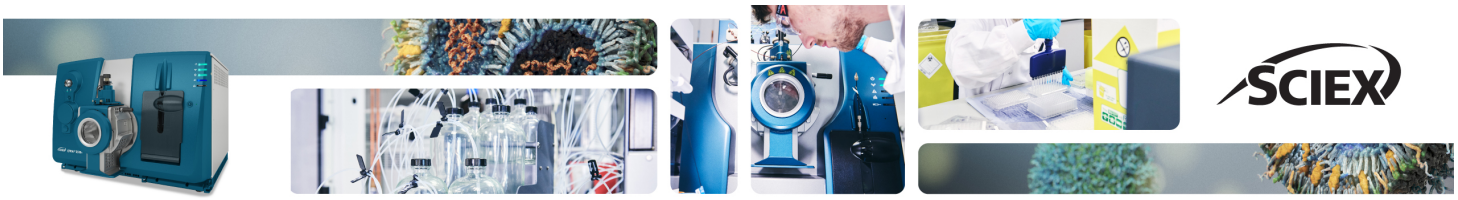

Targeted analysis of serum glycosphingolipids

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In this technical note, the SCIEX 6500+ system was used to measure 84 molecular species of gangliosides and other glycosphingolipids in a targeted assay to analyze the ganglioside metabolome comprehensively. The detection and quantitation of glycosphingolipids has emerged as an important analytical technique due to the significant roles these lipids play in neuronal function, such as myelin maintenance and cell signaling[1-3]. To better understand these compounds' physiological and pathophysiological roles, their metabolites must be accurately detected, quantitated, and put into metabolic context. As with all lipid classes, the sphingolipids comprise multiple molecular species that differ by carbon chain length and the position of double bonds. Glycosphingolipid analysis is further complicated by the addition of multiple sugar moieties attached to the sphingoid base[4]. Although LC-MS/MS methods have been developed to analyze gangliosides quantitatively, none can simultaneously target all glycosphingolipids and provide an integrated view of ganglioside metabolism.

The most efficient and accurate means of comprehensively analyzing glycosphingolipid molecular species is by LC-MS/MS using a targeted, scheduled multiple reaction monitoring (sMRM) scan mode. Here, the SCIEX 6500+ system was used to measure glycosphingolipids in human serum samples using an sMRM method covering 10 classes of gangliosides and 4 other classes of glycosphingolipids in a single run by leveraging the fast polarity switching of the instrument (**Figure 1**). These targets comprise the entire pathway of ganglioside metabolism, and the data generated can provide a metabolic "snapshot" of biological samples.

Key features of targeted analysis of glycosphingolipids

- The SCIEX 6500+ system, using a targeted sMRM method, a method was developed to detect and quantitate 84 glycosphingolipids in serum
- The rapid polarity switch time of the SCIEX 6500+ system (< 5 ms) allows for analysis in both the positive and negative ion modes in a single injection
- The comprehensive method uses a simple extraction protocol and a total analysis time of 15 min to facilitate high-throughput glycosphingolipid analysis

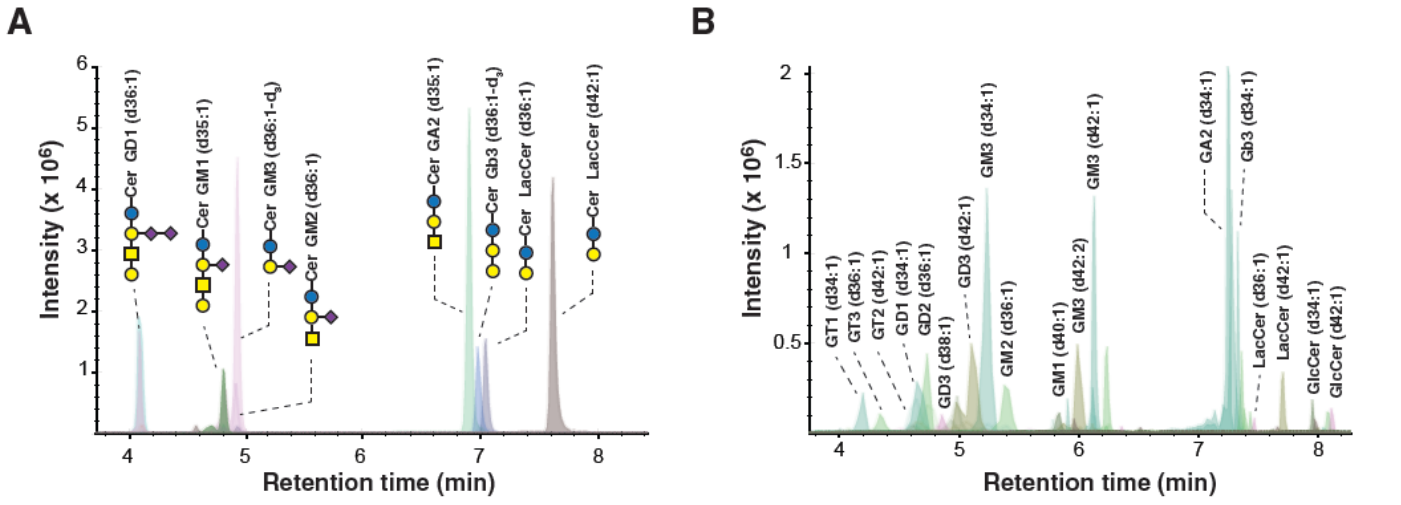

Figure 1. **Detection of gangliosides and other glycosphingolipids by a multiplexed LC-MS/MS method. (A)** Separation of a mixture containing six ganglioside (GM1 d18:1/17:0, GM2 d18:1/18:0, GM3 d18:1/18:0, GD1 d18:1/18:0, GD2 d18:1/18:0, GD3 d18:1/18:0-d3) and four glycosphingolipid (LacCer d18:1/18:0, LacCer d18:1/24:0, Gb3 d18:1/18:0-d3, GA2 d18:1/17:0) standards using a phenyl-hexyl column. **(B)** Extracted ion chromatograms obtained from lipids extracted from normal serum sample targeting various species of gangliosides and other glycosphingolipids.

Introduction

Gangliosides are a sialylated glycosphingolipid comprising a hydrophobic ceramide lipid moiety and an oligosaccharide chain. The diverse sugar composition, the number of sialic acids, and the variations in the length of carbon chains within ceramide contribute to the formation of >200 distinct molecular species of gangliosides. These molecules form functional microdomains, known as lipid rafts, on cell membranes by associating laterally with sphingomyelin, cholesterol and proteins owing to the hydrophobic nature of their tails and their polar glycans. Gangliosides are predominantly found in human brain tissue, where they play vital roles in myelin maintenance, cell signaling and cell-cell interactions[1-3].

Quantitative analysis of gangliosides and related molecules in blood, urine, cerebrospinal fluid, and other relevant samples can provide a sensitive and precise means to evaluate glycosphingolipid metabolism. Various LC-MS/MS methods have been developed to quantitatively determine ganglioside molecular species in various biological specimens, including plasma cells and tissues. . These methods often focus on measuring a limited number of specific ganglioside molecular species or are applied to matrices other than serum[5-7]. Furthermore, none of these LC-MS/MS methods were designed to target a full spectrum of glycosphingolipids simultaneously. To address these deficiencies, this study developed a multiplexed liquid chromatography-tandem mass spectrometry (LC-MS/MS) method targeting 84 individual species of gangliosides and other glycosphingolipids in serum. It targets 10 classes of gangliosides (GM1, GM2, GM3, GD1, GD2, GD3, GT1, GT2, GT3 and GQ1) and 4 classes of glycosphingolipids (GlcCer, LacCer, Gb3 and GA2). The presented method allows for monitoring the entirety of ganglioside metabolic pathways.

Materials and methods

Materials: The following standards were purchased: GM3 (d18:1/18:0), GD3 (d18:1/18:0), GD1a (d18:1/18:0), GT1b (d18:1/18:0), Glucosyl ceramide (d18:1/16:0), Lactosyl ceramide (d18:1/18:0 and d18:1/24:0) and Gb3 (d18:1/18:0) from Avanti Polar Lipids (Alabaster, AL, USA); GM2 (d18:1/18:0) and GM1 (d18:1/18:0) gangliosides from Cayman Chemical (Ann Arbor, MI, USA); GD2 (d18:1/18:0) ganglioside from ChemCruz (Dallas, Texas, USA). Glycosphingolipids (Avanti Polar Lipids Inc., Alabaster, AL, USA) with odd-numbered or deuterated acyl chain were used as internal standards and were added to the serum lipid extract for MRM quantitation: GM3 (d18:1/18:0-d5), GM1 (d18:1/17:0), Glucosyl ceramide (d18:1/16:0-d3) and Lactosyl ceramide (d18:1/18:0-d3). High-performance liquid chromatography (HPLC)-grade solvents (water, acetonitrile, methanol, isopropanol and chloroform) were purchased from Fisher Scientific (Waltham, MA, USA).

Sample preparation: Human serum was obtained from apparently healthy human volunteers following the procedure outlined in the Mayo Clinic institutional review board (IRB) protocol #21-012890. For lipid extraction, 20 µl of serum was mixed with a deuterated standard mixture as an internal standard (IS; GM3-d3 d18:1/18:0, GlcCer-d3 d18:1/16:0 and Gb3-d3 d18:1/18:0), followed by addition of 200 µl of chloroform:methanol (1:2, v/v). The samples were vortexed briefly and incubated in a bath sonicator for 30 min. After centrifugation, the supernatant was collected and dried under pure nitrogen. The dried lipid extract was reconstituted in 20 µl of methanol and mixed before transferring to autosampler vials. A standard mixture was prepared by mixing 5 pmol/µl of the following standards - GM3 d18:1/18:0, GM2 d18:1/18:0, GM1 d18:1/18:0, GD3 d18:1/18:0, GD2 d18:1/18:0, GD1a d18:1/18:0, GT1b d18:1/18:0, GlcCer d18:1/16:0, LacCer d18:1/18:0, LacCer d18:1/24:0 and Gb3 d18:1/18:0. This standard mixture was diluted serially to generate a calibration curve by spiking into serum substitute matrix.

Chromatography: The targeted LC-MS/MS analysis was conducted on a SCIEX QTRAP 6500+ mass spectrometer connected to a SCIEX Eksigent Ekspert LC 400 with a microflow module (5 to 50 µl/min). Separation of gangliosides according to their number of sialic acid and carbon chain length in target lipids was performed using Waters BEH Phenyl-hexyl column (1.0 × 50 mm, 1.7 µm) with a flow rate of 20 µl/min for 15 min using a binary gradient of mobile phase A (water:acetonitrile (9:1, v/v) with 5 mM ammonium hydroxide and 1 mM ammonium formate) and mobile phase B (isopropanol:methanol:acetonitrile (7:1.5:1.5, v/v/v) with 5 mM ammonium hydroxide and 1 mM ammonium formate). Five microliters of samples were injected into the analytical column with 10% of mobile phase B for 1 min. After sample loading, mobile phase B was increased to 70% over 1 min, 100% over 4 min, and maintained at 100% for another 5 min. Thereafter, the mobile phase B was decreased to 10%, and the analytical column was reconditioned for 4 min., as shown in **Table 1**.

Table 1. Gradient elution details

Time (min)	*Mobile phase A (%)*	*Mobile Phase B (%)*
0	*90*	*10*
1	*90*	*10*
2	*30*	*70*
6	*0*	*100*
11	*0*	*100*
11.01	*90*	*10*
15	*90*	*10*

Table 2. Compound parameters for targeted glycosphingolipid analysis. Individual compound parameters for each targeted glycosphingolipid are detailed. Polarity switching within the same run was employed and is indicated by the sign associated with each collision energy (CE) setting. Q1 and Q3 refer the precursor and product ion masses, respectively and RT is the retention time for each compound.

Compound	Q1 (m/z)	Q3 (m/z)	CE (V)	RT (min)	Compound	Q1 (m/z)	Q3 (m/z)	CE (V)	RT (min)
GM3 C16:0	1151.7	290.1	-80	5.18	GD1 C24:1	957.5	290.1	-45	4.95
GM3 C18:0	1179.7	290.1	-80	5.32	GT3 C16:0	866.4	290.1	-50	4.36
GM3 C18:0-d5	1184.7	290.1	-80	5.35	GT3 C18:0	880.4	290.1	-50	4.45
GM3 C20:0	1207.7	290.1	-80	5.55	GT3 C20:0	894.4	290.1	-50	4.54
GM3 C22:0	1235.8	290.1	-80	5.82	GT3 C22:0	908.4	290.1	-50	4.59
GM3 C24:0	1263.8	290.1	-80	5.96	GT3 C24:0	922.5	290.1	-50	4.67
GM3 C24:1	1261.8	290.1	-80	5.89	GT3 C24:1	920.5	290.1	-50	4.63
GM2 C16:0	1354.7	290.1	-80	5.18	GT2 C16:0	967.9	290.1	-50	4.36
GM2 C18:0	1382.8	290.1	-80	5.32	GT2 C18:0	981.9	290.1	-50	4.45
GM2 C20:0	1410.8	290.1	-80	5.55	GT2 C20:0	996.0	290.1	-50	4.54
GM2 C22:0	1438.8	290.1	-80	5.82	GT2 C22:0	1010.0	290.1	-50	4.59
GM2 C24:0	1466.9	290.1	-80	5.96	GT2 C24:0	1024.0	290.1	-50	4.67
GM2 C24:1	1464.9	290.1	-80	5.90	GT2 C24:1	1022.0	290.1	-50	4.63
GM1 C16:0	1516.8	290.1	-80	5.18	GT1 C16:0	1049.0	290.1	-50	4.36
GM1 C17:0	1530.8	290.1	-80	5.24	GT1 C18:0	1063.0	290.1	-50	4.45
GM1 C18:0	1544.8	290.1	-80	5.32	GT1 C20:0	1077.0	290.1	-50	4.54
GM1 C20:0	1572.9	290.1	-80	5.55	GT1 C22:0	1091.0	290.1	-50	4.59
GM1 C22:0	1600.9	290.1	-80	5.82	LacCer C16:0	862.6	264.3	80	6.99
GM1 C24:0	1628.9	290.1	-80	5.96	LacCer C18:0	890.7	264.3	80	7.19
GM1 C24:1	1626.9	290.1	-80	5.90	LacCer C20:0	918.7	264.3	80	7.38
GD3 C16:0	720.8	290.1	-45	4.59	LacCer C22:0	946.7	264.3	80	7.54
GD3 C18:0	734.9	290.1	-45	4.68	LacCer C24:0	974.8	264.3	80	7.7
GD3 C20:0	748.9	290.1	-45	4.77	LacCer C24:1	972.7	264.3	80	7.61
GD3 C22:0	762.9	290.1	-45	4.86	GlcCer C16:0	700.6	264.3	80	7.08
GD3 C24:0	776.9	290.1	-45	5.01	GlcCer C16:0-d3	701.6	264.3	80	7.08
GD3 C24:1	774.9	290.1	-45	4.95	GlcCer C18:0	728.6	264.3	80	7.26
GD2 C16:0	822.4	290.1	-45	4.59	GlcCer C20:0	756.6	264.3	80	7.43
GD2 C18:0	836.4	290.1	-45	4.68	GlcCer C22:0	784.7	264.3	80	7.6
GD2 C20:0	850.4	290.1	-45	4.77	GlcCer C24:0	812.7	264.3	80	7.75
GD2 C22:0	864.4	290.1	-45	4.86	GlcCer C24:1	810.7	264.3	80	7.67
GD2 C24:0	878.4	290.1	-45	5.01	Gb3 C16:0	1024.7	264.3	80	6.94
GD2 C24:1	876.4	290.1	-45	4.95	Gb3 C18:0	1052.7	264.3	80	7.13
GD1 C16:0	903.4	290.1	-45	4.59	Gb3 C18:0-d3	1055.7	264.3	80	7.14
GD1 C18:0	917.4	290.1	-45	4.68	Gb3 C20:0	1080.7	264.3	80	7.32
GD1 C20:0	931.4	290.1	-45	4.77	Gb3 C22:0	1108.8	264.3	80	7.52
GD1 C22:0	945.5	290.1	-45	4.86	Gb3 C24:0	1136.8	264.3	80	7.7
GD1 C24:0	959.5	290.1	-45	5.01	Gb3 C24:1	1134.8	264.3	80	7.57
GT1 C24:0	1105.0	290.1	-50	4.67	GA2 C16:0	1065.7	264.3	80	6.9
GT1 C24:1	1103.0	290.1	-50	4.63	GA2 C17:0	876.4	264.3	80	7.03
GQ1 C16:0	1194.5	290.1	-50	4.02	GA2 C18:0	1093.7	264.3	80	7.1
GQ1 C18:0	1208.5	290.1	-50	4.09	GA2 C20:0	1121.8	264.3	80	7.48
GQ1 C20:0	1222.5	290.1	-50	4.17	GA2 C22:0	1149.8	264.3	80	7.6
GQ1 C22:0	1236.6	290.1	-50	4.27	GA2 C24:0	1177.8	264.3	80	7.75
GQ1 C24:0	1250.6	290.1	-50	4.4	GA2 C24:1	1175.8	264.3	80	7.62
GQ1 C24:1	1248.6	290.1	-50	4.33					

Mass spectrometry: Glycosphingolipid detection was performed using a SCIEX QTRAP 6500+ system (SCIEX, USA). Multiple reaction monitoring (sMRM) analysis was performed in the positive and negative modes in a single experiment.. In all, 74 molecular ions were monitored for gangliosides (GM3, GM2, GM1, GD3, GD2, GD1, GT3, GT2, GT1 and GQ1) and other glycosphingolipid classes (GlcCer, LacCer, Gb3 and GA2). All targeted lipids were quantified by calculating the peak areas of extracted ion chromatograms, followed by normalization using the peak areas of internal standards. The compound parameter settings, including the precursor and product ion masses, the collision energy (CE), and the respective retention times (RTs), are listed in **Table 2.** The polarity in which each glycosphingolipid was measured is indicated by the sign associated with each CE value.

Data processing: LC-MS operation, data acquisition, and data processing were performed using SCIEX Analyst software (SCIEX, USA). The standard mixture spiked into a serum substitute was used to obtain compound-specific calibration curves from which the limits of detection (LOD) and quantification (LOQ) were calculated.

The linearity of the method was evaluated using calibrators across different concentration ranges. The linearity equation and coefficient of determination (r2) were determined based on a calibration curve. The limit of detection was calculated based on the standard deviation of the response (Sy) of the calibration curve and the slope of the calibration curve (S) at levels approximating the LOD according to the formula LOD = 3.3(Sy/S). The method reproducibility was assessed by spiking low and high concentration standards into 20 µl of serum substitute supplement. Extraction efficiency was evaluated by comparing the expected and measured MS response of the standards added to the serum substitute. Intra-assay and inter-assay reproducibility were evaluated by running the low and high-concentration samples five times daily over five days.

Results and Discussion

Development of multiplexed ganglioside and glycosphingolipid targeted method

Targeted analysis for serum gangliosides was performed in a 15 min LC-MS/MS method in the scheduled MRM (sMRM) mode. **Figure 2** shows the principal biosynthetic pathways of gangliosides and other glycosphingolipids. This assay was developed to target all these components to comprehensively analyze the ganglioside metabolome. This multiplexed method applied to the standard mixture and to human serum quantified a total of 84 species of GM3, GM2, GM1, GD3, GD2, GD1, GT3, GT2, GT1 and GQ1 gangliosides and other glycosphingolipids including GlcCer, LacCer, Gb3 and GA2. Using a phenyl-hexyl HPLC column, the gangliosides were separated according to their number of sialic acids, and within a specific class of ganglioside, gangliosides possessing different ceramide carbon chain lengths were separated[8]. The performance of the phenyl-hexyl column is demonstrated by the separation of the mixture of six gangliosides and four glycosphingolipid standards (GM1, GM2, GM3, GD1, GD2, GD3, LacCer, Gb3 and GA2) as shown in **Figure 1A**, which were obtained with 1 pmol of each standard. **Figure 1B** shows the extracted ion chromatograms obtained from lipid extracts of human serum samples by targeting all species of gangliosides and glycosphingolipids, as depicted in **Figure 2**. The elution map of all targeted species detected in normal serum samples is shown in **Figure 3A**. Gangliosides belonging to classes with a higher sialic acid content, GQ1, eluted first, while gangliosides with a single sialic acid, such as GM1, GM2 and GM3, eluted the latest among the gangliosides. Glycosphingolipids exhibited minor variations in

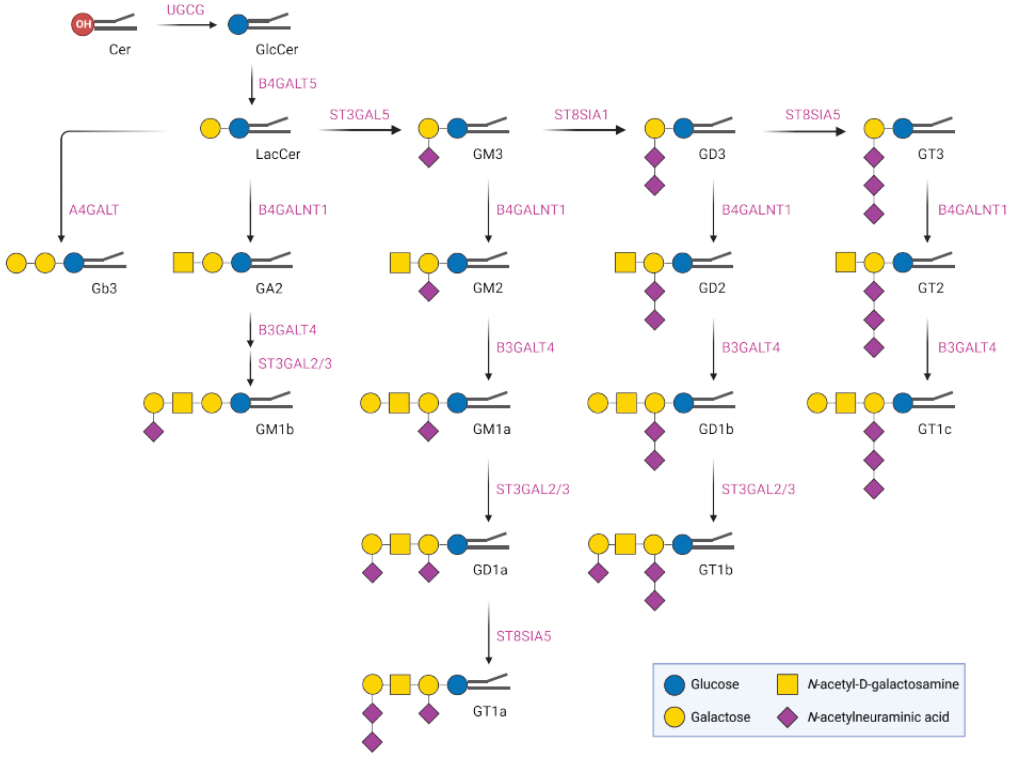

Figure 2 Biosynthesis pathways associated the glycosphingolipids targeted by the multiplexed LC-MS/MS method. Biosynthetic enzymes are labelled in pink. The abbreviations used for analytes and enzymes are as follows: Cer, Ceramide; GlcCer, Glucosyl ceramide; LacCer, Lactosyl ceramide; UGCG, UDP-Glucose Ceramide Glucosyltransferase; B4GALT5, UDP-Gal:Beta-GlcNAc Beta-1,4-Galactosyltransferases 5; ST3GAL5, CMPNeuAc:Lactosylceramide Alpha-2,3-Sialyltransferase; ST8SIA1, ST8 Alpha-N-Acetylneuraminide Alpha-2,8-Sialyltransferase 1; ST8SIA5, ST8 Alpha-N-Acetyl-Neuraminide Alpha-2,8-Sialyltransferases 5; B4GALNT1, UDP-Gal:BetaGlcNAc Beta-1,4 N-Acetylgalactosaminyltransferase 1; B3GALT4, UDP-Gal:BetaGlcNAc Beta 1,3-Galactosyltransferase 4; ST3GAL2/3, CMP-N-Acetylneuraminate-Beta-Galactosamide-Alpha-2,3-Sialyltransferase 2; ST8SIA5, ST8 Alpha-N-Acetyl-Neuraminide Alpha-2,8-Sialyltransferase 5.

Table 3. Method validation for targeted glycosphingolipid analysis. Using 6 ganglioside molecular species, the LOD, the mean standard deviation (SD), and the percent CV were calculated using a substitute serum matrix.

Targeted species	Limit of detection	R^2	QC	Intra-assay (n=5)		Inter-assay (n=5)	
				Mean ± SD	CV%	Mean ± SD	CV%
GM1 (d18:1/18:0)	298 amol	0.995	low	0.15 ± 0.00	2.53%	0.25 ± 0.03	12.24%
			high	5.41 ± 0.18	3.46%	5.12 ± 0.24	4.68%
GM2 (d18:1/18:0)	321 amol	0.996	low	0.25 ± 0.01	3.55%	0.31 ± 0.02	6.37%
			high	6.04 ± 0.47	7.91%	5.98 ± 0.84	14.05%
GM3 (d18:1/18:0)	134 amol	0.995	low	0.34 ± 0.01	1.89%	0.41 ± 0.02	4.75%
			high	10.2 ± 0.21	2.08%	11.0 ± 0.31	2.82%
GD3 (d18:1/18:0)	249 amol	0.998	low	0.15 ± 0.01	4.94%	0.18 ± 0.03	17.04%
			high	6.50 ± 0.35	5.49%	6.72 ± 0.39	5.80%
GD2 (d18:1/18:0)	1054 amol	0.994	low	0.16 ± 0.01	6.80%	0.49 ± 0.02	4.11%
			high	6.56 ± 0.30	4.61%	6.31 ± 0.41	6.49%
GD1a (d18:1/18:0)	576 amol	0.993	low	0.12 ± 0.01	8.24%	0.17 ± 0.01	5.95%
			high	4.15 ± 0.03	0.66%	4.52 ± 0.32	7.07%
GT1b (d18:1/18:0)	188 amol	0.998	low	0.22 ± 0.02	8.20%	0.25 ± 0.03	11.81%
			high	5.57 ± 0.31	5.66%	5.12 ± 0.45	8.78%

retention times based on the number of glycans bound to ceramide. The dehydrated sialic acid fragment (m/z 290.1) was observed as the most dominant product ion when gangliosides were fragmented in the negative ion mode (**Figure 3B**). Thus, the fragment ion at m/z 290 was used as the product and quantifier ion for all GM3, GM2, GM1, GD3, GD2, GD1, GT3, GT2, GT1 and GQ1 gangliosides detected in negative ion mode. Glycosphingolipids other than gangliosides were detected in the positive ion mode using the fragment ion corresponding to

the dehydrated sphingosine backbone (m/z 264.3) as the product and quantifier ion. The standard mixture was spiked into a serum

Table 4. Quantitative, endogenous content of select glycosphingolipids in serum

Sample	Calculated amount (fmol)						
	GM3 (d18:1/18:0)	GM2 (d18:1/18:0)	GM1 (d18:1/18:0)	GD3 (d18:1/18:0)	GD2 (d18:1/18:0)	GD1 (d18:1/18:0)	GT1 (d18:1/18:0)
1	281.6	110.3	90.3	159.3	113.2	101.9	40.3
2	389.5	176.9	85.1	218.9	102.5	99.2	35.6
3	290.1	181.0	60.5	239.8	83.8	159.5	21.5
4	573.2	156.3	76.9	286.9	96.2	78.8	26.7
5	687.3	127.6	121.1	301.2	89.6	54.6	34.1

substitute matrix[9] containing human serum albumin and globulins, but not lipids, to generate a calibration curve and determine the assay's quantitative statistics (**Table 3**). The calibration standards' performance at various concentrations was assessed by calculating the coefficients of determination (r2) from three replicate runs. All r2 values were above 0.99, with a median of 0.994 (**Table 3**). Intra-assay precision showed <10% coefficient of variation for low and high-concentration mixtures. Inter-assay precision was assessed by measuring samples daily for 5 days and was determined to be <18% for both low and high QC mixtures. To evaluate the efficiency of a single-phase extraction using chloroform:methanol (1:2, v/v) as an extraction solvent, the peak areas of analytical standards were compared before and after extraction when added to a serum substitute. The average extraction efficiency for all standards was above 90%, with a median of 97.3% (data not shown).

Endogenous targets measured in samples from human serum samples

As shown in **Figure 1B,** the multiplexed method was used to target and analyze various ganglioside and glycosphingolipid species in lipid extracts from serum. Using the calibration curves derived from the standard mixture spiked into serum substitute (as indicated in **Table 3**), we quantified the endogenous targets detected in the lipid samples from the five individuals, as shown in **Table 4**. Quantitation

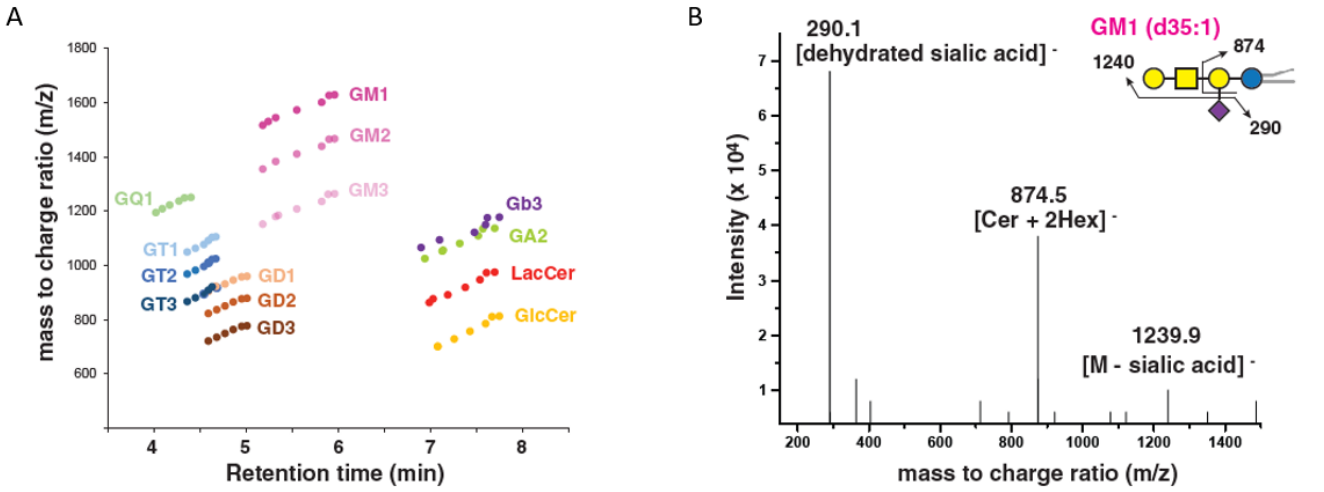

Figure 3 Glycosphingolipid LC-MS/MS method development. (A) Elution map displaying the retention times of the targeted species detected in normal serum samples; **(B)** MS/MS spectrum of GM1 ganglioside (d35:1)

of the total 7 classes of gangliosides (d36:1) revealed that GM3 ganglioside was the most abundant among the gangliosides present in the serum samples of five individuals (average 444.3 fmol). Following GM3, GD3 and GM2 were the subsequent most abundant gangliosides (average 241.2 and 150.4 fmol, respectively, across the five individuals). These data show that this targeted method for glycosphingolipid analysis is suitable for use with biological samples to generate quantitative data.

In summary, a robust multiplexed targeted LC-MS/MS method to simultaneously quantify the 84 molecular species of 10 ganglioside classes (GM1, GM2, GM3, GD1, GD2, GD3, GT1, GT2, GT3, and GQ1) and 4 classes of other glycosphingolipids (GlcCer, LacCer, Gb3, and GA2) in human serum has been developed. Using this multiplexed method, 61 species of gangliosides and glycosphingolipids species that were targeted were detected in human serum samples. Additionally, by testing serum samples, the total amounts of endogenous gangliosides from 7 different classes (GM1, GM2, GM3, GD1, GD2, GD3, GT1) were quantitated using the calibration curves generated from ganglioside standards spiked into a serum substitute matrix.

Conclusions

- The SCIEX 6500+ system has the speed and sensitivity to quantitatively measure 84 glycosphingolipid molecular species in serum to provide a comprehensive understanding of ganglioside metabolism
- Taking advantage of the fast polarity switching of the instrument (< 5ms), all the molecules representing the ganglioside metabolome can be measured in a single run with a total run time of 15 min

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