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An LC-MS/MS Method for Measurement of Sphingolipids in the Plasma of Pediatric Individuals with Disorders of Sphingolipid Metabolism

Joanna Y. Lee[1], Julie D. Saba[1], Yanan yang[2], Hui Zhao[2], Jiyeon Suh[1]

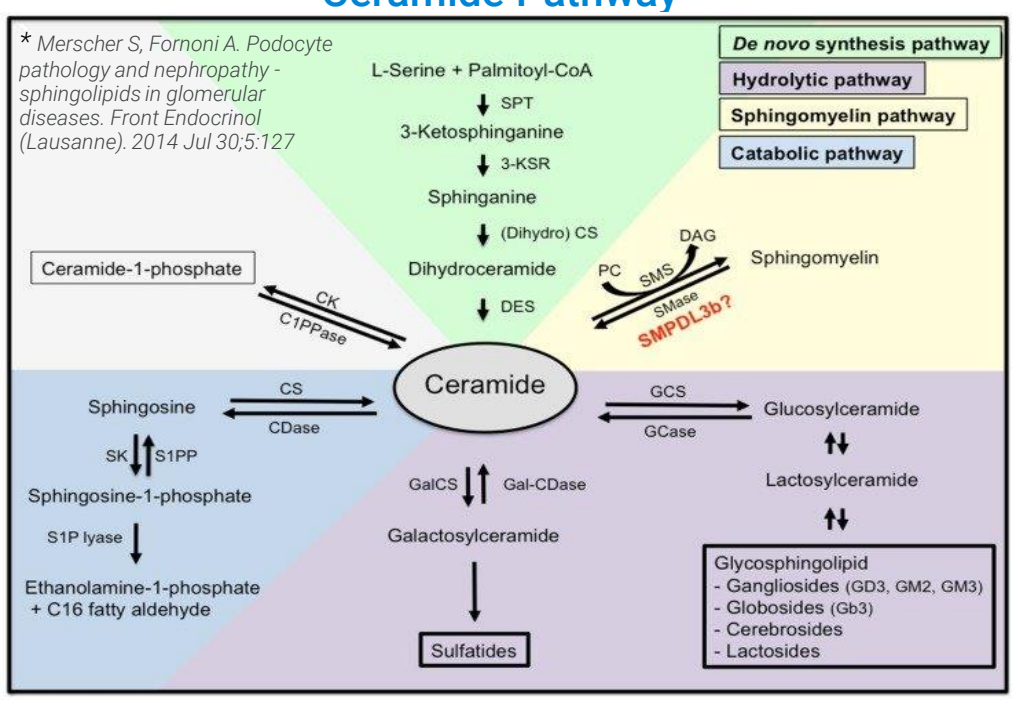
[1]Department of Pediatrics, Division of Hematology/Oncology, University of California-San Francisco

[2]Agilent Technologies, Inc. Santa Clara, CA USA

Introduction

In recent years, there has been a fast-growing body of evidence supporting the contribution of sphingolipids to the pathophysiology of a variety of common diseases. In addition, an ever-growing number of rare disorders of sphingolipid metabolism are being discovered by diagnostic next generation sequencing. In concert with the growing appreciation of the importance of sphingolipids to human health and disease is an exploding demand for fast and reliable quantitative determination of sphingolipids in plasma, and a need to overcome analytical challenges for confident profiling detection. An end-to-end workflow for the extraction, screening, identification, and quantitation of over 80 sphingolipids from 10 classes in plasma by Agilent 1290 Infinity II LC coupled to an Agilent 6495 triple quadrupole LC/MS system was developed. Nine internal standards that represent each of the sphingolipid class were selected to evaluate the method performance by accuracy and precision runs and typically achieving accuracy (70-130%) and precision (RSD < 20%) for all spiking levels, limits of quantitation (LOQ) of 2.5 to 25 nM in plasma, and linear calibration curves with R^2 > 0.99. The method was applied to quantify sphingolipids in pediatric individuals with sphingosine phosphate lyase insufficiency syndrome (SPLIS), ceramidase deficiency, and dihydroceramide desaturase deficiency for comparative analysis of plasma sphingolipids. The collected data was applied to develop pediatric reference ranges, identify gender-specific differences in post-pubertal children and/or age-specific differences, and demonstrate the characteristic derangements in circulating sphingolipids found in three atypical sphingolipid disorders.

Ceramide Pathway*



Experimental

Agilent 1290 Infinity II LC with 6495 Triple Quadrupole LC/MS System.

Chromatographic Conditions

UHPLC: Agilent 1290 Infinity II
Column: Agilent ZORBAX RRHD Eclipse Plus C18, 1.8 µm, 2.1 x 100mm pn: 959758-902
Column oven temperature: 55 ± 2°C
Injection volume: 10 µL
Autosampler: 5 ± 2°C
Flow rate: 0.40 mL/min

Mobile Phase A: 2 mM Ammonium Formate/0.2% Formic Acid in Water
Mobile Phase B: 0.2% Formic Acid in Methanol
Gradient:

Time, min	%A	%B
0	35	65
1.0	25	75
3.0	20	80
7.0	0	100
12	0	100
12.5	35	65
15.0	35	65

MS Conditions-Agilent 6495 Triple-Quadrupole

Parameters	
MS acquisition	Dynamic MRM
Ion source	Agilent Jet Stream electrospray ionization (AJS ESI positive)
Drying gas temperature	270 °C
Drying gas flow	13 L/min
Nebulizer	40 psi
Sheath gas heater	375 °C
Sheath gas flow	11 L/min
Capillary	2500 V
Nozzle voltage	0 V
High pressure RF voltage	150 V
Low pressure RF voltage	60 V

Experimental

Sample Preparation

- ✓ Pipet 50 µL plasma into a 2mL microcentrifuge tube
- ✓ Add 10 µL internal standard mix
- ✓ Extraction
  - Add 1 mL ice cold methanol
  - Vortex and shake at 4°C for 5 min
  - Centrifuge and transfer the supernatant into a new tube; place the tube at -20 °C for 1hr to further precipitate the proteins
  - Centrifuge and dry down
  - Reconstitute and ready for injection

Analyte (Belong to 10 Classes) Specific Conditions*

Name	Transition	Type	ISTD Compound Name	Name2	Transition3	Type	ISTD Compound Name
C12:0 Cer	482.5 -> 264.3	ISTD	<None>	C22:0 DHCer	624.6 -> 266.4	Target	C25:0 Cer
C12:0 Cer1P	562.4 -> 264.3	ISTD	<None>	C24:0 Cer	650.6 -> 264.3	Target	C25:0 Cer
C12:0 GlcCer	644.6 -> 264.4	ISTD	<None>	C24:0 Deoxycer	634.5 -> 256.3	Target	C25:0 Cer
C12:0 LacCer	806.6 -> 264.3	ISTD	<None>	C24:0 DeoxyDHCer	636.5 -> 256.3	Target	C25:0 Cer
C25:0 Cer	664.9 -> 264.3	ISTD	<None>	C24:0 DHCer	652.6 -> 266.4	Target	C25:0 Cer
d17:0 Sa	288.4 -> 60.0	ISTD	<None>	C24:0 DHGlcCer	814.9 -> 266.4	Target	C12:0 GlcCer
d17:0 Sa1P	368.4 -> 252.3	ISTD	<None>	C24:0 DHLacCer	976.8 -> 266.4	Target	C12:0 LacCer
d17:1 S1P	366.2 -> 250.3	ISTD	<None>	C24:0 GlcCer	812.9 -> 264.4	Target	C12:0 GlcCer
d17:1 -So	286.5 -> 268.3	ISTD	<None>	C24:0 LacCer	974.6 -> 264.3	Target	C12:0 LacCer
C14:0 Cer	510.5 -> 264.3	Target	C12:0 Cer	C24:1 Cer	648.6 -> 264.3	Target	C25:0 Cer
C14:0 DHGlcCer	674.9 -> 266.4	Target	C12:0 GlcCer	C24:1 DHGlcCer	812.9 -> 266.4	Target	C12:0 GlcCer
C14:0 DHLacCer	836.6 -> 266.4	Target	C12:0 LacCer	C24:1 DHLacCer	974.7 -> 266.4	Target	C12:0 LacCer
C14:0 GlcCer	672.6 -> 264.4	Target	C12:0 GlcCer	C24:1 GlcCer	810.9 -> 264.4	Target	C12:0 GlcCer
C14:0 LacCer	834.6 -> 264.3	Target	C12:0 LacCer	C24:1 LacCer	972.7 -> 264.3	Target	C12:0 LacCer
C14:0 DHCer	512.5 -> 266.4	Target	C12:0 Cer	C24:1 DHCer	650.6 -> 284.3	Target	C25:0 Cer
C16:0 Cer	538.5 -> 264.3	Target	C12:0 Cer	C26:0 Cer	678.7 -> 264.3	Target	C25:0 Cer
C16:0 DeoxyCer	522.5 -> 256.3	Target	C12:0 Cer	C26:0 DHCer	680.6 -> 266.4	Target	C25:0 Cer
C16:0 DeoxyDHCer	524.5 -> 256.3	Target	C12:0 Cer	C26:0 DHGlcCer	842.7 -> 266.4	Target	C12:0 GlcCer
C16:0 DHGlcCer	702.9 -> 266.4	Target	C12:0 GlcCer	C26:0 DHLacCer	1004.8 -> 266.4	Target	C12:0 LacCer
C16:0 DHLacCer	864.6 -> 266.4	Target	C12:0 LacCer	C26:0 GlcCer	840.7 -> 264.3	Target	C12:0 GlcCer
C16:0 GlcCer	700.7 -> 264.4	Target	C12:0 GlcCer	C26:0 LacCer	1002.8 -> 264.3	Target	C12:0 LacCer
C16:0 LacCer	862.6 -> 264.3	Target	C12:0 LacCer	C26:1 Cer	676.7 -> 264.3	Target	C25:0 Cer
C16:0 DHCer	540.8 -> 266.4	Target	C12:0 Cer	C26:1 DHCer	678.6 -> 266.4	Target	C25:0 Cer
C18:0 Cer	566.6 -> 264.3	Target	C12:0 Cer	C26:1 DHGlcCer	840.7 -> 266.4	Target	C12:0 GlcCer
C18:0 DHGlcCer	730.6 -> 266.4	Target	C12:0 GlcCer	C26:1 DHLacCer	1002.7 -> 266.4	Target	C12:0 LacCer
C18:0 DHLacCer	892.7 -> 266.4	Target	C12:0 LacCer	C26:1 GlcCer	838.7 -> 264.3	Target	C12:0 GlcCer
C18:0 GlcCer	728.9 -> 264.4	Target	C12:0 GlcCer	C26:1 LacCer	1000.8 -> 264.3	Target	C12:0 LacCer
C18:0 LacCer	890.6 -> 264.3	Target	C12:0 LacCer	d18:0/16:0 Cer1P	620.5 -> 264.3	Target	C12:0 Cer1P
C18:0 DHCer	568.6 -> 266.4	Target	C12:0 Cer	d18:0/16:0 DHCer1P	620.5 -> 266.4	Target	C12:0 Cer1P
C18:1 Cer	564.5 -> 264.3	Target	C12:0 Cer	d18:0/18:0 Cer1P	648.5 -> 264.3	Target	C12:0 Cer1P
C18:1 DHCer	566.5 -> 266.4	Target	C12:0 Cer	d18:0/18:0 DHCer1P	648.5 -> 266.4	Target	C12:0 Cer1P
C18:1 DHGlcCer	728.6 -> 266.4	Target	C12:0 GlcCer	d18:0/24:0 Cer1P	732.6 -> 264.3	Target	C12:0 Cer1P
C18:1 DHLacCer	890.7 -> 266.4	Target	C12:0 LacCer	d18:0/24:0 DHCer1P	732.6 -> 266.4	Target	C12:0 Cer1P
C18:1 GlcCer	726.6 -> 264.3	Target	C12:0 GlcCer	d18:1/16:0 Cer1P	618.5 -> 264.3	Target	C12:0 Cer1P
C18:1 LacCer	888.6 -> 264.3	Target	C12:0 LacCer	d18:1/16:0 DHCer1P	618.6 -> 282.3	Target	C12:0 Cer1P
C20:0 Cer	594.6 -> 264.3	Target	C25:0 Cer	d18:1/18:0 Cer1P	646.5 -> 264.3	Target	C12:0 Cer1P
C20:0 DHGlcCer	758.9 -> 266.4	Target	C12:0 GlcCer	d18:1/18:0 DHCer1P	646.5 -> 282.3	Target	C12:0 Cer1P
C20:0 DHLacCer	920.7 -> 266.4	Target	C12:0 LacCer	d18:1/18:1 Cer1P	644.5 -> 264.3	Target	C12:0 Cer1P
C20:0 GlcCer	756.9 -> 264.4	Target	C12:0 GlcCer	d18:1/18:1 DHCer1P	644.6 -> 282.3	Target	C12:0 Cer1P
C20:0 LacCer	918.6 -> 264.3	Target	C12:0 LacCer	d18:1/24:0 Cer1P	730.6 -> 264.3	Target	C12:0 Cer1P
C20:0-dhCer	596.6 -> 266.4	Target	C25:0 Cer	d18:1/24:0 DHCer1P	730.6 -> 282.3	Target	C12:0 Cer1P
C22:0 Cer	622.6 -> 264.3	Target	C25:0 Cer	DeoxySA	286.3 -> 44.3	Target	d17:0 Sa
C22:0 DHGlcCer	786.9 -> 266.4	Target	C12:0 GlcCer	DH-S1P	382.3 -> 266.1	Target	d17:0 Sa1P
C22:0 DHLacCer	948.7 -> 266.4	Target	C12:0 LacCer	DH-Sph	302.3 -> 60.2	Target	d17:0 Sa
C22:0 GlcCer	784.9 -> 264.4	Target	C12:0 GlcCer	S1P	380.2 -> 264.4	Target	d17:1 S1P
C22:0 LacCer	946.6 -> 264.3	Target	C12:0 LacCer	Sph	300.3 -> 282.3	Target	d17:1 -So

** The sphingold base for listed compounds are d18:1 except those specified in the table*

Results and Discussion

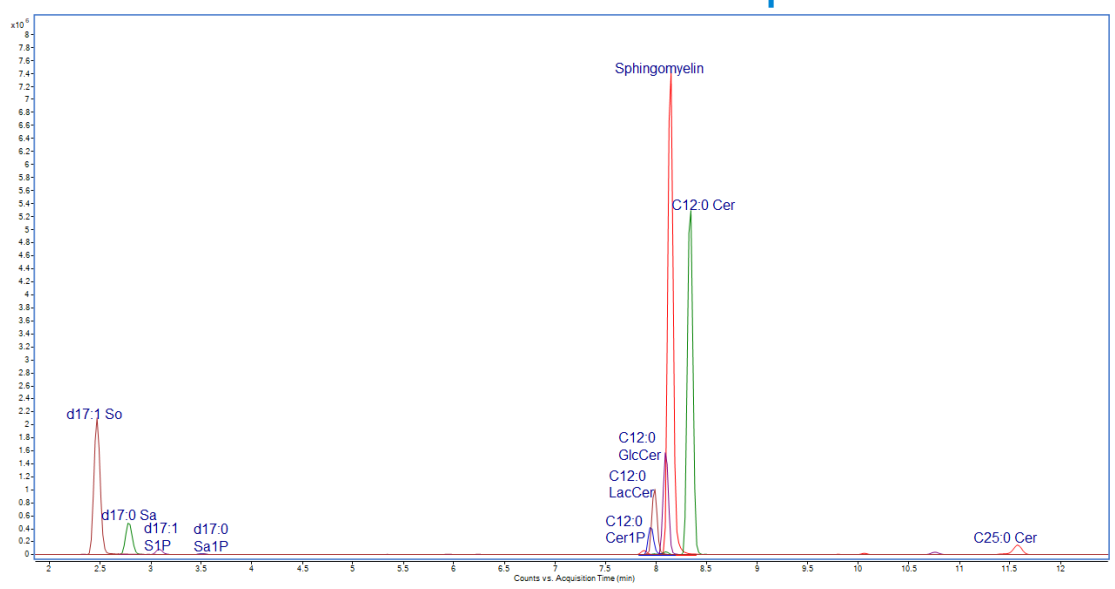
Method Validation Procedure

- ✓ Nine internal standards that represent each of the sphingolipid class were selected to evaluate the method performance
- ✓ Three sets of standards (extracted matrix-matched standards, post-extraction matrix-matched standards and standards in solvent) were prepared to test the calibration curve linearity, limit of quantitation (LOQ), selectivity, accuracy, precision, and matrix effect

Criteria to Accept the Validation Results

- ✓ The calibration curve constructed from extracted matrix-matched standards has a coefficient of determination (r^2) of ≥ 0.99
- ✓ LOQ in plasm determination
  - Accuracy on extraction efficiency within 70-130%
  - Precision (n≥3) within 30%
  - S/N ≥ 7

Elution Profile of Internal Standards Spiked in Plasma



Establishing Reference Range of Plasma Sphingolipids in Healthy Children

- ✓ Blood was collected from 66 healthy pediatric subjects (55% male, 45% female) ages 4 months to 21 years in accordance with an approved UCSF Institutional Review Board protocol
- ✓ Subjects were undergoing elective surgery for benign conditions such as congenital skeletal deformity, gastric tube placement or take down, inguinal hernia, etc. Individuals with malignant, infectious, metabolic, hemolytic, or autoimmune diagnoses were excluded from the study
- ✓ Plasma sphingolipid results were analyzed as a whole, as well as by comparison of results from pre-pubertal males, pre-pubertal females, post-pubertal males and post-pubertal females

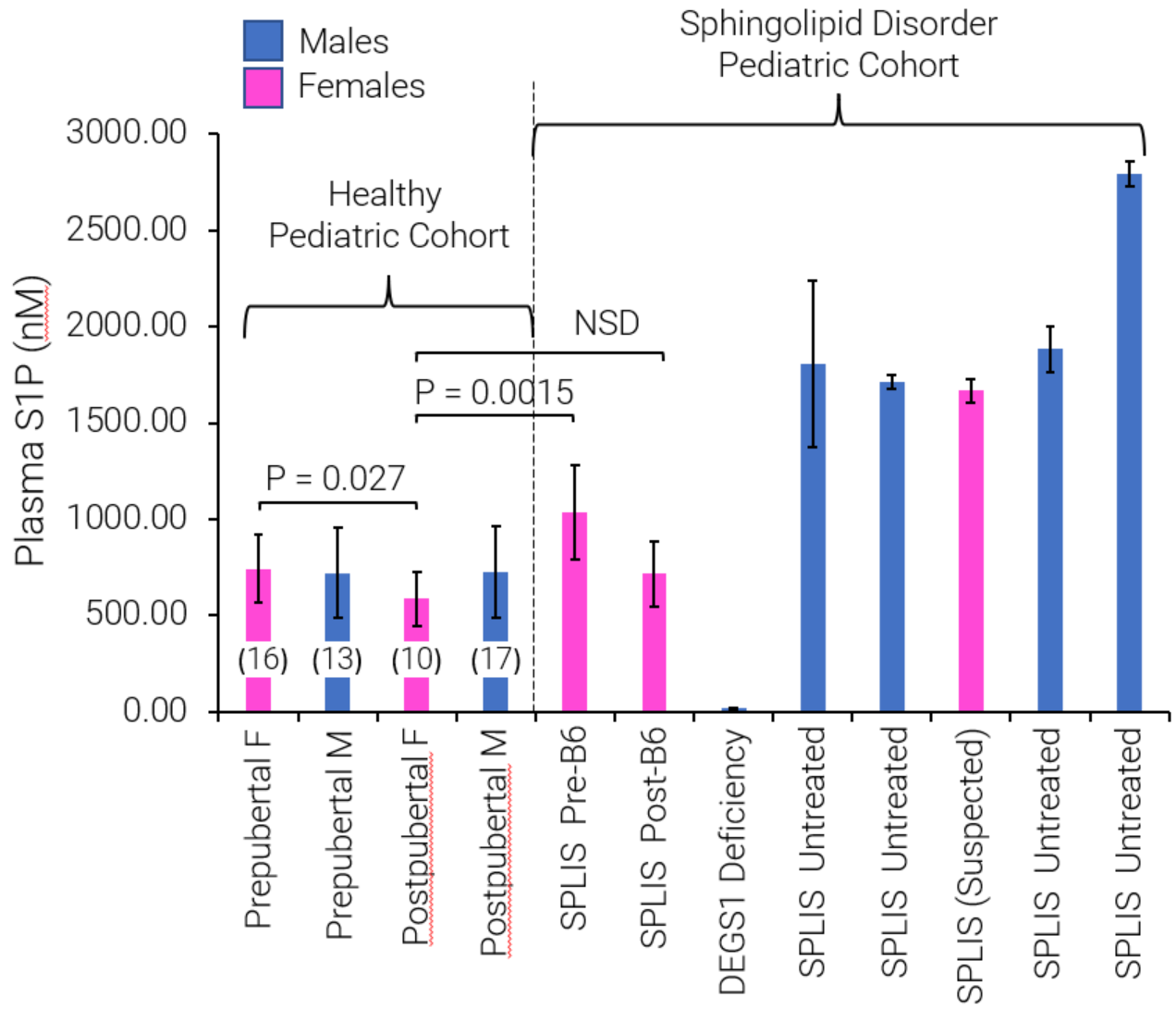
Results and Discussion

Method Evaluation Results

Internal Standard	Extracted matrix-match curve range nM	Coefficients of determination (R^2)	Accuracy range for all calibration points, %	Precision range for all calibration points, %	LOQ in Plasma nM
C12:0 Cer	10-1000	0.9654	70-130	±10	10
C12:0 Cer1P	5-1000	0.9860	85-115	±20	5
C12:0 GlcCer	5-1000	0.9950	75-125	±10	5
C12:0 LacCer	2.5-1000	0.9972	70-130	±15	2.5
C25:0 Cer	25-1000	0.9907	70-130	±15	25
d17:0 Sa	2.5-1000	0.9909	80-120	±10	2.5
d17:1 Sa1P	25-1000	0.9972	80-120	±10	25
d17:1 S1P	10-1000	0.9962	75-125	±5	10
d17:1 So	10-1000	0.9890	80-120	±10	10

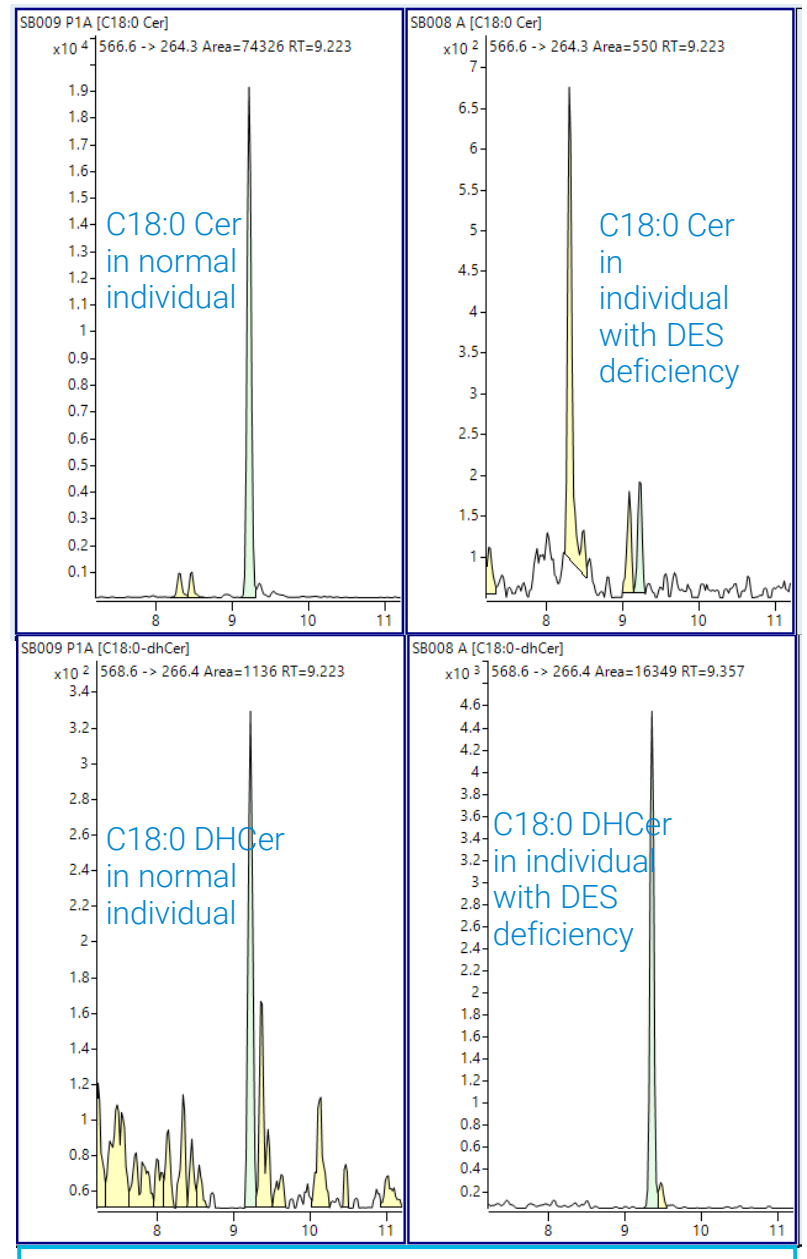
Sphingosine Phosphate Lyase Insufficiency Syndrome (SPLIS)

- ✓ SPLIS is a rare metabolic disorder caused by a deficiency in sphingosine-1-phosphate lyase (SPL), the final enzyme in the sphingolipid degradative pathway
- ✓ SPLIS leads to the accumulation of Sphingosine-1-Phosphate (S1P). SPLIS presentations include fetal hydrops, steroid-resistant nephrotic syndrome (SRNS), primary adrenal insufficiency (PAI), rapid or insidious neurological deterioration, immunodeficiency, acanthosis and endocrine abnormalities



Plasma S1P levels in a healthy pediatric cohort in comparison to children with two types of inborn errors of sphingolipid metabolism. Plasma S1P in postpubertal females (≥13, n=10) were lower than in prepubertal females (<13 years, n=16) and males (<12, n=13) and postpubertal males (≥12, n=17), consistent with lower red cell mass after menarche (red cells being the primary source of plasma S1P). High plasma S1P was observed in all individuals with SPLIS, caused by deficiency in the S1P degrading enzyme S1P lyase. In one young adult female SPLIS individual, plasma S1P levels fell in response to vitamin B6. Nearly undetectable S1P was observed in individuals lacking the ceramide desaturase encoded by DEGS1, consistent with inability to generate sphingosine, the precursor to S1P.

Dihydroceramide Desaturase Deficiency



- ✓ Dihydroceramide desaturase (DES) catalyzes the insertion of a double bond into dihydroceramides (DHCer) to convert them to Cer, both of which are further metabolized to more complex (dihydro) sphingolipids
- ✓ Deficiency in dihydroceramide desaturase leads to the accumulation of DHCer with minimal formation of ceramides, sphingosine and S1P, causing oxidative stress, neuropathy, lipid toxicity, a wide range of cellular processes including cell growth, cell death, autophagy, immune responses, and metabolic diseases

Conclusions

The established LC-MS/MS method:

- Establishes reference ranges for plasma sphingolipids in healthy pre- and post-pubertal children
- Helps reveal the mechanism underlying sphingolipid regulated diseases in children
- Enables sphingolipid biomarker development needed for treating children with inborn errors of of sphingolipid metabolism

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