.* and Belfrage and Vaughn respectively (see Appendix Table A).[37][40]

Consistent with literature reports, rhLAL displays very low activity to its native substrates as seen in Table 2.5 below.[37][39] For the radiometric assays, the best activity is still less than 1 μmol/hr/mg enzyme. Negré *et. al.* reports activities of approximately 3 nmol/hr/mg of lymphocyte or fibroblast pellet for cholesterol oleate and 60 nmol/hr/mg for triolein.[37] Considering that there is significantly less than 1 mg of LAL enzyme in a 3.2 μL of blood (the volume of a DBS), this makes it unlikely that a signal will be detected under radiometric conditions while using a natural highly hydrophobic ester. Later analysis using rhLAL with the Hamilton fluorimetric assay determined that there is only 50-100 pg of LAL enzyme per DBS punch.[36]

Table 2.5. Radiometric rhLAL Assay of cholesterol oleate and triolein.

Substrate	DPM/Assay	Amount rhLAL (μg)	Total DPM	Total activity μmol/hr/mg
Triolein	300,000	0	118	
		2	2628	0.086
		0.4	1841	0.294
		0.04	657	0.921
Cholesterol Oleate*	150,000	0	130.	
		20	6392	0.101
		4	6845	0.540
		2	6295.2	0.992

* obtained due to assistance F. Ghomashchi.

Another feature of the radiometric assay data is the increase in rhLAL activity at lower amounts of enzyme. It is unclear what the source of this trend is. However, the activity detected from the scintillation counter is well within the limits of detection and is only about 1% of the total radioactive material present in the assay at the time of incubation (based on spiking scintillation fluid with half of the total DPMs of cholesterol 14C-oleate added to the initial assay). One can

speculate that the amount of activity present in the samples reflects the relative concentration of substrate and if the substrate is not well solubilized, the effective concentration will be reduced and thus produce a plateau of activity. At 100 µL (the assay volume), micro-precipitates would not be visible and thus solubility of the substrate in the assay could pose a problem.

2.3.3 *Reactive Ester Compound Library*

Our goal was to find a new substrate that was acted upon only by LAL in DBS, thus avoiding the need to carry out 2 assays in parallel in the presence and absence of the LAL-specific inactivator Lalistat2.[36] Using this approximate value and the published specific activities for purified LAL acting on the natural substrates cholesterol oleate and triolein in addition to our own experimentation, we concluded that the amount of products produced with a 3-mm DBS punch and the natural substrates would be well below the detection limits for mass spectrometry.[37] Thus, assays using these natural substrates were not pursued further, focusing instead on a reactive fatty ester, like the initial 4MU palmitate.

Again looking back at the original inactive MS/MS DBS results, we wanted to confirm that our starting assay conditions based on Hamilton *et. al.*'s recent fluorescent DBS LAL assay could replicate their results (see Appendix Table A for assay conditions).[36] Using the fluorescent Compound 2 (4-methylumbelliferone palmitate) substrate and subsequent optimization for ideal substrate solvent (DMSO or DMF) and quench solvent, the original Hamilton *et.al.* assay could be replicated consistently on DBS samples (see Table 2.6).[36]

Table 2.6. Differences in LAL activity between adult and newborn DBS samples.

	Lipase Activity µmol/hr/L blood		**LAL Activity** µmol/hr/L blood	
DBS Type	**Uninhibited**	**Inhibited**		**% LAL Activity**
Newborn N=9	214.4	96.0	118.4	55.2
Adult N=3	597.6	131.8	465.7	77.9

The key takeaway from Table 2.3, is the high amount of non-LAL specific lipase activity, approximately 20% in adults and closer to 45% in newborns. This lack of specificity is the reason for the use of Lalistat2 in the original Hamilton assay.[36] It is this non-LAL activity that we wish to eliminate via a more specific substrate.

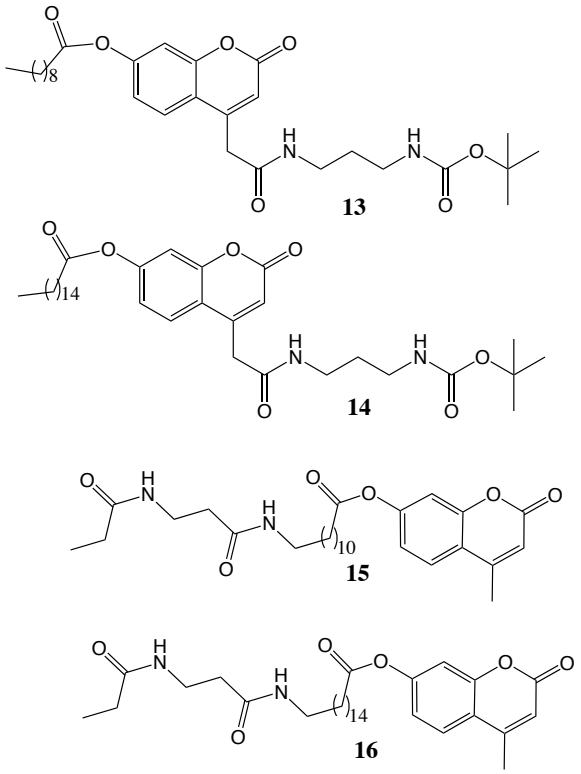


Figure 2.5. Four compounds were synthesized initially to test for LAL specific activity in DBS samples. Compounds **13** and **14** were two different chain-length variants of an existing coumarin-like scaffold. Compounds **15** and **16** were two chain-length 4-methylumbelliferone ester variants with terminal bis-amides.

With a functional fluorimetric DBS assay, what was now needed was a specific substrate assay for LAL using DBS extracts. Four coumarin-based substrates were synthesized and tried initially, as seen in Figure 2.5 Compounds 13 and 14 were synthesized off of an existing lab scaffolds thus structurally similar to earlier Gelb lab MS/MS-compatible leaving groups extending from an alkyl amide chain off the 4th carbon position of 4MU.[45][58] Next, Compounds 15 and 16 were created synthesized to have bis-amides as proton-traps at the omega-terminus of the fatty acyl group of 4MU esters. [11][12][45] Both were tested via the previously

designed MS-compatible assay (refer back to Section 2.2.2 and Appendix A). Unfortunately, no fluorescent LAL activity using DBS samples was detected for any of these four compounds. These studies show that addition of polar groups to the palmitoyl chain abrogated activity toward LAL. Similarly, extension of the 4MU moiety with polar alkyl amide chains was also not tolerated.

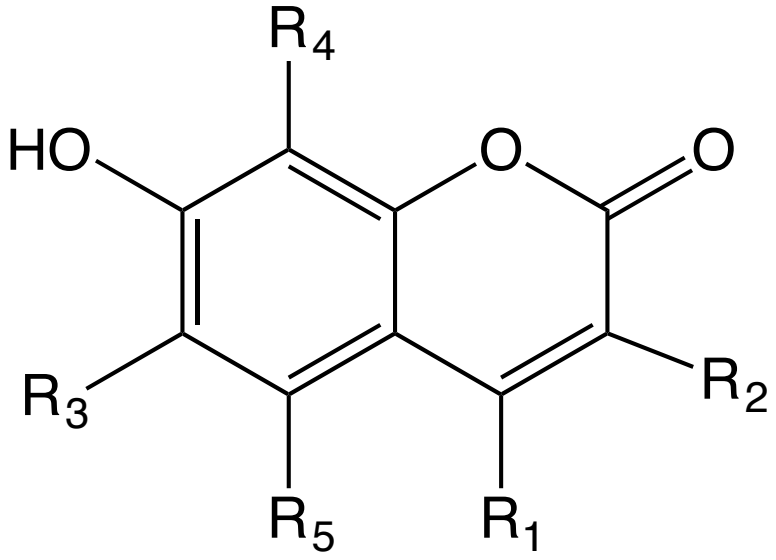
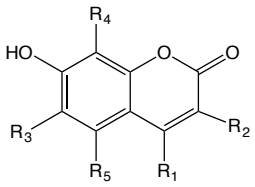


Figure 2.6. Common scaffold of a substituted coumarin, where R_1-R_5 are different possible variable positions available commercially. However, there are no commercial non-proton substituents for site R_5.

Up until this point, only one substrate produced detectable LAL activity using DBS: the original fluorescent non-LAL specific 4-methylumbelliferone palmitate (Compound 2 or 4MU palmitate). This was assumed to be due to the highly reactive nature of the 4MU ester linkage as cleavage produced as stable 4MU free alcohol. Additionally, our work with LAL and experience with the literature on the enzyme stressed the need for strongly hydrophobic substrates. Thus we decided to perform a simple structural variation library study using coumarin analog derivatives of 4-methylumbelliferone palmitate. Twenty-six different substrates were made using the previously mentioned synthesis in Section 2.2.2. Their structures are listed Table 2.7 below.

Table 2.7. List of coumarin analogs structures and their commercial sources for library of potential LAL substrates.



Compound	Groups*	Commercial Source
17	R_1= *n*-Propyl, R_3 = Me	ChemBridge
18	R_1= Me, R_2 = Cl	Sigma
19	R_1= Phenyl	Sigma
20	R_1= R_2 = -$(CH_2)_5$-	Enamine
21	R_1= 4-F-Phenyl	ArkPharm
22	R_1= CH_2OMe	Matrix Scientific
23	R_1 CF_3, R_3 = Cl	Apollo Scientific Limited
24	R_1= *n*-Butyl	ChemBridge
25	R_1= *n*-Butyl, R_4 = Me	Oakwood Chemical
26	R_1= Me, R_3 = Cl	Indofine
27	R_1= R_2 = R_3 = Me	Sigma
28	R_1= Me, R_2 = 2,4-Cl_2-Phenyl	Indofine
29	R_1= N-CH_2-Morphilino	Matrix Scientific
30	R_1= R_2 = -$(CH_2)_4$-, R_3 = Et	Oakwood Chemical
31	R_1= Me, R_2 = Et	Matrix Scientific
32	R_1= Me, R_2 = CH_2Phenyl	Sigma
33	R_1= Me, R_2 = Me	Matrix Scientific
34	R_1= Me, R_3 = Ethyl	Enamine
35	R_2 = Phenyl	Indofine
36	R_1= Et, R_4 = Me	ChemBridge
37	R_1= 4-Pyridyl	Matrix Scientific
38	R_1= -CONH-(4-Pyridyl)	AK Scientific
39	R_1= R_2 = -$(CH_2)_3$-	Sigma
40	R_2 = CN	Indofine
41	R_1= Me, R_2 = Br	Key Organics
4MU	R_1 = Me	Sigma

*R groups not specified are H atoms.

In order to evaluate the library results, the existing fluorimetric assay was adapted to the mass spectrometer via the substitution of the existing detergent for the water-soluble sodium

taurodeoxycholate and an ethyl acetate extraction step was added. These two changes enabled the assay to be screened using MS/MS. Sodium taurodeoxycholate was chosen as primarily due to the fact that it largely remains in the aqueous phase following liquid–liquid extraction with ethyl acetate (thus minimizing injection of large amounts of detergent onto the UPLC column, which is critical to long term usage as for NBS). Replacement of Triton X-100 with 2.5 mmol/L sodium taurodeoxycholate resulted in a 20% decrease of LAL activity, and increasing the detergent concentration further led to an additional loss of activity. Thus, 2.5 mmol/L was chosen for all subsequent studies.

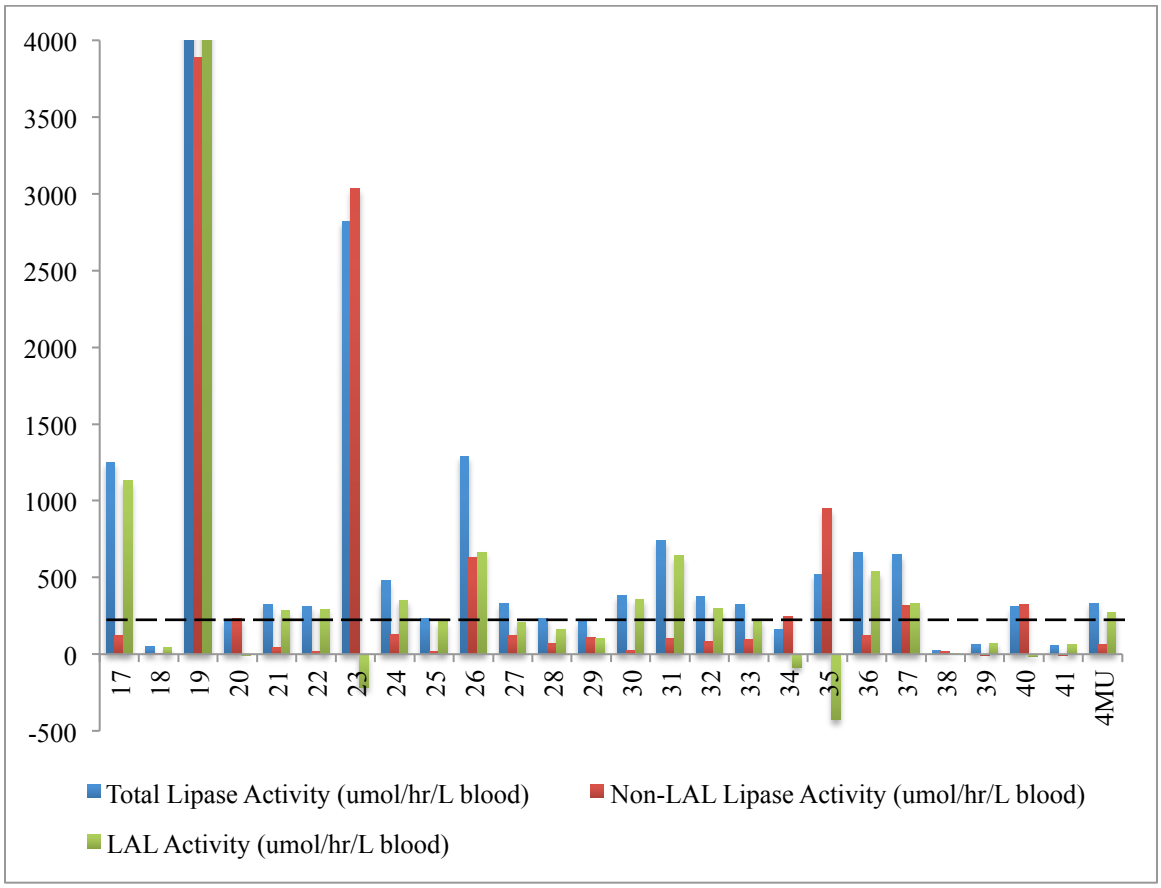


Figure 2.7. Graph showing MS/MS activity of 26 compounds. The total lipase activity is calculated from the uninhibited wells. The non-LAL lipase activity is calculated from the inhibited wells containing Lalistat2. Subtracting the non-LAL lipase activity from the total lipase activity gives the LAL-specific activity. The exact methods for these assays are found in Appendix A.

As seen in Figure 2.7, compounds **18**, **20**, **21**, **22**, **25**, **27**, **28**, **29**, **33**, **34**, **38**, **39**, **40**, and **41** were poor substrates with DBS as the source of LAL compared to 4MU palmitate and were not studied further. In other words, these fourteen compounds had less LAL-specific activity compared to 4MU. Analogs **19**, **23**, **24**, **26**, **32**, **35**, **36**, and **37** showed higher LAL activity than 4MU but addition of Lalistat-2 did not substantially reduced the activity more than what was seen for 4MU palmitate. These eight compounds had lower than 82% LAL specificity (see Appendix Table D) for normal adult DBS samples. This left 3 compounds: **17**, **30**, and **31**. Both **17** and **30** displayed greater than 90% of the LAL-specific activity measured with palmitoyl-4MU. Additionally, compound **17** also had the highest total lipase activity of the three remaining compounds. The combination of high activity and high specificity of Compound **17** over 4MU-palmitate led to its selection as the new lead compound. Compound 17 was then relabeled as palmitoyl 4-propyl-8-methyl-7-hydroxycoumarin (P-PMHC) and was used for all additional studies. A corresponding heavy-labeled internal standard was then synthesized as mentioned in Section 2.2.2.

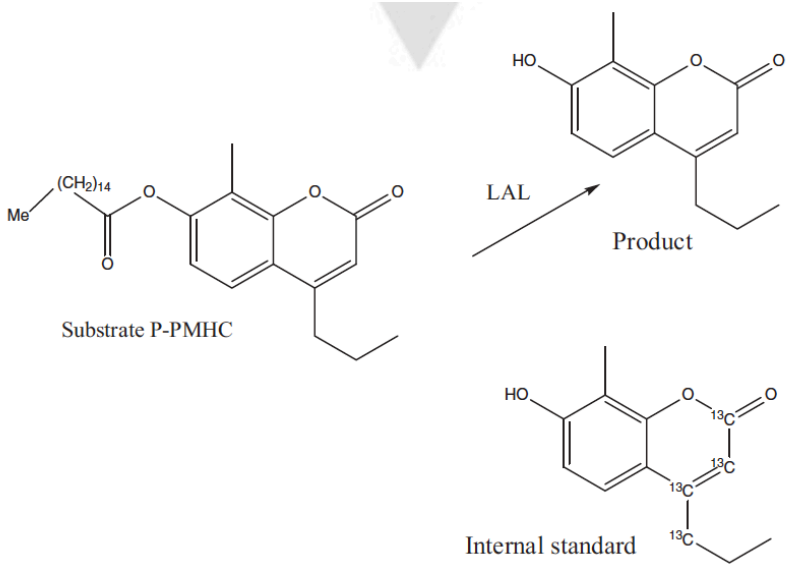


Figure 2.8. Structure and relationship of the LAL substrate, P-PMHC, to its product PHMC and its internal standard.

2.3.4 *Optimization*

Hoping to further streamline the MS/MS assay, we tested a set of seven different phospholipids as replacements for cardiolipin, which was used in the original LAL assay.35 The following phospholipids were tested individually at 0.1 mmol/L: 1,2-dioleoyl-phosphatidylethanolamine, 1,2-dioleoyl-phosphatidylmethanol, 1,2-dioleoyl-phos-phatidylcholine, 1,2-dioleoyl-phosphatidylserine, brain phosphatidylethanolamine, 1,2-heptadecyl-phosphatid-ylcholine, and 1,2-lauryl-phosphatidylcholine. None of these phospholipids led to any substantial increase in LAL over that measured with cardiolipin (data not shown); thus, we continued our studies with the latter.

Table 2.8. Effect of DMSO age on LAL activity in DBS punches.

OLD DMSO		**Lipase Activity**		**Activity of LAL**
Lalistat2	**Sample**	**nmol/hr/µL**	**pmol/hr/spot**	**pmol/hr/spot**
Uninhibited	DBS	0.0463	48.05	53.37
Inhibited	DBS	0.0296	94.69	
NEW DMSO		**Lipase Activity**		**Activity of LAL**
Lalistat2	**Sample**	**nmol/hr/µL**	**pmol/hr/spot**	**pmol/hr/spot**
Uninhibited	DBS	0.534	1709.53	1400.34
Inhibited	DBS	0.0966	309.19	

As mentioned earlier in the original Hamilton assay, DMSO was used as a concentrated substrate solvent to prepare the 4MU palmitate and Lalistat-2 stock solutions.[36] The final concentration of DMSO in the LAL assay was 2.4%[36]. Early on during the course of our studies, we noted that the quality of the DMSO was critical to being able to detect LAL activity in DBS. Previously opened bottles of DMSO stored for approximately 1 month or longer led to

essentially complete loss of LAL activity measured in DBS as shown in Table 2.8. Based on this result, we decided to replace DMSO initially with dimethylformamide (DMF) for preparation of the substrate stock solution, and only ethanol to prepare the Lalistat-2 solution (however, with the use of LAL-specific P-PMHC, Lalistat-2 was no longer used). In our final assay conditions, we used ethanol as the only solvent other than water.

The LAL pH rate profile was studied from pH 3.5 to 7.0 in 0.1 mol/L succinate or from pH 3.5 to 5.5 in 0.1 mol/L sodium acetate (Fig. 2.8) to determine the pH optimum for LAL using P-PMHC. Based on the analysis shown below, we chose 0.1 mol/L sodium acetate at pH 4.5 to maximize LAL activity.

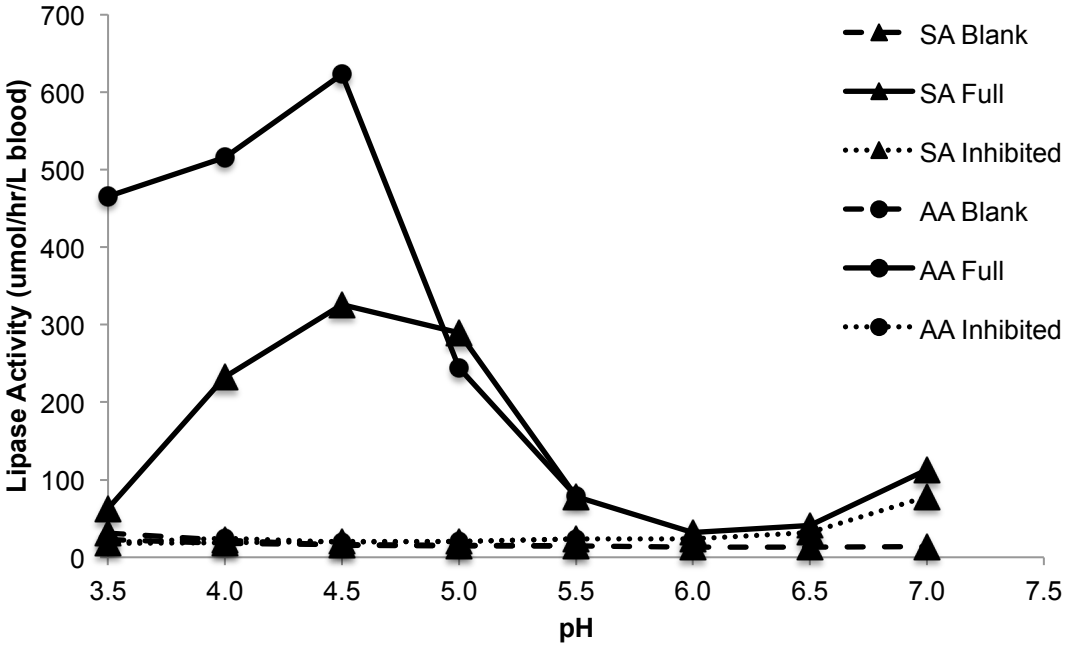


Figure 2.9. pH optimization of P-PMHC LAL substrate assay where ▲ refers to assays performed in a Succinic acid (SA) buffer and ● refers to assays performed in an Acetate (AA) buffer. Both inhibited and blank assays had 300 µM Lalistat2 while only the full and inhibited assays contained DBS extract.

We settled on a concentration of P-PMHC in the mixture of 3 mmol/L based on a good combination of high LAL activity and minimal background (no DBS or blank control, data not shown). The Hamilton assay used 40 µL of an aqueous extract of a 3-mm DBS added to 160 µL

of assay mixture.[36] To keep the solvent volumes for the liquid–liquid extraction to a minimum, thus avoiding long times for solvent removal, we added only 10 µL of an aqueous extract of a 3-mm DBS punch to 30 µL of assay mixture.

With the final assay conditions chosen, we obtained a specific activity of 874 µmol/h/L using a 3-mm punch of a DBS from a healthy adult. The activity was measured by UPLC-MS/MS after extraction of the reaction mixture with ethyl acetate. The tandem mass spectrometry multiple reaction monitoring response was converted to micromoles of product with the use of a chemically identical but isotopically differentiated internal standard (carbon 13-labeled 4-propyl-8-methyl-7-hydrdoxycoumarin) (Fig. 2.7). The use of the internal standard accounts for all product losses owing to sample processing and analysis. Using a 3-mm punch from an identical DBS and the previous fluorometric LAL assay with and without Lalistat-2, we obtained a specific activity of 466 µmol/h/L (within the range of values reported previously) for the LAL component, which is 1.9-fold lower than the activity measured with the new UPLC-MS/MS assay.35

Table 2.9. Effect of Lalistat2 on MS/MS assay LAL activity for substrate P-PMHC.*

DBS	Activity without Lalistat2 µmol/h/L	Activity with 10 µM Lalistat2, µmol/h/L	Activity with 100 µM Lalistat2, µmol/h/L
Adult 1 (triplicate)	554,582,582	10,9,10	6,4,5
Adult 2 (triplicate)	519, 539, 473	5,2,3	2,0,1
Blank (15 measurements)		16.8-26.2	

*Activity values for adult DBS measurements were blank corrected. Blank measurements were obtained by using an equal volume of water instead of aqueous DBS extract.

The LAL substrate P-PMHC was highly specific for LAL in DBS as seen in Table 2.6. When extracts from 2 healthy persons' DBS were pre-incubated with 10 µmol/L Lalistat-2, 98% of the activity toward P-PMHC was inhibited. This increases to 99% inhibition if 100 µmol/L Lalistat-2 was used (Table 2.9). In contrast, when the same DBS extract was analyzed using the previously reported Hamilton assay with 4MU palmitate, 78% of the total lipase activity was

blocked by 10 μmol/L Lalistat-2 (not shown).[36] Under the assumption that Lalistat2 was completely selective for LAL, the data showed that P-PMHC, but not 4MU palmitate, was highly specific for LAL in DBS.

Because hydrolysis of P-PMHC leads to the fluorescent product 4-propyl-8-methyl-7-hydroxycoumarin, this novel LAL substrate is still compatible with fluorescent assays using a standard plate reader fluorometer to measure LAL activity in DBS. Table 2.10 shows LAL activities measured with UPLC-MS/MS and by fluorometry on an identical set of DBS from 10 adults. Agreement between the two assays generally showed <30% difference in activity values for all but 1 pair, for which the difference approached 50%.

The analytical range is an important assay parameter that is defined as the ratio of assay response measured with the quality control high sample (typical of a healthy person) divided by the assay response for all elements independent of LAL.[59] The larger the analytical range, the greater the activity values will be spread out, leading to greater accuracy especially when activity is low. The mean analytical range for the UPLC-MS/MS assay was 44 compared with a value of 14 for the fluorometric assay.

Table 2.10. LAL activity in DBS measured by UPLC-MS/MS and fluorometric assays.[53]

LAL activity in DBS measured by UPLC-MS/MS and fluorometric assays.				
Type	Samples	LAL activity, μmol/h/L blood[a]	Mean	%CV
Fluorescence				
Adult	1	746, 707, 596, 599	662	9.94
	2	541, 751, 675, 519, 803	658	17.08
	3	618, 592, 583, 628	605	3.05
	4	933, 1683, 1073, 938, 1261, 1348	1206	21.81
	5	809, 917, 901, 768, 815	842	6.83
	6	1198, 1635, 1361, 1382, 1284	1372	10.67
	7	758, 854, 841, 645, 781	794	9.95
	9	427, 100, 376, 239	286	44.54
	10	767, 857, 1150, 1158, 900	980	15.89
	12	989, 843, 792, 918, 604, 703	808	15.89
Blank[b]		48–78	58	19.03
UPLC-MS/MS				
Adults	1	598, 555, 653, 578, 608	598.5	5.5
	2	712, 701, 674, 632, 588, 636	657.1	6.5
	3	492, 499, 506, 480, 453, 465	482.2	3.9
	4	962, 842, 862, 848, 1016, 849	896.3	7.5
	5	846, 852, 852, 864, 923, 907	874.0	3.4
	6	990, 979, 1129, 1152, 929	1035.8	8.5
	7	613, 555, 488, 536, 514, 470	529.0	8.9
	9	316, 330, 220, 247, 292, 236	273.4	15.2
	10	938, 796, 818, 627, 1112, 740	838.3	18.3
	12	424, 534, 376, 496, 368, 385	430.4	14.7
Blank[b]		6.3–24.9	15	36.4

[a] Activity values are blank corrected.
[b] Range of blank values given for 12 and 56 repeats of the fluorometric and tandem mass spectrometry assays, respectively.

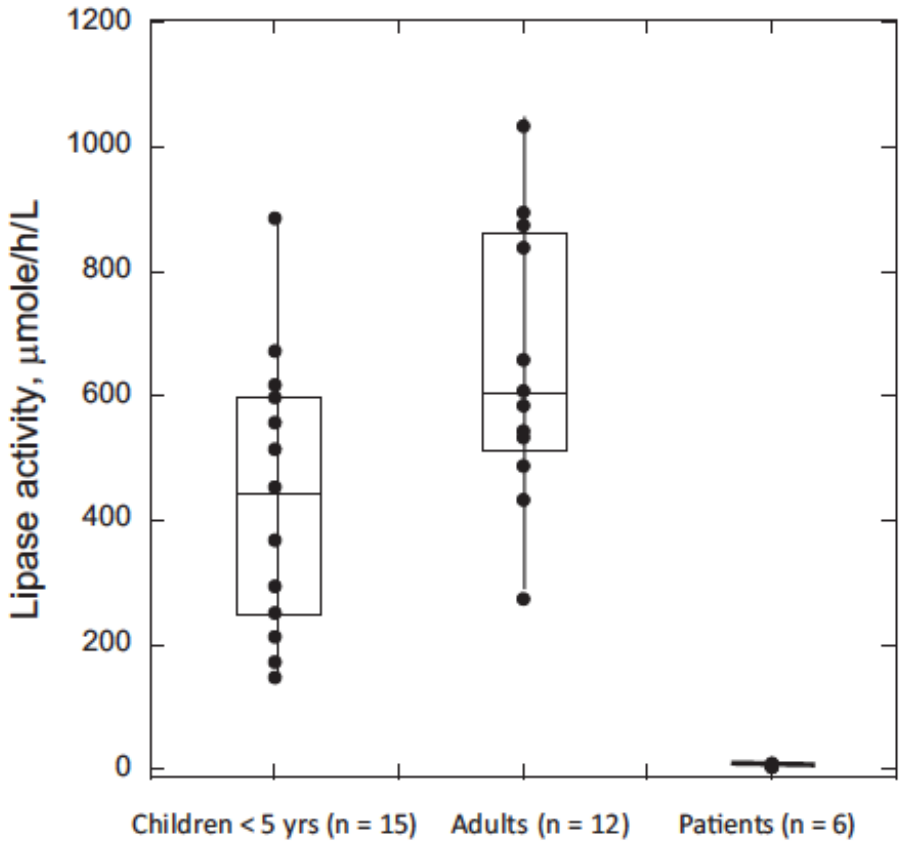


Figure 2.10. LAL activity in DBS measured with substrate P-PMHC and UPLC-MS/MS. The bottom of each box is the value of the first quartile, the middle line is the medium value, and the top line is the third quartile. All values are blank corrected. Individual patient values are given in the Appendix A.[53]

Fig. 2.9 shows the LAL activity data in DBS from 15 healthy children (<5 years old), 12 healthy adults, and 6 patients previously shown to be LAL-deficient. All patients had symptoms consistent with LAL deficiency, and these 6 patients were shown to be LAL-deficient by the original LAL assay method on DBS.[36] The patients are de-identified, and we have no additional information. All assays were carried out with UPLC-MS/MS using the optimized conditions with substrate P-PMHC in the absence of Lalistat2 (thus, only a single assay per patient was needed). All healthy persons showed LAL activity well separated from that measured with LAL-deficient patients. The fact that LAL activity was close to zero in all 6 LAL-deficient patients supplies further evidence that substrate P-PMHC was LAL-specific.

2.4 DISCUSSION

Early work with LAL quickly revealed the need to avoid natural unactivated fatty ester substrates should we desire a high-resolution robust NBS assay. Since LAL deficiencies can have no residual activity (Wolman's) or slight residual activity of only 2 to 5% (CESD), it is necessary to be able to distinguish low activity patient samples. With a more reactive activated ester substrate, LAL activity signal will be higher as compared to an unactivated ester thus improving the resolution for low activity samples. This resolution will help clinicians identify potential patients who require immediate life-saving intervention from those who will have a more gradual disease progression.

This high-resolution separation of low LAL activities is also greatly affected by the use of the Lalistat2 inhibitor. When using the inhibitor, two runs are performed in parallel and the difference between the uninhibited and the inhibited DBS assay responses is translated into the LAL-specific activity. As the two values are significant in value, essentially the propagation error is doubled for all downstream calculations possibly resulting in an elevated LAL cutoff. When the cutoff is raised and thus a narrower separation between affected and unaffected populations, the risk for false positives also increases, again harming the practicality of an inhibitor-based LAL assay. Thus, removing the use of Lalistat2 in LAL DBS assays will be critical in developing a useable NBS assay for general LAL activity screening which is only possible with the specific substrate P-PMHC. More importantly, the lack of Lalistat2 inhibitor means that the assay can now be performed as a single incubation of a single sample, further reducing equivalent assay costs by half compared to the original Hamilton assay.[36]

One challenge faced in the development of this assay was the sensitivity of LAL to both solvents and detergents. As LAL is a serine esterase, it is possible that the DMSO decomposition products after air and UV light exposure are in sufficient quantities to affect the active site of LAL.[60][61] The solution to this problem is the use of an organic solvent that lacks reactive thiol decomposition products as the solvent for the LAL substrate. By using ethanol as both the solvent for the cardiolipin and the substrate, the assay is both simplified and made more robust as it is now possible to expand the solubility of the substrate in the assay cocktail.

A NBS assay will enable patients of WD and CESD to have access to life-sustaining or life-prolonging care at an early age before cholesterol ester deposition becomes fatal to the liver and vasculature. For WD patients, misdiagnosis is rapidly fatal and can lead to delaying of needed liver or bone marrow transplants. In the case of CESD, patients often are not given the early and aggressive screening to monitor for atherosclerosis until it is too late. An assay for LAL would thus provide a needed addition to newborn screening that would have consequences for the fields of neonatal medicine in WD and the shared areas of pediatrics, internal medicine, and cardiology for cases of CESD. Additionally, if implemented nationally, an accurate account of the number of individuals suffering from reduced LAL activity can be gathered, potentially changing how the treatment of early-onset hypercholesterolemia is managed.

It has been shown by comparison of large pilot new-born screening studies of lysosomal storage diseases that the tandem mass spectrometry enzymatic activity method gives a substantially lower number of positive screenings than the fluorometric method when compared at equivalent cutoff values.[55] This may be because the analytical range of the tandem mass spectrometry assays is >3-fold greater than that of the corresponding fluorometric assays.[59] Thus, the use of UPLC-MS/MS may be the method of choice for newborn screening of LAL deficiency, but this

re-mains to be determined. Finally, it should be possible to multiplex the new UPLC-MS/MS LAL assay with other tandem mass spectrometry assays for lysosomal storage dis-eases simply by addition of substrate P-PMHC to a single assay mixture containing a collection of additional substrates and internal standards and performing a single UPLC-MS/MS in which all products and internal standards are detected by multiple reaction monitoring mode.

2.5 CONCLUSION

In short, LAL is a critical enzyme for the process of intracellular lipid metabolism whose absence results in early mortality. Due to its low concentration in blood, it is important to take into account all modifiers, no matter how insignificant, of LAL during assay design. Reactive ester substrates are necessary for sufficient detection of enzyme activity. Finally, the ability to do a single sample, direct enzyme functional assay has significant improvements in simplicity over an indirect method. By working with reactive esters made from coumarin analogs, it is possible to gain specificity despite the general esterase-activity of LAL. This is the first assay for LAL designed for MS/MS use. With this assay, it is now possible to evaluate the feasibility of NBS for lysosomal acid lipase.

Chapter 3. ASSAYS OF N-ACETYLGALACTOSAMINE-6-SULFATASE AND ARYLSULFATASE B

The following text is adapted from the Clinical Chemistry article (doi:10.1373/clinchem.2015.242560) and reproduced with permission from the American Association for Clinical Chemistry.[59]

3.1 INTRODUCTION

As discussed previously in Chapter 1, lysosomal storage disorders (LSDs) are rare and thus detection of these diseases becomes more pressing as treatment options become available.[13] The use of assays of enzymatic activity can be done both for the general public in the form of newborn screening (NBS) and for specific diagnostic purposes when a disease is suspected.[4] In either case, the enzymatic activity assays were initially developed using the fluorophore 4-methylumbelliferone (4MU) analogs of the natural enzymatic substrate and in starting in the last two decades, tandem mass spectrometry (MS/MS).[62][63] The Gelb lab specifically has piloted the use of tandem mass spectrometry as a primary means of multiplexing or combining assays of multiple different diseases combined into a single analysis, which is now being used around the United States and the world.[63]

This article focuses on the development and validation of a multiplexed tandem MS/MS assay for three mucopolysaccharidoses (MPS), MPS types II, IVA, and VI.[41] The deficient enzyme in MPS type II, iduronide-2-sulfatase (I2S) historically and presently assayed with a fluorescent 4MU glycoside assay using iduronic acid-2-sulfate 4MU where the 4MU is released by α-L-iduronidase (IDUA) with the removal of iduronic acid following action by I2S to cleave off the 2-sulfate group.[49][50] Similarly the enzymes of MPS types IVA and VI remove the sulfate

group of monosulfated 4MU glycoside of galactose-6-sulfate or 4MU-sulfate after action of the enzymes GALNS and ARSB respectively.[42][43][44][51] The measured free 4MU is released by a bacterial β-galactosidase for GALNS assays of MPS IVA.[44]

We report the development of MS/MS assays for I2S, GALNS, and ARSB that give a much higher assay response in the mass spectrometer than previously reported assays. These new reagents lead to a larger lysosomal enzyme assay analytical range, which we defined as the ratio of assay response with the high QC DBS, because of the relevant enzymatic reaction, divided by the response for nonenzymatic processes. Increasing the analytical range is important for NBS and diagnosis of LSDs because this is predicted to lead to a more accurate enzyme activity value at the low end. This is expected to lead to better differentiation between disease-affected patients and those with pseudodeficiencies and, in general, lead to a lower rate of false positives. For diagnosis, it may lead to better prediction of disease severity. We also compared the analytical range of 6 MS/MS assays to those measured fluorometrically with 4MU-substrates.

3.2 Materials and Methods

All methods, including the synthesis of the substrates, are located in Appendix B.

3.3 Results and Discussion

3.3.1 *FIA-MS/MS Assays for GALNS and ARSB*

Our original MS/MS substrate for GALNS consisted of a Gal-6-sulfate linked to an analog of 4MU bearing a hydrophobic chain.[43] Although this assay distinguished between healthy and MPS-IVA samples, the MS/MS signal for the product in assays with random newborns was 50-fold less than for our other MS/MS assays for lysosomal enzymes.[9][11] This original assay is not sufficiently robust for NBS, and the only way forward is to find a higher activity substrate or

to increase the MS/MS response of the product.[43] GALNS is thought to be responsible for removal of sulfate from Gal-6-sulfate and GalNAc-6-sulfate in mucopolysaccharides.[41] Thus, we explored the consequence of replacing Gal-6-sulfate in our previous substrate with GalNAc-6-sulfate. We also replaced the 4MU-based aglycone in our original GALNS substrate with an aglycone containing the 4-acetamido-phenol moiety because this aglycone change results in an improved assay MS/MS response per mole for the product derived from our new I2S substrate.[50] The structure of our new GALNS substrate, GalNAc-6-S-C6/C6-benzoyl group (Bz), is shown in Figure 3.1. The name derives from the presence of the N-hexanoyl group (C6), the hexamethylene linker (C6), and the Bz in the aglycone. Calculations suggest that the 2 carbonyl groups in the aglycone serve as a site of facile protonation (Figure 3.1), which allows for higher-yield ionization in the electrospray source.[12] We also pursued new assays for ARSB using GalNAc-4-S-C5/C5-Bz (Figure 3.1).

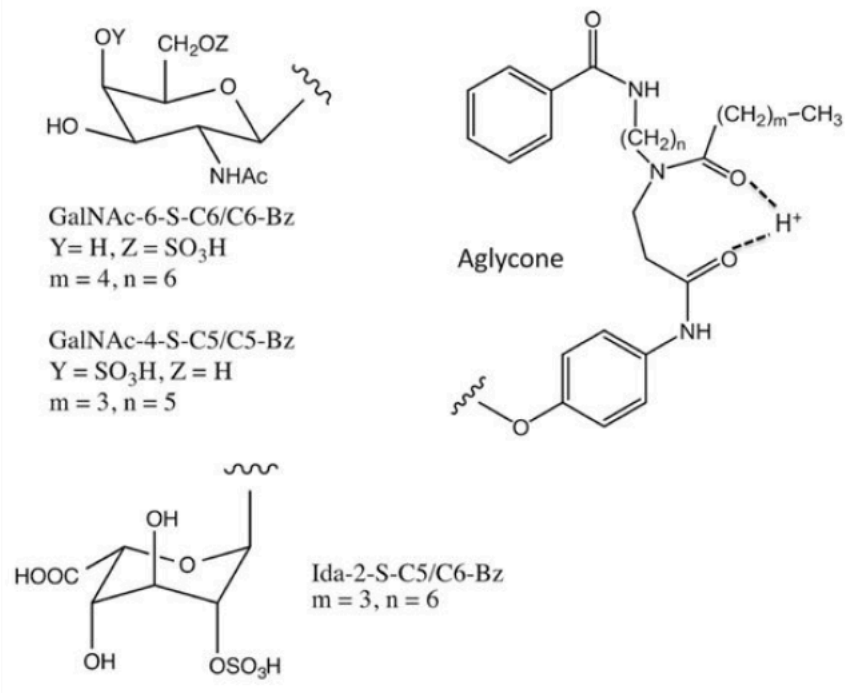


Figure 3.1. Structures of the substrates for assaying GALNS, ARSB, and I2S
The proton shown hydrogen bonded to the 2 carbonyl groups is the proposed site of gas phase protonation in the electrospray ion source. Internal standards lack the sulfate and contain 5 deuteriums on the benzoyl group.

Appendix B Table 3 shows that the kcat/Km for GalNAc-6-S-C6/C6-Bz was 81-fold larger than that for Gal-6-S-C7/C6-Bz, indicating that GALNS greatly preferred the GalNAc substrate (we did not have the comparator compound Gal-6-S-C6/C6-Bz, but it is unlikely that GalNAc distinguishes between the number of methylenes in the aglycone). These studies justified the selection of our new GALNS GalNAc-6-S-C6/C6-Bz for continued studies.

The first version of the MS/MS assay was based on flow injection analysis (FIA). We considered the possibility of adding an enzyme that cleaved the glycoside only after the sulfatase removed the sulfate, thus generating the aglycone (Figure 3.2). If the aglycone product gave a stronger MS/MS response per mole than that for the immediate sulfatase product (desulfated glycoside), use of the coupled enzymatic process would yield a gain in analytical range. Furthermore, buffer salt removal by liquid–liquid extraction was required for FIA-MS/MS to prevent suppression during the ionization process. In the case of sulfatases the initial product contains the sugar, which is hydrophilic and reduced the solvent extraction yield. Conversion to the aglycone will increase the transfer of analyte to the organic layer.

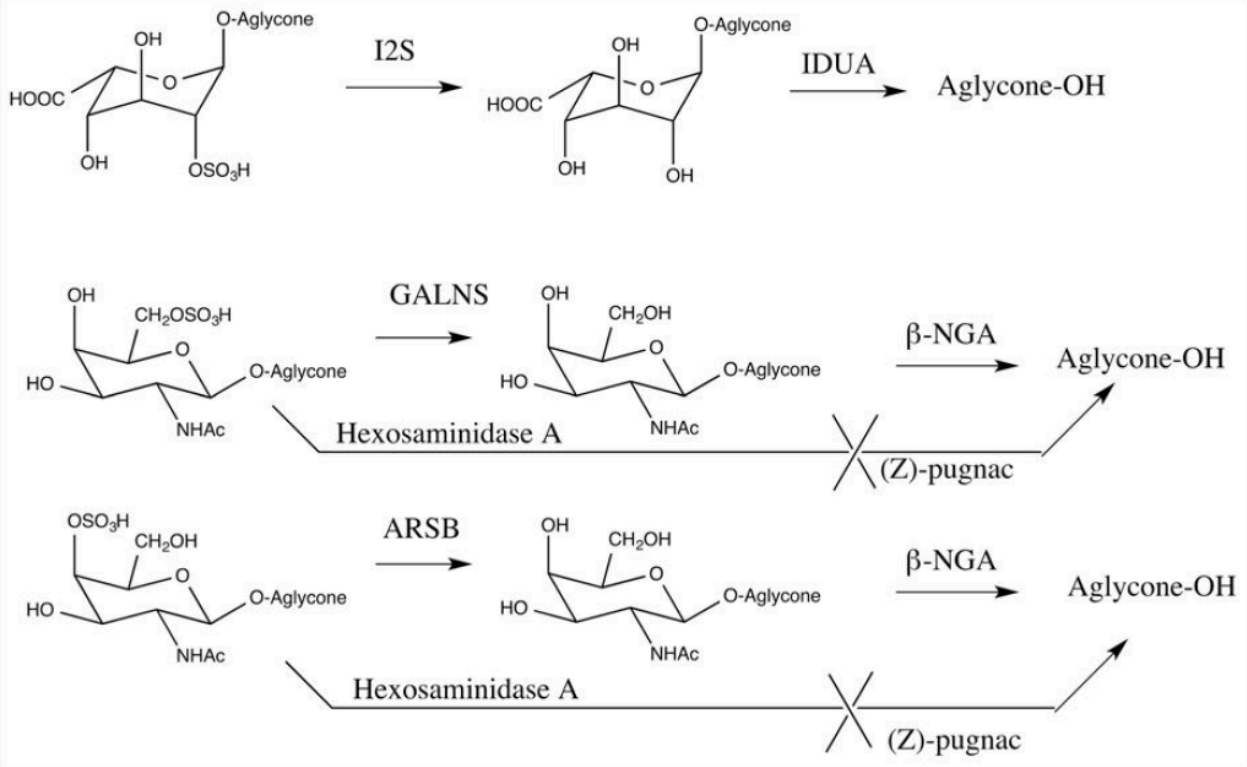


Figure 3.2. Enzymatic reactions for I2S, GALNS, and ARSB
Coupling enzymes to convert the direct product of the lysosomal enzyme to the free aglycone are shown (this step is omitted for some variations of the assays described in the main text). The use of (Z)-Pugnac to block the action of human hexosaminidase A on the GALNS and ARSB substrates is also shown.

The ion response per mole of the GALNS- and ARSB-generated aglycones, C6/C6-Bz and C5/C5-Bz, were both found to be 2.4-fold larger than that for the corresponding glycosides GalNAc-6-S-C6/C6-Bz and GalNAc-4-S-C5/C5-Bz. This finding, along with the ethyl acetate consideration, argued for use of a coupled assay with enzymatic removal of the desulfated sugar. A disadvantage of this approach became apparent when we studied the pH dependence of the nonenzymatic hydrolysis of GalNAc-6-sulfate-C6/C6-Bz to the aglycone (see Appendix B Figure B.1). Glycoside hydrolysis in the absence of DBS increased with a drop in pH, showing that it was acid catalyzed. It is known that glycosides of 2-acetamido sugars are more acid sensitive than those with a 2-hydroxyl because of neighboring group participation by the acetamido group.[64] This acid-catalyzed pathway gave rise to aglycone, thus increasing the

background of the assay. With DBS, enzymatic formation of the sulfatase product GalNAc-C6/C6-Bz was maximal at pH approximately 5.0, and thus going to pH >5.0 would not help. Similar pH-dependent observations were seen with the ARSB substrate GalNAc-4-S-C5/C5-Bz (see Appendix B Figure B.1).

The use of GalNAc-4-S-C5/C5-Bz and GalNAc-6-S-C6/C6 required complete inhibition of hexosaminidase A because this enzyme can cleave the glycoside of GalNAc residues containing a 6-sulfate (Figure 3.2, also see Appendix B Table 4).[65] Recombinant human hexosaminidase A cleaved GalNAc-4-S-C5/C5-Bz, showing that the enzyme tolerated 4-sulfation (see Appendix B Table 5). Endogenous hexosaminidase A present in DBS cleaved these glycosides (see Appendix B Table 5). The hexosaminidase A inhibitor (Z)-Pugnac at 1 mmol/L blocked almost all of the activity of recombinant hexosaminidase A and enzyme in DBS (see Appendix B Table 6).[66] In subsequent assays we used 2 mmol/L (Z)-Pugnac to ensure complete hexosaminidase A inhibition. To generate the aglycone from the desulfated glycoside, we explored the bacterial enzyme β-N-acetylgalactosaminidase (β-NGA).[52] The data in Appendix B Tables 6 and 7 show that β-NGA did not generate aglycones from the sulfated substrates, and β-NGA catalyzed hydrolysis of the non-sulfated GALNS and ARSB products were not sensitive to (Z)-Pugnac. This allowed us to develop the new FIA-MS/MS assay of GALNS and ARSB based on the natural GalNAc-4/6-sulfate substrates (described above).

The internal standard for GALNS is GalNAc-C6/C6-Bz with 5 deuteriums in the benzoyl group. Thus, any incomplete β-NGA reaction is accounted for by using the glycoside internal standard. We found that increasing the amount of β-NGA beyond that in our standard assays did not increase the overall rate of aglycone formation (data not shown), showing that the reaction was limited by the sulfatase as desired. Selective reaction monitoring was used with FIA-MS/MS to

measure the amount of nondeuterated product aglycone and deuterated internal standard aglycone after collision-induced dissociation of precursor ions.

Table 3.11. Performance of MS/MS Assays of GALNS, ARSB, and I2S.

Assay	MS/MS product response (peak area)	Analytical range[a]	Mean (%CV) activity for 5 replicates of the QC DBS, $\mu mol \cdot h^{-1} \cdot L^{-1}$[a]
GALNS FIA-MS/MS with β-NGA	Filter paper, 17 340 PE QC H, 220 050	23.4 (PE QC H)	No data
GALNS LC-MS/MS without β-NGA	Filter paper, 525 CDC QC H, 138 460 PE QC H, 230 905	120 (CDC QC H) 198 (PE QC H)	Filter paper, 0.02 (9.1%) CDC QC B, 0.08 (1.8%) CDC QC L, 0.22 (8.7%) CDC QC M, 1.36 (12.8%) CDC QC H, 2.46 (12.3%) PE QC L, 0.29 (19.0%) PE QC M, 2.70 (12.0%) PE QC H, 4.07 (7.2%)
ARSB FIA-MS/MS with β-NGA	Filter paper, 20 520 PE QC H, 360 020	22.4 (PE QC H)	No data
ARSB LC-MS/MS without β-NGA	Filter paper, 2750 CDC QC H, 1 209 484 PE QC H, 1 406 271	170 (CDC QC H) 226 (PE QC H)	Filter paper, 0.12 (6.1%) CDC QC B, 0.22 (3.6%) CDC QC L, 1.02 (10.2%) CDC QC M, 11.76 (6.4%) CDC QC H, 20.51 (11.2%) PE QC L, 1.96 (9.5%) PE QC M, 18.11 (5.7%) PE QC H, 27.39 (3.7%)
I2S FIA-MS/MS with IDUA	Filter paper, 9902 CDC QC H, 1 379 295 PE QC H, 3 297 559	257 (CDC QC H) 387 (PE QC H)	Filter paper, 0.05 (63.9%) CDC QC B, 1.05 (6.2%) CDC QC L, 1.72 (4.8%) CDC QC M, 8.50 (7.2%) CDC QC H, 11.64 (6.1%) PE QC L, 1.17 (3.8%) PE QC M, 9.90 (3.5%) PE QC H, 17.50 (2.8%)
I2S LC-MS/MS with IDUA	Filter paper, 4146 CDC QC H, 2 207 739 PE QC H, 3 104 262	424 (CDC QC H) 721 (PE QC H)	Filter paper, 0.03 (60%) CDC QC B, 1.31 (6.3%) CDC QC L, 2.19 (4.3%) CDC QC M, 10.35 (2.7%) CDC QC H, 12.93 (6.4%) PE QC L, 1.34 (7.0%) PE QC M, 11.42 (5.8%) PE QC H, 19.40 (3.3%)
I2S LC-MS/MS without IDUA	Filter paper, 1756 CDC QC H, 1 578 629 PE QC H, 1 905 935	521 (CDC QC H) 681 (PE QC H)	Filter paper, 0.04 (145.7%) CDC QC B, 1.10 (6.4%) CDC QC L, 2.20 (3.6%) CDC QC M, 12.60 (4.7%) CDC QC H, 18.66 (5.6%) PE QC L, 1.53 (2.2%) PE QC M, 14.29 (3.1%) PE QC H, 24.41 (6.4%)

[a]Values calculated as described in the online Data Supplement. QC samples from the CDC are base pool (B, 0% whole blood), low (L, 5% whole blood), medium (M, 50% whole blood), and high (H, 100% whole blood). Same for the PerkinElmer (PE) samples except there is no base pool.

Table 3.11 lists the analytical range of the FIA-MS/MS assays for GALNS and ARSB. The analytical range was defined as the ratio of assay response for the QC high sample due to the

relevant enzyme divided by the response for nonenzymatic processes (calculated as described in Appendix B).

3.3.2 *LC-MS/MS ASSAYS FOR GALNS AND ARSB*

Given the multiple factors that contribute to analytical range in the FIA-MS/MS assay using β-NGA, we also studied a second assay for GALNS and ARSB based on coupled LC-MS/MS. In this case, sample desalting occurred on the liquid chromatography (LC) column, and thus conversion of the glycoside product to the aglycone with β-NGA and liquid–liquid extraction with ethyl acetate were not required. The assay incubation was quenched with acetonitrile, and precipitated macromolecules were pelleted by centrifugation. The supernatant was diluted with water and subjected to LC-MS/MS. Because we detected the desulfated products GalNAc-C5/C5-Bz and GalNAc-C6/C6-Bz rather than the aglycones, we were not concerned with acid-catalyzed glycoside hydrolysis. We were also not concerned with enzyme-independent desulfation of substrates in the electrospray ion source because substrate and product had distinct LC retention times (see Appendix B Figure B.2), and we integrated only the region of the chromatograph in which product and internal standard eluted. (Z)-Pugnac was still added because a substantial amount of the internal standard was cleaved without the hexosaminidase A inhibitor (not shown). To minimize the instrumental complexity, we used a normal-pressure LC column and developed an isocratic solvent system to separate the GALNS and ARSB products from their corresponding substrates. In this way we simply inserted the LC column into the solvent delivery system that was used for FIA-MS/MS. Furthermore, in our previous studies of LC-MS/MS for LSD assays, several thousand assays could be obtained without column replacement.[11]

Table 3.11 lists the analytical ranges for the GALNS and ARSB LC-MS/MS assays. Given the higher analytical ranges, we carried out additional studies using LC-MS/MS rather than FIA-MS/MS assays. To demonstrate the reproducibility of the assay and the linearity with respect to the amount of enzyme in the DBS, we carried out 5 independent replicate assays on a series of QC DBS containing various amounts of leukocyte-depleted and whole blood (Table 3.11; linearity plots provided in Appendix B Figure B.3).

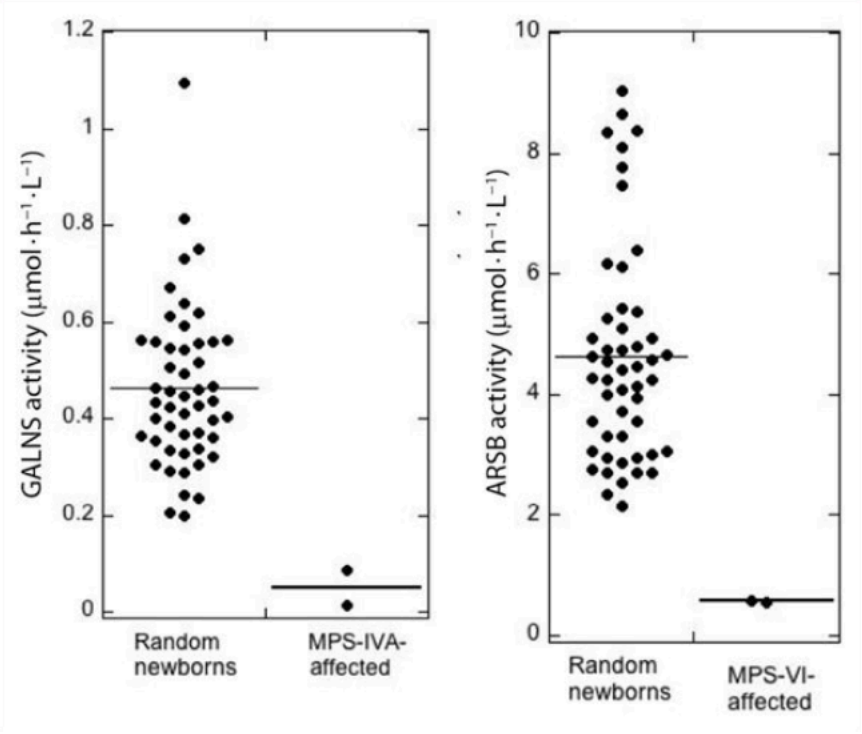


Figure 3.3. Enzyme activities in random newborns and LSD-affected patients measured using LC-MS/MS
Note the difference in *y*-axis scales. Mean activity is shown as a horizontal line.

Assay results for 50 random newborns and MPS-IVA– and MPS-VI–affected patients are provided in Figure 3.3 (tabular data in found in Appendix B Tables 8 and 9), showing that this LC-MS/MS assay readily distinguished between 50 healthy newborns and MPS-IVA or MPS-VI patients.

3.3.3 *MS/MS ASSAYS FOR I2S*

We explored a variation of our previous MS/MS assay for I2S by adding IDUA to generate the aglycone after desulfation (Figure 3.2). We found that the glycoside IdA-C5/C6-Bz gave an approximately 5-fold lower MS/MS response per mole than did its aglycone (not shown). We carried out 3 assay variants: (a) coupled assay with IDUA, ethyl acetate extraction, FIA-MS/MS; (b) IDUA, protein precipitation with acetonitrile, LC-MS/MS; and (c) no IDUA, acetonitrile, LC-MS/MS. Table 3.11 summarizes the results with QC DBS. All 3 methods gave similar analytical ranges. LC traces and linearity data are shown in Appendix Figures B.2 and B.3.

3.3.4 *TRIPLEX ASSAY FOR MPS-II, -IVA, AND –VI*

After exploring several possible options, we put all 3 MPS assays into a single triplex assay using a single cocktail incubated with a single 3-plex DBS punch. For solubility reasons we reduced the concentration of the MPS-II substrate to 0.5 mmol/L and kept the other 2 substrates at 1 mmol/L (I2S had the highest activity). We included citric acid in the quench step so that the MPS-II product containing the iduronic acid group would protonate on its carboxylate to facilitate extraction into ethyl acetate during the liquid–liquid extraction. The extract was submitted to LC-MS/MS using the above isobaric conditions under low pressure. Ethyl acetate extraction was found to provide a more robust assay because it led to a large reduction in the amount of substrate in the mixture applied to the LC column, which in turn reproducibly ensured complete separation between substrates and product/internal standards. Results are summarized in Table 3.12, and linearity data using QC standards is shown in Appendix B Figure B.3.

Table 3.12. Performance of the triplex assay for I2S, GALNS, and ARSB.

Enzyme	MS/MS product response (peak area)	Analytical range[a]	Mean (%CV) activity for 5 replicates of the QC DBS, μmol · h^{-1} · L^{-1}[a]
I2S	Filter paper, 4100	430 (CDC QC H)	Filter paper, 0.028 (5.0%)
	CDC QC H, 2 151 806	577 (PE QC H)	CDC QC B, 0.732 (5.7%)
	PE QC H, 2 737 395		CDC QC L, 1.50 (5.1%)
			CDC QC M, 8.49 (5.7%)
			CDC QC H, 11.93 (11.3%)
			PE QC L, 1.19 (8.2%)
			PE QC M, 10.22 (2.5%)
			PE QC H, 16.01 (1.7%)
GALNS	Filter paper, 3096	85 (CDC QC H)	Filter paper, 0.024 (4.7%)
	CDC QC H, 248 713	119 (PE QC H)	CDC QC B, 0.077 (3.7%)
	PE QC H, 353 333		CDC QC L, 0.203 (9.4%)
			CDC QC M, 1.26 (8.3%)
			CDC QC H, 2.05 (10.3%)
			PE QC L, 0.236 (7.7%)
			PE QC M, 2.10 (3.2%)
			PE QC H, 1.86 (5.8%)
ARSB	Filter paper, 3265	143 (CDC QC H)	Filter paper, 0.058 (4.5%)
	CDC QC H, 453 074	188 (PE QC H)	CDC QC B, 0.094 (5.9%)
	PE QC H, 584 751		CDC QC L, 0.47 (9.3%)
			CDC QC M, 5.34 (8.1%)
			CDC QC H, 8.21 (14.0%)
			PE QC L, 0.90 (8.8%)
			PE QC M, 7.90 (3.2%)
			PE QC H, 10.83 (6.6%)

[a]Values calculated as described in the online Data Supplement. QC samples from the CDC are base pool (B, 0% whole blood), low (L, 5% whole blood), medium (M, 50% whole blood), and high (H, 100% whole blood). Same for the PerkinElmer (PE) samples except there is no base pool.

3.3.5 *ANALYTICAL RANGE FOR FLUOROMETRIC ENZYME ASSAYS BASED ON 4MU SUBSTRATES*

We used the 4MU substrates in Table 3 to assay lysosomal enzymes IDUA, acid α-glucosidase, I2S, GALNS, and ARSB. The principle of these assays is based on enzymatic release of 4MU from the substrate conjugate (i.e., 4MU-glycosides). The mixture was quenched with a buffer with a pH of approximately 10 to cause ionization of the hydroxyl group of 4MU to its phenolate

(4MU-OH to 4MU-O−). The pKa of 4MU-OH is 7.6, and the 4MU-O−/4MU-OH fluorescence emission ratio is approximately 16.66. We confirmed these findings by spectral titration of 4MU (not shown). The above analysis showed that the neutral species 4MU-OH had finite fluorescence, and thus we explored the fluorescence of 4MU-glycoside substrates. Table 3.13 lists the percentages of 4MU present as an impurity in the substrates and the ratio (4MU-O− emission per mole)/(4MU-glycoside emission per mole). These ratios were in the range 2200–5400 (the outlier was for 4MU-sulfate, ratio = 58700) and accounted for the increase in fluorescence when 4MU-glycosides were acted on by lysosomal enzymes. Although this ratio was much higher than 1, in assays with DBS, the percentage of total 4MU-substrate that was converted to 4MU was typically only approximately 1%. Thus, the intrinsic fluorescence of the 4MU-glycoside substrate greatly increased the background and reduced the analytical range.

The analytical ranges of these 4MU assays were defined as above (details of the calculation provided in the online Data Supplement) and listed in Table 3.13. Background-corrected enzyme activities are also listed in Table 3.13.

Table 3.13. Fluorimetry assays with 4MU glycosides.[a]

4MU-glycoside/enzyme	Amount of 4MU contaminant (mol %)	4MU-O⁻/4MU-glycoside emission ratio	Analytical range	Enzyme activity, μmol · h^{-1} · L^{-1}	Percent substrate converted to product
4MU-*α*-L-iduronide/IDUA	0.067	2744	16.3 (CDC High) 12.3 (PE High)	12.5 (CDC High) 9.6 (PE High)	2.1 (CDC High) 1.6 (PE High)
4MU-*α*-D-glucose/acid *α*-glucosidase	0.023	2245	16.6 (CDC High) 12.3 (PE High)	17.5 (CDC High) 10.1 (PE High)	2.9 (CDC High) 1.7 (PE High)
4MU-*α*-L-iduronide-2-sulfate/I2S 4MU-sulfate/ARSB	0.025 0.032	2520 58 700	11.4 (PE High) 34.0 (PE High)	8.7 (PE High) 5.0 (PE High)	1.4 (PE High) 0.16 (PE High)
4MU-*β*-*N*-GalNAc-4-sulfate/ARSB	0.010	5390	7.8 (PE High)	5.94 (PE High)	0.33 (PE High)
4MU-*β*-*N*-GalNAc-6-sulfate/GALNS	0.016	4120	6.5 (PE High)	9.14 (PE High)	0.51 (PE High)

[a]Values calculated as described in the online Data Supplement. PE, PerkinElmer.

3.3.6 *Conclusion*

For rare diseases like LSDs, care should be taken to develop the most discriminating and cost-effective analysis. In order accomplish this goal, the Gelb lab has determined that their use of

mass spectrometry based assays provide significantly higher analytical range when compared to their fluorescent counterparts. In addition to the gain in sensitivity (and thus a reduction in false positives), the MS/MS-assays require less time to process and thus take further steps toward generalized NBS screening for Mucopolysaccharidoses Types II, IVA, and VI.

Chapter 4. CONCLUSION

Newborn screening for lysosomal storage disorders is critical to improving the long-term health of population. In addition, expanded newborn screening will provide insight on rates of rare diseases like LALD and mucopolysaccharidoses. With expanded genetic testing, we will also be able to further understand the underlying biochemical pathways affected in lysosomal storage disorders.

The Gelb lab has provided a framework for developing assays for both glycosaminoglycan and lipid lysosomal enzymes. For most glycosaminoglycan enzymes, both the original biological context and a finely tuned analytical handle must be evaluated in concert to develop the best newborn screening substrates. For lipid metabolic enzymes, the specifics of the lysosomal enzyme's active site are critical and often require more complex matrices and detergents for development of specific substrates to account for their difficult solubilities.

The end goal of any mass spectrometry-based newborn screening assay is to provide reliable, low-cost tests to improve the health of the community, starting with its newborns.

BIBLIOGRAPHY

[1] Bennett, M. J. Ed. (2008). *Laboratory Medicine Practice Guidelines: Follow-up Testing for Metabolic Diseases Identified by Expanded Newborn Screening Using Tandem Mass Spectrometry*. Retrieved May 24, 2018 from https://www.aacc.org/science-and-practice/practice-guidelines/expanded-newborn-screening

[2] Moyer, V.A., Calonge, N., Teutsch, S.M., & Botkin, J.R. (2008) Expanding Newborn Screening: Process, Policy, and Priorities. *The Hastings Center Report, 38*(3), 32-39. doi:10.1353/hcr.0.0011

[3] Rousseau, F., Giguère, Y., Berthier, M., Guérrette, D., Girard, J., & Déry, M. (2012). Newborn Screening By Tandem Mass Spectrometry: Impacts, Implications and Perspectives. In Dr Jeevan Prasain (Ed.), *Tandem Mass Spectrometry - Applications and Principles*. Retrieved March 2, 2015 from http://www.intechopen.com/books/tandem-mass-spectrometry-applications-and-principles/newborn-screening-by-tandem-mass-spectrometry-impacts-implications-and-perspectives

[4] Parenti, G., Andria, G., & Ballabio, A. (2015). Lysosomal Storage Diseases: From Pathophysiology to Therapy. *Annu. Rev. Med. 66*, 471-486. doi:10.1146/annurev-med-122313-085916

[5] Kilakkathi, V. (2012). *Newborn Screening In America: Problems and Policies*. Council for Responsible Genetics. Retrieved March 2, 2015 from http://www.councilforresponsiblegenetics.org/pageDocuments/WNMAKEPP1P.pdf

[6] National Newborn Screening & Global Resource Center. National Newborn Screening Status Report. Retrieved November 2, 2014 from http://genes-r-us.uthscsa.edu/sites/genes-r-us/files/nbsdisorders.pdf

[7] De Jesus, V.R., Zhang, K., Keutzer, J., Bodamer, O.A., Mühl, A., Orsini, J.J., Caggana, M., Vogt, R.F., & Hannon, W.H. (2009). Development and Evaluation of Quality Control Dried Blood Spot Materials in Newborn Screening for Lysosomal Storage Disorders. *Clin Chem*. *55:1*,158-164. doi:10.1373/clinchem.2008.111864

[8] Federal Advisory Committees Health Resources & Services Administration. Recommended Uniform Screening Panel. Retrieved February 27, 2018 from https://www.hrsa.gov/advisory-committees/heritable-disorders/rusp/index.html

[9] Turecek F., Scott C.R., & Gelb M.H. (2007). Tandem Mass Spectrometry in the Detection of Inborn Errors of Metabolism for Newborn Screening. *Methods in Molecular Biology, 359*, 143-157. doi:10.1385/1-59745-255-6:143

[10] Parkinson-Lawrence, E., Shandala, T., Prodoehl, M., Plew, R., Borlace, G., & Brooks, D. (2010). Lysosomal Storage Disease: Revealing Lysosomal Function and Physiology. *Physiology, 25*(2), 102-115. doi:10.1152/physiol.00041.2009

[11] Spacil, Z., Tatipaka, H., Barcenas, M., Scott, C. R., Turecek, F. & Gelb, M. H. (2013). High-throughput assay of 9 lysosomal enzymes for newborn screening. *Clin Chem, 59*(3), 502-511. doi:10.1373/clinchem.2012.189936

[12] Spacil Z., Hui R., Gelb M.H., & Turecek F. (2011). Protonation sites and dissociation mechanisms of t-butylcarbamates in tandem mass spectrometric assays for newborn screening. *J Mass Spectrom, 46*(10), 1089-1098. doi:10.1002/jms.1993

[13] Wang, R.Y., Bodamer, O.A., Watson, M.S., & Wilcox, W.R. (2011). Lysosomal storage diseases: Diagnostic confirmation and management of presymptomatic individuals. *Genetics in Medicine, 13*(5), 457-484. doi:10.1097/GIM.0b013e318211a7e1

[14] Saftig, P., & Klumperman, J. (2009). Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function. *Nature Reviews Mol Cell Bio*, *10*(9), 623-635. doi:10.1038/nrm2745

[15] Boustany R.M. (2013). Lysosomal storage diseases-the horizon expands. *Nature Rev Neurol*, *9*(10), 583-598. doi:10.1038/nrneurol.2013.163

[16] UF Maegawa Lab Department of Pediatrics. Lysosomal Storage Disease Program. Retrieved October 30, 2017 from https://maegawa.research.pediatrics.med.ufl.edu/lsd-program/

[17] Assmann G., & Seedorf U. Acid lipase deficiency, Wolman disease and Cholesterol ester storage disease. (2001). In: Scriver C., Beaudet A., Sly W., & Valle D. (Eds). *The metabolic and molecular bases of inherited disease*. pp.3551-72. New York: McGraw-Hill.

[18] Aslanidis, C., Ries, S., Fehringer, P., Büchler, C., Klima, H., & Schmitz, G. (1996). Genetic and Biochemical Evidence That CESD and Wolman Disease Are Distinguished by Residual Lysosomal Acid Lipase Activity. *Genomics, 33*(1), 85-93. doi:10.1006/geno.1996.0162

[19] Muntoni, S., Wiebusch, H., Jansen-Rust, M., Rust, S., Seedorf, U., Schulte, H., Berger, K., Funke, H., & Assmann, G. (2007). Prevalence of Cholesteryl ester storage disease. *Arterioscler Thromb Vasc Biol, 27*(8), 1866-1868. doi:10.1161/ATVBAHA.107.146639

[20] Reynolds, T. (2013). Cholesterol ester storage disease: a rare and possibly treatable cause of premature vascular disease and cirrhosis. *J Clin Pathol*, *66*(11), 918-923. doi:10.1136/jclinpath-2012-201302

[21] Freudenberg, F., Bufler, P., Ensenauer, R., Lohse, P., & Koletzko, S. (2013). Cholesteryl Ester Storage Disease: an Easily Missed Diagnosis in Oligosymptomatic Children. *Z Gastroenterol, 51*(10), 1184-1187. doi:10.1055/s-0033-1350463

[22] Hulkova H., & Elleder, M. (2012). Distinctive histopathological features that support a diagnosis of cholesterol ester storage disease in liver biopsy specimens. *Histopathology, 60*(7), 1107-1113. doi:10.1111/j.1365-2559.2011.04164.x

[23] Thelwall, P.E., Smith, F.E., Leavitt, M.C., Canty, D., Hu, W., Hollingsworth, K.G., …, Quinn, A.G. (2013). Hepatic cholesteryl ester accumulation in lysosomal acid lipase deficiency: Non-invasive identification and treatment monitoring by magnetic resonance. *Journal of Hepatology, 59*(3), 543-549. doi:10.1016/jhep.2013.04.016

[24] Roussel, A., Canaan, S., Egloff, M., Riviere, M., Dupuis, L., Verger, R., & Cambillau, C. (1999) Crystal Structure of Human Gastric Lipase and Model of Lysosomal Acid Lipase, Two Lipolytic Enzymes of Medical Interest. *J Bio Chem, 274*(24), 16995-17002. Retrieved from www.jbc.org.

[25] Rajamohan, F., Reyes, A.R., Ruangsiriluk, W., Hoth, L.R., Han, S., Caspers, N., …, Kurumbail, R.G. (2015). Expression and functional characterization of human lysosomal acid lipase gene (LIPA) mutation responsible for cholesteryl ester storage disease (CESD) phenotype. *Prot Expr Purif, 110*, 22-29. doi:10.1016/j/pep.2014.12.009

[26] Zschenker, O., Till, I., & Ameis, D. (2006). Overexpression of Lysosomal Acid Lipase and Other Proteins in Atherosclerosis. *The Journal of Biochemistry, 140*(1), 23-38. doi:10.1093/jb/mvj137

[27] Balwani, M., Breen, C., Enns, G.M., Deegan, P.B., Hozik, T., Jones, S.; …, Quinn, A.G. (2013). Clinical Effect and Safety Profile of Recombinant Human Lysosomal Acid Lipase in Patients with Cholesteryl Ester Storage Disease. *Hepatology, 58*(3), 950-957. doi:10.1002/hep.26289

[28] Groener, J.E., Bax, W., Stuani, C., & Pagani, F. (2000). Difference in substrate specificity between human and mouse lysosomal acid lipase: low affinity for cholesteryl ester in mouse lysosomal acid lipase. *Biochimica et Biophysica Acta, 1487*(2-3), 155-162. doi:10.1016/S1388-1981(00)00091-3

[29] Schaffner, T., Elner, V.M., Bauer, M., & Wissler, R. W. (1978). Acid lipase: a histochemical and biochemical study using triton X100-naphtyl palmitate micelles. *J Histochem Cytochem, 26*(9), 696-712. doi:10.1177/26.9.30799

[30] Negre A., Salvayre, R., Durand, P., Lenoir, G., & Douste-Blazy, L. (1984). Enzyme Studies on Epstein-Barr Virus-Transformed Lymphoid Cell Lines from Wolman's Disease: Lipases,

Cholesterol esterase and 4-Methylumbelliferyl acyl ester hydrolases. *Biochimica et Biophysica Acta, 749*(1), 89-95. doi:10.1016/005-2760(84)90301-1

[31] Sheriff, S., Hong, D., & Grabowski, G. (1995). Characterization of Lysosomal Acid Lipase by Site-directed Mutagenesis and Heterologous Expression. *J Bio Chem, 270*, 27766-27772. doi:10.1074/jbc.270.46.27766

[32] Zschenker, O., Oezden, D., & Ameis, D. (2004). Lysosomal Acid Lipase as a Preprotein. *J. Biochem, 136*,(1), 65-72. doi:10.1093/jb/mvh093

[33] Du, H., Sheriff, S., Bezerra, J., Leonova, T., & Grabowski, G. (1998). Molecular and Enzymatic Analyses of Lysosomal Acid Lipase in Cholesteryl Ester Storage Disease. *Molecular Genetics and Metabolism 64*(2), 126-134. doi:10.1006/mgme.1998.2707

[34] Sando, G.N., & Rosenbaum, L.M. (1985). Human Lysosomal Acid Lipase/Cholesteryl Ester Hydrolase: Purification and Properties of the Form Secreted by Fibroblasts in Microcarrier Culture. *The Journal of Biological Chemistry, 260*(28), 15186-15193. Retrieved from www.jbc.org.

[35] Fourchier, S.W., & Defesche J.C. (2013). Lysosomal acid lipase A and the hypercholesterolaemic phenotype. *Curr Opin Lipidol. 24*(4), 332-338. doi:10.1097/MOL.0b013e328361f6c6.

[36] Hamilton J., Jones I., Srivastava R., & Galloway P. (2012). A new method for the measurement of lysosomal acid lipase in dried blood spots using the inhibitor Lalistat 2. *Clin Chim Acta, 413*(15-16), 1207-1210. doi:10.1016/j.cca.2012.03.019

[37] Negré, A., Salvayre, R., Dagan, A., Borrone, C., & Gatt, S. (1985). New spectrophometric Assays of Acid Lipase and Their Use in the Diagnosis of Wolman and Cholesteryl Ester Storage Disease. *Analytical Biochemistry* 145(2), 398-405. doi:10.1016/0003-2697(85)90380-X

[38] Rosenbaum, A.I., Cosner, C.C., Mariani, C.J., Maxfield, F.R., Wiest, O., & Helquist, P. (2010). Thiadiazole carbamates: Potent Inhibitors of Lysosomal Acid Lipase and Potential Niemann-Pick Type C Disease Therapeutics. *J Med Chem, 53*(14), 5281-5289. doi:10.1021/jm100499s

[39] Coates, P.M., Cortner, J.A., Hoffman, G.M., & Brown, S.A. (1979). Acid Lipase Activity of Human Lymphocytes. *Biochimica et Biophysica Acta, 572*(2), 225-234. doi:10.1016/0005-2760(79)930038-9

[40] Belfrage, P., & Vaughan, M. (1969). Simple liquid partition system for isolation of labeled oleic acid from mixtures with glycerides. *J Lipid Res, 10*(3), 341-344. Retrieved from www.jlr.org.

[41] Neufeld E.F., & Muenzer J. (2001). The Mucopolysaccharidoses. In: Scriver X., Beaudet A., Sly W., Valle D. (Eds). *The Metabolic and Molecular Bases of Inherited Diseases*. pp 3421-3452. New York: McGraw-Hill.

[42] Duffey T.A., Sadilek M., Scott C.R., Turecek F., & Gelb M.H. (2010). Tandem mass spectrometry for the direct assay of lysosomal enzymes in dried blood spots: Application to screening newborns for Mucopolysaccharidosis VI (Maroteaux-Lamy Syndrome). *Anal Chem, 82*(22), 9587-9591. doi:10.1021/ac102090v

[43] Khaliq T., Sadilek M., Scott C.R., Turecek F., & Gelb M.H. (2011). Tandem mass spectrometry for the direct assay of lysosomal enzymes in dried blood spots: Application to screening newborns for Mucopolysaccharidosis IVA. *Clin. Chem, 57*(1), 128-131. doi:10.1373/clinchem.2010.149880

[44] Camelier, M.V., Burin, M.G., De Mari, J., Vieira, T.A., Marasca G., & Giugliani R. (2011). Practical and reliable enzyme test for the detection of mucopolysaccharidois IVA (Morquio Syndrome type A) in dried blood samples. *Clin Chim Acta, 412*(19-20), 1805-1808. doi:10.1016/j.cca.2011.06.001

[45] Wolfe B.J., Blanchard S., Sadilek M., Scott C.R., Turecek F., & Gelb M.H. (2011). Tandem mass spectrometry for the direct assay of lysosomal enzymes in dried blood spots: application to screening newborns for mucopolysaccharidosis II (Hunter syndrome). *Anal Chem, 83*(3), 1152-1156. doi:10.1021/ac102777s

[46] Sanford, M., & Lo, J.H. (2014). Elosulfase alfa: First Global Approval. *Drugs, 74*(6), 713-718. doi:10.1007/s40265-014-0210-z

[47] Horovitz, D.D.G., Magalhaes, T.S.P.C., Acosta, A., Ribeiro, E.M., Giulani, L.R., Palhares, D.B., …, Llerena, J.C.Jr. (2013). Enzyme replacement therapy with galsulfase in 34 children younger than five years of age with MPS VI. *Mol Genet Metab, 109*(1), 62-69. doi:10.1016/j.ymgme.2013.02.014

[48] Tomatsu, S., Mackenzie, W.G., Theroux, M.C., Mason, R.W., Thacker, M.M., Shaffer, T.H., …, Orii, T. (2012). Current and emerging treatments and surgical interventions for

Morquio A syndrome: a review. *Res Rep Endocr Disord, 2*, 65-77. doi:10.2147/RRED.S37278

[49] Voznyi, Y.V., Keulemans, J.L., & van Diggelen O.P. (2001). A fluorometric assay for diagnosis of MPS II (Hunter disease). *J Inherit Metab Dis, 24*(6), 675-680. doi:10.1023/A:1012763026526

[50] Chennamaneni, N.K., Kumar, A.B., Barcenas, M., Spacil, Z., Scott, C.R., Turecek, F., & Gelb, M.H. (2014). Improved reagents for newborn screening of mucopolysaccharidosis types I, II, and VI by tandem mass spectrometry. *Anal Chem, 86*(9), 4508-4514. doi:10.1021/ac5004135

[51] Civallero, G., Michelin, K., de Mari, J., Viapiana, M., Burin, M., Coelho, J.C., & Giugliani, R. (2006). Twelve different enzyme assays on dried-blood filter samples for detection of patients with selected inherited lysosomal storage diseases. *Clin Chim Acta, 372*(1-2), 98-102. doi:10.1016/j.cca.2006.03.029

[52] Sumida, T., Fujimoto, K., & Ito, M. (2011). Molecular cloning and catalytic mechanism of a novel glycosphingolipid-degrading beta-N-acetylgalactosaminidase from Paenibacillus sp. Ts12. *J Biol Chem, 286*(16), 14065-14072. doi:10.1074/jbc.M110.182592

[53] Masi S., Chennamaneni, N., Turecek, F., Scott, R.C., & Gelb, M.H. (2018). Specific Substrate for the Assay of Lysosomal Acid Lipase. *Clin Chem, 64*(4), 690-696.

[54] Jones S.A., Rojas-Caro S., Quinn A.G., Friedman M., Marulkar S., Ezgu F., …,Valayannopoulos, V. (2017). Survival in infants treated with sebelipase alfa for

lysosomal acid lipase deficiency: an open-label, multicenter, dose-escalation study. *Orphanet J Rare Disl 12*, 25. doi:10.1186/s13023-017-0587-3

[55] Elliot S., Buroker N., Cournoyer J., Potier A., Trometer J., Elbin C., …, Scott, C.R. (2016). Pilot study of newborn screening for six lysosomal storage diseases using tandem mass spectrometry. *Mol Genet Metab, 118*(4), 304-309. doi:10.1016/j.ymgme.2016.05.015

[56] Ebdrup S., Refsgaard H.H.F., Fledelius C., & Jacobsen P. (2007). Synthesis and structure-activity relationship for a novel class of potent and selective carbamate-based inhibitors of hormone selective lipase with acute in vivo antilipolytic effects. *J Med Chem, 50*(22), 5449-5456. doi:10.1021/jm0607653

[57] Long, J.Z., & Cravatt, B.F. (2011). The metabolic serine hydrolases and their functions in mammalian physiology and disease. *Chem Rev, 111*(10), 6022-6063. doi:10.1021/cr200075y

[58] Li, Y., Scott, C.R., Chamoles, N.A., Ghavami, A., Pinto, B.M., Turecek, F., & Gelb, M.H. (2004). Direct multiplex assay of lysosomal enzymes in dried blood spots for newborn screening. *Clin Chem, 50*(10), 1785-1796. doi:10.1373/clinchem.2004.035907

[59] Kumar, A.B., Masi, S., Ghomashchi, F., Ito, M., Scott, C.R., Turecek, F., Gelb, M.H., & Spacil, Z. (2015). Tandem mass spectrometry has a larger analytical range than fluorescence assays of lysosomal enzymes: application to newborn screening and diagnosis of mucopolysaccharidoses types II, IVA, and VI. *Clin Chem, 61*(11),1363-1371 doi:10.1373/clinchem.2015.242560

[60] Lohse, P., Chanrokh-Zadeh, S., Lohse, P., & Seidel, D. (1997). Human Lysosomal acid lipase/cholesteryl ester hydrolase and human gastric lipase: identification of the catalytically active serine, aspartic acid, and histidine residues. *J Lipid Research, 38*(5), 892-905. Retrieved from www.jlr.org.

[61] Lohse, P., Lohse, P., Chanrokh-Zadeh, S., & Seidel, D. (1997). Human lysosomal acid lipase/cholesteryl ester hydrolase and human gastric lipase: site-directed mutagenesis of Cys227 and Cys236 results in substrate-dependent reduction of enzymatic activity. *J Lipid Research, 38*(9), 1896-1905. Retrieved from www.jlr.org.

[62] Chamoles, N.A., Blanco, M., & Gaggioli, D. (2001). Fabry disease: enzymatic diagnosis in dried blood spots on filter paper. *Clin Chim Acta, 308*(1-2), 195-196. doi:10.1016/S0009-8981(01)00478-8

[63] Gelb, M.H., Scott, C.R., & Turecek, F. (2015). Newborn screening for lysosomal storage diseases. *Clin Chem, 61*(2), 335-346. doi:10.1373/clinchem/2014.225771

[64] Jennings, H.J., & Lugowski, C. (1980). Facile cleavage of some 2-acetamido-2-deoxy-β-D-gluco- and galactopyranosides using aqueous HF. *Can J Chem, 58*, 2610-2612. Retrieved from http://www.nrcresearchpress.com/journal/cjc.

[65] Bayleran, J., Hechtman, P., & Saray, W. (1984). Synthesis of 4-methylumbelliferyl-beta-D-n-acetylglucosamine-6-sulfate and its use in classification of GM2 gangliosidosis genotypes. *Clin Chim Acta*, *143*(2), 73-89. doi:10.1016/0009-8981(84)90215-8

[66] Kim, E.J., Perreira, M., Thomas, C.J., & Hanover, J.A. (2006). An O-GlcNAcase-specific inhibitor and substrate engineered by the extension of the N-acetyl moiety. *J Am Chem Soc. 128*(13), 4234-4345. doi:10.1021/ja0582915

[67] Chen, R.F. (1968). Fluorescent pH indicator. Spectral changes of 4-methylumbellferone. *Anal Lett. 1*(7), 423-428. doi:10.1080/0003271680851147

[68] Kakkis, E.D., Joans, M.A., & Neufeld, E.F. (1994). Overexpression of the human lysosomal enzyme alpha-l-iduronidase in chinese hamster ovary cells. *Protein Expr Purif*, *5*(3), 255-232. doi:10.1006/prep.1994.1035

[69] Blanchard, S., Sadilek, M., Scott, C.R., Turecek, F., & Gelb, M.H. (2008). Tandem mass spectrometry for the direct assay of lysosomal enzymes in dried blood spots: Application to screening newborns for Mucopolysaccharidosis I. *Clin Chem, 54*(), 2067-70. Retrieved from clinchem.aaccjnls.org.

[70] Kumar, A. H., Spacil, Z., Ghomashchi, F., Masi, S., Sumida, T., Ito, M., Turecek, F., Scott, C. R., & Gelb, M. H. (2015). Fluorimetric Assays for N-Acetylgalactosamine-6-Sulfatase and Arylsulfatase B Based on the Natural Substrates for Confirmation of Mucopolysaccharidoses Types IVA and VI. *Clin Chem Acta, 451*(Pt B), 125-128. doi:10.1016/j.cca.2015.08.010

APPENDIX A

Table of Contents:

I. Various LAL Assays during Assay Development

Table A: LAL Assay Conditions

Assay	Assay Type	Assay Cocktail	Sample Type and Preparation
Compound 7 Assay Variation 1	MS/MS	0.69 mM Substrate 1 10 μM IS pH 4 0.15 M sodium acetate,1% Triton-X	DBS All healthy adult control DBS samples vs Filter paper (FP) controls.
Procedure: Either 10 μL of 30 μM Lalistat2 solution or H_2O was added to the inhibited or uninhibited tubes respectively. The assay was incubated at 37˚C for 16 hours shaking at 250 rpm (New Brunswik Incubator). The assay was quenched with 120 μL ACN then the protein and blood particulates were precipitated via centrifugation for 5 min at 3000 rpms. 120 μL of the water-ACN supernatant was transferred into a clear 320 μL round bottom 96-well plate. The samples were diluted with 60 μL of Milli-Q H_2O then analyzed by LC MS/MS			
Compound 12 Assay Variation 2	MS/MS	300 μM Substrate 2 0.15 M sodium acetate pH 4 1% Triton-X buffer and 0.5% cardiolipin stock solution (14:1 volume ratio)	DBS Quality control low and high samples were used in addition to healthy adult control. DBS and Filter paper punches were incubated 200 μL of Millipore H_2O.
Procedure: 20 μL of this DBS solution added to 75 μL of LAL assay cocktail and incubated shaking for 12 hrs. Samples were Filter Paper, Base Pool, Quality Control High, and Normal DBS done in duplicate. Quenched with120 μL ACN quench, centrifuged 3000 rpm for 5 min subsequently diluted with 120 μL water then read via LC-MS/MS.			
Compound 12 and Coumarin Analog Assay Variation 3	MS/MS	3.7 mM substrate (4-MUb palmitate) 0.5% m/v cardiolipin stocks in MeOH/EtOH/iPrOH were warmed to 37˚C along with the assay buffer (0.15 M Sodium Acetate pH 4 2.5mM Sodium Taurodeoxycholate). Final assay cocktail ratio is 1.4 Buffer solution : 0.14 alcohol solution.	DBS Sample resuspended in 200 μL of Milli-Q H_2O for 1 hour at room temperature shaken at 200 rpm in a plate shaker
Procedure: 40 μL of rehydrated blood mixture is transferred per assay into a 1.5 mL Eppendorf tube containing either 10 μL of 30 μM Lalistat 2 solution or 10 μL of H_2O. The samples were preincubated for 15 min at 37˚C at 250 rpm. 150 μL of assay cocktail was added to sample and was capped for 3 hr of incubation at 37˚C at 250 rpm. The assay was quenched by flash-freezing the samples in -80˚C then added 400 μL of ethyl acetate. The samples were then vortexed and spun down at 2700 rcm. 200 μL of the ethyl acetate layer was removed and placed in a clear V-bottom polypropylene 350 μL 96-well plate. The organic layer was blown dry under N2 then resuspended in 1:1 H2O:MeOH before mass spectrometry analysis. The controls used were 1) DBS extract with inhibitor incubated separately, 2) DBS extract with inhibitor spiked with 2.5 nmol of free coumarin analog or 4-methylumbelliferonem and 3) DBS extract with inhibitor quenched immediately.			

Table A (cont.)

Assay	Assay Type	Assay Cocktail	Sample Type and Preparation
Natural Substrates Assays	Radiometric	Substrates: Cholesterol oleate/ Triolein Mixture of substrate and 14C-radiolabeled equivalent was dried under N_2 with egg yolk phosphatidyl choline 50 nmol cold substrate and 150 or 300 thousand DPMs of radiolabeled substrate (cholesterol oleate and triolein) respectively per assay 0.2 M citrate-phosphate buffer, 1% Triton-X, 0.5 mM egg yolk phosphatidylcholine	rhLAL Added in 2 µL aliquots of various concentrations.
Procedure: 2 µL of various concentrations of rhLAL were added to 100 µL of assay cocktail containing radio-substrates and incubated for 1 hour at 37˚C. Samples were extracted via addition of 1.625 mL MeOH/$CHCl_3$/n-Heptane mixture (1.41:1.25:1) per 100 µL of assay cocktail and 0.525 mL pH 10 0.05 M Na Carbonate buffer. Samples were then centrifuge for 15 min then counted in scintillation counter (0.5 mL to 18 mL scintillation fluid) after resting in dark overnight. Based on procedure and extraction by Negré *et. al.* and Belfrage and Vaughn respectively.[33,35]			
Compound 2 Assay Variation 1	Fluorimetric	13.3 mM substrate (4-MUb palmitate) DMSO or DMF stocks were warmed to 37˚C along with the assay buffer (0.15 M Sodium Acetate pH 4 1% Triton-X). 0.5% m/v cardiolipin/methanol solution was kept chilled until use. Final assay cocktail ratio is 1.4 Buffer solution : 0.1 Methanol solution : 0.04 DMSO solution.	DBS Sample resuspended in 200 µL of MilliQ H_2O for 1 hour at room temperature shaken at 200 rpm in a plate shaker
Procedure: 40 µL of rehydrated blood mixture is transferred per assay into a black 96-well plate. Otherwise same as rhLAL fluorimetric variation 1 procedure. The fluorimetric calibration curve was established using the same procedure except with base pool DBS samples, 8 µL of H_2O and 2 µL of 4-methylumbelliferone product solution (ranging from 0 to 5 nmol total product added).			
Compound 2 Assay Variation 2	Fluorimetric	13.3 mM substrate (4-Mub palmitate) DMSO or DMF stocks were warmed to 37˚C along with the assay buffer (0.15 M Sodium Acetate pH 4 1% Triton-X). 0.5% m/v cardiolipin/methanol solution was kept chilled until use. Final assay cocktail ratio is 1.4 Buffer solution : 0.1 Methanol solution : 0.04 DMSO/DMF solution.	rhLAL Various concentrations delivered in 2 µL aliquots
Procedure: Various dilutions of rhLAL were used. For inhibitor studies, 10 µL of either H_2O or 30 uM aqueous Lalistat2 stock (freshly prepared from 300 µM DMSO stock) was added to a black 96-well plate then 2 µL of rhLAL was added at various dilutions then pre-incubated at RT for 10 min while shaking at 200 rpm. 100 µL or 150 µL of fluorimetric assay cocktail (as stated previously) was added to each well. Samples were incubated for 3 hours and quenched were either 100 µL of 15 mM $HgCl_2$ or H_2O. Samples were read immediately on fluorometer (excitation wavelength 355 nm, emission wavelength 460 nm).			

Table A. (cont.)

Assay	Assay Type	Assay Cocktail	Sample Type and Preparation
Compound 2 Assay Variation 3	Fluorimetric	3.7 mM substrate (4-MUb palmitate) 0.5% m/v cardiolipin stocks in MeOH/ EtOH/iPrOH were warmed to 37˚C along with the assay buffer (0.15 M Sodium Acetate pH 4 1% Triton-X). Final assay cocktail ratio is 1.4 Buffer solution : 0.14 alcohol solution.	DBS Punch resuspended in 200 µL of MilliQ H_2O for 1 hour at room temperature shaken at 200 rpm in a plate shaker

Procedure:

40 µL of rehydrated blood mixture is transferred per assay into a black 96 well plate containing either 10 µL of 30 µM Lalistat 2 solution or 10 µL of H_2O. The samples were preincubated for 15 min at 37˚C at 400 rpm. 150 µL of assay cocktail was added to sample and was capped for 3 hr of incubation at 37˚C at 400 rpm. The assay was quenched by adding 100 µL of MeOH. The controls used were 1) DBS extract with inhibitor incubated separately and 2) DBS extract with inhibitor spiked with either 1.25 nmol or 2.5 nmol of free coumarin analog or 4-methylumbelliferone to establish a fluorimetric calibration curve. Samples were read immediately on fluorometer (excitation wavelength 355 nm, emission wavelength 460 nm).

II. UPLC ASSAY CONDITIONS

UPLC-MS/MS parameters**.** UPLC was carried out as described in the main text. Ten µL of sample was injected in full-loop mode. The weak needle wash was water/acetonitrile (90/10) with 0.1% formic acid, and the strong needle wash was 100% acetonitrile with 0.1% formic acid.

Table B. MS/MS Parameters and settings.

Parameter	Value
Capillary Voltage	2.95 kV
Extractor Voltage	3 V
Desolvation Temperature	450 °C
Source Temperature	150 °C
Desolvation Gas Flow	850 L/hr
Cone Gas Flow	30 L/hr
Collision Gas Flow	0.15 mL/min
LM Resolution 1	2.82
HM Resolution 1	14.92
Ion Energy 1	0.8
LM Resolution 2	2.88
HM Resolution 2	14.7
Ion Energy 2	1.1
Aperture	0.1
Entrance	0.5
Exit	0.5
Gain	1

Table C. Multiple Reaction Monitoring Parameters.*

Compound	Parent (m/z)	Daughter (m/z)	Dwell (s)	Cone (V)	Collision (V)
Product	219.1	190	0.05	40	22
Internal Standard	223.1	194	0.05	40	22

*All data for activity of substrate P-PMHC was determined from the ratio of the traces of Product over Internal Standard.

III. REACTIVE ESTER COMPOUND ASSAYS

These compounds were tested for LAL activity as follows:

Compound 7. Assay cocktail contained 0.69 mM **7** (synthesis not provided), 10 μM internal standard (analog of the product containing an *n*-hexanoyl amide tail instead of the *n*-pentanoyl amide tail) in 0.15 M sodium acetate, pH 4.02, containing 0.1% Triton X-100. Assay cocktail (30 mL) was added to a 3 mm DBS punch from a normal adult and 10 μL of 30 μM aqueous Lalistat-2 or H_2O was added, and the mixture was incubated at 37°C for 16 hrs in an Eppendorf tube with orbital shaking at 250 rpm. The assay was quenched by addition of 120 μL of acetonitrile. The sample was centrifuged for 5 min at 3000 x g, and 120 μL of supernatant was transferred to an autosampler plate. Water (60 μL) was added to the well, and 10 μL was injected for UPLC-MS/MS. Compound **7** product was analyzed by UPLC-MS/MS using the authentic product as a standard to establish UPLC and MS/MS conditions.

Compound 12. Assay cocktail contained 0.3 mM **12** (synthesis not provided, from a 10 mM DMSO stock) in a 14:1 solution of 0.15 M sodium acetate pH 4.0 1% Triton-X-100 and 0.5% m/v cardiolipin in methanol. For DBS tests, 20 μL of rehydrated blood mixture (from a 3 mm DBS punch re-suspended in 200 μL of H_2O for 1 hr at RT with orbital shaking) was added to 75 μL of assay cocktail and incubated at 37˚C for 12 hrs in an Eppendorf tube with orbital shaking at 250 rpm. For recombinant LAL tests, 1 μL of diluted enzyme solution (prepared from a 2 mg/mL stock diluted serially by 10-fold twice in H_2O) was added to 19 μL of H_2O. The process was then the same as for the DBS version. The assay was quenched by addition of 120 mL of acetonitrile. The sample was centrifuged for 5 min at 3000 x g, and 120 μL of supernatant was transferred to an autosampler plate. Water (120 mL) was added to the well, and 10 μL was

injected for UPLC-MS/MS. Compound **9** product was analyzed by UPLC-MS/MS using the authentic product as a standard to establish UPLC and MS/MS conditions.

Compound 13-16. Assay cocktail contained 0.345 mM **13-16** (synthesis not provided, from a 13.3 mM DMSO or DMF stock) in a 14:1:0.4 solution of 0.15 M sodium acetate pH 4.0 1% Triton-X-100 (warmed to 37˚C), 0.5% m/v cardiolipin in methanol, and DMSO/DMF substrate stock (warmed to 37˚C) respectively. For DBS assays, 40 µL of rehydrated blood mixture (from a DBS punch re-suspended in 200 µL of H_2O for 1 hr at RT with orbital shaking) was added to 10 µL of either H_2O or 30 µM aqueous Lalistat-2 solution (freshly prepared from a 0.3 mM DMSO stock) were pre-incubated for 10 min at 37 ˚C in a black, 96-well microtiter plate. Assay cocktail (100 or 150 µL) was added, and samples were incubated at 37˚C for 3 hrs. For recombinant-LAL, assays, 2 µL of enzyme solutions at various concentrations (from serial dilutions of a 2 mg/mL stock stored at 4˚C) were added to 10 µL of H_2O or 30 µM Lalistat-2 solution and incubated at RT for 10 min with 200 rpm of orbital shaking. The process was then same as the DBS version. The assay was quenched by addition of 100 µL of H_2O. Samples were immediately read on a plate-reader fluorometer with an excitation wavelength of 355 nm and emission wavelength of 460 nm. The fluorimetric calibration curve was established using the same procedure as above using rehydrated base pool blood (blood devoid of leukocytes) except for the addition of 8 µL of H_2O and 2 µL of 4MU standard solution (ranging from 0 – 2.5 nmol).

The library of 4MU palmitate analogs was tested as LAL substrates as follows. Assay cocktail was a solution of 10:1 buffer to substrate stock, each warmed to 37˚C. The substrate stock contained 3.7 mM substrate and 0.5% cardiolipin in ethanol. The buffer contained 0.15 M sodium acetate pH 4.0 and 2.5 mM sodium taurodeoxycholate. Rehydrated blood mixture (40

μL) was combined with 10 μL of H_2O or 30 μM Lalistat-2 in an Eppendorf 1.5 mL polypropylene tube and pre-incubated for 15 min at 37˚C with orbital shaking at 250 rpm. Assay cocktail (100 or 150 μL) was added and samples were incubated for 3 hrs at 37˚C with orbital shaking at 250 rpm. Assay was quenched by first flash freezing to -80˚C followed by subsequent addition of 400 mL of ethyl acetate and thawing. Samples were then vortexed and centrifuged at 2700 x g for 5 min. Top ethyl acetate layer (200 μL) was transferred to an autosampler plate and blown dry under N_2 gas then resuspended in 1:1 H_2O:methanol solution, and 10 μL per well injected for analysis by UPLC-MS/MS. The LC-MS/MS data was converted into mmole of product by using an external standard of 2.5 nmole (the individual corresponding coumarins) spiked into 40 μL rehydrated blood in an 1.5μL Eppendorf polypropylene tube incubated separately from the assay cocktail, which were quenched immediately following incubation. Additional controls included a Lalistat-2 inhibited rehydrated DBS solution incubated separately from the assay cocktail then combined and immediately quenched as a blank. Unique duplicate spiked controls and blank controls were used for each coumarin analog.

Appendix Table D. SAR Compound Library MS/MS Assay Results.

Structure	Full Assay Ion Counts	Blank Ion Counts	Analytical Range Ratio	Full Lipase activity umol/hr/L blood	Inhibited Lipase Activity umol/hr/L blood	LAL-specific activity	% LAL specificity
17	428379.5	55117	7.8	1248.89	119.05	1129.84	90.5
18	76148	26788	2.8	49.01	5.45	43.56	88.9
19	507273.3	306888.36	1.7	19687.72	3887.5	15800.22	80.3
20	2353581.5	2151025	1.1	227.55	235.21	-7.66	-3.4
21	733949	142570	5.1	325.59	43.14	282.45	86.8
22	716379.38	263808.81	2.7	311.95	18	293.95	94.2
23	598667	469293	1.3	2816.75	3034.32	-217.57	-7.7
24	975999	62126	15.7	479.16	129.28	349.88	73.0
25	268673.5	23394	11.5	232.93	19.62	213.31	91.6
26	741746	77310.5	9.6	1291.05	628.74	662.31	51.3
27	34611	31636	1.1	329.06	121.34	207.72	63.1
28	261699.88	151492.98	1.7	234.98	71.13	163.85	69.7
29	303236.5	118550.5	2.6	211.03	109.59	101.44	48.1
30	15711.5	1158	13.6	379.79	23.28	356.51	93.9
31	638168	22489.5	28.4	743.28	103.41	639.87	86.1
32	65086	10002.5	6.5	378.31	83.38	294.93	78.0
33	311104.5	30185	10.3	324.48	95.67	228.81	70.5
34	1721073.5	1677845	1.0	162.88	248.79	-85.91	-52.7
35	327700.5	255262	1.3	521.33	946.32	-424.99	-81.5
36	1358200.19	455514.06	3.0	661.73	124.35	537.38	81.2
37	535159	244593	2.2	647.35	317.37	329.98	51.0
38	220167	110119	2.0	21.25	16.33	4.92	23.1
39	66133	26034.5	2.5	65.27	-5.83	71.1	108.9
40	742623.5	531052	1.4	308.86	322.17	-13.31	-4.3
41	51135	22264.5	2.3	55.54	-6.36	61.9	111.5
4MU	133720.43	33359.48	4.0	331.35	60.32	271.03	81.8

As shown below in Appendix Table D, compounds **18**, **20**, **21**, **22**, **25**, **27**, **28**, **29**, **33**, **34**, **38**, **39**, **40**, and **41** were poor substrates with DBS as the source of LAL compared to 4MU palmitate and were not studied further (all had LAL activity below 270 µmol/hr/L blood). Analogs **19**, **23**, **24**, **26**, **32**, **35**, **36**, and **37** showed higher activity but where poorly LAL-specific based on the observation that addition of Lalistat-2 did not substantially reduced the activity more than what

was seen for 4MU palmitate (all had % LAL specificity below 82% as shown in Appendix Table D). This left 3 compounds: **17**, **30**, and **31**. Both **17** and **30** displayed greater than 90% of the LAL-specific activity measured with palmitoyl-4MU. Compound **17** was found to be more active than 4MU-palmitate and thus it was pursued due to the highest combination of total lipase activity and LAL-specificity (see main text for studies of its LAL specificity). The final optimized protocol for LAL assays using substrate **17** is given in the main text. Appendix Table D also shows the analytical range of all the library compounds. This was calculated by using the ratio of full assay (without inhibitor Lalistat2) ion counts over blank assay ion counts. The % LAL specificity was calculated by using the percentage ratio of the LAL-specific activity (the difference between the full uninhibited assay and inhibited assay activities) over the full assay activity.

IV. Final LAL Assay Table.

Appendix Table E. LAL activity measured with substrate **17** by UPLC-MS/MS.[1]

Type	Sample	LAL Activity (μmol/hr/L blood)	Mean	CV
Children under 5 years	1	101, 171, 116, 195, 146	145.9	23.6
	2	542, 581, 619, 642, 660	609.1	7.0
	3	310, 368, 406, 393, 346, 332	359.2	9.4
	4	301, 295, 276, 291, 306, 275	290.8	4.0
	5	580, 546, 527, 550, 554	551.3	3.0
	6	560, 713, 407, 584, 414, 628	551.1	20.0
	7	267, 244, 264, 241, 228, 224	244.9	6.6
	8	346 358, 400, 395, 344, 322	361.0	7.8
	9	556, 612, 584, 612, 582, 637	597.4	4.4
	10	140, 211, 159, 187, 105, 202	167.6	22.0
	11	158, 181, 237, 148, 286, 259	211.7	24.6
	12	933, 934, 1034, 790, 898, 698	881.1	12.4
	13	525, 544, 485, 508, 538, 440	506.8	7.0
	14	621, 581, 676, 671, 718, 722	665.1	7.6
	15	332, 530, 497, 506, 400, 487	458.6	15.2
Adults	1	598, 555, 653, 578, 608	598.5	5.5
	2	712, 701, 674, 632, 588, 636	657.1	6.5
	3	492, 499, 506, 480, 453, 465	482.2	3.9
	4	962, 842, 862, 848, 1016, 849	896.3	7.5
	5	846, 852, 852, 864, 923, 907	874.0	3.4
	6	990, 979, 1129, 1152, 929	1035.8	8.5
	7	613, 555, 488, 536, 514, 470	529.0	8.9
	8	567, 547, 435, 644, 564, 488	540.7	12.2
	9	316, 330, 220, 247, 292, 236	273.4	15.2
	10	938, 796, 818, 627, 1112, 740	838.3	18.3
	11	447, 526, 695, 595, 596, 684	590.4	14.6
	12	424, 534, 376, 496, 368, 385	430.4	14.7
LALD Patients	1	5, 6, 4, 3, 5	4.5	22.1
	2	15, 12, 10, 11, 13, 9	11.8	15.4
	3	1, 2, 0, 1, 2	1.4	53.3
	4	3, 1, 0, 1, 0	0.8	190.2
	5	25, 12, 11, 31, 37, 17	22.2	43.5
	6	8, 6, 7, 6,7	6.8	8.5
Blank		6.3 - 24.9 (n = 56)	15.0	36.4

[1]DBS samples were all blank corrected.

APPENDIX B

Table of Contents:

I. Materials.

(Z)-Pugnac ((Z)-O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino N-phenylcarbamate) was from Santa Cruz Biotechnology (Cat. sc-204415). Ammonium formate (Cat. 156264-2.5KG), ammonium acetate (A1542-250G), barium acetate (243671-100G), and cerium acetate (529559-10G) were from Sigma. Quality control DBS were obtained from the CDC [7] or from Perkin Elmer Life Sciences. Solvents for flow injection- and LC-MS/MS and formic acid are Optima grade from Fisher Scientific. DBS fro random newborns were obtained from Prof. Christiane Auray-Blais, University of Sherbrooke, Quebec. The DBS were kept at ambient temperature for 1 week after generation, then stored at -20°C for ~2 years. DBS from MPS-affected patients were obtained with the help of the MPS Society. DBS were generated and received at the Univ. of Washington within 3 days, then stored at -20°C for up to 1 yr. All human samples were obtained and processed in compliance with our IRB Protocol at the Univ. of Washington.

II. Preparation of recombinant human IDUA.

Cell growth**.** CHO cells overexpressing human IDUA into the culture medium were obtained from Prof. E. Neufeld (UCLA).[68] Growth medium is 1:1 DMEM:F12-Ham (Invitrogen Cat. 11885084 and 11765054) containing 10% heat inactivated fetal bovine serum, 20 mM HEPES, non-essential amino acids (1 mM final, Sigma M7145), 300 mg/mL Geneticin (Invitrogen Cat. 10131-035) Pen-Strep, nucleosides (10 mg/mL each of guanosine, adenosine, uridine, cytidine, hypoxanthine, thymidine (Sigma G6264, A4036, U3003, C4654, H9636, and T1895), pH adjusted to 6.8 and filter sterilize. Production medium is growth medium but with fetal bovine serum reduced to 2.5% and additives (10 mg/L human insulin (Santa Cruz Sc-360248), 6.7 mg/L selenium, added as sodium selenite, Sigma S9133, 2 mg/L ethanolamine). The stock solution of sodium selenite was prepared according to the manufactuer's instructions.

Gelatin coated microcarrier beads were prepared as follows. Beads (1.2 gram at 1 g/L, Sigma M9418) were suspended in PBS without calcium and magnesium and left for 1 hr then autoclaved at 120 °C for 20 min. The beads were allowed to settle by gravity and washed with 100 mL of PBS and then repeated with growth medium. Store the suspension at 4 °C for up to 30 days (keep sterile).

CHO cells were grown to 7-90% confluency in growth medium in an incubator (37 °C, 5% CO_2) in 10 cm Nunc dishes (split with trypsin-EDTA). In a 3L spinner flask 600 mL of pre-warmed growth medium plus 1.2 g of beads was added followed by 1.2-2.4 x 10^8 cells. This was stirred at 30-40 rpm. About 28 hr later another 600 mL of pre-warmed growth medium was added to bring the final volume to 1.2 L. We continued to culture the cells, counting them every other day. To count, 1 mL of medium with beads was removed and the supernatant removed after the beads settled in the tube. The beads were washed once with pre-warmed PBS, then add 0.45 mL of pre-warmed trypsin-EDTA was added, and the mixture incubated 37 °C in a water bath for 30 min (or until all cells detach from beads). A aliquot of the supernatant was submitted to cell counting with Trypan blue. The cells should reach a density of ~10^6 in 1 week. After that, the beads were allowed to settle for ~20 min, and 800 ml of medium was removed and replaced with 800 ml of pre-warmed production medium. After removing 800 mL of medium, it was added to 1 M sodium phosphate, pH 5.8 to give 10 mM final. The solution was stored at 4 °C. About 9-12 hours after adding production medium, the beads were allowed to settle, and 800 mL of medium was removed and replaced with 800 mL production medium as above. Phosphate was added to the harvested medium as above. Each batch of production medium was checked

for IDUA enzymatic activity as described below, and the above process was continued until the level of activity started to fall (typically we collected 800 mL of the original growth medium and 4x800 mL of production medium.

Partial purification of IDUA. Each 800 mL portion of medium (see above) was filtered with a Nalgene filter sterilizer unit to remove particulate. All batches of filtered medium were combined and dialyzed (30 kDa MW cutoff tubing) at 4 °C against 10 mM sodium phosphate, 20 mM NaCl, pH 5.8 (7-9 volumes of buffer, 3 times). The dialyzed medium is lyophilized. To the powder was added 75 mL of purified water (Milli-Q, Millipore Corp.) per 400 mL of dialyzed medium. The solution was centrifuged at ~6,000xg at 4 °C for 20 min and the supernatant filtered through a Nalgene sterilizing filter unit. Store remaining lyophilized powder at -80 °C until processed.

The ice-cold solution is loaded at 1.2 mL/min with a peristaltic pump onto a Hi-Trap Heparin Sepharose column (5 mL, Pharmacia 17-0407-01) (the column was on the bench at room temperature but the sample solution and buffers were chilled in ice). The column was pre-equilibrated with buffer A (10 mM sodium phosphate, 100 mM NaCl, pH 5.8). After sample loading, the column was washed with 50 mL of buffer A, then with 25 mL of buffer A with 200 mM NaCl (both at 1.2 mL/min). Elute the IDUA with buffer A with 0.6 M NaCl at 1.0 mL/min. Most of the IDUA elutes, but we typically run an additional 15 mL of this buffer to make sure all IDUA is obtained. Fractions are assayed for IDUA (see below), pooled and concentrated and buffer exchanged by ultrafiltration (Amicon Ultra15, 30 kDa MW cutoff) into 10 mM sodium phosphate, 100 mM NaCl, pH 5.8 (3 rounds of buffer exchange, the column eluant was concentrated 5-fold). This partially purified IDUA was stored in aliquots at -20 °C. The column matrix can be re-used 4-5 times if it is recycled according to the manufacturer's procedure. We typically use up to 10 mL of Heparin Sepharose to process CHO cell medium from 4 L of culture. The yield of partially purified IDUA is 4.5-5.0 million Units (see below) from 800 mL of growth medium plus the 4x800 mL portions of production medium (see above).

IDUA assay. The 4MU substrate has been published.[69] An aliquot of stock solution in methanol was transfered to a glass tube, and solvent was completely removed with a stream of oil-free air or using a centrifugal vacuum concentrator. The residue was taken up in assay buffer (50 mM sodium formate, pH 2.8) to give 0.2 mM substrate. To 40 mL of this assay cocktail was added 1-2 mL of CHO cell medium. The mixture was incubated at 37 °C for 1 hr in a capped Eppendorf tube. The reaction was quenched by adding 0.25 mL of glycine-carbonate buffer, pH 10.5 (to 85 mM glycine, adjust to pH 10.5 with sodium carbonate). The fluorescence was read with excitation at 365 nm and emission at 450 nm in a plate reader. The assay was calibrated by adding known amounts of free 4MU (Sigma M1381) to buffer. 1 Unit of IDUA is the amount that generates 1 nmole of product per hr under the above conditions.

III. Preparation of recombinant b-NGA.

Bacterial growth and enzyme purification. A synthetic *Nde*I/*Xho*I gene fragment for b-NGA (sequence shown below) was prepared by Genscript corporation. The restriction fragment was ligated into the pET28C expression vector . After transfection of into *E. coli* BL21-DE3, cells were grown on LB medium containing 50 mg/mL kanamycin. The plasmid encodes b-NGA but with the peptide segment MVNRKQKTISFQLLVWSMMMILILQPLC deleted from the N-terminus (to improve protein expression).

E. coli was grown at 25 °C with swirling in 1 L of medium plus antibiotic until the OD600 reached ~2.0. Medium (250 mL) was removed and replaced with 250 mL of fresh medium containing 1 mM IPTG, and the culture was continued for 4 hr. Cells were pelleted by centrifugation at 5000-6000xg for 15 min at 4 °C, and the pellet was immediately lyzed. To the pellet was added 50 mL of ice-cold lysis buffer A (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.5) containing one EDTA-free protease inhibitor table (Pierce 88266), 1 mM PMSF and 5 mM b-mercaptoethanol. The pellet was stirred in lysis buffer for 10 min on ice, and then sonicated with a probe sonicator (10 times using a cycle of 20 pulses on then 10 sec off). The sample was centrifuged at 11,000xg for 20 min at 4 °C. The supernatant was loaded onto 4-5 ml of packed Ni-NTA gel (Qiagen) that was pre-equilibrated in lysis buffer in a 2.6 cm diameter glass column. The column was capped and mixed end-over-end on a wheel at 4 °C overnight. The column was mounted vertically to allow the resin to settle. The outlet of the column was opened to allow the excess buffer to elute. The resin was washed with 50-60 mL of buffer A plus 1 mM PMSF and 5 mM b-mercaptoethanol (both freshly added). The column was then washed with 30 mL of buffer A plus 50 mM imidazole plus 5 mM b-mercaptoethanol (freshly added). The enzyme was eluted with buffer A plus 250 mM imidazole plus 5 mM b-mercaptoethanol (freshly added). Fractions were analyzed by SDS-PAGE, and those containing b-NGA were pooled (the Ni-NTA resin can be regenerated according to the manufacturer's instructions). The enzyme pool was dialyzed against 25 mM HEPES, 150 mM NaCl, 1 mM DTT, pH 7.5 at 4 °C. The dialyzed enzyme was stored in aliquots at -20 °C. The yield was ~13 mg per L of culture. The purity is >90% as judged by SDS-PAGE.

Enzyme assay. The activity of b-NGA was measured using 0.2 mM of the MPS-IVA internal standard compound[42] in 50 mM sodium acetate, pH 6.0 for 1 hr at 37 °C. The mixture was quenched with 250 mL of glycine-carbonate, pH 10.5 buffer (see above), and fluorescence was measured as above. The assay was calibrated with free 4MU. One Unit of enzyme is the amount that generates 1 nmole of product per hr.

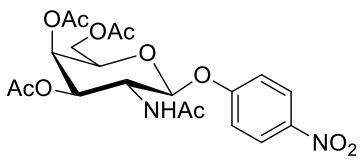
NdeI/XhoI gene fragment coding for b-NGA:

CATATGCCGACATCCGTCGGCGCAGCCGAAGAAGAGACGCAGACGTTCATTATTAA
CGATGCGGATATCGGAGGCGGGATAAACAAATTCCAATATTTCGGAACCTGGGGTA
CAAGCACCAACATAGCGGGCCTTTATAACGGTGATGAGCATTGGTCCAATTCGGCG
AATTGGACTGATCCGAGCAGCATTTATTTTACCGTCCGGTTTGAAGGCGAGCGGCTG
GAATTGTACGCGATTAAGGACCCGAGACATGGGATATACGCGGTATCCGTCGACGG
AGGACCGGAGGTTGAAGTAGATGCTTACGCCTCGGTTCGAACCTTCGATCAGTTAAT
ATATGACAGCGGCAAGCTGGCGAGCGGCGAGCATCTGGTGAAGGTCAGAGCAACCG
GCAAAAAAACCGGGGGCGCTCCGGATATGCAAATCGATTACGCCAAAGTAACGAAG
AAGATTATCGCCGTTACCGGCATTGAAATGGAAGCCGGCGAACTGACCGTCGAAGA
CGGCTCGATTACGCAATTGAATGCGGCTGTATTGCCGGCCAATGCGAGCAATCAAAC
GATTCGCTGGTCGACGTCGGACGATCAAGTAGCGAAGATCGACGCGCAAGGCAGGC
TGTCGGCCGAGGCGGTCGGCACGGTTCAGGTAACCGCGACGTCTGAAGACGGGGGA
TTTCAGGCAACGAGGAAAGTCGTCGTCGCGCCGGCTTCCCAATATTTGAAAGGAGCT
GTCGGAACGACCGACATGCATTATGTCAAGGAGAGTCCGTTCGATGACTATAAGCG
CATCGACTATATGGAAATTGCCCAGATGAATGAAAAAGTATGGACGGGCAATGCGT
GGAAAGGAGATCGGGCAAGCGCCCAGTTCGTGCTGTGGACGACAAACAGAAAGGA
AGACCAGGTTACGTTGTCGATCGGAGACTTGGTTGGGGAGCATAACCAAAGCATTG
CCTCCTCCAATTTATCCGTGCATTTTGTCAAAACGACAAAAGCGGCGCGAGGAAATC

CTTCTCAAGGAAAACCGCAGGAGCTCATCCCGGATATTCTTGGCTCGGCAGATCCTG
TGAGCATCGATAAATTCGGCGTCCAGCCGATCTGGGTATCGATTGATGTGCCGAAAG
ACGCCAAAGCGGGCAATTACAGCGGCCAATTGACGGCAAGCTCGGCTTCGGGAGAA
GAAGTGAGCTTCACCCTTCAATTGGAAGTGCTGGATCTGACCTTGCCAGATGTGAAG
GATTGGGGATTTGAGCTTGATCTGTGGCAGAATCCCTATGCTGTGGCGAGAGTGAAC
GGAATAACCCAGGATCAGCTTTGGACCAAGGCGCATTTCGACGCGATGAAGCCGCA
TTATGAAATGCTCGCCGCCGCCGGACAGAAGGTTATTACGACAACGGTAACGTATG
ATCCCTGGAATTCCCAAACGTATGACGTTTACGACACCATGGTGAAATGGACGAAG
AAGGCGGACGGGACTTATACTTTTGATTTTGAGATTTTTGACAAATGGGTTCAATTT
ATGATGGATCTGGGGATCGACAGCCAGATTGACGCGTACAGCATGGTTTCCTGGGC
GAGCAAAATCAAATATTATGATGAAGTCCAGAAGAAGGATGTTATAGAAACGGTTC
AAACTAATAATCCGAAATGGGCAGTGATGTGGCGGGCGTTCCTGGAAGCCTTCGTCC
CTCATCTCGAAGCTAAGGGCTGGCTCGACAAAACCTATATGGCTAATGATGAGAGA
GCGCTGAACGACTTGCTTATTGCCGCAGACTTGATCGAGGAAGTGTCGGGCGGCAA
GCTCAAAATTGCGGCGGCGATGAACGTCAACAGCTTGTCCGACTCGCGTCTGGATCG
TCTTCATAAAATATCGGTCGGGCTTTATCATGTCAATCATGAGAACAAGCAATTGAC
GAATACGTCTGAGCATCGCAGACAGCTTGGGCTGATTACGACCATTTATAACTGCGT
GGGGCACTATCCCAACAGCTTTGCCCGTTCCAATCCCGCCGAGCCGGTATGGGTCAT
CTGGGATACGATGCGGCATCAAACGGACGGATACTTGAGATGGGCGTTCGACTCTTT
CGTGAAAGATCCGTATGAAACGACCGATTTCAAAACATGGGAATCCGGCGATTCCG
CTCAAGTCTATCCGGGTGCCGTCAGCTCCGTACGCTTCGAAAGAATGAAGGAAGGA
ATCCGAGACGCCGAGAAGGTCAGATACTTGACCGAGCACAATGCCGAGATAGGCGA
AGAAATCGCGGCGGCGGCCTGGCAGATGCAGAATGTCGGCTACGCTCTCGATCCTT
ATAACGGCGTTAAAGATCCCGGCATTGTCAATATTCCGCAAGAGGTCAATCGTTTAA
AGGCTTCATTGGATCAGGCGGCGCGAAAATATTTGGATCTGATACGGGAAGAGATG
AAGGACGAAATTGTGCTAGTCGGTCCGGACGCTGTTCAATCCGATGAAAGCTTTGCG
GTGAAATTCGGACTGAACCGGTTAAGCAAGCCTGTTCAGGCGCTGGATATTACGCTC
CGTTATTCGGCAGACCGGTTCGAGTTCATCGGAGCCGCATCCGCCGATGAACATGTT
CAGATCGTCGATACGATCAAGCAAGGGGACGGCAAGCTCCGCCTGATCGTCGCGGC
GCTAGGCAGCGAGCATGCCATTCATGCGGACAGTTTATTCCTGGAGCTGAGCTTCCG
CGCCAAAGCTGCAGATCAATCCGCGCAGGGGATTGTGGAAGCTGCTGAAGTCATCG
TCAGCGGAAGCGATGGCGTGGAGTCCAGCCTGTCTCCAGTCTCTATCGCAATCGACG
TCCTGCCGGTTGATCCATTCGATCGTCTGGATGTGAACCGTGACGGAAAAATCAGCA
TCGGCGACCTTAGCATAACCGCCGCGCATTACGGCAAAACGGATCAAAGCCCGGAT
TGGGAGATGATCAAGAACGCCGATGTGAATAGAGACGGTAAAATTGACATCGTCGA
CCTTACGGAA

IV. Synthesis of MS/MS substrates, products and internal standards for GALNS and ARBS assays.

Synthesis of MS/MS substrates, products and internal standards for GALNS and ARBS assays.

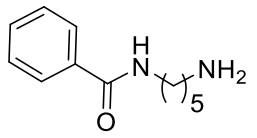


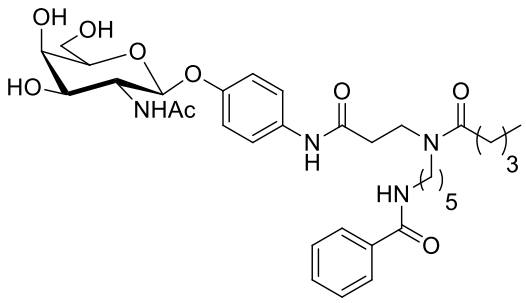
(2R,3R,4R,5R,6S)-5-acetamido-2-(acetoxymethyl)-6-(4-nitrophenoxy)tetrahydro-2*H*-pyran-3,4-diyl diacetate (1)

Pyridine (60 mL) was added to nitrogen back flushed flask containing D-galactosamine hydrochloride (5 g, 23.2 mmol) and the resultant slurry was cooled on an ice bath. To the cooled mixture acetic anhydride (25 g, 245 mmol) was added dropwise and allowed to warm to room

temperature followed by stirring at this temperature for 16 hours. The reaction mixture was quenched with the addition of methanol (15 mL) and let to stir for 20 minutes. The resultant mixture was concentrated under reduced pressure and the residue was dissolved in 20% methanol in chloroform with the aid of warming the mixture. This solution was washed with 1N HCl solution followed by brine solution. The resultant organic layer was dried using anhydrous sodium sulfate and concentrated under reduced pressure. The residue was taken in nitrogen back flushed flask equipped with a dropping funnel. Anhydrous dichloromethane (100 mL) was added to this residue and the resultant slurry was cooled on an ice bath. In the dropping funnel titanium(IV) chloride (6.5 g, 42.1 mmol) was dissolved in anhydrous dichloromethane (40 mL) and the resulting solution was added dropwise to the cooled solution. The reaction mixture was warmed to 50°C in an oil bath and left to stir at this temperature for 48 hours. The reaction mixture was cooled back on an ice bath and saturated sodium bicarbonate solution was added dropwise with vigorous shaking. The resultant mixture was extracted between chloroform and saturated sodium bicarbonate solution. The organic layer was dried using anhydrous sodium sulfate and concentrated under reduced pressure. The resultant residue was dissolved in acetone (60 mL) and added slowly to a cooled mixture of 4-nitrophenol (16.1 g, 116 mmol) in acetone (130 mL) and 4N KOH aqueous solution (23.2 mL) at 0°C. The reaction was left to stir at room temperature for 48 hours and concentrated under reduced pressure to dryness. The residue was redissolved in 10% methanol in chloroform by the aid of warming the mixture. This solution was extracted between 1N NaOH and chloroform and the chloroform layer was further washed with brine solution. The organic layer was dried using anhydrous sodium sulfate and concentrated under reduced pressure. The crude product thus obtained was purified by silica flash chromatography using 3% methanol in dichloromethane as the elution mixture. The fractions with the desired compound, as determined by TLC, were combined and concentrated under reduced pressure to get **1** (3.29 g, 30%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.20 (d, J = *9.1 Hz*, 2H), 7.09 (d, J = *9.1 Hz*, 2H), 5.61 (d, J = *8.0 Hz*, 1H), 5.56 – 5.39 (m, 3H), 4.32 – 4.07 (m, 4H), 2.18 (s, 3H), 2.07 (s, 3H), 2.04 (s, 3H), 1.97 (s, 3H). MS (ESI^+) for $[M + Na]^+$; calculated: 491.1, found: 491.2.

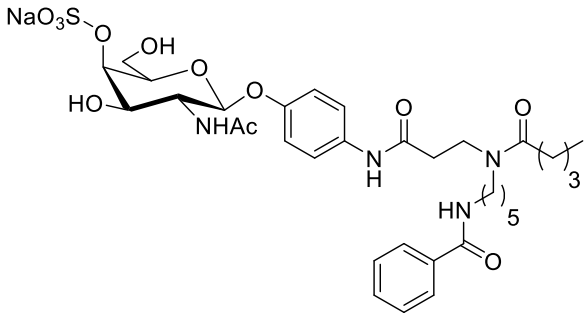
***N*-(5-aminopentyl)benzamide (2).** To methyl benzoate (1.0 g, 7.34 mmol), pentane-1, 5-diamine (0.75 g, 7.34 mmol) and water (0.37 mL) were added, and the mixture was heated to 100°C for 24 hours under constant stirring. The reaction mixture was cooled to room temperature and directly loaded on to a short silica column. Upon elution with 10 to 20% of methanol (with 5% aq. NH_4OH solution) in chloroform the desired mono-benzoylated product **2** was obtained (0.80 g, 53%) as a pale yellow oil. ^{1}H NMR (300 MHz, MeOD) δ 7.83 (d, J = 7.4 Hz, 2H), 7.58 – 7.31 (m, 4H), 3.41 (t, J = 7.0 Hz, 2H), 2.76 (t, J = 7.2 Hz, 2H), 1.74 – 1.21 (m, 6H). MS *m/z* 207.2 $(M+H)^+$.





***N*-(5-(*N*-(3-((4-(((2S,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)phenyl)amino)-3-oxopropyl)pentanamido)pentyl)benzamide (GalNAc-C5/C5-Bz, ARSB Product)** To a solution of **1** (3.5 g, 7.47 mmol) in anhydrous methanol (90 mL), cooled on an ice bath, 0.5 M sodium methoxide solution in methanol (3 mL, 1.50 mmol) was added dropwise and allowed to warm to room temperature.

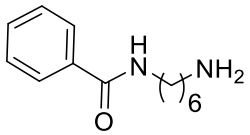
After 2 hours formic acid (0.1 mL) was added to the reaction mixture and concentrated to dryness under reduced pressure. To the resulting residue methanol (135 mL), water (15 mL) and 10% palladium on activated carbon (125 mg) was added and let to stir under a hydrogen atmosphere at room temperature for 16 hours. Water was added dropwise to the reaction mixture till the entire white residue was completely dissolved. The reaction mixture was filtered and the filtrate was cooled on an ice bath. To it pyridine (2 mL) was added and followed by the dropwise addition of a solution of acryloyl chloride (2.1 g, 23.2 mmol) in dichloromethane (50 mL). The reaction was let to stir on the ice bath for 30 minutes and then warmed to room temperature and continued for 2 hours. Sodium carbonate powder (3.0 g) was added to the reaction mix and let to stir for 15 minutes and filtered. The filtrate was concentrated under reduced pressure and further dried under high vacuum. The residue was dissolved in 2-propanol (50 mL) and water (6.6 mL) mixture and to it *N*-(5-aminopentyl)benzamide **2** (2.0 g, 9.69 mmol) was added and let to stir for 40 hours at 65°C. The reaction mixture was concentrated to dryness under reduced pressure and redissolved in methanol (70 mL). Upon cooling this mixture on an ice bath, triethylamine (2.5 mL) was added followed by the dropwise addition of a solution of pentanoyl chloride (2.7 g, 22.4 mmol) in dichloromethane (50 mL). The reaction was left to stir on the ice bath for 30 minutes and then warmed to room temperature and continued for 16 hours. The reaction mixture was concentrated under reduced pressure and subjected to purification by silica flash chromatography using 15% methanol in dichloromethane as the elution mixture to yield **GalNAc-C5/C5** (2.96 g, 60%). ^{1}H NMR (300 MHz, MeOD) δ 7.84 (d, *J = 7.1 Hz*, 2H), 7.59 - 7.35 (m, 5H), 7.09 – 6.91 (m, 2H), 5.00 (d, *J = 8.4 Hz*, 1H), 4.28 – 4.09 (m, 1H), 3.97 – 3.55 (m, 7H), 3.46 - 3.24 (m, 4H), 2.72 – 2.51 (m, 2H), 2.49 - 2.28 (d, *J = 7.3 Hz*, 2H), 2.02 (s, 3H), 1.78 – 1.49 (m, 6H), 1.49 -1.22 (m, 4H), 0.94 (t, *J = 7.1 Hz*, 3H). MS (ESI$^+$) for $[M + H]^+$; calculated: 657.3, found: 657.5.



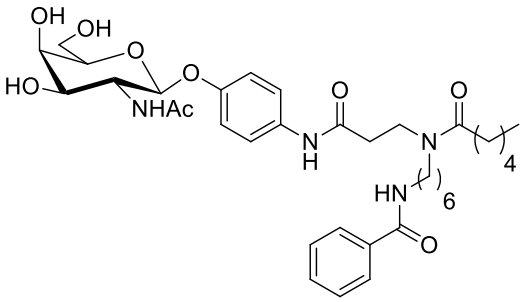
sodium (2R,3R,4R,5R,6S)-5-acetamido-6-(4-(3-(*N*-(5-benzamidopentyl)pentanamido)propanamido) phenoxy)-4-hydroxy-2-(hydroxymethyl)tetrahydro-2*H*-pyran-3-yl sulfate (GalNAc-4-S-C5/C5-Bz, ARSB Substrate).

To a cooled (0°C) solution of **ARSB Product** (25 mg, 38.1 µmol) in anhydrous pyridine (0.5 mL), benzoyl chloride (4.9 µL, 41.9 µmol) was added. After 1 hour at room temperature the solution was cooled back to 0°C and another portion of benzoyl chloride (4.9 µL, 41.9 µmol) was added and left to stir for 2 hours at room temperature. Upon checking the TLC and mass spec analysis of the reaction mixture, more small portions of benzoyl chloride can be added until most of the starting material was converted to the dibenzoylated product. The reaction was extracted between 1 M HCl solution and chloroform. The chloroform layer was further washed with a mixture of water and brine solution (1:1). The organic layer was concentrated and purified by flash silica column chromatography using 5% methanol in DCM as the eluent. The desired fractions were concentrated under reduced pressure and further under high vacuum. The resultant residue was dissolved in anhydrous pyridine and sulfur trioxide pyridine complex (8.3 mg, 52.1 µmol) was added to it at room temperature. The resulting mixture was heated to 45°C for 16 hours followed by the addition of methanol (0.5 mL) and stirred for further 10 min. The reaction mixture was concentrated under reduced pressure and

further under high vacuum. The resulting residue was re-dissolved in anhydrous methanol (5.0 mL) and cooled to 0°C. To this cooled solution 0.5 M solution of sodium methoxide in methanol (0.5 mL) was added dropwise and let stir for 16 hours. The reaction was quenched by the addition of 1 M aqueous solution of sodium phosphate monobasic (1.0 mL) and subjected to semi-preparative reverse phase HPLC purification (gradient water/methanol system) to yield **GalNAc-4-S-C5/C5-Bz** (5.8 mg, 20%). The **GalNAc-4-S-C5/C5-Bz** was further purified by ion-exchange chromatography. A fritted thin column was loaded with 2 mL of Q-Sepharose fast flow (GE Healthcare, product code: 17-0510-01) and washed with methanol (50 mL). A solution of the **GalNAc-4-S-C5/C5-Bz** in methanol (2 mL) was loaded on to this column and the column was further washed with methanol (100 mL). The **GalNAc-4-S-C5/C5-Bz** was then eluted with 1M ammonium formate in methanol solution (100 mL) and concentrated under reduced pressure at temperature less than 25°C. The resultant residue was dissolved in DI-water and loaded on a C18 cartridge (Resprep cat # 26034, which was pre-activated with methanol and washed with DI-water) by applying negative pressure in the bottom. The cartridge was further washed with DI water (4x25 mL) and the substrate was eluted from the cartridge using methanol (4x25 mL). The methanol fraction was concentrated under reduced pressure at temperature less than 25°C to afford the pure **GalNAc-4-S-C5/C5-Bz**. ^{1}H NMR (300 MHz, MeOD) δ 7.80 (dd, *J* = 7.0, 1.2 Hz, 2H), 7.58 – 7.38 (m, 5H), 7.03 – 6.94 (m, 2H), 5.01 (dd, *J* = 8.4, 1.1 Hz, 1H), 4.75 (d, *J* = 3.1 Hz, 1H), 4.13 (dd, *J* = 10.9, 8.4 Hz, 1H), 3.95 – 3.61 (m, 6H), 3.45 – 3.34 (m, 4H), 2.61 (dd, *J* = 16.1, 7.0 Hz, 2H), 2.47 – 2.31 (m, 2H), 1.97 (s, 3H), 1.73 – 1.49 (m, 6H), 1.43 – 1.23 (m, 5H), 0.91 (td, *J* = 7.3, 2.2 Hz, 3H). MS *m/z* 735.4 $[M - Na^+]^-$.

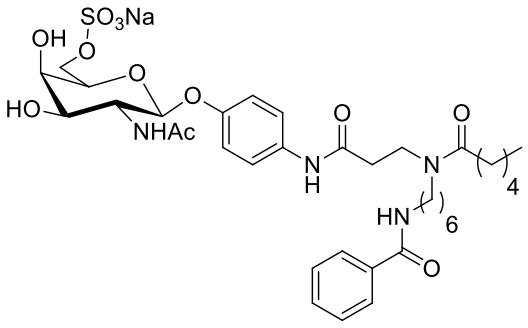


N-(6-aminohexyl)benzamide (3). To methyl benzoate (25.0 g, 183.6 mmol), hexane-1,6-diamine (21.3 g, 183.6 mmol) and water (9.25 mL) were added and the mixture was heated to 100°C for 24 hours under constant stirring. The reaction mixture was cooled to room temperature and directly loaded on to a short silica column. Upon elution with 10 to 20% of methanol (with 5% aq. NH_4OH solution) in chloroform the desired mono-benzoylated product **3** was obtained (19.8 g, 49%) as a pale yellow oil. ^{1}H NMR (300 MHz, MeOD) δ 7.81 – 7.78 (m, 2H), 7.52 – 7.40 (m, 3H), 3.38 (t, *J* = 7.1 Hz, 2H), 2.78 – 2.69 (m, 2H), 1.64 – 1.38 (m, 8H). MS *m/z* 221.1 $(M+H^+)$.



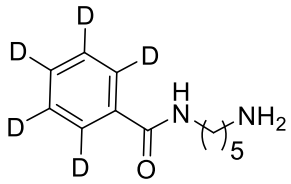
***N*-(6-(N-(3-((4-(((2S,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)phenyl)amino)-3-oxopropyl)hexanamido)hexyl)benzamide (GalNAc-C6/C6-Bz, GALNS Product)** To a solution of **1** (0.27 g, 0.576 mmol) in anhydrous methanol (9 mL), cooled on an ice bath, 0.5 M sodium methoxide solution in methanol (0.3 mL, 0.15 mmol) was added dropwise and allowed to warm to room temperature. After 2 hours formic acid (10 μL) was added to the reaction mixture and concentrated to dryness under reduced pressure. To the resulting residue methanol (13.5 mL), water (1.5 mL) and 10% palladium on activated carbon (12.5 mg) were added and let to stir under a hydrogen atmosphere at room temperature for 16 hours. Water was added dropwise to the reaction mixture till the entire white residue was completely dissolved. The reaction mixture was filtered and the filtrate was cooled on an ice bath. To it pyridine (0.16 mL) was added and followed by the dropwise addition of a solution of acryloyl chloride (0.16 g, 1.76 mmol) in dichloromethane (4 mL). The

reaction was let to stir on the ice bath for 30 minutes and then warmed to room temperature and continued for 2 hours. Sodium carbonate powder (0.3 g) was added to the reaction mix and let to stir for 15 minutes and filtered. The filtrate was concentrated under reduced pressure and further dried under high vacuum. The residue was dissolved in 2-propanol (6.3 mL) and water (0.7 mL) mixture and to it *N*-(6-aminohexyl)benzamide **3** (0.17 g, 0.77 mmol) was added and let to stir for 40 hours at 65°C. The reaction mixture was concentrated to dryness under reduced pressure and redissolved in methanol (12 mL). Upon cooling this mixture on an ice bath triethylamine (0.25 mL) was added followed by the dropwise addition of a solution of hexanoyl chloride (0.24 g, 1.78 mmol) in dichloromethane (4 mL). The reaction was left to stir on the ice bath for 30 minutes and then warmed to room temperature and continued for 16 hours. The reaction mixture was concentrated under reduced pressure and subjected to purification by silica flash chromatography using 15% methanol in dichloromethane as the elution mixture to yield **GalNAc-C6/C6-Bz** (0.23 g, 58%). ^{1}H NMR (300 MHz, MeOD) δ 7.80 (d, J = *6.9 Hz*, 2H), 7.56 – 7.39 (m, 5H), 6.99 (d, J = *9.1 Hz*, 2H), 4.96 (d, J = *8.4 Hz*, 1H), 4.17 (dd, J = *10.7, 8.4 Hz*, 1H), 3.96 – 3.51 (m, 7H), 3.44 – 3.33 (m, 4H), 2.69 – 2.50 (m, 2H), 2.48 – 2.28 (m, 2H), 1.98 (s, 3H), 1.72 – 1.49 (m, 6H), 1.49 – 1.21 (m, 9H), 0.89 (dt, J = *8.7, 4.8 Hz*, 3H). MS (ESI$^+$) for [M + H]$^+$; calculated: 685.4, found: 685.5.

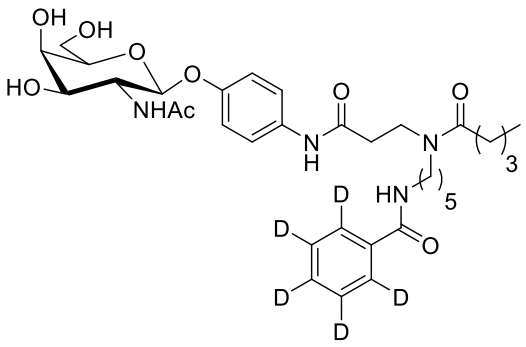


Sodium ((2R,3R,4R,5R,6S)-5-acetamido-6-(4-(3-(*N*-(6-benzamidohexyl)hexanamido)propanamido)phenoxy)-3,4-dihydroxytetrahydro-2*H*-pyran-2-yl)methyl sulfate (GalNAc-6-S-C6/C6-Bz, GALNS Substrate) To **GALNS Product** (99 mg, 0.144 mmol) under nitrogen, anhydrous pyridine (5 mL) was added. To this solution sulfur trioxide pyridine complex (34 mg, 0.214 mmol) was added and let to stir for 5 hours at room temperature. The reaction was quenched with the addition of methanol (0.5 mL) and stirring for 30 minutes. The reaction mixture was concentrated under reduced pressure and redissolved in water and subjected to reversed phase (C18) HPLC purification using water-methanol gradient system to get **GalNAc-6-S-C6/C6-Bz, GALNS Substrate** (36 mg, 32%). The **GalNAc-6-S-C6/C6-Bz** was further purified by ion-exchange chromatography. A fritted thin column was loaded with 2 mL of Q-Sepharose fast flow (GE Healthcare, product code: 17-0510-01) and washed with methanol (50 mL). A solution of **GalNAc-6-S-C6/C6-Bz** in methanol (2 mL) was loaded on to this column and the column was further washed with methanol (100 mL). The **GalNAc-6-S-C6/C6-Bz** was then eluted with 1M ammonium formate in methanol solution (100 mL) and concentrated under reduced pressure at temperature less than 25^{o}C. The resultant residue was dissolved in DI-water and loaded on a C18 cartridge (Resprep cat # 26034, which was pre-activated with methanol and washed with DI-water) by applying negative pressure in the bottom. The cartridge was further washed with DI water (4x25 mL) and the substrate was eluted from the cartridge using methanol (4x25 mL). The methanol fraction was concentrated under reduced pressure at temperature less than 25^{o}C to afford the pure **GalNAc-6-S-C6/C6-Bz**. ^{1}H NMR (300 MHz, MeOD) δ 7.81 (d, J = *6.9 Hz*, 2H), 7.59 – 7.35 (m, 5H), 7.00 (d, J = *9.0 Hz*, 2H), 4.93 (d, J = *8.4 Hz*, 1H), 4.32 – 4.10 (m, 3H), 4.03 – 3.90 (m, 2H), 3.83 – 3.60 (m, 3H), 3.44 – 3.33 (m, 4H), 2.61 (q, J = *7.0 Hz*, 2H),

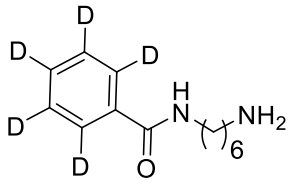
2.49 – 2.29 (m, 2H), 1.98 (s, 3H), 1.72 – 1.49 (m, 6H), 1.48 – 1.22 (m, 9H), 0.98 – 0.81 (m, 3H). MS (ESI$^-$) for [M –Na$^+$]$^-$; calculated: 763.3, found: 763.7.



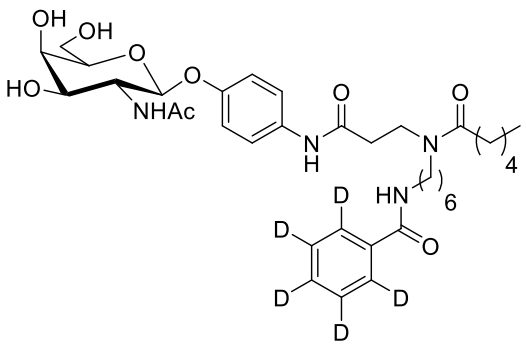
***N*-(5-aminopentyl)benzamide-2,3,4,5,6-d_5 (6)** To a solution of *t*-butyl (5-aminopentyl)carbamate (0.15 g, 0.741 mmol) and triethylamine (0.3 mL) in dichloromethane (10 mL), cooled on an ice bath, benzoyl chloride-d_5 (119 mg, 0.817 mmol) was added dropwise and warmed to room temperature. This mixture was let to stir for 4 hours and quenched with addition of methanol (1 mL) and concentrated to dryness under reduced pressure. The residue was resuspended in dichloromethane (2 mL) and 4M HCl in dioxane (0.75 mL, 3 mmol) was added to it dropwise and let to stir at room temperature for 16 hours. The reaction mixture was concentrated to dryness under reduced pressure and redissolved in methanol (10 mL) and to it sodium bicarbonate powder (0.3 g) was added and let to stir for 15 minutes. The resultant slurry was filtered and the filtrate was concentrated to dryness under reduced pressure to yield **6** and used for next step without further purification.



***N*-(5-(*N*-(3-((4-(((2S,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)phenyl)amino)-3-oxopropyl)pentanamido)pentyl)benzamide-2,3,4,5,6-d_5 (ARSB Internal Standard)** By extending the synthetic procedure outlined for the synthesis of **ARSB Product** and substituting the use of *N*-(5-aminopentyl)benzamide **2** with the d_5-deuterated amine **6** the desired **ARSB Internal Standard** (142 mg, 51%) was obtained as a white amorphous solid. ^{1}H NMR (300 MHz, MeOD) δ 7.43 (dd, *J* = 9.0, 2.1 Hz, 2H), 6.99 (dd, *J* = 9.1, 2.4 Hz, 2H), 4.96 (d, *J* = 8.4, 1H), 4.17 (dd, *J* = 10.7, 8.4 Hz, 1H), 3.89 (d, *J* = 3.1 Hz, 1H), 3.85 – 3.59 (m, 6H), 3.44 – 3.33 (m, 4H), 2.61 (dd, *J* = 15.4, 7.0 Hz, 2H), 2.47 – 2.29 (m, 2H), 1.98 (s, 3H), 1.73 – 1.49 (m, 6H), 1.46 – 1.23 (m, 5H), 0.90 (td, *J* = 7.3, 1.8 Hz, 3H). MS (ESI$^+$) for [M + H]$^+$; calculated: 662.4, found: 662.7.



***N*-(6-aminohexyl)benzamide-2,3,4,5,6-d_5 (7).** To a solution of Boc-1,6-diaminohexane·HCl salt (0.2 g, 0.791 mmol) and triethylamine (0.4 mL) in methanol (5 mL), cooled on an ice bath, benzoyl chloride-d_5 (345 mg, 2.36 mmol) solution in anhydrous dichloromethane (1 mL) was added dropwise and warmed to room temperature. This mixture was let to stir for 4 hours and concentrated to dryness under reduced pressure. The residue was redissolved in 10% methanol in DCM and the resulting solution was washed with 1M aqueous NaOH solution and followed by brine-water (1:1) mixture. The organic layer thus obtained was concentrated to dryness under reduced pressure. The resultant residue was resuspended in dichloromethane (2 mL) and 4M HCl in dioxane (0.75 mL, 3 mmol) was added to it dropwise and let to stir at room temperature for 16 hours. The reaction mixture was concentrated to dryness under reduced pressure and redissolved in methanol (10 mL) and to it sodium bicarbonate powder (0.3 g) was added and let to stir for 15 minutes. The resultant slurry was filtered and the filtrate was concentrated to dryness under reduced pressure to yield **7** and used for next step without further purification.



***N*-(6-(*N*-(3-((4-(((2S,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)phenyl)amino)-3-oxopropyl)hexanamido)hexyl)benzamide-2,3,4,5,6-d_5 (GALNS Internal Standard)** By extending the synthetic procedure outlined for the synthesis of **GALNS Product** and substituting the use of *N*-(6-aminohexyl)benzamide **3** with the d_5-deuterated amine **7** the desired **GALNS Internal Standard** (39.5 mg, 52%) was obtained as a white amorphous solid. ^{1}H NMR (300 MHz, MeOD) δ 7.43 (d, J = 9.0, 2H), 6.99 (dd, J = 9.1, 2.4 Hz, 2H), 4.96 (d, J = 8.4 Hz, 1H), 4.24 – 4.12 (m, 1H), 3.89 (d, J = 3.1 Hz, 1H), 3.85 – 3.60 (m, 6H), 3.42 – 3.33 (m, 4H), 2.61 (q, J = 6.8 Hz, 2H), 2.47 – 2.29 (m, 2H), 1.98 (s, 3H), 1.70 – 1.49 (m, 6H), 1.49 – 1.21 (m, 9H), 0.89 (td, J = 6.7, 4.0 Hz, 3H). MS (ESI$^+$) for $[M + H]^+$; calculated: 690.4, found: 690.5.

V. Quantification of compounds by ^{1}H-NMR.

We used NMR to accurately determine the amount of reagent in stock solutions so that the comparison between MS/MS and fluorimetric assays could be done starting from assay cocktails with known concentrations of substrates. This also allowed us to determine relative ionization efficiencies (MS/MS ion counts in the appropriate single reaction monitoring chanel) when the same number of moles of 2 different analytes were analyzed by LC-MS/MS. The reagent was dissolved in 0.75 mL of CD_3OD and 6-9 mL of 1 or 10% DMF in CD_3OD was added as an internal standard. ^{1}H-NMR was acquired with an extended time between pulses of 15 sec to obtain accurate peak integrals. The integrals of the NMR peaks for the analyte and internal standard were used to determine the absolute moles of analyte. After quantitative NMR, the same vials were concentrated to dryness using a vacuum centrifuge and re-dissolved in methanol to give 5 mM stock solutions. The samples were serially diluted in 10-fold increments to give 500 nM solutions, which were submitted to flow injection MS/MS to measure the single reaction monitoring ion signals for the analytes in triplicate. Single reaction monitoring parameters (cone voltage and collision energy) were optimized for each analyte.

V. MS/MS assays.

Flow-injection analysis (FIA)-MS/MS assays of GALNS and ARSB with b-NGA and ethyl acetate extraction.

Substrates and internal standards were prepared as stock solutions in methanol and stored in glass vials with Teflon-lined screw caps at -20 °C. After transferring stock solution to a glass tube, methanol was completely removed in a vacuum centrifuge (important to remove all solvent) prior to adding assay buffer. The assay buffer is 50 mM ammonium acetate, 7.5 mM $Ba(OAc)_2$ and 5.0 mM $Ce(OAc)_3$, pH 5.0 (adjusted with acetic acid after metal salts added, stored 4 °C). The final cocktail, made fresh, contained 2 mM (Z)-Pugnac (added from a stock in water), 8 Units/mL b-NGA, 1 mM substrate, and 5 mM internal standard. Assays were carried out with 3 mm DBS punches in 30 mL of assay cocktail in polypropylene 96-well plates (Sigma Cat. CLS3363) sealed with a storage mat (Sigma Cat. CLS3080) and shaken at 250 rpm at 37 °C

for 16 hr. Assays were quenched by adding 0.3 mL of 1 g/L sodium taurocholate (to prevent emulsions), followed by 0.4 mL of reagent grade ethyl acetate. After mixing up and down ~5 times with the pipettor the plate was centrifuged (Allegra X-12R, Beckmann) for 20 min at ~2100g to cause complete separation of the solvent layers. A 0.2 mL aliquot of the upper phase was transfered to a new 96-well plate, and solvent was completely removed at room temperature with a stream of nitrogen. Residues were taken up in 0.1 mL of 1:1 water:acetonitrile for FIA-MS/MS. The FIA solvent is 80% acetonitrile/20% water/5 mM ammonium formate.

LC-MS/MS assays of GALNS and ARSB.

Samples for LC-MS/MS were setup as for FIA assays except b-NGA was omitted. Incubated assays were quenched by adding 120 mL of acetonitrile. The plate was sealed as above and centrifuged at ~3,000 rpm for 5 min. Purified water (120 mL) was added to wells of a new 96-well plate, and 120 mL aliquots of the supernatant from the centrifuged plate was transfered to the wells containing water. The LC column is a Chromolith FastGradient RP-18 encapped 50-2 HPLC column (EMD Millipore Cat. 152007) fitted with a Uniguard Cartridge Holder (Thermo Scientific Cat. 850-00) and a Hypersil Drop-In Guard Cartridge C18 (3 mm, 10L x 2.1 mm I.D., Fisher Scientific 30103-012101). The LC flow rate was 0.4 mL/min. Ten mL of sample were injected per run. The isocratic LC solvent is 45% water/25% methanol/30% acetonitrile/0.1% formic acid, prepared from Optima grade solvents (Fisher Scientific).

MPS-II Assays

The assay cocktail (made fresh) contained 1 mM substrate, 0.8 Units/mL IDUA, 5 mM internal standard in the same buffer as above. The assay was setup, processed and analyzed as described above for FIA-MS/MS. LC-MS/MS assays were carried out as above in the presence and absence of IDUA.

Triplex Assays

The assay cocktail (made fresh) contained 0.5 mM I2S substrate, 1 mM ARSB and GALNS substrates, 5 mM of each of the three internal standards, and other buffer components as above for the LC-MS/MS assay of ARSB and GALNS. After incubation with the DBS punch as above, the mixture was quenched with 0.1 mL of methanol/ethyl acetate (1/1). After mixing up and down 4-5 times with the Pipettor, the liquid was transferred to a new deep-well, 96-well plate followed by addition of 0.4 mL of ethyl acetate then 0.2 mL of 44 mM citric acid in water. After mixing up and down 5-10 times with the Pipettor, the plate was sealed as above and centrifuged for 5 min at 3,000 rpm at room temperature. A 0.2 mL portion of the top layer was transferred to a new plate, and solvent was removed with a jet of oil-free nitrogen. Residues were taken up in 0.1 mL of acetonitrile/water (1/1) with 0.02% formic acid. After mixing 5-10 times with the Pipettor, samples were subjected to LC-MS/MS as above.

VI. 4MU fluorimetric assays.

a-L-Iduronidase, acid a-glucosidase, I2S, GALNS, ARSB

a-L-Iduronidase assay buffer is 100 mM sodium formate, 75 mM D-saccharic acid-1,4-lactone (Santa Cruz Biotechnology Cat. ss-221521), pH 3.4. Acid *a*-glucosidase buffer is 89 mM succinic acid, 8 *m*M acarbose (Carbosyn Cat. OA00002), pH 4.7. I2S buffer is as for MS/MS buffer (maint text) except without (Z)-Pugnac. Assay cocktails were prepared fresh from methanol stock solutions of substrates. Methanol was completely removed by vacuum centrifuge or a stream of N_2 before buffer addition. The substrates are: 1) 1 mM 4MU-*a*-L-iduronide (Carbosynth Cat. EM58440); 2) 1 mM 4MU-*a*-D-glucopyranoside (Toronto Research Chemicals Cat. M334495); 3) 1 mM of the 4MU-containing I2S substrate published previously.[45]

A 3 mm DBS punch was incubated with 30 mL of assay cocktail in sealed 96-well plates (see main text). Reactions were quenched with 0.25 mL of 0.1 M sodium bicarbonate, pH 10.7, the plates were centrifuged for 5 min at 3,000 rpm, 0.2 mL of supernatant was transfered to a black, 96-well plate, and the fluorescence was measured (355 nm excitation and 460 nm emission). If bubbles were seen in the plate wells, the black plate was centrifuged as above to remove bubbles.

Controls were carried out by adding 15 mL of buffer without substrate to a well containing a 3 mm DBS punch. A second well contained 30 mL of complete assay cocktail (buffer + substrate) but substrate was 2 mM and the well lacked a DBS punch. Samples were incubated as above, then 15 mL of the 30 mL well was transferred to the well with the DBS punch. Samples were immediately quenched and processed as above. All assays were calibrated using standard 4MU replacing 4MU-substrate and prepared and processed as for the control reactions. In this way, the calibration contains blood, which significantly quenches the 4MU fluorescence, and this is representative of the quenching of 4MU in the enzymatic assays.

GALNS and ARSB fluorimetric assays with 4MU-GalNAc-6-S and 4MU-GalNAc-4-S were carried out as described.[70] ARSB assays with 4MU-sulfate were prepared as follows. Assay buffer (15 mL, same as I2S buffer above) containing 5 mM 4MU-sulfate was added to a 3 mm DBS punch in a well of a 96-well, polypropylene plate. After 16 hr incubation at 37 ° with shaking (250 rpm), a 15 mL portion of assay buffer (no substrate) was added, and the mixture was quenched with 200 mL of 0.1 M sodium bicarbonate, pH 10.7. 4MU fluorescence was read as above.

VIII. Conversion of assay response to enzyme activities.

Enzyme activities in units of mmol product per hour per L of blood were calculated using the amount of product formed in the complete assay with the DBS punch minus that formed in the control assay divided by the incubation time in hours and divided by the volume of blood in the DBS punch (taken as 3.1×10^{-6} L).

IX. Calculation of analytical range values in main text Table 1.

For MS/MS assays, possible contributors to the enzyme-independent assay response are: 1) buffer alone; 2) product present as an impurity in the substrate; 3) breakdown of substrate in buffer without blood to give the product; 4) cleavage of the substrate in the electrospray ionization source to give product; 5) components of the blood other than product that give rise to

the same MS/MS response in the product channel. Contributor 5 is taken as zero since we do not see any detectable signal in the product MS/MS channel if we leave substrate out of the assay with DBS. Contributors 1-4 will occur in a control in which substrate is incubated in buffer without DBS. Thus the analytical range is calculated as [(assay response in the complete assay) - (assay response in the no DBS control)] divided by (assay response in the no blood control). Note that the no blood control lacks compounds in the blood that suppress the product MS/MS signal, whereas such suppressors are present in the complete assay with DBS. Since the internal standard is chemically identical to the product but contains deuterium, suppression of product and internal standard has to be identical. We take the assay response as the MS/MS signal for the product divided by that for the internal standard, so differential suppression in complete and control assays does not contribute to the calculated analytical range.

X. Calculation of the values in main text Table 2.

Percent free 4MU in the 4MU-substrates.

The fluorescence of 4-MU glycosides has to be pH independent in the range of 3 to 10.7 since there are no functional groups in the molecules that can ionize in this range. Any increase in fluorescence observed when the pH is shifted from 3 to 10.7 has to be due to free 4MU contamination (the presence of other fluorescent impurities was ruled out by showing that the emission and excitation wavelength maxima fit those published for 4MU, not shown). The increase in fluorescence for the pH 3 to 10.7 shift was used along with the fluorescence per mole of the anion 4-MU-O^- at pH 10.7 (measured with standard 4MU) to obtain the moles of free 4MU present in the 4MU-substrate. The mole % of free 4MU is then calculated using the moles of 4MU-substrate present.

Using the mole % of free 4MU and the 4MU-OH/4MU-O^- emission ratio (see main text), the contribution to the fluorescence of 4MU-glycoside at pH 3.0 from the 4MU-OH contaminant is obtained. This value is used to correct the observed fluorescence of 4MU-glycoside at pH 3.0 to obtain the fluorescence of the pure 4MU-glycoside. From these values we obtain the ratio (4MU-O^- emission per mole)/(4MU-glycoside emission per mole).

XI. *Calculation of the analytical range of the enzyme assays.*

IDUA, acid a-glucosidase, I2S

The complete assay is set up by adding 30 mL of buffer plus substrate to a 3 mm quality control high DBS punch and incubating for the desired time. A second DBS punch is mixed with 15 mL of buffer without substrate (sample A), and a second sample is set up containing 30 mL of buffer without a DBS punch and with substrate at a 2-fold higher concentration than in the complete assay (sample B). Samples A and B are incubated along with the complete assay sample. After incubation, 15 mL of sample B is transferred to sample A, and the mixture is quenched immediately with pH 10.7 buffer. The complete assay sample is also quenched with pH 10.7 buffer. In this way, both the complete and control assays contain the 4MU-glycoside, any 4MU present in 4MU-glycoside as a contaminant and any 4MU formed from non-enzymatic hydrolysis of 4MU-glycoside in assay buffer during the incubation. The fluorescence for the

complete assay is due to fluorescence from the enzyme-dependent and -independent events, whereas the fluorescence for the control is due only to the enzyme-independent events, thus the analytical response is obtained as:
[(fluorescence of complete assay) - (fluorescence of control assay)]/(fluorescence of control assay).
Note that the analytical range is not influenced by quenching of fluorescence by blood components such as hemoglobin since blood is present in both the complete and control assays at the same concentration. The fluorescence measured for 20 mM 4MU in buffer with blood is only 21.7% of that measured in buffer alone. Since this quenching is substantial, we did not measure the enzyme-independent assay response by incubating 4MU-substrate in buffer without blood since the signal would be anomalously high when compared to that for the complete assay, which contains blood.

GALNS and ARSB assays.

To measure the analytical range, we measured the fluorescence in complete assay with blood, substrate, (Z)-Pugnac and b-NGA to that measured in an identical solution but lacking b-NGA. The enzyme b-NGA releases 4MU only after the sulfate is removed from the GALNS and ARSB substrates.[70] Only in the presence of b-NGA do the GALNS and ARSB sulfatases contribute to the increase in fluorescence. Any 4MU present as an impurity in the substrates or formed by non-enzymatic breakdown of the substrates occurs in the absence of b-NGA. Both the plus and minus b-NGA samples contain blood and thus are quenched to the same extent. If there is non-sulfated 4MU-GalNAc present as an impurity in the substrates or if the sulfated substrates can lose sulfate in the absence of sulfatases during incubation, these factors will lead to an increase in fluorescence in the plus b-NGA sample (but not in the minus b-NGA sample) that is sulfatase-independent, and thus the analytical range will be overestimated. To measure the amount of non-sulfated 4MU-GalNAc in the substrates and the amount of non-enzymatic substrate desulfation, we measured the increase in fluorescence when the substrates in buffer without blood were treated with b-NGA since this enzyme liberates 4MU only from the non-sulfated material (see above). No detectable increase in fluorescence was observed with the GALNS and ARSB substrates (not shown) indicating essentially no non-sulfated material in the substrates or formed by non-enzymatic desulfation during incubation. This justifies the use of the minus b-NGA assay response to calculate the analytical range. Thus, the analytical range is calculated as: [(assay response with b-NGA)-(assay response without b-NGA)](assay response without b-NGA).

Appendix B Table 1. MS/MS instrumentation parameters.

MS/MS was carried out on a Waters Xevo TQD (Waters, Milford, MA) using separate instrumentation settings for flow injection and LC MS/MS. Ion count peak areas were determined using the Waters TargetLynx software. FIA and LC were done on a Waters Acquity UPC with 2D technology (Sample Manager, Binary Solvent Manager, Column Manager).

FIA and LC-MS/MS assays.

Parameter (units)	**FIA**	**LC-MS/MS**
Polarity	ES+	ES+
Capillary voltage (V)	3000	3500
Extractor (V)	3.00	3.00
Source temperature (°C)	90	150
Desolvation temperature (°C)	150	500
Cone Gas Flow (L/h)	50	30
Desolvation Gas Flow (L/h)	450	1000
LM 1 Resolution	2.6	2.9
HM 1 Resolution	15.0	15.0
Ion Energy 1	0.5	0.0
Collision Cell Entrance Potential (V)	0.50	0.5
Collision Cell Exit Potential (V)	0.50	0.5
LM 2 Resolution	2.8	2.8
HM 2 Resolution	14.7	14.7
Ion Energy 2	0.6	0.6
Multiplier (V)	493.04	493.04
Collision Gas	Argon	Argon

Appendix B Table 2. MS/MS Single reaction monitoring values for I2S, GALNS and ARSB.

Compound	Alternate name[1]	Precursor [*m*/*z*]	Product [*m*/*z*]	Cone [V]	Collision energy [eV]
C5/C5-Bz aglycone	ARSB P aglycone	454.27	345.21	25	17
C5/C5 Bz-d5 aglycone	ARSB IS aglycone	459.30	350.25	25	17
C5/C6-Bz aglycone	I2S P aglycone	468.29	359.23	26	17
C5/C6-Bz-d5 aglycone	I2S IS aglycone	473.32	364.26	26	17
C6/C6-Bz aglycone	GALNS P aglycone	482.30	373.19	27	17
C6/C6-Bz-d5 aglycone	GALNS IS aglycone	487.33	378.28	27	17
Ida-C5/C6-Bz	I2S P	644.32	359.23	34	23
Ida-C5/C6-Bz-d5	I2S IS	649.35	364.26	34	23
GalNAc-C5/C5-Bz	ARSB P	657.35	345.21	26	24
GalNAc-C5/C5-Bz-d5	ARSB IS	662.38	350.25	26	24
GalNAc-C6/C6-Bz	GALNS P	685.38	373.25	26	25
GalNAc-C6/C6-Bz-d5	GALNS IS	690.41	378.28	26	25
Ida-2-S-C5/C6-Bz	I2S S	724.27	359.23	20	25
GalNAc-4-S-C5/C5-Bz	ARSB S	737.31	345.21	15	24
GalNAc-6-S-C6/C6-Bz	GALNS S	765.34	373.25	15	24

[1]S, P, and IS stand for substrate, product, and internal standard, respectively.

Appendix B Table 3. Comparison of Gal-6-S versus GalNAc-6-S substrates.

Substrate	Substrate concentration	GALNS activity (mmole hr^{-1} L^{-1})
Gal-6-S-C7/C6-Bz	1.0 mM	0.039
GalNac-6-S-C6/C6-Bz	1.0 mM	1.90
Gal-6-S-C7/C6-Bz + GalNAc-C6/C6-Bz	0.5 mM each	0.0053 (Gal-6-S) 1.13 (GalNAc-6-S)

Injection of 0.5 pmole each of Gal-C7/C6-Bz and GalNAc-C6/C6-Bz showed that the former gave a 1.26-fold higher response by LC-MS/MS than the latter.

Appendix B Table 4. Action of recombinant human-HexA on GalNAc-4-S-C5/C5-Bz

Recombinant human-HexA	(Z)-Pugnac	GalNAc-C5/C5-Bz ion counts	Aglycone-C5/C5-Bz ion counts
0 ng	0 mM	174	2789
3.1 ng	0 mM	138	26128
3.1 ng	0.5 mM	708	6744
3.1 ng	1 mM	154	3562

All assays were done with standard assay conditions and analyzed by LC-MS/MS.

Appendix B Table 5. Action of HexA in DBS on GalNAc-4-S-C5/C5-Bz and GalNAc-6-S-C6/C6-Bz.

Substrate	Sample	(Z)-Pugnac	GalNAc-C5/C5-Bz ion counts	Aglycone-C5/C5-Bz ion counts
GalNAc-4-S-C5/C5-Bz	Filter paper	0 mM	3426	33818
GalNAc-4-S-C5/C5-Bz	DBS	0 mM	423801	380478
GalNAc-4-S-C5/C5-Bz	Filter paper	1 mM	3368	28376
GalNAc-4-S-5/C5-Bz	DBS	1 mM	327075	102466
GalNAc-6-S-C5/C5-Bz	Filter paper	0 mM	820	14466
GalNAc-6-S-C5/C5-Bz	DBS	0 mM	110155	1106009
GalNAc-6-S-C5/C5-Bz	Filter paper	1 mM	757	15363
GalNAc-6-S-5/C5-Bz	DBS	1 mM	110828	55262

All assays were done with standard assay conditions and analyzed by LC-MS/MS. DBS was obtained from a single adult and filter paper is a blood-free newborn screening card, a 3 mm punch was used in both cases.

Appendix B Table 6. Action of b-NGA on GalNAc-C5/C5-Bz

Substrate	b-NGA	(Z)-Pugnac	GalNAc-C5/C5-Bz ion counts	Aglycone-C5/C5-Bz ion counts
GalNAc-C5/C5-Bz	0 Units	0 mM	206941	25465
GalNAc-C5/C5-Bz	118 Units	0 mM	101781	372629
GalNAc-C5/C5-Bz	118 Units	0.5 mM	110088	352827
GalNAc-C5/C5-Bz	118 Units	1 mM	123159	379031

All assays were done with standard assay conditions using a 3 mm punch of filter paper (no blood) and analyzed by LC-MS/MS.

Appendix B Table 7. Action of b-NGA on GalNAc-4-S-C5/C5-Bz and GalNAc-6-S-C5/C5-Bz

Substrate	b-NGA	GalNAc-C5/C5-Bz ion counts	Aglycone-C5/C5-Bz ion counts
GalNAc-4-SC5/C5-Bz	0 Units	1842	13395
GalNAc-4-S-C5/C5-Bz	118 Units	132	22281
GalNAc--6-S-C5/C5-Bz	0 Units	1097	16405
GalNAc-6-S-C5/C5-Bz	118 Units	253	19787

All assays were done with standard assay conditions using a 3 mm punch of filter paper (no blood) and analyzed by LC-MS/MS.

Appendix B Table 8. LC-MS/MS assay results for GALNS.

Sample	P ion cts	IS ion cts	P/IS	Activity mmole h^{-1} L^{-1}
NB ctrl #01	11053	59182	0.187	0.555
NB ctrl #02	7923	69934	0.113	0.333
NB ctrl #03	10904	69926	0.156	0.462
NB ctrl #04	7489	75152	0.100	0.292
NB ctrl #05	7105	86012	0.083	0.240
NB ctrl #06	12341	95225	0.130	0.382
NB ctrl #07	24468	89978	0.272	0.813
NB ctrl #08	18621	93486	0.199	0.593
NB ctrl #09	31676	86738	0.365	1.095
NB ctrl #10	17443	102301	0.171	0.506
NB ctrl #11	12341	107864	0.114	0.336
NB ctrl #12	17457	92381	0.189	0.562
NB ctrl #13	17332	144382	0.120	0.354
NB ctrl #14	20983	147035	0.143	0.422
NB ctrl #15	31981	155581	0.206	0.612
NB ctrl #16	15532	150387	0.103	0.303
NB ctrl #17	27872	148073	0.188	0.560
NB ctrl #18	29502	157291	0.188	0.558
NB ctrl #19	19284	155663	0.124	0.365
NB ctrl #20	20031	144771	0.138	0.409
NB ctrl #21	21438	149363	0.144	0.425
NB ctrl #22	23597	151711	0.156	0.461
NB ctrl #23	19127	153239	0.125	0.368
NB ctrl #24	20282	164934	0.123	0.362
NB ctrl #25	14227	143961	0.099	0.289
NB ctrl #26	32529	151650	0.215	0.639
NB ctrl #27	22224	163931	0.136	0.400
NB ctrl #28	25289	152062	0.166	0.493
NB ctrl #29	18617	167033	0.111	0.328
NB ctrl #30	29607	160807	0.184	0.547
NB ctrl #31	28039	161098	0.174	0.517
NB ctrl #32	28062	153535	0.183	0.543
NB ctrl #33	23924	155191	0.154	0.457
NB ctrl #34	19715	156159	0.126	0.372
NB ctrl #35	42090	167638	0.251	0.750
NB ctrl #36	25194	171485	0.147	0.435
NB ctrl #37	20196	148481	0.136	0.402
NB ctrl #38	16065	146980	0.109	0.321
NB ctrl #39	11852	166717	0.071	0.205
NB ctrl #40	11564	166942	0.069	0.200
NB ctrl #41	16736	160962	0.104	0.305

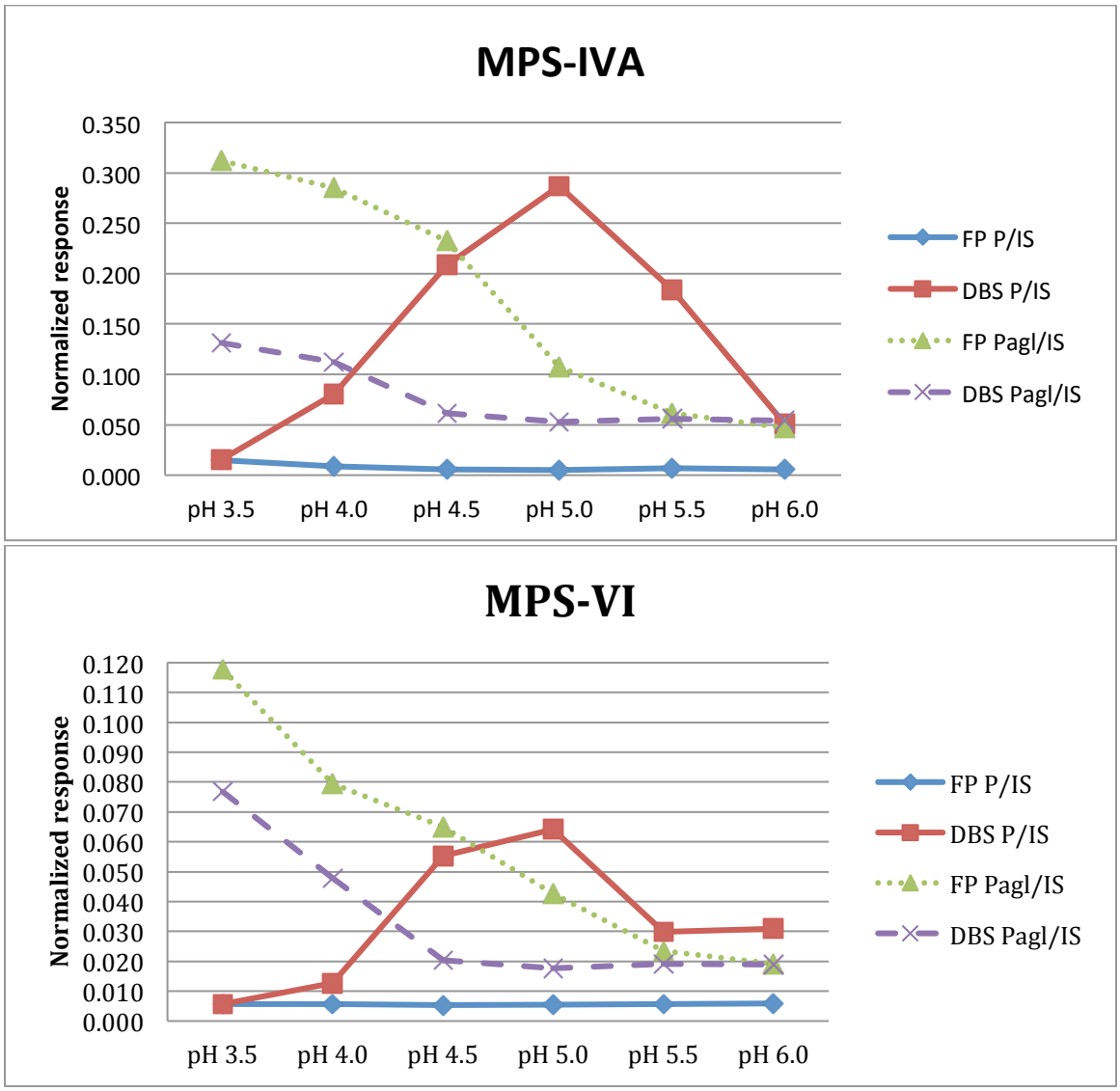
NB ctrl #42	25585	163185	0.157	0.465
NB ctrl #43	39940	163277	0.245	0.730
NB ctrl #44	30127	159231	0.189	0.563
NB ctrl #45	36823	163590	0.225	0.671
NB ctrl #46	23234	172584	0.135	0.398
NB ctrl #47	33232	160093	0.208	0.618
NB ctrl #48	25397	168030	0.151	0.448
NB ctrl #49	23307	159626	0.146	0.432
NB ctrl #50	13939	171796	0.081	0.236
MPS_4A pat#01	963	136974	0.007	0.012
MPS_4A pat#02	4230	135619	0.031	0.085

Appendix B Table 9. LC-MS/MS assay results for ARSB.

Sample	P ion cts	IS ion cts	P/IS	Activity mmole h^{-1} L^{-1}
NB ctrl #01	334134	121063	2.760	8.337
NB ctrl #02	122755	121034	1.014	3.058
NB ctrl #03	107617	112816	0.954	2.875
NB ctrl #04	99382	117635	0.845	2.545
NB ctrl #05	293926	118904	2.472	7.466
NB ctrl #06	167472	114661	1.461	4.408
NB ctrl #07	180650	121997	1.481	4.469
NB ctrl #08	181599	128933	1.408	4.250
NB ctrl #09	313116	116856	2.679	8.094
NB ctrl #10	193886	122340	1.585	4.783
NB ctrl #11	104020	116572	0.892	2.689
NB ctrl #12	228105	126872	1.798	5.428
NB ctrl #13	183396	121898	1.504	4.540
NB ctrl #14	164821	120633	1.366	4.122
NB ctrl #15	142788	120927	1.181	3.561
NB ctrl #16	177605	116091	1.530	4.617
NB ctrl #17	157385	120535	1.306	3.939
NB ctrl #18	193366	125628	1.539	4.645
NB ctrl #19	205698	131130	1.569	4.734
NB ctrl #20	180305	127868	1.410	4.255
NB ctrl #21	134713	122656	1.098	3.312
NB ctrl #22	225243	126645	1.779	5.369
NB ctrl #23	161685	114446	1.413	4.263
NB ctrl #24	253135	124759	2.029	6.127
NB ctrl #25	116840	118962	0.982	2.961
NB ctrl #26	364299	127322	2.861	8.643
NB ctrl #27	149503	150249	0.995	3.000
NB ctrl #28	202392	128559	1.574	4.752
NB ctrl #29	97083	136801	0.710	2.137
NB ctrl #30	159646	120631	1.323	3.993
NB ctrl #31	283990	138661	2.048	6.184
NB ctrl #32	267620	126674	2.113	6.380
NB ctrl #33	219169	129646	1.691	5.103
NB ctrl #34	122823	137218	0.895	2.697
NB ctrl #35	401611	134195	2.993	9.041
NB ctrl #36	234728	134686	1.743	5.261
NB ctrl #37	145171	95604	1.518	4.583
NB ctrl #38	91795	90175	1.018	3.069
NB ctrl #39	70944	91099	0.779	2.346
NB ctrl #40	86576	94464	0.916	2.762
NB ctrl #41	105875	108698	0.974	2.936

NB ctrl #42	120056	88728	1.353	4.082
NB ctrl #43	212687	82549	2.576	7.782
NB ctrl #44	124531	101207	1.230	3.712
NB ctrl #45	198482	121683	1.631	4.923
NB ctrl #46	124756	106206	1.175	3.543
NB ctrl #47	284389	102605	2.772	8.373
NB ctrl #48	90606	100861	0.898	2.707
NB ctrl #49	220360	134542	1.638	4.944
NB ctrl #50	142504	129641	1.099	3.315
MPS_6 pat#01	24035	123199	0.195	0.580
MPS_6 pat#03	32688	173161	0.189	0.561

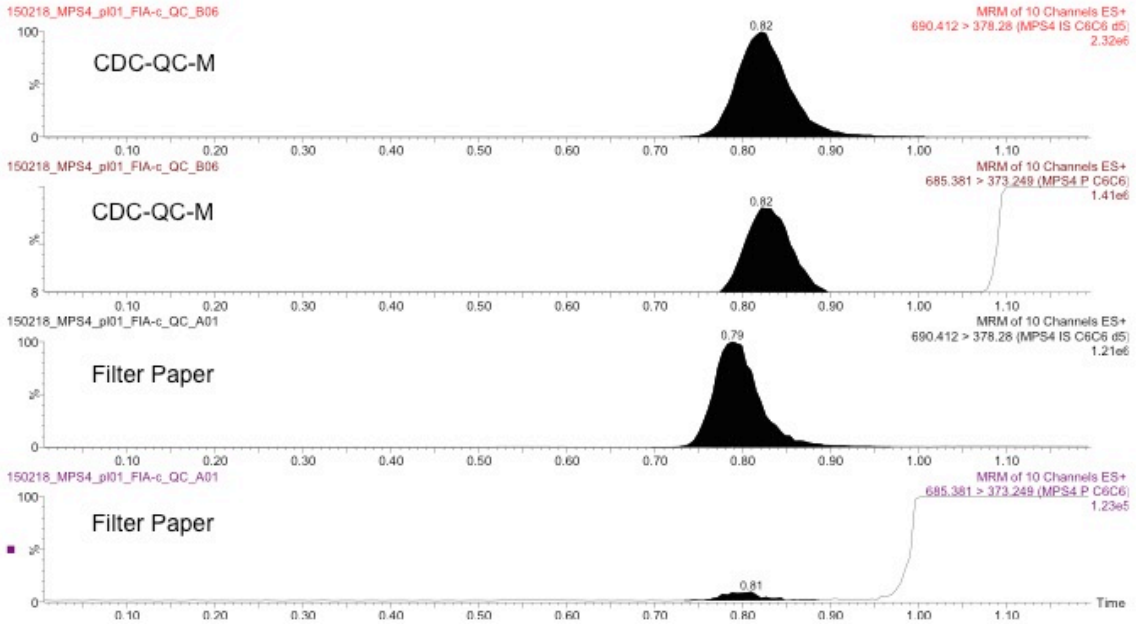
Appendix B Figure 1. pH Dependence of (Top Panel) GalNAC-6-S-C6/C6/-Bz and (Bottom Panel) GalNAc-4-S-C5/C5-Bz enzyme-independent and -dependent reactions.
Shown is the ion count peak area for GalNAc-containing product divided by that of the internal standard (P/IS) or the ion count peak area for aglycone divided by that of the internal standard (Pagl/IS) as a function of the indicated buffer pH during incubation for samples that contained a filter paper punch (FP) or a DBS punch (from a healthy adult). Buffers were 50 mM ammonium acetate adjusted to the desired pH with 5% acetic acid. Samples were analyzed by LC-MS/MS.



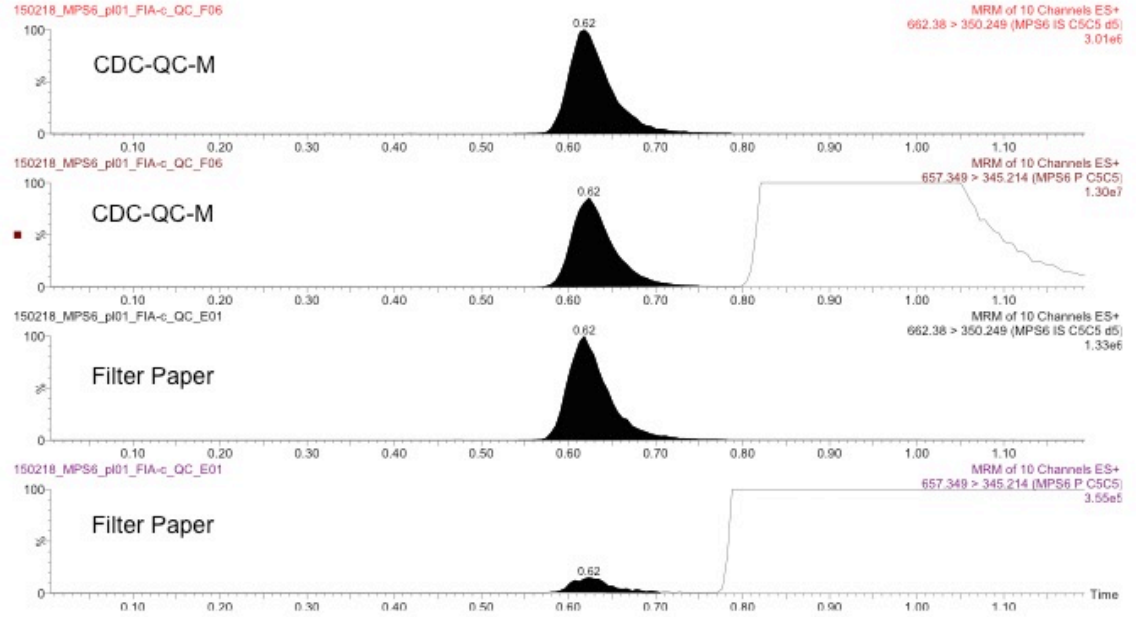
Appendix B Figure 2. LC-MS/MS ion traces. MPS-IVA (LC) and MPS-VI (LC) are the selective MRM traces for GALNS and ARSB LC-MS/MS assays, respectively. For both, the bottom panel is the product MRM trace for filter paper, second from bottom is internal standard MRM for filter paper, third from bottom is product for the CDC QC M DBS and top is the internal standard MRM for the CDC QC M DBS. MPS-II (FIA-IDUA) is the flow injection-MS/MS assay for I2S using IDUA, MPS-II (LC-IDUA) is the I2S LC-MS/MS assay with IDUA,

and MPS-II (LC) is the I2S LC-MS/MS assay without IDUA. The substrate peaks are visible in the ion traces. The retention times are: MPS-IVA (1.14 min); MPS-VI (0.93 min); MPS-II IDUA method (1.04 min); MPS-II (1.17 min).

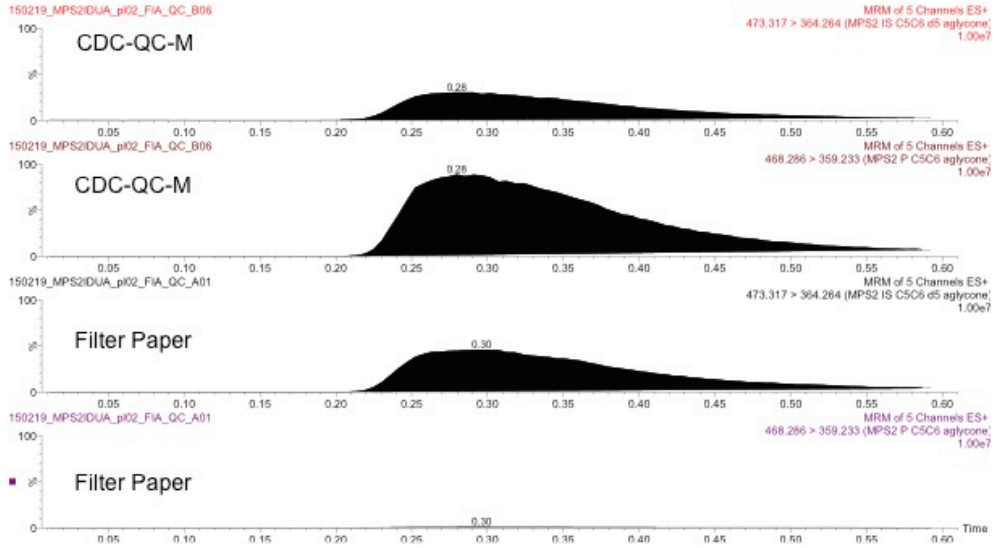
MPS-IVA (LC)

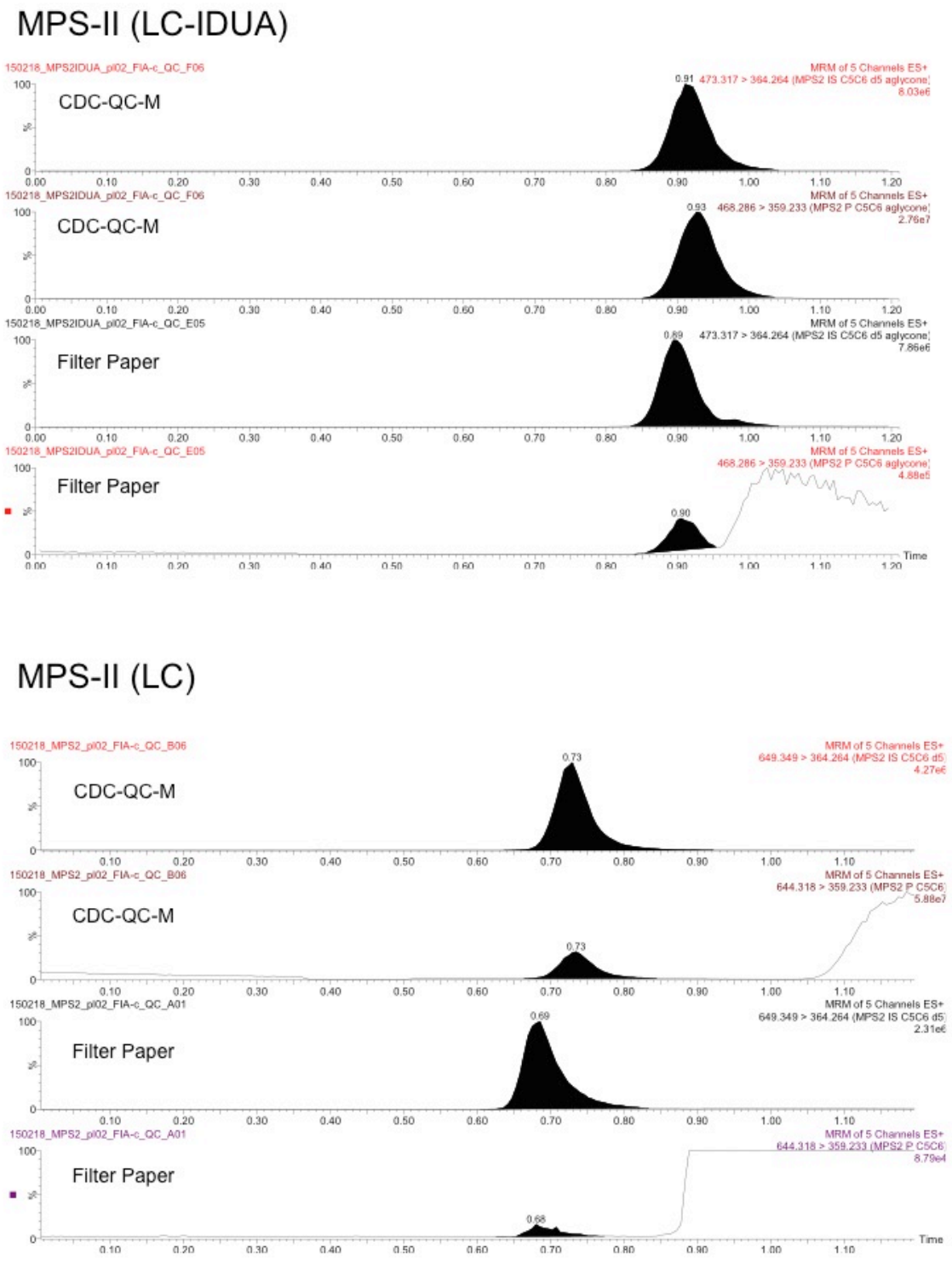


MPS-VI (LC)



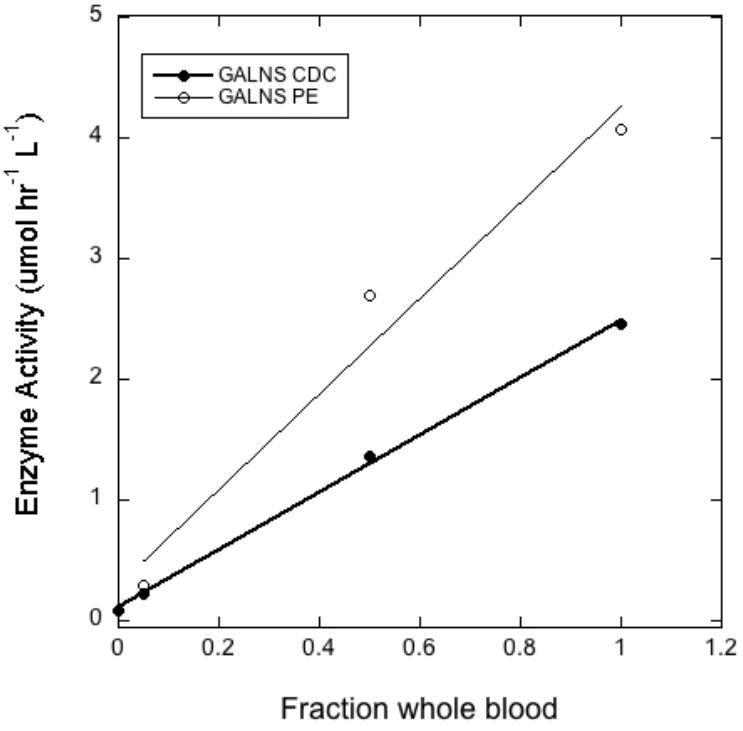
MPS-II (FIA-IDUA)

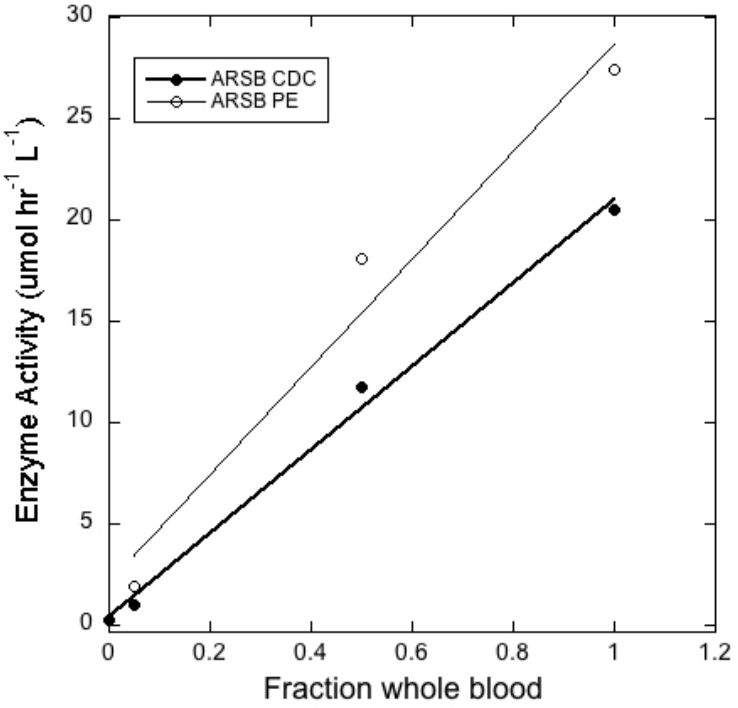


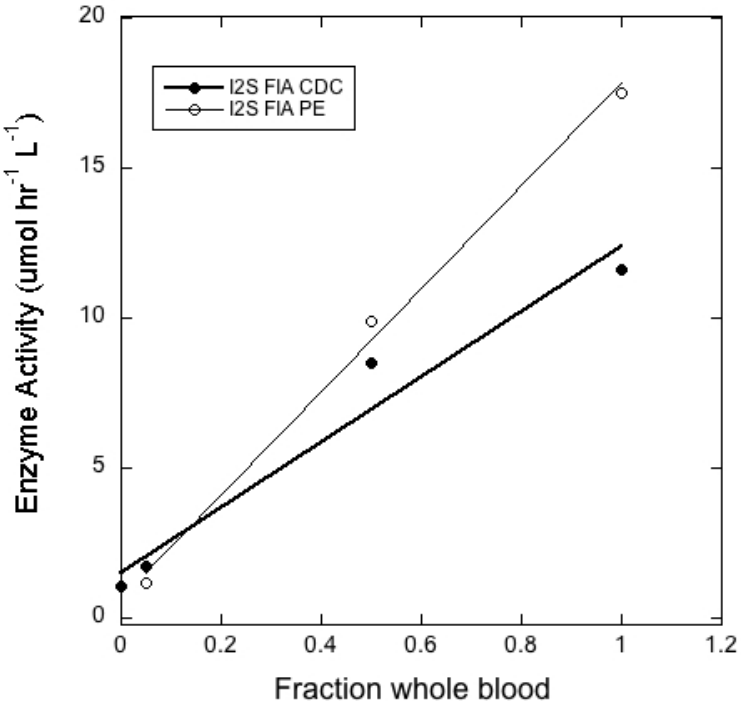


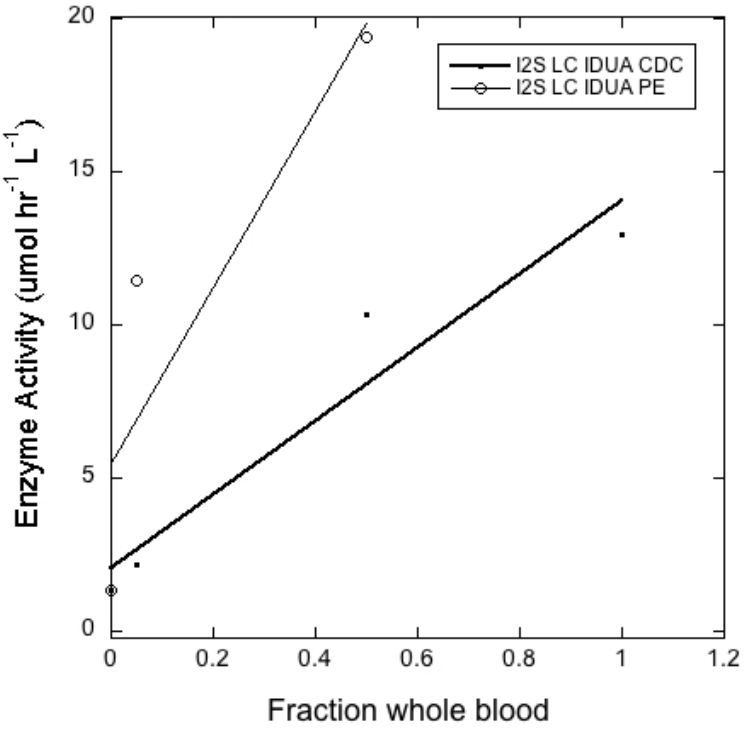
Appendix B Figure 3. Enzyme activity versus the fraction of whole blood for the quality control DBS. The DBS are made by mixing leukocyte-depleted blood with whole blood

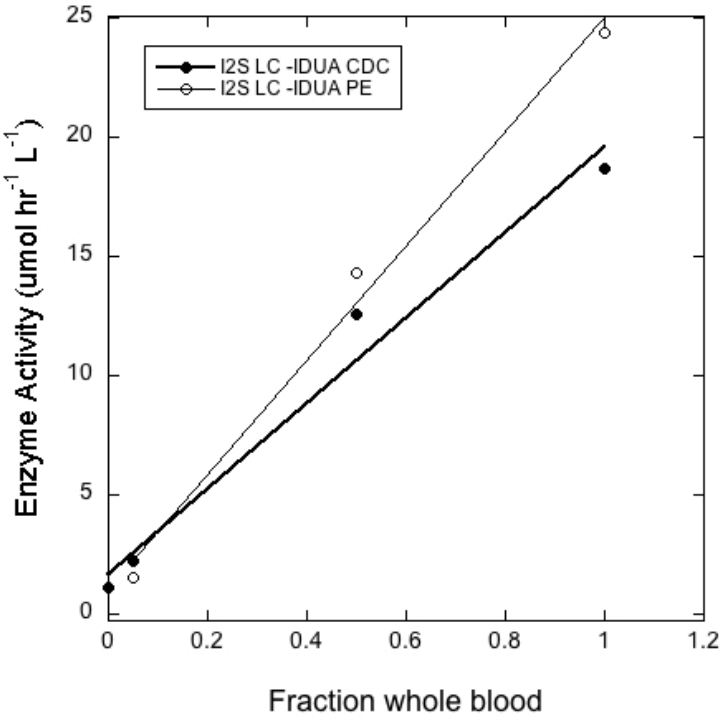
(fraction given on the X-axis). In the case of the CDC quality control DBS, there is finite activity at 0 fraction whole blood showing that there is some residual lysosomal enzyme in the leukocyte-depleted blood. In the case of the Perkin Elmer (PE) quality control DBS, no 0 fraction whole blood samples were available. First panel is GALNS LC-MS/MS assay, second panel is ARSB LC-MS/MS assay, third panel is I2S flow injection-MS/MS assay with IDUA, fourth panel is I2S LC-MS/MS assay with IDUA, fifth panel is I2S LC-MS/MS assay without IDUA, and sixth panel is the triplex for I2S, GALNS, and ARSB using ethyl acetate liquid-liquid extraction followed by LC-MS/MS.

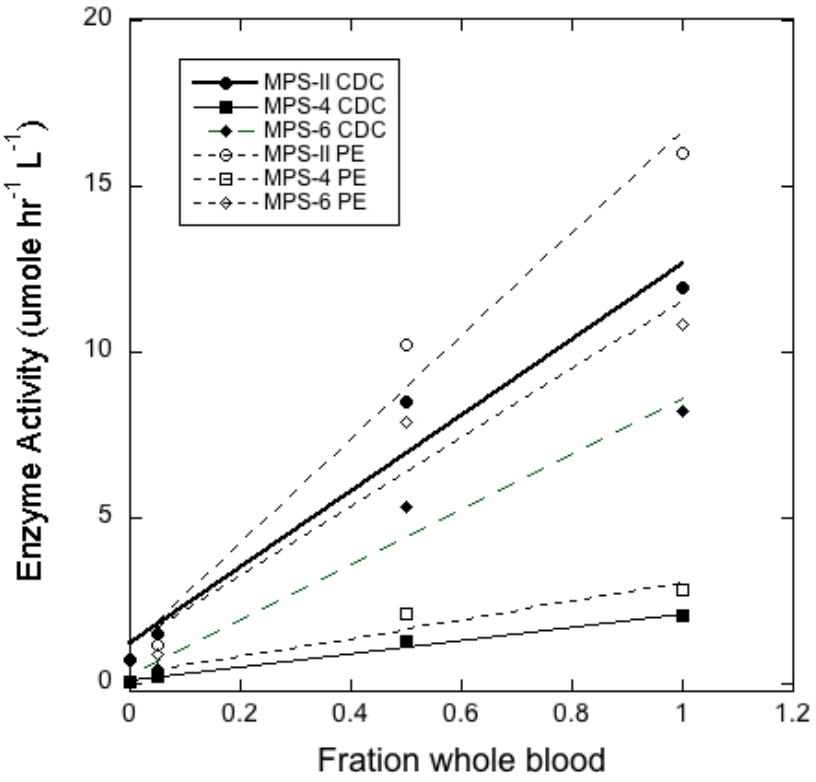



ARSB CDC
ARSB PE
Enzyme Activity (umol hr-1 L-1)
Fraction whole blood
0
5
10
15
20
25
30
0
0.2
0.4
0.6
0.8
1
1.2


I2S FIA CDC
I2S FIA PE
Enzyme Activity (umol hr-1 L-1)
Fraction whole blood
0
5
10
15
20
0
0.2
0.4
0.6
0.8
1
1.2


I2S LC IDUA CDC
I2S LC IDUA PE
Enzyme Activity (umol hr-1 L-1)
Fraction whole blood
0
5
10
15
20
0
0.2
0.4
0.6
0.8
1
1.2


I2S LC -IDUA CDC
I2S LC -IDUA PE
Enzyme Activity (umol hr-1 L-1)
Fraction whole blood
0
5
10
15
20
25
0
0.2
0.4
0.6
0.8
1
1.2


MPS-II CDC
MPS-4 CDC
MPS-6 CDC
MPS-II PE
MPS-4 PE
MPS-6 PE
Enzyme Activity (umole hr-1 L-1)
Fration whole blood
0
5
10
15
20
0
0.2
0.4
0.6
0.8
1
1.2

VITA

Sophia Masi
1 (941) 780-5497
masis@uw.edu

Education

BA Washington University in St. Louis Major in Chemistry, Anthropology	08/2007-05/2011
PhD Candidate University of Washington Chemistry	08/2016-present

Experience

2nd Year Medical School Curriculum Committee Member	09/2012-06/2014
Windermere North Condominiums HOA Board Member	02/2016-02/2017

Publications/Presentations

- Harms MB, Ori-McKenney KM, Scoto M, Tuck EP, Bell S, Ma D, **Masi SM**, Allred P, Al-Lozzi M, Reilly MM, Miller LJ, Jani-Acsadi A, Pestronk A, Shy ME, Muntoni F, Vallee RB, Baloh RH. Mutations in tail domain of DYNC1H1 cause dominant spinal muscular atrophy. *Neurology*. **78:22**, 1714 (2012) (PMID: 22459677).
- Kumar AB, **Masi SM**, Ghomashchi F, Chennamaneni NK, Ito M, Scott CR, Turecek F, Gelb MH, Spacil Z. Tandem Mass Spectrometry Has a Larger Analytical Range than Fluorescence Assays of Lysosomal Enzymes: Application to Newborn Screening and Diagnosis of Mucopolysaccharidoses Types II, IVA, and VI. *Clin Chem*. **61:11**, 1363 (2015) (PMCID: 4737431)
- Kumar AB, Spacil Z, Ghomashchi F, **Masi SM**, Sumida T, Ito M, Turecek F, Scott CR, Gelb MH. Fluorimetric Assays for N-Acetylgalactosamine-6-Sulfatase and Arylsulfatase B Based on the Natural Substrates for Confirmation of Mucopolysaccharidoses Types IVA and VI. *Clin Chim Acta*. **451**, 125 (2015) (PMCID: 465091)
- Masi S, Chennamaneni N, Turecek F, Scott RC, Gelb MH. Specific Substrate for the Assay of Lysosomal Acid Lipase. *Clin Chem*. **64:4**, 690 (2018)

Research Experience

Undergraduate Research Assistant for R. Baloh, MD PhD	06/2009-05/2011
Graduate Research Assistant for Prof. M. Gelb	06/2013-05/2018

Teaching Experience

Teaching Assistant for Dr. R. Steiner	10/2013-05/2014
Teaching Assistant for Prof. M. Gelb	01/2015-03/2015
Teaching Assistant for D. Wiegand	03/2015-06/2015

Interests

- When she is not at work, she is de-stressing, cuddling her cat, and complaining about her lack of free time.
- She enjoys cute things, chocolate, and Zelda.
- After completing her PhD, Sophia expects to continue on to medical clerkships, where she will learn how to treat patients as humans instead of science projects.