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Development and Validation of UPLC/MS/MS Methods for Quantification of Gangliosides in the Clinical Study of Ganglioside GM3 Synthase Deficiency

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DEVELOPMENT AND VALIDATION OF UPLC/MS/MS METHODS FOR QUANTIFICATION OF GANGLIOSIDES IN THE CLINICAL STUDY OF GANGLIOSIDE GM3 SYNTHASE DEFICIENCY

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July 12th, 2016

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DEVELOPMENT AND VALIDATION OF UPLC/MS/MS METHODS FOR QUANTIFICATION OF GANGLIOSIDES IN THE CLINICAL STUDY OF GANGLIOSIDE GM3 SYNTHASE DEFICIENCY

QIANYANG HUANG

ABSTRACT

Gangliosides are a large subclass of glycosphingolipids that structurally characterized by the addition of mono- or poly-sialyated carbohydrate moieties onto the ceramide scaffold. They are presented at significant abundance in the central nervous system of vertebrates. Their interactions with trans-membrane receptors, especially receptor tyrosine kinase, are believed to play critical roles in modulating signal transduction events during cell proliferation, differentiation, migration, and adhesion. Furthermore, the disruption and dysfunction on their metabolism have been found to be associated with various neurodegenerative disorders, such as Alzheimer disease, Parkinson's disease, and GM3 synthase deficiency. GM3 synthase deficiency (GSD) is a newly identified neurological disorder that has been prevalently found in the Amish population in the United States. Although the pathological mechanism remains to be understood, the condition is severe. It is characterized by infantile onset of severe irritability, developmental stagnation, profound intellectual disability and intractable seizures. In order to advance our understanding on the etiology of these neurodegenerative disorders, especially GSD, analytical assays with sufficient specificity, sensitivity, and throughput are urgently demanded to practically assist the

implementation of relevant clinical studies. In this thesis, three projects have been conducted separately to benefit the clinical studies from individual aspects. In project 1, owing to the increased complexity of molecular assays in screening and diagnosing patients affected by GSD associated with increased numbers of identified nonsense/missense mutations, we have developed and validated a novel UPLC/MS/MS method with promising sensitivity, specificity, and throughput for four major ganglioside species, GM2, GM3, GD2, and GD3, in human plasma to facilitate the diagnosis and screening of GSD. In project 2, in order to obtain more informative results from GSD patients undergoing clinical trial in relation to the development of related therapeutic intervention, we have developed and validated a new UPLC/MS/MS method in human plasma for quantification of monosialogangliosides in combination with DMTMM&PAEA chemical derivatization for analysis with superior sensitivity and specificity. In project 3, we have implemented a clinical study aiming at developing a therapeutic intervention strategy based on oral administration of commercialized ganglioside-enrich formula to the affected patients.

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LIST OF ABBREVIATIONS

Abbreviation	Full Name
GSD	Ganglioside GM3 Synthase Deficiency
GEM	Glycolipid-Enriched Microdomain
RTK	Receptor Tyrosine Kinase
EGFR	Epidermal Growth Factor Receptor
FGFR	Fibroblast Growth Factor Receptor
PDGFR	Platelet-Derived Growth Factor Receptor
TrkA	Nerve Growth Factor Receptor
IR	Insulin Receptor
Gal	Galactose
Glu	Glucose
GluCer	Glucosylceramide
LacCer	Lactosylceramide
GalNeu	Acetylgalactosamine
HPLC	High-Performance Liquid Chromatography
UPLC	Ultra-Performance Liquid Chromatography
MS	Mass Spectrometry
MRM	Multiple Reaction Monitoring
SAP	Spiked Analyte-Free Plasma
UPS	Unknown Plasma Sample
SA	Standard Addition

Neu5Ac	N-Acetylneuraminic Acid
Neu5Gc	N-Glyconeuraminic Acid
KDN	Deaminated Neuraminic Acid
DMTMM	4-(4, 6-Dimethoxy-1, 3, 5-triazin-2-yl)-4-methylmorpholinium chloride
PAEA	2-(2-Pyridilamino)-ethylamine
LLOQ	Lower Limit of Quantitation
MeOH	Methanol
CAN	Acetonitrile
FA	Formic Acid
AA	Ammonium Acetate

CHAPTER Ⅰ

INTRODUCTION

1.1 Gangliosides

Gangliosides are a large subfamily of glycosphingolipids that abundantly present in the central nervous system of living organisms. Up to date, over 180 ganglioside species with distinctive structures have been identified in vertebrates [1]. Similar to other glycosphingolipid species, they possess hydrophilic carbohydrate moieties being added onto the ceramide backbone except for the presence of sialic acid residues, N-Acetylneuraminic acid (Neu5Ac), on carbohydrate moieties (Fig.1). This unique combination between the hydrophobic ceramide portion and hydrophilic carbohydrate moiety with negatively charged Neu5Ac residues confers them distinctive amphiphilic properties, accounting for their characteristic behaviors and diverse functionalities in the physiological systems of living organisms.

Gangliosides are generally classified and nomenclated by the degree of

sialylation and the monosaccharide makeup of the carbohydrate moieties. In addition to the diversity of their carbohydrate moieties, the presence of long alkyl chain fatty acids with variable chain length and saturation degree in the ceramide portion generates an extra degree of heterogeneity of their structures [2], implying their physiological versatility in regulating the central nervous system of living organisms.

Gangliosides have been found to be predominantly localized on the outer leaflet of plasma membrane of neuronal and glial cells. Generally, they are settled onto the cell membrane by embedding their hydrophobic ceramide portions inward into the transmembrane section of the lipid bilayer and protruding their hydrophilic oligosaccharide moieties outward into the extracellular matrix. This characteristic cell surface configuration not only allows the ceramide scaffold to act as a functional lipid anchor to clinch them against the cell membrane with a confined orientation in a very secure manner, but also enables complete exposure of the oligosaccharide moiety to its surrounding to facilitate direct interactions with binding ligands from extracelluar matrix in a very accessible environment [3].

They exert their fundamental functionalities in maintaining integration and organizing dynamics of lipid bilayer skeleton primarily by serving as ubiquitous components of characteristic regions, known as glycolipid-enriched microdomains (GEMs) or lipid rafts, and interacting with other integral membrane components, such as transmembrane proteins, sphingomyelins, and cholesterols, on the mammalian cell membrane [4]. They present ubiquitously at significant abundance in the central nervous system of vertebrates. Mounting evidence has demonstrated that their biosynthesis and

metabolism physiologically play pivotal roles for the appropriate development and eventual maturation of central nervous system in vertebrate during embryogenesis [5]. Moreover, their cell surface expressions and interactions with transmembrane receptors, especially receptor tyrosine kinase are believed to be extensively implicated in modulating the signal transduction during a wide variety of essential cellular events, including cell differentiation [6-8], proliferation [9-12], migration [13-16], and adhesion [17-20]. However, the depletion, disruption, and malfunction on their biosynthetic pathway have been illustrated to be pathologically associated with the pathogenesis of a number of neurodegenerative disorders, including Parkinson's disease [21-27], Alzheimer disease [28-34], and GM3 synthase deficiency [35-37], all of which are neurological syndromes featured by devastating neurodegenerative impacts with no therapeutic cures available for effective treatment nowadays. Since the etiologies attributed to those currently incurable neurodegenerative disorders and the pathological roles played by gangliosides during their pathogenesis have not been fully understood, considerable numbers of studies related to this area are currently being undertaken towards the complete elucidation of underlying mechanisms by which abnormalities on ganglioside biosynthetic and metabolic pathways are etiologically related to the development of such clinically devastating disorders.

Fig.1 Structures of various sphingolipids.

1.2 Gangliosides' Nomenclature and Classification

Gangliosides are generally nomenclated by the "two-letter & one-digit" rule well-defined by Svennerholm [1]. In this nomenclature system, Svennerholm clearly stated that for a particular ganglioside species GXN_y, the first letter "G" denotes the word "ganglioside", the second letter "X" serves as a direct indication of the sialylation degree of the carbohydrate moiety where "M" represents the monosialylation, "D" represents the disialylation, "T" represents the trisialylation, and "Q" represents the quasialylation, and the sole digit "N" in this naming system represents different combinations of monosaccharide units composing the main carbohydrate chain regardless of the number of sialic acid residues attached. In this regard, the number "4" suggests the oligosaccharide moiety is solely consisted of β-D-glucose that linked directly to the ceramide portion, the number "3" illustrates the oligosaccharide moiety is essentially a disaccharide that composed by β-D-glucose and β-D-galactose linked through 2,5-glycosidic bond, the number "2" indicates trisaccharide made up by β-D-glucose, β-D-galactose, and β-D-N-acetylgalactosamine through two sequential 2,5-glycosidic linkages is the oligosaccharide moiety, and the number "1" describes the oligosaccharide moiety is built with β-D-glucose, β-D-galactose, β-D-N-acetylgalactosamine, and β-D-galactose linked via three consecutive 2,5-glycosidic bonds to obtain a quasaccharide carbohydrate template with highest degree of structural complexity. Furthermore, with this quasaccharide carbohydrate template, a number of sialylation sites on the template, including C3 positions on two β-D-galactoses and C6 position on β-D-N-acetylgalactosamine, have been made available for the sialic acid residue to be physically bound, which necessitates the

application of an additional subscript "y" followed by the digit dedicated for the further discrimination and classification of GX1 species with sialic acid residues being located onto disparate monosaccharide units along the carbohydrate platform. Overall, in consideration of all possible combinations and outcomes on their oligosaccharide moieties, over 180 ganglioside species with characteristic carbohydrate entities have been identified in the vertebrates. Detailed information on their nomenclature is shown in Fig.2.
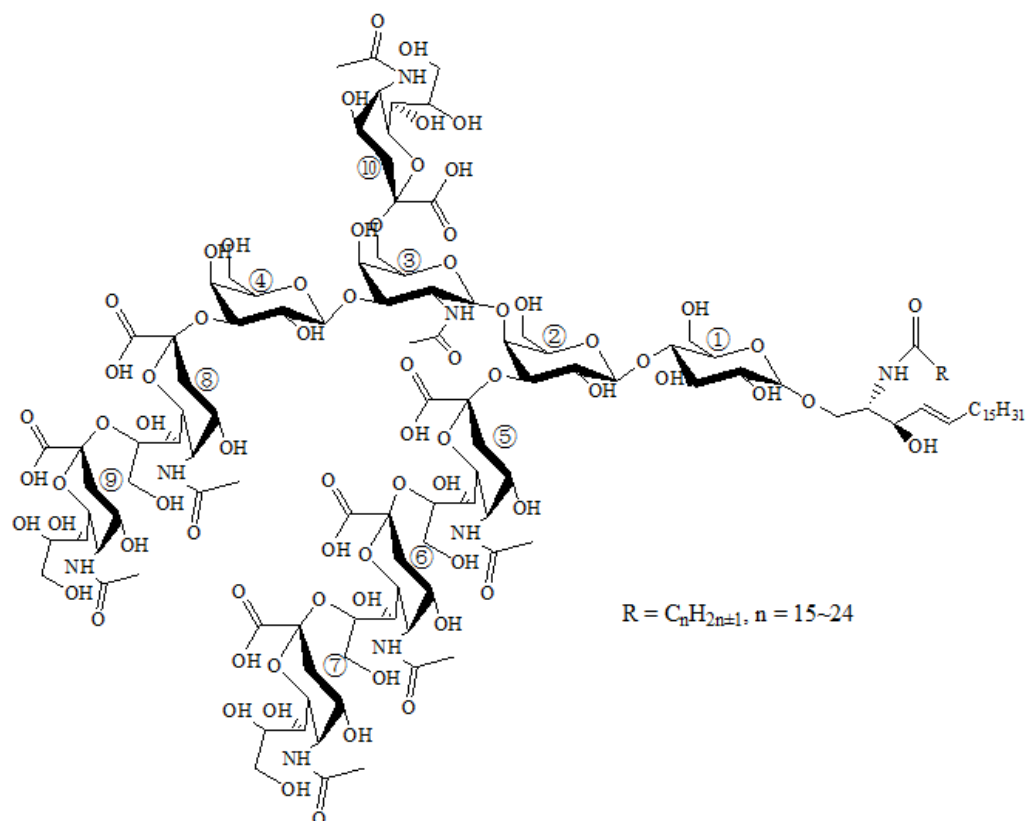
GM3	①②⑤	GT1a	①②③④⑤⑧⑨
GM2	①②③⑤	GT1b	①②③④⑤⑥⑧
GM1a	①②③④⑤	GT1c	①②③④⑤⑥⑦
GM1b	①②③④⑧	GT1α	①②③④⑤⑥⑩
GD3	①②⑤⑥	GQ1α	①②③④⑤⑥⑧⑩
GD2	①②③⑤⑥	GQ1b	①②③④⑤⑥⑧⑨
GD1a	①②③④⑤⑧	GQ1c	①②③④⑤⑥⑦⑧
GD1b	①②③④⑤⑥		

Fig.2 Classification and nomenclature of basic gangliosides.

Glucose residue: ①; Galactose residue: ②, ④; N-acetylgalactosamine residue: ③; N-acetylneuraminic acid residue: ⑤, ⑥, ⑦, ⑧, ⑨, ⑩.

In addition to their vast structural diversity from the carbohydrate moieties, the amide-bonded fatty acid chain from the C2 position of ceramide backbone also appears to possess variable chain length and saturation degree that altering in a very uniform manner. The amide-bonded fatty acids from the ceramide portion generally start with the length as short as 14-carbon and gradually lengthens in a 2-carbon increment manner to up to 24 carbons to generate a huge series of long alkyl chain fatty acids with 2-carbon differences from each other in the overall profile. The amided-bonded long alkyl chain fatty acids are predominantly unsaturated on 16- and 18-carbon species. According to previous studies on fatty acid profiling in gangliosides, the individual contribution of a variety of fatty acids from the ceramide portion to the total abundance of a single ganglioside species could be varying significantly from one sample specimen to another or even the same sample specimen at different developmental stages, suggesting differing roles possibly played by different fatty acid species in different physiological systems [38]. In combination with their structural diversity on the carbohydrate moiety, this variability on the ceramide scaffold generates extra degree of structural heterogeneity on gangliosides, conferring them with great structural complexity as well as physiological versatilities for the mediation of various cellular events in the central nervous system of living organisms.

1.3 Gangliosides' Biosynthesis

Gangliosides can be classified into four series, 0-, a-, b-, and c-series, corresponding to different degrees of sialyation on the lactosylceramide and disparate levels

of complexity on their structures. Simple gangliosides, mostly 0- and a-series, are predominantly expressed in normal human tissues during embryogenesis, whereas the complex gangliosides from b- and c-series are essentially observed in developing tissues during later developmental stages. As demonstrated by Fig.3, the de-novo biosynthesis of gangliosides usually starts off with the addition of a glucose residue onto the ceramide backbone via the catalysis by ceramide β-glucosyltransferase [39]. Thereafter, the glucosylceramide (GlcCer) is converted into the synthetic precursor of four series of gangliosides, lactosylceramide (LacCer), through a single-step reaction catalyzed by GlcCer β-1, 4-galactosyltransferase (LacCer synthase) [40-41]. Afterwards, specific sialyltransferases ST3Gal V (GM3 synthase), ST8Sia I (GD3 synthase), and ST8Sia V (GT3 synthase) catalyze the transfer of Neu5Ac residues onto the LacCer scaffold [42], and synthetic LacCer, GM3, GD3, and GT3 act as precursors for the downstream biosynthesis along the individual pathway following the stepwise manner to generate 0-, a-, b-, and c-series gangliosides, respectively, upon the sequential addition of Gal, GalNAc, and Neu5Ac residues onto the skeletons from previous synthetic step under the catalysis by the β-1,3-galactosyltransferase IV [43], β- 1,4-N-acetylgalactosaminyltransferase I [44], and sialyltransferases ST3Gal II [45-47], ST8Sia V [48], or ST6GalNAc III/V[49-50].

The early biosynthetic stages mainly occur in the cis/median-Golgi, whereas the later metabolic stages mostly take place in the trans-Golgi and its corresponding organelle network [51]. The activity of glycosyltransferases is specifically regulated by their locations on the particular tissue/organ at the transcriptional level [52], leading to their tissue-specific gene expressions, which could be exemplified with the essential expression of GM2/GD2

synthase in embryonic tissue and adult brain, whereas the GM3 synthase is universally present in all human tissues [53-54]. In addition, post-translational modifications also regulate the biosynthesis of four series of gangliosides catalyzed by glycosyltransferase via processes including N-glycosylation, phosphorylation, and dephosphorylation, as protein kinases A and C are capable of activating the activity of GM2/GD2 synthase while inhibiting the others [55-57].
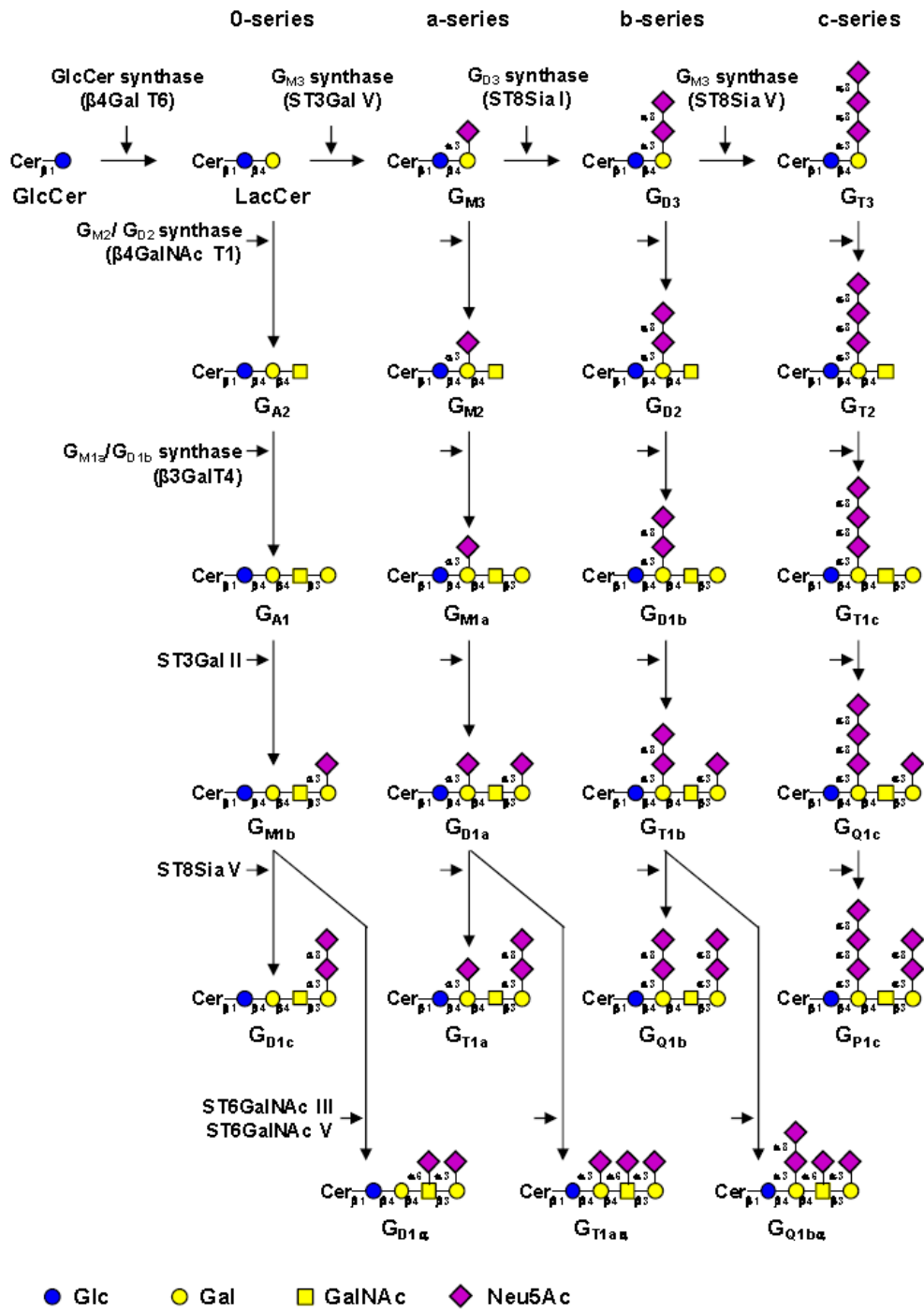
Fig.3 The stepwise biosynthetic pathways for four ganglioside series [68].

1.4 Gangliosides' Interactions with Receptor Tyrosine Kinase (RTK)

Gangliosides are a big family of mono- and poly-sialylated glycosphingolipids that present abundantly at the outer leaflet of plasma membrane of neuron and gial cells. They exert the majority of their physiological functionalities by either directly or indirectly interacting their carbohydrate moieties, which are being placed on an orientation that is protruding outward into the extracellular matrix, with the extracellular domain of a wide variety of receptor tyrosine kinases, including including epidermal growth factor receptor (EGFR) [58], platelet-derived growth factor receptor (PDGFR) [59-62], fibroblast growth factor receptor (FGFR) [63], nerve growth factor receptor (TrkA) [64-66], and insulin receptor (IR) [67], as reviewed previously by S. Julien et al. [68]. Receptor tyrosine kinases (RTK) are proteins that critically involved in the receptor-mediated biological regulation of numerous essential cellular events, such as cell survival, proliferation, differentiation, migration, and adhesion. Up-to-date, 58 types of RTKs have been totally identified and characterized in humans, all of which are sharing high degree of structural homologies that consisting of three characteristic regions, an extracellular domain containing the ligand-binding site, a featured transmembrane domain, and a cytoplasmic domain containing the entire biological machinery of tyrosine kinase. In general, RTKs are activated upon the binding of the ligands to induce the dimerization of the extracellular receptor domain, which is usually followed by the subsequent autophosphorylation of the cytoplasmic domain on the tyrosine residue to activate the downstream signaling cascade. RTKs are predominantly localized in GEMs along with other integral lipid raft components, such as gangliosides, choresterols, and sphingomyolins, and associated proteins, such as

integrins, tetraspanins, and plexins, to maintain organization and integrity of the GEMs. Within the GEMs, depending upon the expression pattern of gangliosides, cell type, and experimental conditions, the signaling of RTKs can be positively/negatively regulated by gangliosides through either direct or indirect interactions as summarized in Fig.4 [69-70]. Furthermore, alterations on the expression patterns and levels of gangliosides are implicated in regulating the activity of RTKs by reorganizing/excluding RTKs from GEMs to affect their relative abundance to be presented in GEMs through modifications on molecular composition and 3-dimensional structure of GEMs [71-73]. Finally, the presence of gangliosides was also demonstrated to be one of the determining factors for the crosstalk between RTK subunits and other lipid raft associated proteins to be physiologically undertaken and appropriately regulated.

Based on previous studies, three different mechanisms have been identified and described to be critically involved in the regulation of RTKs by gangliosides: the regulation on the degree of ligand binding to the targeted receptors by gangliosides as in the case of fibroblast growth factor (FGF)/GM1 interaction where the binding of FGF to fibroblast growth factor receptor (FGFR) is down-regulated by increased level of GM1, the regulation of receptor dimerization as in the case of epidermal growth factor receptor (EGFR)/GM3 interaction where the usual dimerization of EGFR upon the binding of epidermal growth factor (EGF) is inhibited in the presence of GM3 to prevent the tyrosine residue from being subsequently phosphorelated, and the regulation of RTKs activity by affecting their localization in GEMs as in the case of IR/GM3 where the degree of insulin receptor (IR) delocalization in GEMs is up-regulated to reduce the ligand/receptor

interaction by decreasing binding sites from IR available for the binding of insulin in the presence of GM3 in GEMs [69, 74]. Generally, these three mechanisms predominantly account for the majority of known interactions and pathways through which the signaling and activity of RTKs could be potentially regulated by gangliosides.
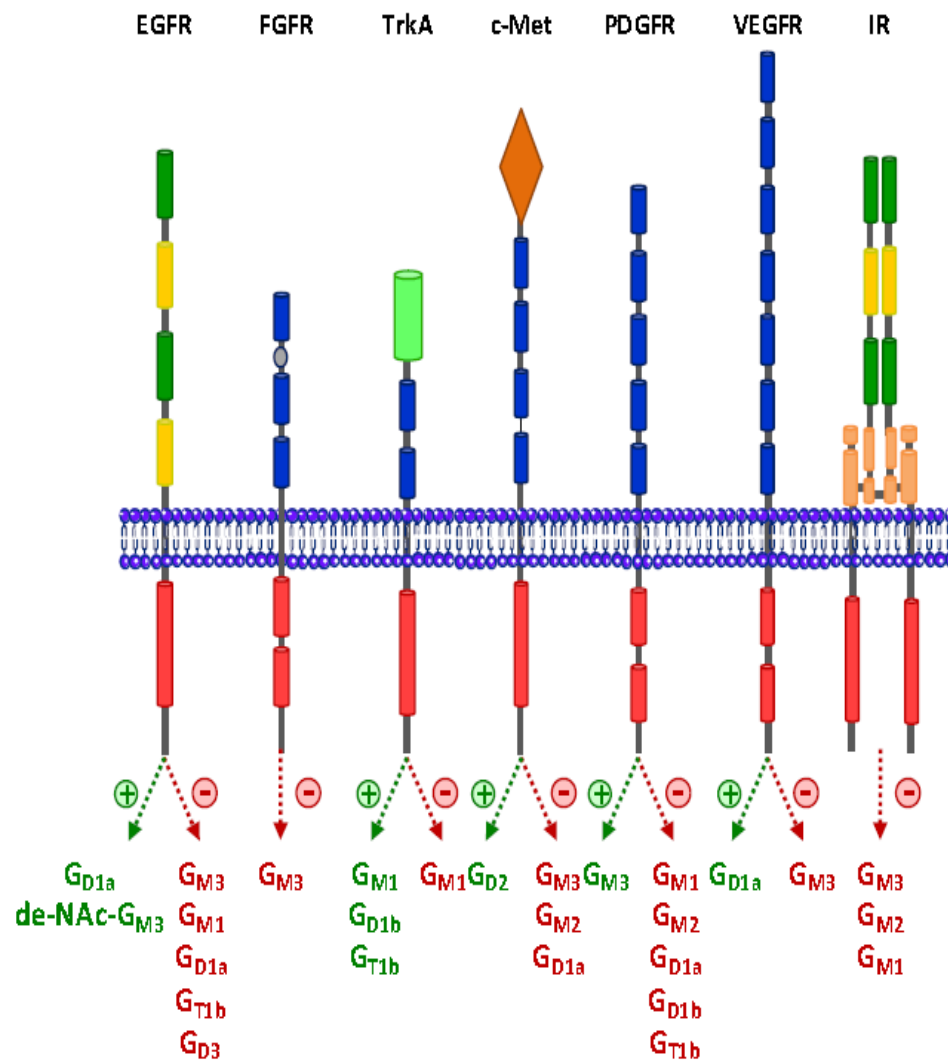
Fig.4 Biological regulations on different RTKs by gangliosides [68].

1.5 Ganglioside GM3 Synthase Deficiency (GSD)

GM3 synthase, or so-called lactosylceramide α-2, 3-sialyltransferase, is a ubiquitously expressed protein that is predominantly localized in the Golgi apparatus and presented at particularly high abundance in the central nervous system of vertebrates. This protein is responsible for catalyzing the enzymatic transferring of Neu5Ac onto the lactosylceramide backbone via α-2, 3-glycosidic linkage to generate GM3 ganglioside, which is the first step in the de-novo biosynthesis of complex ganglioside species a- and b-series as shown in Fig.5. This enzyme is encoded by ST3Gal5/SIAT9 gene which is located on chromosome 2p11.2 and composed of 14 exons that encoding a 419 amino acid protein in human with minor variance in the amino acid sequence among different species. The full-length GM3 synthase protein is found to be containing three characteristic regions, a single short transmembrane region and two sialyl motifs, long and short, each of which possesses a highly-conserved cysteine residue that has been demonstrated by previous studies to be essential for the enzyme to be able to exert its normal catalytic activity for the biosynthesis of GM3 ganglioside [75].

Recently, a single-nucleotide polymorphism has been identified and described in a large Old Order Amish pedigree, which is believed to be associated with an autosomal recessive mutation occurred on the GM3 synthase to affect its normal catalytic activity, leading to the complete loss-of-function on the enzymatic activity of GM3 synthase and thus premature biosynthetic termination of GM3 ganglioside in the individual affected by homozygous mutation. Based on previous studies, this autosomal nonsense mutation has been found to be geographically located at the exon 8 of ST3Gal5/SIAT9 gene right

between the long and short sialyl motifs and genetically associated with a single nucleotide substitution on original C base in unaffected individuals to T base in homozygous mutants at position 694 of the genome, which predictably switches the original "CGA" codon representing the translation of amino acid arginine to mutated "TGA" codon representing the immediate termination of the translation machinery, leading to the untranslated short sialyl motif on the mutated protein with truncated primary sequence, the complete loss of enzymatic capability of catalytically converting lactosylceramide to GM3 ganglioside on the functional protein, and thus the premature biosynthetic termination of GM3 ganglioside in individuals affected by this homozygous autosomal recessive mutation.

Unfortunately, as shown by the biosynthetic pathway of gangliosides in Fig. Ⅴ, since the biosynthesis of GM3 ganglioside essentially serves as the initial step for the stepwise de-novo biosynthesis of more complex a- and b-ganglioside series from lactosylceramide, the enzymatic dysfunction on GM3 synthase not only terminates the biosynthesis of GM3 ganglioside itself, but also physiologically depletes the formation of more complex a- and b-ganglioside series in the biological system of homozygous mutants, which has been found to be clinically associated with devastating manifestations as the phenotypes accounted for this unique mutation. The syndrome resulted from this autosomal recessive mutation has been illustrated by previous studies to be an infantile-onset symptomatic epilepsy that characterized by the development of cortical blindness, choreoathetosis, flaccid weakness, seizures, profound developmental stagnation, and become completely caregiver dependent as its primary clinical outcomes [35, 76-77].

However, although the evidence on casual role played by altered ganglioside

levels in the etiology of this severe infantile-onset neurological disorder and implicate genes involved in ganglioside biosynthesis as candidates for causing other related human diseases has been well documented, the actual cause on whether the epileptic phenotype is attributable to either the physiological deficiency of GM3 and its downstream derivatives or the accumulation of ganglioside biosynthetic precursors caused by lack of flux through the a- and b-series ganglioside pathways remains to be further elucidated and understood by future studies.
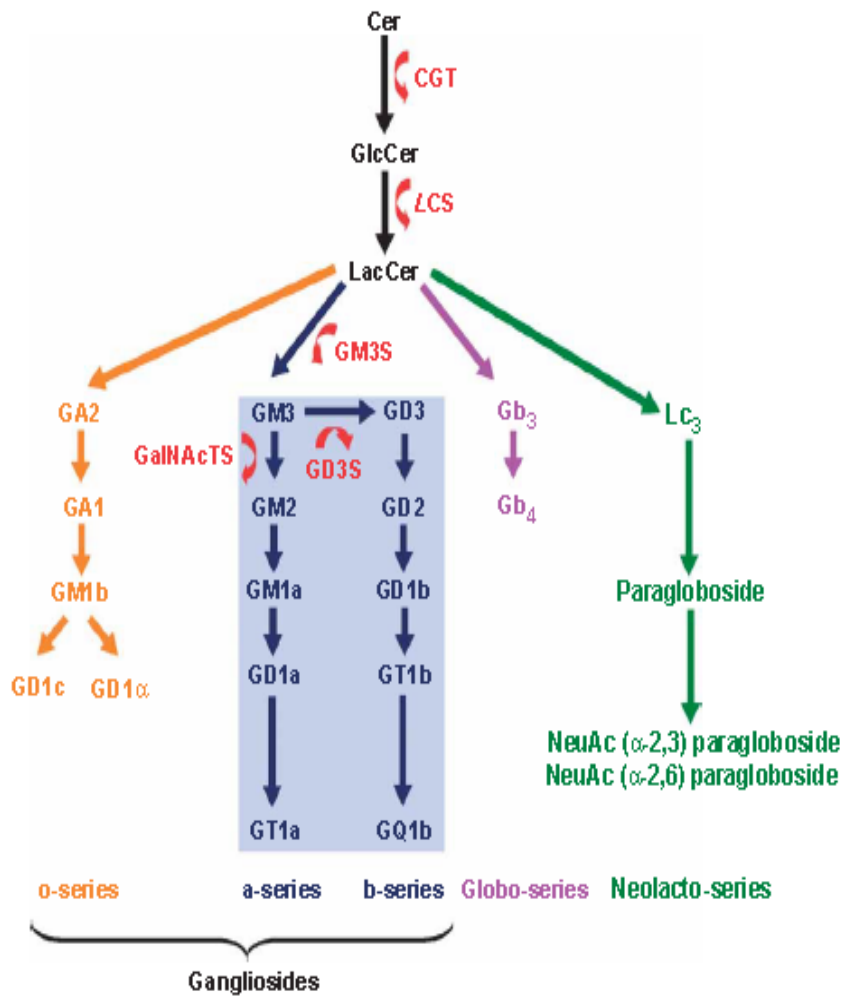
Fig.5 Ganglioside biosynthesis and GM3 synthase deficiency.

The darkened box indicates all gangliosides that cannot be synthesized in the absence of GM3 synthase. CGT: Ceramide glucosyl transferase; LCS: LacCer synthase; GM3S: GM3 synthase; GD3S: GD3 synthase; GalNAcT: GalNAc transferase. The major glycosphingolipid (GSL) are color coded and the major ganglio- (blue; a- and b-series, brown; o-series) and alternative globo- (pink) and neolacto- (green) series GSLs derived from the immediate precursor, LacCer, are delineated. The a- and b-ganglio series (blue) are absent in the plasma of the affected children [35].

1.6 Hypothesis

Due to the lack of effective cure for GSD, the individuals affected by this homozygous mutation seem to be continuously suffering from this condition even after our current understanding on the disease etiology has been well described and established. However, we have always believed that this neurological condition is practically treatable from the time it was identified. In fact, we have noticed a very distinguished phenomenon that the affected kids appear to be fairly normal for approximately eight weeks after their birth, which hints us to speculate that the affected children might have acquired sufficient amount of gangliosides from maternal circulation before their birth to make the symptoms remain undeveloped during the early age after birth and then gradually develop related symptoms after the endogenous gangliosides have eventually been depleted. Based on this rationale, it becomes rather reasonable that if we can replace the deficient gangliosides caused by this genetic defect in those affected kids with continuous supplement of exogenous gangliosides from handful sources, we might be able to correct their pathological stigmas and partially or even completely restore the normal ganglioside biosynthetic pathway, which might eventually lead to a potential cure.

So, in the past few years, extensive searching has been performing worldwide for the available sources of supplemental gangliosides that are compatible with our intended clinical trial. Initially, the animal tissues with relatively more abundant ganglioside content appeared to be the most enriched and accessible natural source we could find for the study. Therefore, three affected newborns were continuously given with a pork brain formula once they were diagnosed with DNA mutation analysis soon after birth.

This formula essentially provided approximately 10 mg GM3 per day, with incremental amounts given along with their weight gain. Incredibly, all infants being treated showed no early signs or symptoms of the disease except hypotonia when they were fed on the pork brain formula, although the effects seemed to be not comparably dramatic when the similar therapy was conducted on the older children. Unfortunately, all young children involved in the study started to develop an allergic reaction against the pork brain formula by 9 months of age, thus this pork brain formula-based therapy had to be discontinued on those affected kids from that point, and our searching for new ganglioside-enriched supplemental products continued to move on.

In the course of searching for new treatments, we identified a highly-concentrated and formulated dairy ganglioside product called G500 manufactured by Fonterra in New Zealand. This product primarily contains GM3 and GD3, and has been used as a nutritional supplement in normal infants in the previous study [78]. By developing a collaborative partnership with this company, the project needs of acquiring large-scale nutritional ganglioside-enriched supplement product have become practical and feasible for the subsequent clinical studies.

Considering the promising preliminary results we have observed in the younger children fed with pig brain formula, to begin with, we hypothesize that the orally administered gangliosides may be adequately absorbed and taken up into the circulation of affected patients, and utilized by their metabolic pathway. In addition, we further hypothesize that the gangliosides can be delivered across the blood-brain barrier, used by the brain of affected patients, and compensate for metabolically deficient gangliosides in

their physiological system. Finally, we hypothesize that if the absorption and utilization of administered GM3 is a reversible pathological process, some of the manifestations and symptoms associated with this neurological disorder might get partially or even completely relieved by introducing adequate amounts of GM3 into their body, particularly into the CNS, which may potentially lead to a normal life in these children when the exogenous GM3 in adequate quantity was given before they started to develop the related symptoms.

From the perspective of evaluating the effectiveness of new therapy as well as the recovery stage of studied patients, it will be very essential to periodically collect bodily fluid samples from the physiological circulations of treated patients and accordingly implement the quantitative analysis on those collected samples to determine the absolute levels of gangliosides presented in their bodily fluids as the most important criteria for assessing bioavailability and metabolism of administered exogenous gangliosides from G500. Unfortunately, the established quantitative assays with sufficient detection sensitivity and specificity to meet the stringent requirements of the clinical study have not yet been reported on the literature up to date. As a result, the need of establishing a sensitive and reliable quantitative assay dedicated for this study has been urgently demanded.

In addition, based on our current understanding on the disease etiology, this disease is essentially an inherited metabolic disorder associated with compromised GM3 biosynthetic capability and premature endogenous ganglioside depletion, implying the pattern of disease progression is very likely to be critically age-dependent and the timeframe is one of the most important determining factors pertaining to the success of the

therapy. As a result, younger patient might be subjected to shorter exposure of physiological damages associated with premature endogenous ganglioside depletion and thus standing a better chance of getting cure and recovered by the proposed therapy, enforcing the disease to be ambiguously diagnosed as early as birth. Unfortunately, despite the characterization of disease, the prevalence of the disease remains unknown, and we believe most of the affected individuals are, with no doubt, under-diagnosed due to the complex and time-consuming nature of the DNA mutation analysis. Therefore, a rapid quantative method with promising sensitivity, specificity, and ruggedness dedicated for the early-on newborn screening and clinical diagnosis of GSD is also in urgent need.

Hence, the projects aiming at developing quantitative method in human plasma for rapid clinical screening and on-birth diagnosis of GSD by UPLC/MS/MS, developing highly-sensitive method for quantification of monosialogangliosides in biological fluids UPLC/MS/MS, and elucidating the potential efficacy and efficiency of the G500 therapy with intended clinical studies by a combination of different methodologies have been described in detail in the following chapters.

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CHAPTER Ⅱ

DEVELOPMENT AND VALIDATION OF UPLC/MS/MS METHOD IN HUMAN

PLASMA FOR RAPID SCREENING AND DIAGNOSIS OF GSD

2.1 Introduction to Current Diagnostic Method of GSD and High Performance Liquid Chromatography Interfaced to Mass Spectrometry (LC-MS)

Currently, the molecular-based DNA mutation analysis has been the only commercially available method for diagnosing individuals affected by this unique nonsense mutation associated with the pathogenesis of GSD. Since the time when the etiology and pathology of GSD were described by Simpson et al [1] in 2004, with the assistance of the molecular-based DNA mutation analysis, more than 50 patients have been diagnosed to be affected by GSD from the Amish population around the US. Recently, cases with positive diagnosis of GSD affection have also been reported in other populations, including African American and European[2,3], suggesting that the actual prevalence of GSD in the US as well as worldwide might be much broader than we speculated and have been highly

underestimated. As a result, the practical need of implementing a screening and diagnostic study has been extremely urgent for the precise determination of actual prevalence of GSD. However, based on the GSD cases reported in African American as well as European population, it has been discovered that the occurrence of those cases is etiologically associated with alternative homozygous nonsense mutation 862C>T located at exon6 of ST3Gal5/SIAT9 in European, whereas another homozygous missense mutation 994G>A located at exon7 was identified in cases from African American, both of which are known to be capable of causing the major enzymatic dysfunction of GM3 synthase. With the increased number of mutations that determined to be associated with the pathogenesis of GSD, the workflow of molecular-based DNA mutation analysis could be potentially complicated, which might significantly reduce the analytical throughput to complete the screening and diagnosis of GSD, making this molecular diagnostic technique targeting DNA mutation being no longer suitable for implementing this study in a very efficient and dynamic fashion. So, the development of alternative methods with desired sensitivity, specificity, and throughput has been urgently demanded by this particular study.

HPLC is a chromatographic separation technique where the sample is forced by a liquid at high pressure (the mobile phase) through a column that is packed with a stationary phase generally composed of irregularly or spherically shaped particles chosen or derivatized to accomplish particular types of separations. HPLC methods are historically divided into two different categories based on stationary phases and the corresponding required polarity of the mobile phase. Use of octadecylsilyl (C18) and related organic-modified particles as stationary phase with pure or pH-adjusted water-organic

mixtures such as water-acetonitrile and water-methanol are used in techniques termed reversed phase liquid chromatography (RP-LC). The materials such as silica gel as stationary phase with neat or mixed organic mixtures are used in techniques termed normal phase liquid chromatography (NP-LC). RP-LC is most popularly used technique to introduce samples prior to MS detection in LC-MS instrumentation.

Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio of charged particles. It is generally used for measuring masses of particles, determining the elemental composition of a sample, and elucidating the chemical structures of molecules. In general, MS instrumentations consist of three modules, a ionization source, a mass analyzer, and a detector. In this setup, the ionization source enables the direct transformation of molecules in gaseous/liquid phase into gaseous ions that being detectable by the mass analyzer. Within the mass analyzer, the gaseous ions would be separated and analyzed based on their mass-to-charge ratios through a wide variety of mechanisms, such as quadrupole mass filter and magnetic mass sector, to allow subsequent detection by the detector. In the course of detection, the detector magnifies the ion counts into the electronic flow and generates a data set correlates the mass-to-charge ratio to the intensity of electronic flow, which is known as mass spectrum.

Recently, the emerging and advance of electrospray ionization technique, which rapidly desolvates and ionizes molecules directed from liquid flow into gaseous phase with minimal degree of induced in-source decay, has given rise to the combination of HPLC to MS that physically incorporates the separation capabilities of HPLC into the mass analysis capabilities of MS to achieve the on-line separation and simultaneous detection of

different molecules in very complex sample mixture. With the advent of LC-MS, the excellent quantitative perform featured by promising sensitivity and resolution for the determination of gangliosides has been enabled to achieve analysis with small sample requirement and high throughput. Gu et al. established a method for simultaneous quantification of GM1 and GM2 gangliosides in human cerebrospinal fluid using reverse phase LC/MS [4]. Sorensen et al. reported a liquid chromatographic approach interfaced with tandem mass spectrometry for the quantification of gangliosides GD3 and GM3 in bovine milk and infant formula [5].

However, LC/MS/MS methods established for plasma samples, one of the handiest biological samples in terms of collection, with desirable speed of analysis for the study has not been reported by previous studies. As a result, in the present work, we have developed and validated a UPLC/MS/MS method for the measurement of four common ganglioside species including GM2, GM3, GD2, and GD3 in human plasma to facilitate the early-on newborn screening and clinical diagnosis of GSD. This assay employs a simple protein precipitation strategy for sample extraction, UPLC for chromatographic separation, and tandem mass spectrometry in the MRM mode for detection to achieve high sensitivity and specificity for rapid analysis. Moreover, due to the limitation of availability of analyte-free plasma, a three-level standard addition calibration rather than seven-level conventional calibration was utilized to establish the matrix-matched analytical platform as required by the FDA for the quantification of small molecules in biological fluids. This method has been successfully applied to measurements of these four common ganglioside species in plasma samples from normal adults. Considering the sensitivity, specificity, and

quickness of this method, we think it is very suitable for the rapid clinical screening and diagnosis of GSD as well as other ganglioside-related disorders during the early stage of the disease development.

2.2 Materials and Methods

2.2.1 Materials

Ganglioside standards GM3 and GD3 were purchased from Avantilipids (Alabaster, AL). GM2 and GD2 ganglioside standards were obtained from EMD Chemicals (Billerica, MA) and Enzo Life Sciences (Farmingdale, NY), respectively. The internal standard (IS) N-omega-CD3-Octadecanoyl monosialoganglioside GM3 (GM3-D3) was purchased from Matreya LLC (Pleasant Gap, PA). HPLC grade methanol and isopropanol were obtained from EMD Milipore (Billerica, MA). Deionized water was prepared through the Barnstead Nano-PURE Water Purification System (Asheville, NC). Ammonium acetate was from VWR (Bridgeport, NJ).

2.2.2 Human Plasma

The study was approved by the DDC Clinic Institutional Review Board, and written informed consent was obtained from their legal guardians. Clinical information was collected when the patients received medical services at DDC Clinic. Human plasma samples

were stored at -20℃before analysis.

2.2.3 Stock and Working Solutions

Stock solutions of GM2, GM3, GD2, GD3, and IS $^2D_3$-GM3 were prepared by dissolving the original materials individually into 80% MeOH to obtain a concentration of 0.25 mg/ml for each, and were stored at -20 ℃ before use. Three working solutions were prepared in 80% MeOH from the stock solutions to obtain ganglioside concentrations as following: 3, 13.6, 0.125, 2 μg/ml (WS-QC); 4.5, 20.4, 0.125, 3 μg/ml (WS-1), and 18, 81.6, 0.5, 12 μg/ml (WS-2) for GM2, GM3, GD2, GD3, respectively. The IS stock solution also served as a working solution, named as WS-IS. These working solutions were used for preparation of spiked plasma samples as below.

2.2.4 Plasma Sample Preparation

For method validation, known-concentration samples were prepared by spiking ganglioside standards into the analyte-free plasma from patients with GM3 Synthase Deficiency. In brief, 30 μL of analyte-free plasma was transferred into each of three 0.6-mL centrifuge vials (a, b, and c) followed by individual addition of 10 μL WS-QC to obtain the plasma concentrations of 1.00, 4.53, 0.04, and 0.67 g/ml for GM2, GM3, GD2, and GD3, respectively. Thereafter, 10 μL of 80% methanol, WS-1, and WS-2 were spiked into the vials a, b, and c, respectively. Afterwards, 10 μL of WS-IS was spiked into each of the three samples, and this set of samples were named as spiked analyte-free plasma (SAP), Standard

Addition 1 (SA-1), and Standard Addition 2 (SA-2), respectively.

For measurement of the gangliosides in unknown plasma samples from normal human subjects, similar procedure as described above was followed except that WS-QC was not added. The three samples in each set were named as unknown plasma sample (UPS), SA-1 and SA-2, respectively. All the plasma samples were extracted as described below prior to LC/MS/MS analysis.

2.2.5 Sample Extraction

Following the plasma sample preparation, 240 μL of methanol was added into each of them followed by vigorous vortexing. Then, the spiked samples were subjected to centrifugation at 14,000 G for 15 min at 4 ℃ for protein precipitation. The supernatants were transferred into auto-sampler vials with micro-inserts for LC/MS analysis.

2.2.6 LC/MS Instrumentation

The UPLC system was composed of a DGU-20A$_{3R}$ degasser, two LC-30AD pumps, a SIL-30AC autosampler, a CTO-10A column oven and a CBM-20A system controller from Shimadzu (Columbia, MD). The UPLC system was interfaced to a Qtrap 5500 mass spectrometer equipped with an electrospray ionization source and a built-in Valco switch valve from AB SCIEX (Framingham, MA). The Multiple Reaction Monitoring (MRM) mode was utilized for quantitation. Data acquisition and chromatographic peak integration were conducted using Analysis 1.6.1 software package from AB SCIEX.

2.2.7 LC/MS/MS Procedure

After extraction, 30 μl of each sample was injected onto a Kinetex-C18 UPLC column (1.7 μm, 50 mm × 2.1 mm; Phenomenex, Torrance, CA) guarded with a C18 Security Cartridge. The mobile phase was a mixture of 2% MeOH plus 5 mM ammonium acetate (A) and MeOH-Isopropanol (1:1) plus 5 mM ammonium acetate (B) using the gradient program shown in Table 1. The LC flow at 0.3 mL/min was introduced to the waste within the first 2 minutes, and then switched to the mass spectrometer between 2.1 and 9 minutes while all the monitored ganglioside components were gradually eluted out. Afterwards, the LC flow at 0.6 mL/min was directed to the waste again, during which the column was cleaned up and re-equilibrated for next run. The column temperature was constantly controlled at 35 ℃ during the analysis.

Table 1 Optimized UPLC conditions for the separation of gangliosides

Time Minute	B%	Flow rate (ml/min)	Stage	Valco Switch Valve
0	30			
1	30			To waste
2	70		Elution	
2.01	70	0.3		To MS
9	95			
9.01	95		Cleanup	
15.5	95	0.6		
15.51	30			
19.5	30		Equilibration	To waste
19.51	30	0.3		
20.0	30			

A: 2% MeOH + 5 mM Ammonium Acetate

B: MeOH - Isopropanol (1:1) + 5 mM Ammonium Acetate

2.2.8 Calibration Curve

Quantitation of the gangliosides in the spiked analyte-free plasma (SAP) and unknown plasma samples (UPS) was implemented using standard addition calibration based on three levels, including one to-be-measured sample with nil addition and two standard addition calibrators (SA-1 and SA-2). For each measured ganglioside specimen, the total peak area (TPA) was obtained by summing the individual peak area from every MRM transition channel in the corresponding sample from a single injection.

The three-level calibration curve for each ganglioside sample was then established by plotting its normalized TPA by the internal standard as y-axis versus the plasma concentration of standard addition as x-axis. The linear regression with a weighting factor of $1/y^2$ was employed. The calculation of each ganglioside plasma concentration in a sample was conducted using the corresponding equation obtained from the regression line as its intercept with x-axis through extrapolation. Exemplified standard addition calibration curves for each of the four measured gangliosides from the measurement of healthy individuals were shown in Fig.6.
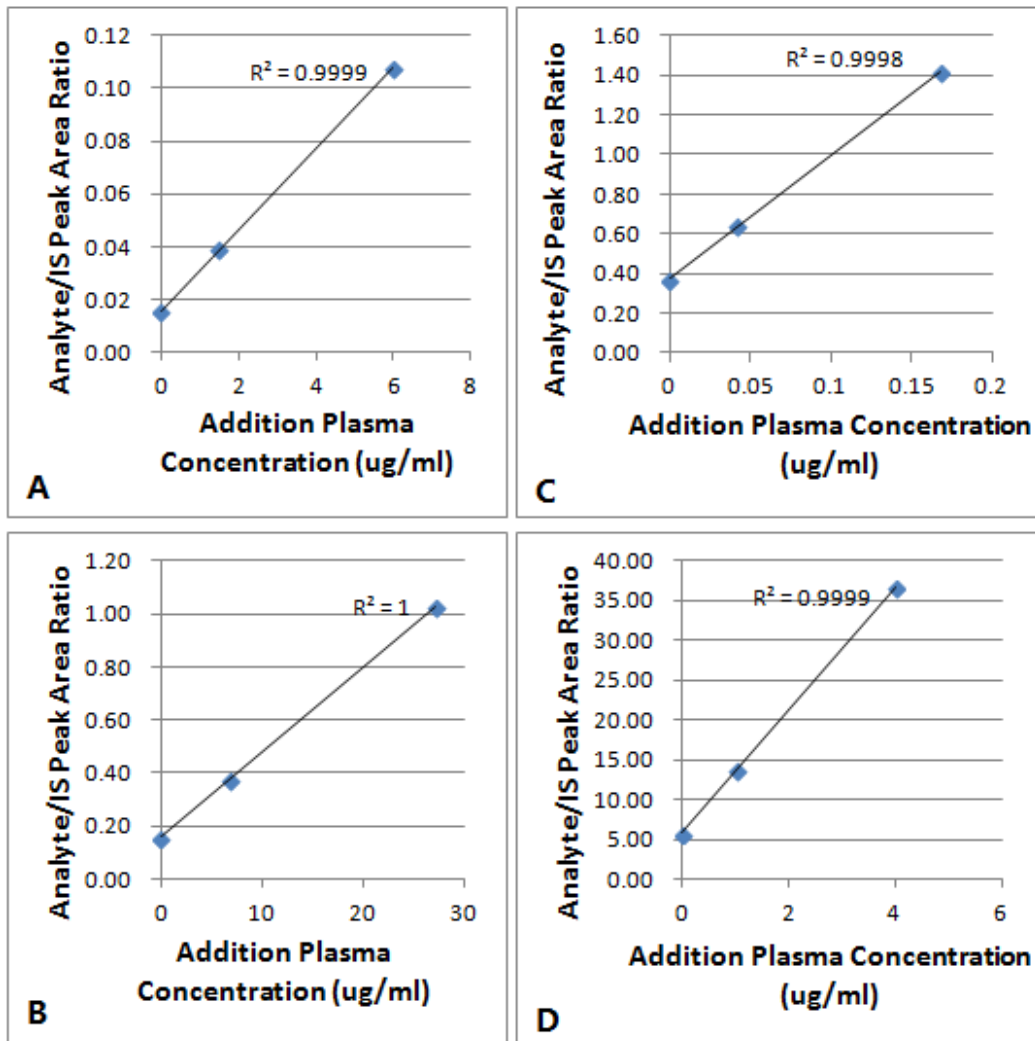
Fig.6 Exemplified calibration curves for GM2 (A), GM3 (B), GD2 (C), and GD3 (D)

from the measurement of a healthy subject.

2.3 Results and Discussions

2.3.1 Product Ion Mass Spectra

The representative product ion mass spectra of D18:1-20:0 ganglioside components for the four ganglioside species from added standards were shown in Fig.7. Apparently, fragmentation pattern relevance can be observed within GM series and GD series, including the common product ions m/z = 274 and 292 for GM series, and m/z = 290 and 581 for GD series. Owing to their relatively greater molecular masses, the singly-charged ions of gangliosides are beyond the detection ranges of our mass spectrometer and many other commercially available triple quadruple instruments. Therefore, the selection of doubly-charged precursor ions and their appropriate daughter ions as MRM transition channels has been necessitated for quantitation in this assay.

For GD series, two proton-donating carboxyl groups are attached to their sialic acid moieties in the carbohydrate portion, the generation of doubly-charged ions had been observed to be more favorable in the negative than that in a positive ionization mode. For GM series that contain only one proton-donating carboxyl group, the signal responses from doubly-charged precursor ions were practically undetectable in a negative ionization mode under various conditions we studied during the method development. Since the positive mode was found to be more effective in yielding doubly-charged precursor ions from GM2 and GM3, positive ionization has been selected for determining GM series.
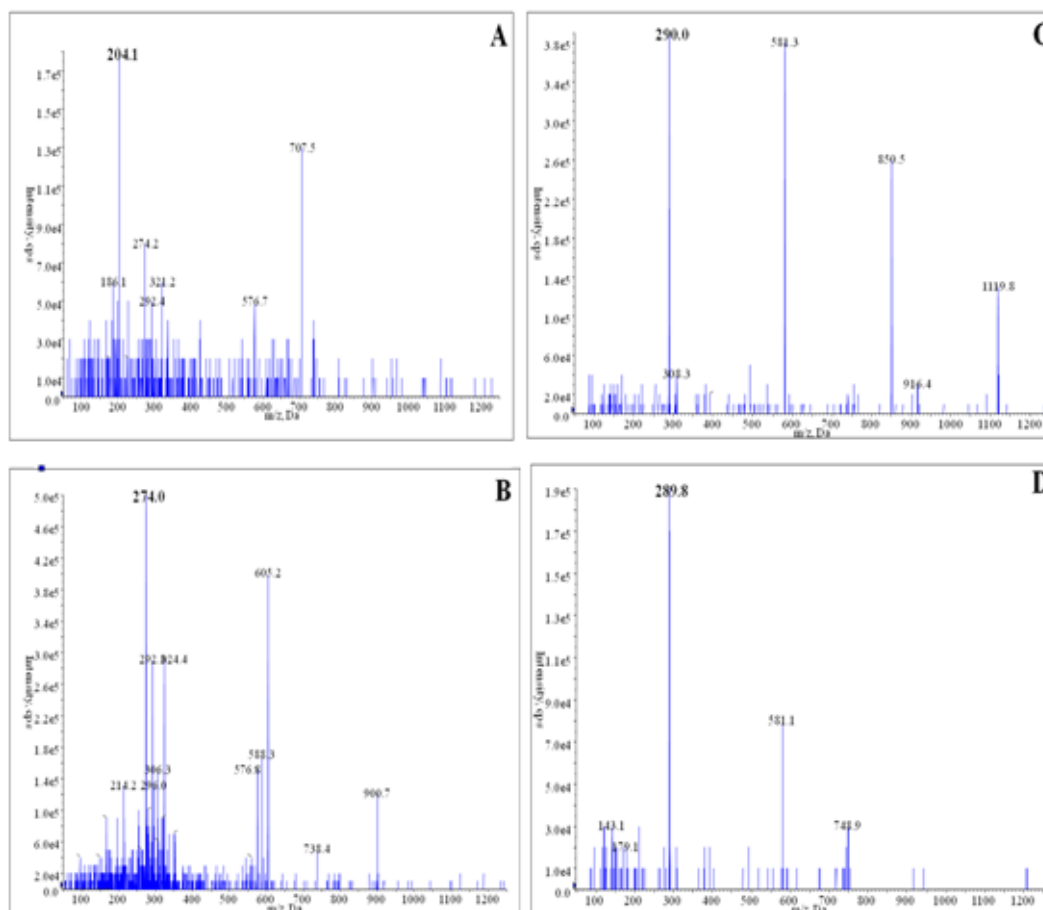
Fig.7 The product ion (MS/MS) spectra for D18:1-20:0 components of GM2 (A), GM3 (B), GD2 (C), and GD3 (D).

After evaluating different pairs of precursor $\rightarrow$ product ions for quantitation, it was revealed that the following MRM transitions provided the optimal sensitivity and selectivity: $[GM2+2H^+]^{2+} \rightarrow 204$, $[GM3+2H^+]^{2+} \rightarrow 274$, $[GD2-2H^+]^{2-} \rightarrow 290$, and $[GD3-2H^+]^{2-} \rightarrow 290$. The fragmentation mechanisms from the precursor to the daughter ions have been interpreted and elaborated in Fig.8.

2.3.2 Selection of MRM Transitions

As known, each ganglioside species include multiple components that share the same oligosaccharide moiety but differ in fatty acid chain length within their ceramide portion. For accurate quantitation, all the major ganglioside components from each ganglioside species with appreciable abundance in the plasma and commercial standards need to be detected in the analysis. Therefore, we first carried out a screening study on predominant ganglioside components of individual ganglioside species in both normal human plasma and commercial standards using the predicted MRM channels through LC/MS/MS analysis. The selected MRM transitions corresponding to all the significant ganglioside components observed by such analysis for the quantitation of the four ganglioside species are listed in Table 2. In addition to the quantitation MRM channels, quality assurance MRM channels with transitions from their precursor ions to m/z 274, 292, 290, and 290 were also employed for GM2, GM3, GD2, and GD3, respectively. Our experiments showed that the response ratio for each pair of MRM channels was practically constant in standard-spiked solvent and unknown plasma extracts, confirming the identity

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of the monitored components. The optimized MS parameters for individual analytes are

given in Table 3.
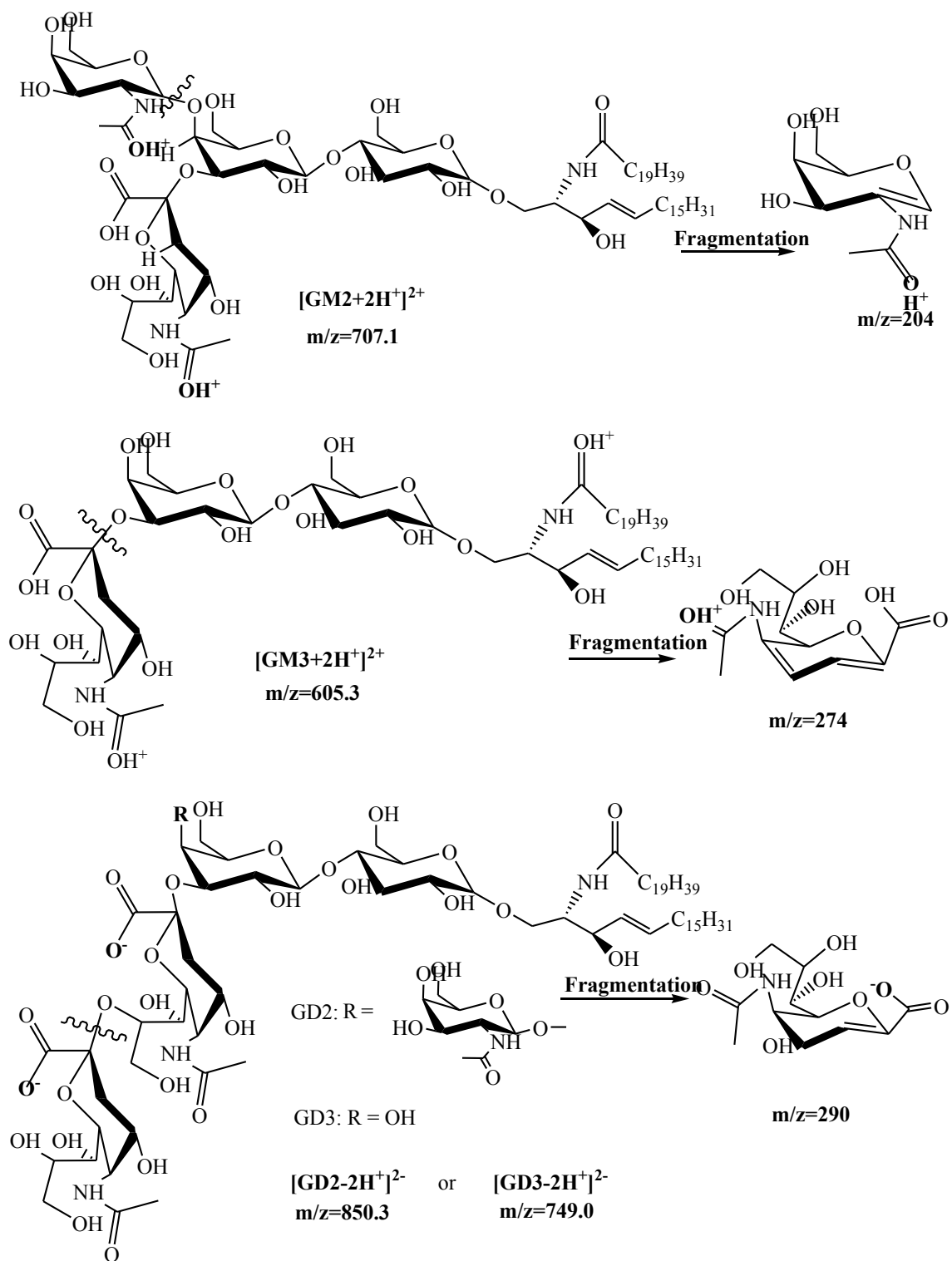
Fig.8 Proposed fragmentation mechanisms for D18:1-20:0 components of GM2, GM3, GD2, and GD3 gangliosides.

Table 2 The MRM transitions for quantification

Positive Mode			Negative Mode		
Species	**Component**	**MRM Transition**	**Species**	**Component**	**MRM Transition**
GM2	D18:1-14:0	665.1/204	GD2	D18:1-14:0	808.3/290
	D18:1-16:1	678.1/204		D18:1-16:0	822.3/290
	D18:1-16:0	679.1/204		D18:1-18:1	835.3/290
	D18:1-18:1	692.1/204		D18:1-18:0	836.3/290
	D18:1-18:0	693.1/204		D18:1-19:0	843.3/290
	D18:1-20:0	707.1/204		D18:1-20:0	850.3/290
GM3	D18:1-12:0	549.3/274		D18:1-21:0	857.3/290
	D18:1-14:0	563.3/274		D18:1-22:0	864.3/290
	D18:1-16:1	576.3/274	GD3	D18:1-12:0	693.0/290
	D18:1-16:0	577.3/274		D18:1-14:0	707.0/290
	D18:1-18:1	590.3/274		D18:1-15:0	714.0/290
	D18:1-18:0	591.3/274		D18:1-16:1	720.0/290
	D18:1-20:0	605.3/274		D18:1-16:0	721.0/290
	D18:1-21:0	612.3/274		D18:1-17:0	728.0/290
	D18:1-22:0	619.3/274		D18:1-18:1	734.0/290
	D18:1-23:0	626.3/274		D18:1-18:0	735.0/290
	D18:1-24:0	633.3/274		D18:1-19:0	742.0/290
				D18:1-20:0	749.0/290
				D18:1-21:0	756.0/290
				D18:1-22:0	763.0/290
				D18:1-23:0	770.0/290
				D18:1-24:0	777.0/290
				D18:1-25:0	784.0/290

Table 3 Optimized MS parameters for individual ganglioside species

Analyte MS Setting	Positive Mode		Negative Mode	
	GM2	GM3	GD2	GD3
Curtain Gas (Psi)	35.0	35.0	35.0	35.0
Collision Gas (Psi)	High	High	High	High
IonSpray Voltage (eV)	5400.0	5400.0	-4300.0	-4300.0
Temperature (℃)	500.0	500.0	600.0	600.0
Ion Source Gas 1 (Psi)	45.0	45.0	45.0	45.0
Ion Source Gas 2 (Psi)	45.0	45.0	45.0	45.0
Declustering Potential (eV)	65.0	65.0	-39.7	-45.7
Entrance Potential (eV)	7.0	7.0	-10.0	-10.0
Collision Energy (eV)	25.0	25.0	-50.0	-47.0
Collision Cell Exit Potential (eV)	20.0	20.0	-23.6	-21.5

2.3.3 HPLC Conditions

Since multiple MRM transitions have been exploited to monitor each of the four ganglioside species, a sufficient chromatographic separation between ganglioside molecules and plasma interferences is essential to eliminate the potential ionization suppression influence from the matrix for the acquisition of high quality quantification during the analysis. Based on our preliminary studies during the method development, a C18 reverse-phase UPLC column was found to provide the best resolution for multiple ganglioside components. Under our optimized LC conditions, all the ganglioside components were eluted out and spread within a retention time window from 3 to 9 minutes. In general, gangliosides could be strongly retained by the C18 stationary phase due to the hydrophobic nature of their ceramide portion. We observed that the use of Methanol-Isopropanol (1:1) as the solvent of mobile phase B could elute the gangliosides from the C18 stationary phase more effectively than the use of methanol only, and lead to shorter chromatographic running time and better peak shapes. The MRM chromatograms for the major components of GM3 ganglioside are shown in Fig.9.

It is noteworthy that the addition of 5 mM ammonium acetate in the mobile phases considerably reinforced the peak intensities from all MRM channels in both positive and negative ionization modes. This experimental phenomenon can be reasonably ascribed to ammonium acetate's ability to stabilize the pH at neural range, enabling both the proton-donating interaction between ammonium group and gangliosides in the positive mode and proton-accepting interaction between acetate group and gangliosides in the negative mode to be undertaken for the promotion of ionizing efficiency during the

electrospray ionization. While ammonium formate or formic acid was added into the mobile phases, the responses from positive ionization was enhanced to a certain extent, whereas substantial signal suppression was occurred in the negative ionization, which was apparently attributable to intensified proton-donating tendency of formic acid and ammonium formate as compared with ammonium acetate.
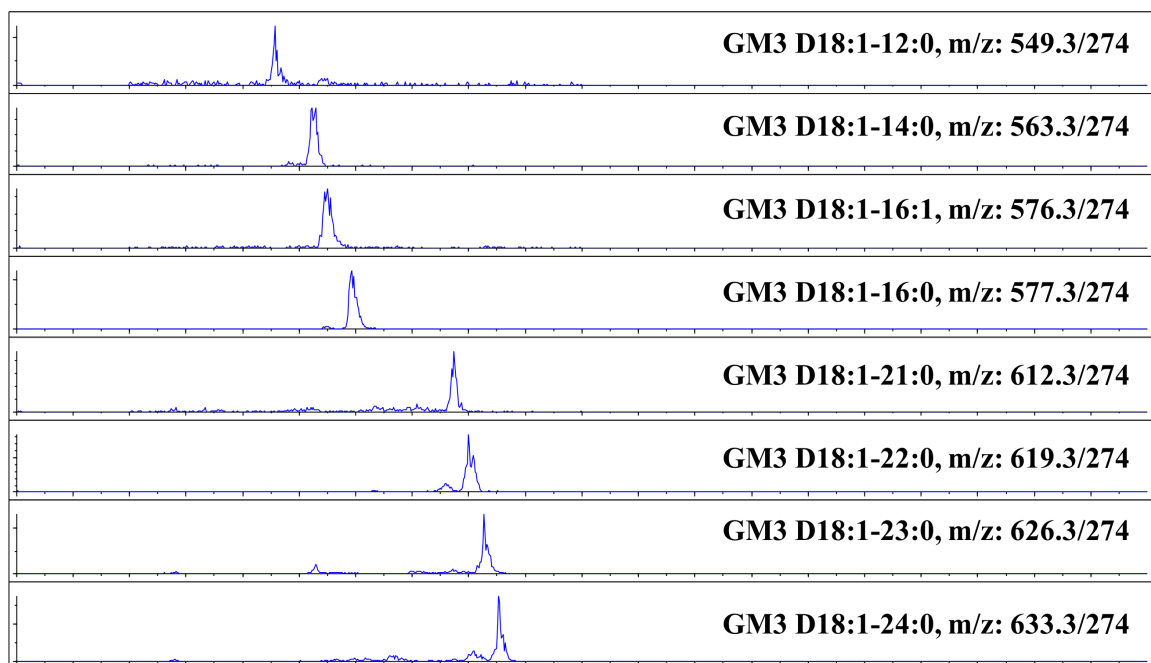
Fig.9 Representative chromatograms for major components of GM3 in plasma extract from a healthy subject.

2.3.4 Sample Extraction Procedure

The most commonly used method for the isolation of gangliosides from biomatrices is a liquid-liquid partition strategy that first reported by Svennerholm and Fredman [6] for the treatment of human brain samples, which has been used or modified later by other investigators [7, 8] for extracting gangliosides from milk and other matrices. In brief, the workflow is constructed by the steps as following: (a) mixing the biomatrix with chloroform/methanol/water (4:8:3) by vigorous shaking; (b) transferring the ganglioside-enriched methanol/waster upper phase into a new container without disturbing the middle and lower phase; (c) drying the crude extract with inert gas purging, and (d) reconstituting the residues with methanol to a certain volume for quantitative analysis. To compare the extraction recovery by using our simple protein-precipitation procedure and the existed extraction method, we performed a series of parallel studies. In these assays, analyte-free plasmas were spiked with the gangliosides followed by extraction with two different extraction methods. As expected, our protein precipitation method was not only easy-handling and time-saving, but also provided full recovery for all of the four ganglioside species (Table 4). In contrast, the reported method generated insufficient recovery for GM2 and GM3 gangliosides. According to the literatures, protein-precipitation from plasma samples was commonly conducted by the addition of 3 to 4-fold of methanol or acetonitrile, followed by high speed centrifugation. However, the widely adopted condition generated unsatisfactory recovery of gangliosides from plasma. Based on modest polarity of gangliosides from their hydrophilic oligosaccharide moiety and hydrophobic ceramide portion, we attempted to achieve the best extraction recovery through adjustment

of the polarity by increasing portion of methanol. Nearly 100% extraction recovery was achieved for all of the four ganglioside species by the addition of 8-fold methanol into plasma for protein precipitation.

Table 4 Comparison of extraction recoveries between the reported method and our

protein-precipitation procedure (n=3)

Species	Extraction Recovery (%)			
	Protein-precipitation procedure		Reported method	
	Recovery (Mean ± SD) (%)	CV (%)	Recovery (Mean ± SD) (%)	CV (%)
GM2	100 ± 8	7.83	66 ± 6	9.70
GM3	94 ± 7	7.93	41 ± 4	9.12
GD2	106 ± 9	8.69	103 ± 5	4.46
GD3	99 ± 5	4.98	102 ± 9	9.23

2.3.5 Standard Addition Calibration

According to the FDA guidelines for bioanalytical method validation [9], the same type of matrix as actual samples should be used for the preparation of calibrators and QCs during the quantitative method development and validation whenever feasible concerning the potential pronounced signal interference imposed by the complex matrices. For the determination of exogenous compounds that do not present in intended matrices, the conventional standard calibration is usually employed to generate calibration curves based on six or more levels of analytes spiked into analyte-free matrices. However, for the measurement of endogenous compounds that originally present in the sample biomatrices at significant concentrations, the standard addition calibration has been widely used for the establishment of matrix-matched calibration. Commonly, each standard calibration curve is constructed upon a set of 3-4 different concentrations prepared by addition (including a nil addition) of known amounts of analyte standard into aliquots of a sample to be measured. Recent publications involving the application of standard addition calibration include the quantification of bile acids [10], sialic acids [11], gangliosides [5, 12], and other endogenous compounds [13, 14, 15] in various biomatrices. In most of these studies, including determination of gangliosides in milk and buttermilk, three or four points were used to construct the calibration curves.

As indicated earlier, one of our objectives for the development of this assay is to screen the ganglioside levels in plasma from patients with GM3 synthase deficiency during clinical treatments. We have chosen three-level standard addition upon such a consideration that these patients are mostly children or even infants, and that they can only

provide small amounts of plasma for the study. As shown in Fig.6, the exemplified calibration curves had R^2 values in a range of 0.9998 - 1 for all four ganglioside species, demonstrating a good linear relationship between the analyte response and concentration. For the method validation, we prepared known-concentration samples by spiking ganglioside standards into aliquots of analyte-free plasma from GSD patients. Analysis of such known samples has shown that our assay employing the three-level standard addition offered satisfactory precision and accuracy for the measurement of all the four ganglioside species in plasma, which is detailed below.

2.3.6 Precision and Accuracy

The precision and accuracy for both intra-assay and inter-assay were assessed by measuring analyte-free plasma samples spiked with the ganglioside standards in triplicate. As shown in Table 5, the CV % for intra- and inter-assay of all measured ganglioside species was within a range from 0.4 to 6 % with the relative error below 9 %. Conclusively, the precision and accuracy of our quantitative method well fulfill the 15 % error and variation tolerance as required by the FDA guidelines for bioanalytical method validation [9].

Table 5 Intra- and inter-assay precision and accuracy for the measurement of

gangliosides spiked in analyte-free human plasma from GSD patients (n=3)

Species	Spiked conc (ug/ml)	Intra-assay			Inter-assay		
		Measured (Mean±SD) (ug/ml)	Precision (CV %)	Accuracy (R.E. %)	Measured (Mean±SD) (ug/ml)	Precision (CV %)	Accuracy (R.E. %)
GM2	1.00	0.99±0.025	2.52	-0.60	1.04±0.059	5.67	4.45
GM3	4.53	4.45±0.019	0.42	-1.81	4.60±0.156	3.40	1.50
GD2	0.04	0.05±0.002	3.82	8.45	0.04±0.002	3.27	7.02
GD3	0.67	0.69±0.043	6.13	3.82	0.70±0.040	5.75	4.35

2.3.7 Stability

The pre- and post- extraction stabilities of gangliosides in plasma under various storage conditions were evaluated via the analysis of samples prepared from the analyte-free plasma spiked with the gangliosides after undergoing the studied storage conditions, and the results are listed in Table 6. The stability of "4-hour pre-extraction storage at room temperature" was assessed by spiking gangliosides into the thawed analyte-free plasma, letting the spiked samples stand at room temperature for 4 hours, and then analyzing the samples via LC/MS immediately following the extraction procedure. The stability of "8-hour post-extraction storage at room temperature" was evaluated by spiking gangliosides into analyte-free plasma, extracting the spiked samples, letting the extracts stand at room temperature for 8 hours, and then injecting them into LC/MS for analysis. The measured stability values for the four ganglioside species range from 86 to 110 % under three different storage conditions, including one month pre-extraction at -20 ℃, 4-hour pre-extraction at room temperature, and 8-hour post-extraction at room temperature. Apparently, the gangliosides were stable in the plasma at the stages of frozen storage, sample treatment, and analysis.

Table 6 Stabilities of gangliosides in human plasma under various storage conditions

(n=3)

Storage conditions	Recovery (Mean ± SD) (%)			
	GM2	GM3	GD2	GD3
One month pre-extraction storage at -20 ℃	104 ± 9	90.5 ± 8	84.2 ± 5	86.5 ± 4
4-hour pre-extraction storage at room temperature	96.3 ± 7	98.6 ± 2	86.2 ± 7	88.8 ± 6
8-hour post-extraction storage at room temperature	101 ± 3	106 ± 1	106 ± 5	110 ± 6

2.3.8 Matrix Effect

The matrix effect for mass spectrometry detection of gangliosides was assessed through comparable analysis of analyte-free plasma extracts spiked with ganglioside standards and the pure solvent spiked with such standards at the same contractions in triplicate. It was found that the matrix impurities substantially suppressed the ganglioside signals, and the LC/MS/MS peak areas from the plasma extracts were only 61.3, 36.0, 68.3, and 49.0% in comparison to the pure solvent for ganglioside GM2, GM3, GD2, and GD3, respectively. By using the standard addition calibration, the matrix effect practically had no influence on the measurement because the same matrix was utilized for the calibrators and the relevant samples, which was proven by the high linearity of calibration, precision, and accuracy.

2.3.9 Method Application

The validated LC/MS/MS assay has been successfully applied to determine ganglioside concentrations in human plasma from different specimens, including 20 normal human subjects and 6 GSD patients. As shown in Table 7, the averaged ganglioside levels in the plasma samples from 20 normal human subjects (include 12 males and 8 females) were 2.37, 9.72, 0.01, and 1.80 μg/ml for GM2, GM3, GD2, and GD3, respectively. In contrast, the analyte-free plasma from 6 patients with GSD had no gangliosides presented at the measurable level, which was consistent with the pathological manifestation of the neurodegenerative disorder. The averaged GM3 concentration in plasma samples from

eight healthy human subjects determined by a published LC/MS method [13] was 7.2 µg/ml, which was quite comparable to our result (9.72 µg/ml).

2.4 Conclusion

This study detailed the development and validation of a sensitive LC/MS/MS method for the determination of gangliosides GM2, GM3, GD2, and GD3 in human plasma. This method utilized a simple protein-precipitation procedure for rapid extraction of the gangliosides from the matrix, UPLC for high resolution chromatographic separation, and multiple MRM transitions for sensitive and specific mass spectrometric detection, which had been successfully applied to determine the level of gangliosides in plasma from normal human subjects. Considering the quickness, sensitivity, and specificity of this assay, we believe that it is also applicable to other potential biomatrices with some appropriate modifications.

Table 7 The ganglioside plasma concentrations from human specimens

Subjects	Total	Averaged Levels			
		GM2 (Mean ± SD) (µg/ml)	GM3 (Mean ± SD) (µg/ml)	GD2 (Mean ± SD) (µg/ml)	GD3 (Mean ± SD) (µg/ml)
Normal Adults	20	2.37 ± 0.79	9.72 ± 3.68	0.01 ± 0.01	1.80 ± 0.64
GSD Patients	6	NM	NM	NM	NM

NM – Not Measurable

2.5 Future Direction

The quantitative method established in this study has been known to be the first LC-MS/MS method for quantification of gangliosides in human plasma with full method validation under FDA guideline for bioanalytical method development. It utilizes a simple protein precipitation procedure for the analyte extraction and a matrix-matched standard addition calibration based on three levels for the measurement of analytes in the plasma matrix. However, a number of factors have practically limited this method from being broadly applied into the routine analysis in the clinical laboratory.

To begin with, depending on the sugar components present in their carbohydrate moieties, monosialoganglioside molecules generally have molar mass ranging from 1200 to 1600 Da, making the majority of their singly charged molecular ions being undetectable by our instrument (dynamic range 50~1250 m/z). Considering carboxyl group on the sialic acid residue serves as the sole ionization site on their chemical structures, their chances of undergoing dual protonation/deprotonation to generate doubly charged molecular ions have been found to be extremely difficult during the electrospray ionization, leading to their very low detection sensitivities during the analysis. So, from the clinical perspective, this method would be very promising and reliable for clinical diagnosis on the screening of ganglioside-related disorders from newborns, but not quite applicable to pharmacokinetic studies during the clinical trial where sufficient detection sensitivity from the analysis is typically required to draw solid and unarguable conclusions.

In addition, the clinical study usually requires certain degree of throughput from the analytical method to ensure collected samples would be handled in a very timely

manner and facilitate the decision-making as well as problem solving. Unfortunately, due to the nature of standard addition calibration method, a single sample is required to be analyzed repetitively in triplicate for this case in order to establish the calibration curve that dedicated for this particular sample, which is extremely laborious and time-consuming, making this method more preferable for diagnostic purpose rather than routine clinical analysis in terms of analytical throughput.

Last but not the least, the introduction of protein precipitation strategy for sample extraction significantly simplifies the experimental procedure through which the plasma sample is processed and reduces the time needed for sample preparation prior to the analysis. However, the simplicity of protein precipitation strategy might also adversely affect the quality of analysis by retaining the majority of the matrix interferences in the plasma extract along with the analyte. As can be seen during the application of this method, the increased abundance of matrix interferences in the post-extraction extract tends to accumulatively build up on the analytical column over several hundreds of injections and thus jeopardize the separation performance over time, leading to the significantly increased analytical cost as the column has to be replaced in a more frequent manner.

As a result, since this method was initially developed and validated solely for facilitating the screening and diagnosis of GSD on the upcoming newborns from the Amish families, the sample preparation method, chromatographic separation method, and the mass spectrometric detection method have been thoroughly simplified in order to accomplish the rapidness and efficiency of the analysis according to the time frame needed by clinical studies. However, the flip side of this method is that it maintains the analytical quickness of

the method while sacrifices the certain degree of the detection sensitivity and specificity, which impedes us from further applying this method to answering the major questions remaining in the ongoing clinical studies. In order to further overcome those challenging issues, improvements on the method performance are needed to take place in order to successfully circumvent the major challenges imposed by stringent requirements of clinical studies on specificity, sensitivity, and throughput of bioanalytical methodology.

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CHAPTER Ⅲ

QUANTIFICATION OF MONOSIALOGANGLIOSIDES IN HUMAN PLASMA THROUGH CHEMICAL DERIVATIZATION FOR SIGNAL ENHANCEMENT IN LC-ESI-MS

3.1 Introduction to Chemical Derivatization Methods for Sialic Acid Neu5Ac and DMTMM&PAEA-Based Amidation

Sialic acids are a big family of N-/O-substituted derivatives of neuraminic acids that characterized by the presence of nine-carbon scaffold as their structural backbone. Currently, there have been over 43 derivatives from the sialic family identified to exist freely in the nature, three of which act as the predominant species in the entire population, N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc), and 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN). Based on up-to-date knowledge, Neu5Ac has been found to be the only sialic acid species that virtually presenting in the carbohydrate terminus of glycosphingolipids, especially gangliosides. Their individual

chemical structures can be found in Fig.10. Depending on the identity of monosaccharide unit to which Neu5Ac is bound, it could be linked with neighboring monosaccharide units through either α2-3 or α2-6 linkage in gangliosides. Biologically, sialic acids usually serve as a surface clutch at the terminal position of carbohydrate moiety of glycoprotein receptors to mediate the intracellular signaling cascade triggered by extracellular ligand-binding events. Its biological role in acting as a cell surface regulator to extracellular signaling has been prevalently found in a wide variety of essential cellular events, including cell-cell recognition [1-3], cell differentiation [4-6], and antigen-mediated immunological response [7-9].
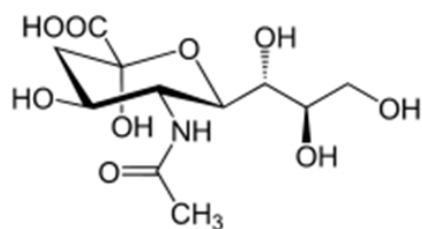
N-Acetylneuraminic acid
(Neu5Ac)
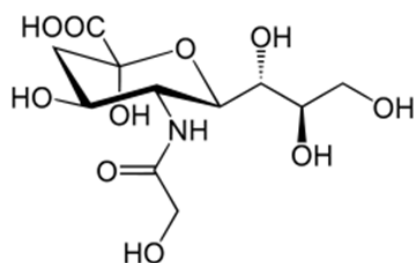
N-Glycolylneuraminic acid
(Neu5Gc)
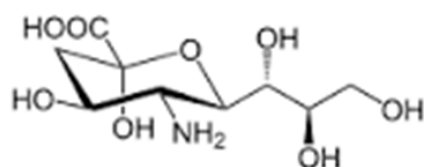
2-keto-3-deoxy-D-glycero-D-
galacto-nononic acid
(KDN)

Fig.10 Structures of sialic acid Neu5Ac, Neu5Gc, and KDN

Owing to its biological versatility in the physiological system, substantial studies have been done on the analytical methodology to enable the quantitative analysis of this fascinating molecule with promising sensitivity and specificity in various biological systems under different physiological conditions in the past decades. However, some of the early trials were failed to reach the concentration at which the level of Neu5Ac with biological significance could be reliably measured because its highly hydrophilic nature makes it fairly easy to interfere with other structural analogs during the analysis. Fortunately, the advent of mass spectrometry interfaced to high performance liquid chromatography in recent years has given rise to improved sensitivity and specificity of the analysis, whereas its mass spectrometric analysis still remain challenging and problematic. As a result, a number of derivatization methods have also been developed and introduced to practically circumvent the challenges faced by analytical chemist and analytically facilitate its mass spectrometric analysis.

As reviewed previously by Huan, Nie et al [10], the both bound- and unbound-Neu5Ac could undergo derivatization to obtain enhanced ionization in its mass spectrometric analysis. Particularly, for the analysis of bound-Neu5Ac, same as in the case for the analysis of gangliosides, esterification and amidation are two most commonly used reactions for the derivatization of Neu5Ac-containing molecules as shown in Fig.11.
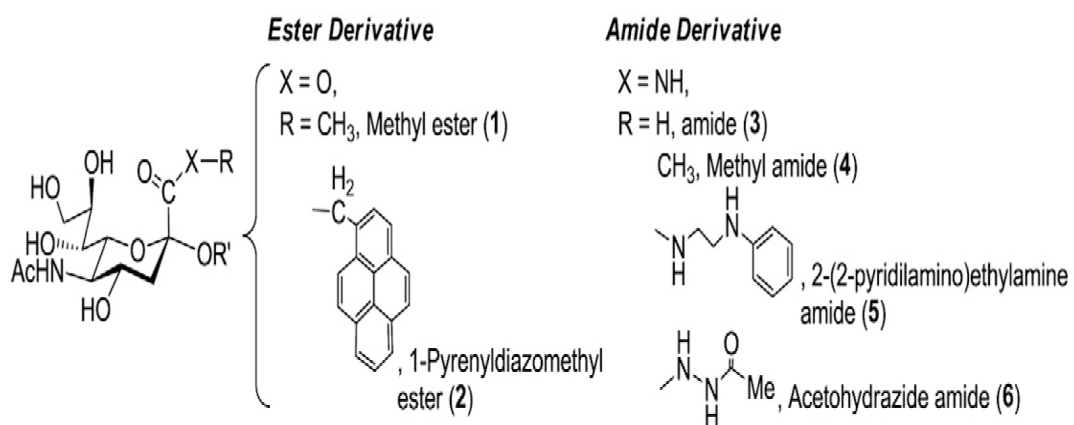
Fig.11 Structures of sialic acid derivatives [10].

In consideration of our urgent demand in significantly enhancing the detection sensitivities from monosialogangliosides on the LC-ESI-MS, the amidation reactions with the option to attach additional proton acceptors/donors onto the chemical structures of monosialogangliosides would thus be a more preferable and advantageous choice over the esterification reaction from the sensitivity enhancement perspective. Among multiple available options for amidation reactions, 2-(2-Pyridilamino)-ethylamine (PAEA) & 4-(4, 6-Dimethoxy-1, 3, 5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) amidation characterized by simultaneous attachment of two proton-accepting groups following the reaction confers the capability of this reaction to convert singly-charged-favorable monosialogangliosides to doubly-charged-favorable PAEA-monosialogangliosides in ESI positive through a one-step chemical modification, suggesting its tremendous potential in resolving this particular problem.

DMTMM&PAEA-based amidation reaction was first introduced by Shin-ichi Endo et al. [11] to derivatize a group of Neu5Ac-containing molecules, including 3'-sialyllactose, 6'-sialyllactose, and ganglioside GM3, and enhance their detection signals in MALDI-TOF. Based on the molecular mechanism of the reaction, DMTMM chemically serves as an activator of the carboxylic acid group by forming the DMTMM-Neu5Ac active ester intermediate through nucleophilic substitution, which allows the occurrence of the subsequent amidation on the esterified carboxylic acid group through another round of necleophilic substitution to form an amide bond with the head aliphatic amine group from PAEA. The alkaline pyridylamine group at the other end of PAEA confers the capability to Neu5Ac residue for undergoing double pronation under either ESI+ or MALDI+ by

increasing its ability to capture dual protons during the ionization, which shifts its ionization profile from predominantly singly charged to primarily doubly charged in terms of its charging pattern. In addition, since the pyridylamine group is less alkaline than the alphatic amines group on PAEA, this disparate property on alkalinity gives rise to the orientational selectivity on the reaction, which also prevents the further coupling of another carboxylic group onto the amidated PAEA. From this study, they have demonstrated that multiple times reinforcements on MS responses were observed from PAEA derivatives of 3'-sialyllactose, 6'-sialyllactose, and ganglioside GM3. Based on their results, the DMTMM&PAEA-based amidation was able to be undertaken through a one-pot reaction under mild conditions at room temperature with continuous stirring. However, unsatisfactorily, the reaction yield they ended up with 3-hour reaction in the 90% isopropanol system for 3'-sialyllactose, 6'-sialyllactose, and ganglioside GM3 were 45%, 60%, and 30%, respectively, which leaves a plenty of room for further improvement on the conditions under which the reaction takes place before it could virtually be applied to mass spectrometrically analyze the monosialoganglioside from the quantitative standpoint.

As a result, in the present work, we have developed and validated a new UPLC-MS/MS method with enhanced sensitivity for the measurement of three monosialoganglioside species (GM1, GM2, and GM3) in human plasma using PAEA&DMTMM-based derivatization. The conditions under which the PAEA&DMTMM reaction occurs have been re-optimized based on solvent, reaction time, reagent concentration, and reaction temperature to achieve above 90% reaction yield for monosialogangliosides. This method employed reverse phase UPLC for chromatographic

separation and tandem mass spectrometry in the MRM mode for superior detection sensitivity and specificity. The ESI-MS responses of the three monosialoganglioside species were enhanced by more than 15 times following PAEA&DMTMM-based derivatization. We have successfully applied this method to the determination of monosialoganglioside levels in human plasma from GSD patients, carriers and normal adults.

3.2 Materials and Methods

3.2.1 Materials

Derivatization reagents 4 - (4, 6 – Dimethoxy - 1, 3, 5 – triazin – 2 - yl) – 4 - methylmorpholinium chloride (DMTMM) and 2 - (2 - Pyridilamino) ethylamine (PAEA) were purchased from Sigma Aldrich (St. Louis, MO) and Santa Cruz (Dallas, TX), respectively. Calibration standards GM1, GM2, and GM3 were obtained from EMD Chemicals (Billerica, MA) and Avantilipids (Alabaster, AL). Internal standards (ISs) N-omega-CD3-Octadecanoyl monosialogangliosides GM1 ($^2D_3$-GM1), GM2 ($^2D_3$-GM1), and GM3 ($^2D_3$-GM3) were purchased from Matreya LLC (Pleasant Gap, PA). HPLC grade methanol, acetonitrile, and isopropanol were obtained from EMD Milipore (Billerica, MA). HPLC grade formic acid was obtained from Fisher Scientific (Pittsburgh, PA). Deionized water was prepared using the Barnstead Nano-PURE Water Purification System (Asheville, NC). All materials were used directly without further purification.

3.2.2 Human Plasma

The study was approved by the DDC Clinic Institutional Review Board, and written consents were obtained from the study subjects or their legal guardians. The genotypes of the studied patients were determined through DNA sequencing and mutation analysis. The human plasma samples were stored at $-20℃$ before analysis.

3.2.3 Stock and Working Solutions

Stock solutions of DMTMM and PAEA were prepared by dissolving the lyophilized powders in 1:1 MeOH/Isopropanol to obtain concentrations of 1 M, and their working solutions with concentrations of 100 mM were obtained by serial dilution with 1:1 MeOH/Isopropanol.

Stock solutions of GM1, GM2, and GM3 were prepared by dissolving the lyophilized powders in 90% MeOH to obtain equal concentrations of 0.250 mg/mL. The seven-level working solutions, which also served as calibrators, were prepared though serial dilution with 90% MeOH to obtain concentrations of 10.0, 20.0, 50.0, $1.00×10^2$, $4.00×10^2$, $1.00×10^3$, $2.00×10^3$ ng/mL for GM1, 10.0, 20.0, 50.0, $1.00×10^2$, $4.00×10^2$, $1.00×10^3$, $2.00×10^3$ ng/mL for GM2, and 80.0, $1.60×10^2$, $4.00×10^2$, $8.00×10^2$, $3.20×10^3$, $8.00×10^3$, $1.60×10^4$ ng/mL for GM3. The preparation of three-level QC working solutions was carried out in a similar manner to obtain concentrations of 25.0, $2.50×10^2$, $1.60×10^3$ ng/mL for GM1, 25.0, $2.50×10^2$, $1.60×10^3$ ng/mL for GM2, and $2.00×10^2$, $2×10^3$, $1.28×10^4$ ng/mL for GM3. Likewise, stock solutions of Iss $^2D_3$-GM1, $^2D_3$-GM1, and $^2D_3$-GM3 were

prepared by dissolving the lyophilized powders in 90% MeOH to obtain concentrations of 0.250 mg/mL. The IS working solution was prepared by dilution of the corresponding stock solution with 90% MeOH to provide a concentration of 5.00×10^2 ng/mL for each IS.

The low plasma QC working solution was prepared by pooling plasma samples from multiple GSD patients and dividing the pool into 20 µL aliquots. The high plasma QC working solution was prepared by pooling plasma samples from normal adults and dividing the pool into 20 µL aliquots.

All prepared stock and working solutions were kept at -20℃ before use.

3.2.4 Sample Preparation

For plasma sample, 20 µL of plasma was taken and spiked with 10 µL of IS working solution. Afterwards, the spiked samples were subjected to sample extraction phase described below.

For calibration, each of calibrators was prepared by individually mixing 10 µL of a corresponding calibrator working solution with 10 µL IS working solution. The calibrators were then subjected to sample derivatization phase described below.

During the sample extraction phase, the spiked plasma samples underwent protein precipitation with the addition of 190 µL methanol as described previously [12]. The supernatant was transferred into a 1.7-mL plastic centrifuge vial for subsequent extraction. Thereafter, 200µL of water and 160 µL of chloroform were added to the supernatant. After vortexing, the mixture was centrifuged at 10,000 g for 3 min to separate

the liquid into two phases. The upper phase was transferred into a new 1.7-mL centrifuge vial. Subsequently, the extraction was repeated twice for the lower phase with 200 μL of MeOH and 200 μL of water for each trial, and the extracts were combined and subjected to the derivatization phase.

During the sample derivatization phase, each sample (plasma extract/calibrator) was dried under a continuous nitrogen stream, and reconstituted with 40 μL of isopropanol. Thereafter, 12 and 48 μL of PAEA and DMTMM working solutions, respectively, were added into the reconstituted sample. Then, the reaction mixture was vortexed to mix thoroughly, and incubated at 80℃ on the heat block for 40 min. After the incubation, the sample vial was removed and placed onto the ice bath to terminate the reaction. Once cooled, 100 μL of isopropanol was added to dilute the reacted mixture to a total volume of 200 μL. Following vortexing, the mixture was centrifuged at 5,000 g for 1 min, and the supernatant was transferred into an auto-sampler vial with a micro-insert for LC/MS analysis.

3.2.5 LC/MS Instrumentation

The UPLC system was composed of a DGU-20A$_{3R}$ degasser, two LC-30AD pumps, a SIL-30AC autosampler, a CTO-10A column oven and a CBM-20A system controller from Shimadzu (Columbia, MD). The UPLC system was interfaced to a Qtrap 5500 mass spectrometer equipped with an electrospray ionization source and a built-in Valco switch valve from AB SCIEX (Framingham, MA). The Multiple Reaction

Monitoring (MRM) mode was utilized for quantitation. Individual MRM transitions have been given in Table 8. The MS parameters for individual PAEA-monosialogangliosides have been given in Table 9. Data acquisition and chromatographic peak integration were conducted using the Analysis 1.6.1 software package from AB SCIEX.

Table 8 The MRM transitions for quantification of PAEA-monosialogangliosides

Species	Collision Energy eV	Component	MRM Transition	Species	Collision Energy eV	Component	MRM Transition
PAEA-GM1	39.9	d18:1-14:0	805.6/411.2	PAEA-GM3	33.0	d18:1-14:0	622.9/411.2
		d18:1-16:1	818.6/411.2			d18:1-16:1	635.9/411.2
		d18:1-16:0	819.6/411.2			d18:1-16:0	636.9/411.2
		d18:1-18:1	832.6/411.2			d18:1-18:1	649.9/411.2
		d18:1-18:0	833.6/411.2			d18:1-18:0	650.9/411.2
		d18:1-20:0	847.6/411.2			d18:1-20:0	664.9/411.2
PAEA-GM2	34.9	d18:1-16:0	738.7/411.2			d18:1-21:0	671.9/411.2
		d18:1-18:1	751.7/411.2			d18:1-22:0	678.9/411.2
		d18:1-18:0	752.7/411.2			d18:1-23:0	685.9/411.2
		d18:1-20:0	768.7/411.2			d18:1-24:0	692.9/411.2

Table 9 MS parameters for individual PAEA-monosialogangliosides

Analytes / MS Parameters	PAEA-GM1	PAEA-GM2	PAEA-GM3
Curtain Gas (Psi)	30.0	30.0	30.0
Collision Gas (Psi)	High	High	High
IonSpray Voltage (eV)	5000.0	5000.0	5000.0
Temperature (℃)	600.0	600.0	600.0
Ion Source Gas 1 (Psi)	35.0	35.0	35.0
Ion Source Gas 2 (Psi)	35.0	35.0	35.0
Declustering Potential (eV)	83.0	93.0	57.9
Entrance Potential (eV)	8.1	11.1	12.6
Collision Energy (eV)	39.9	34.9	33.0
Collision Cell Exit Potential (eV)	36.0	34.5	32.7

3.2.6 LC-MS/MS Procedure

Following chemical derivatization, 10 μL of sample was injected onto a Kinetex-C8 UPLC column (1.7 μm, 50 mm × 2.1 mm; Phenomenex, Torrance, CA) guarded with a C8 Security Cartridge. The mobile phases used were a mixture of 2% MeOH with 1% formic acid (A) and ACN-MeOH (6:4) with 1% formic acid (B). The chromatographic separation was implemented employing a gradient elusion program. Briefly, the initial B% was maintained at 50% for 1 min and steeply increased to 75% at 1.5 min followed by a linear increase to 95% at 7 min. Thereafter, the B% was held constant at 95% for an additional 1.5 min and then dropped immediately to 50% for remainder of the run. In addition, the LC eluent at 0.3 mL/min was directed into the waste during the first 4.5 minutes and then introduced into the electrospray ionization source from 4.6 to 8.0 minutes as all derivatized monosialoganglioside components were sequentially eluted and detected. Thereafter, the flow rate was increased to 0.6 mL/min to rapidly clean up and re-equilibrate the column for the next run. The temperatures of auto-sampler and column oven were maintained at 4 and 35℃, respectively, throughout the analysis.

3.2.7 Calibration Curve

Quantitation of the gangliosides in plasma samples was conducted using calibration curves based on seven levels. For each of the three monosialoganglioside species, the total peak area was calculated by summing up the IS-normalized peak areas of individual components based on a single injection.

The seven-level calibration curves were then established by plotting the IS-normalized total peak area of each measured monosialoganglioside species as the y-axis against the concentration levels of seven-level calibrators as the x-axis. The linear regression with a weighting factor of $1/y^2$ was employed. The calculated equation was applied to the measurement of monosialoganglioside levels in plasma sample. The standard calibration curves for measured monosialoganglioside species have been shown in Fig.12.
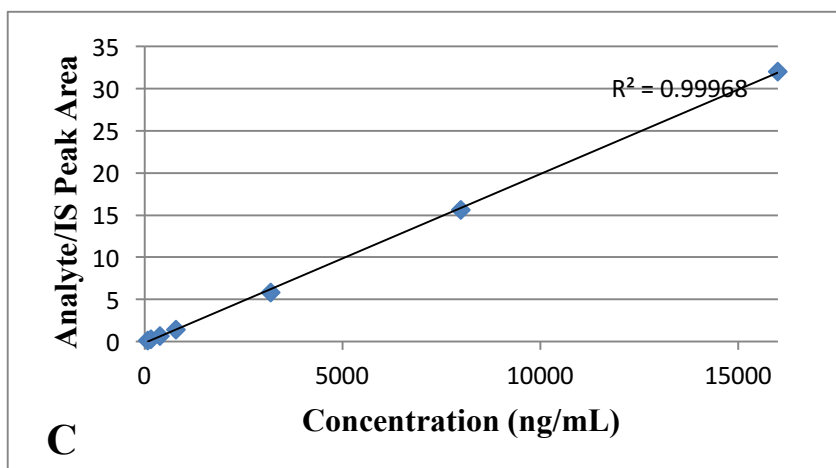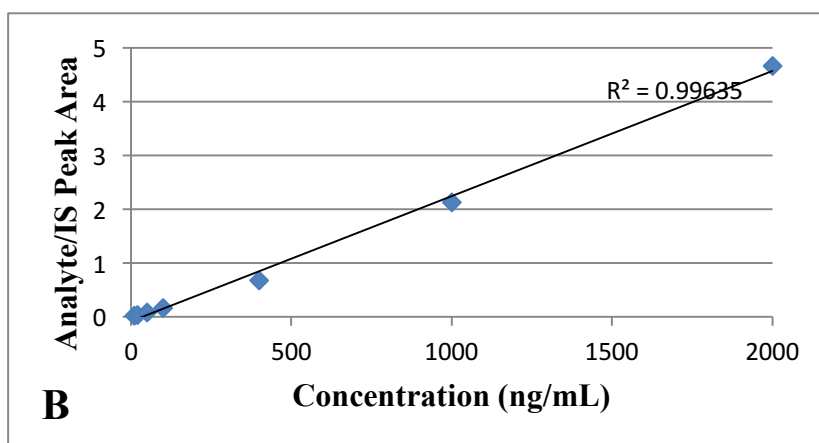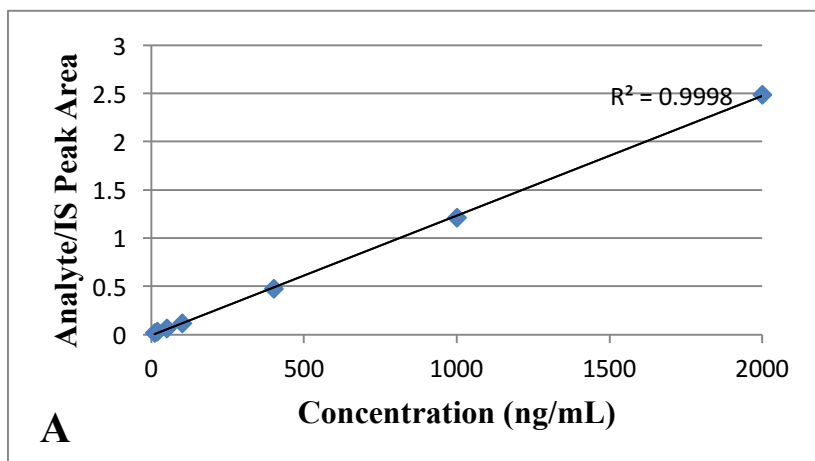
Fig.12 Calibration curves for GM1 (A), GM2 (B), and GM3 (C).

3.3 Results and Discussion

3.3.1 Derivatization Conditions

Since the carboxylic acid group on the sialic acid residue bears high preference to lose a proton, negative ESI is thus a more common option in generating singly charged molecular ions of monosialogangliosides than positive ESI in terms of ionization efficiency. However, considering their high molecular weight, the m/z values of singly charged molecular ions of major monosialoganglioside species are virtually beyond the detection range of many commercially available triple-quadrupole mass spectrometers, including our instrument. Therefore, the selection of corresponding doubly charged molecular ions with detectable m/z values has been a more practical approach to analyze these molecules with such instruments. Based on our preliminary studies, the absence of an additional proton donating/accepting group on native structures of monosialogangliosides impedes them to undergo the dual proton transferring pathway during ionization, which accounts for the low tendency of accepting/donating two protons by monosialogangliosides in ESI and the insufficient generation of doubly charged molecular ions in their MS profile, resulting in limited detection sensitivity. In order to overcome the limitation, modifications on their native structures are needed to increase the efficiency in generating corresponding doubly charged molecular ions in ESI.

The PAEA&DMTMM-based derivatization was first reported by Shin-ichi Endo et al. [11] for signal enhancement in the analysis of sialic acid Neu5Ac and

ganglioside GM3 using MALDI-TOF. From this study, multiple times reinforcement on MS response was observed from the PAEA-GM3. However, it was discovered that the derivatization yields of GM1, GM2, and GM3 were extremely poor (< 35%) under the reported conditions. In order to overcome this limitation, we optimized reaction conditions in terms of reagent concentration, reaction time, reagent ratio, reaction solvent, as well as instrumental conditions to achieve a maximal LC/MS peak area from the analytes. As shown in Fig.13, we found that 60mM PAEA and 60mM DMTMM provided the maximal LC peak area for all measured monosialoganglioside species. In addition, 40min was found to be the most favorable time for the reaction. Moreover, in consideration of the reaction mechanism, amidation is essentially a typical endothermic reaction with the stringent threshold on the temperature under which the reaction is undertaken, suggesting the directly proportional relationship between the reaction yield and temperature. Based on this rationale, the reaction temperature was optimized to 80 ℃ in accordance to the boiling point of the solvent mixture. Under the optimized conditions, the reaction yield was observed to be above 90%. With this derivatization, we observed over 15-time signal enhancement compared to the previous method [12] due to the abundance of doubly charged molecular ions in positive ESI. This beneficial effect could be attributed to the addition of a pyridylamine group, a preferable dual-proton acceptor. The reaction scheme has been elaborated in Fig.14.
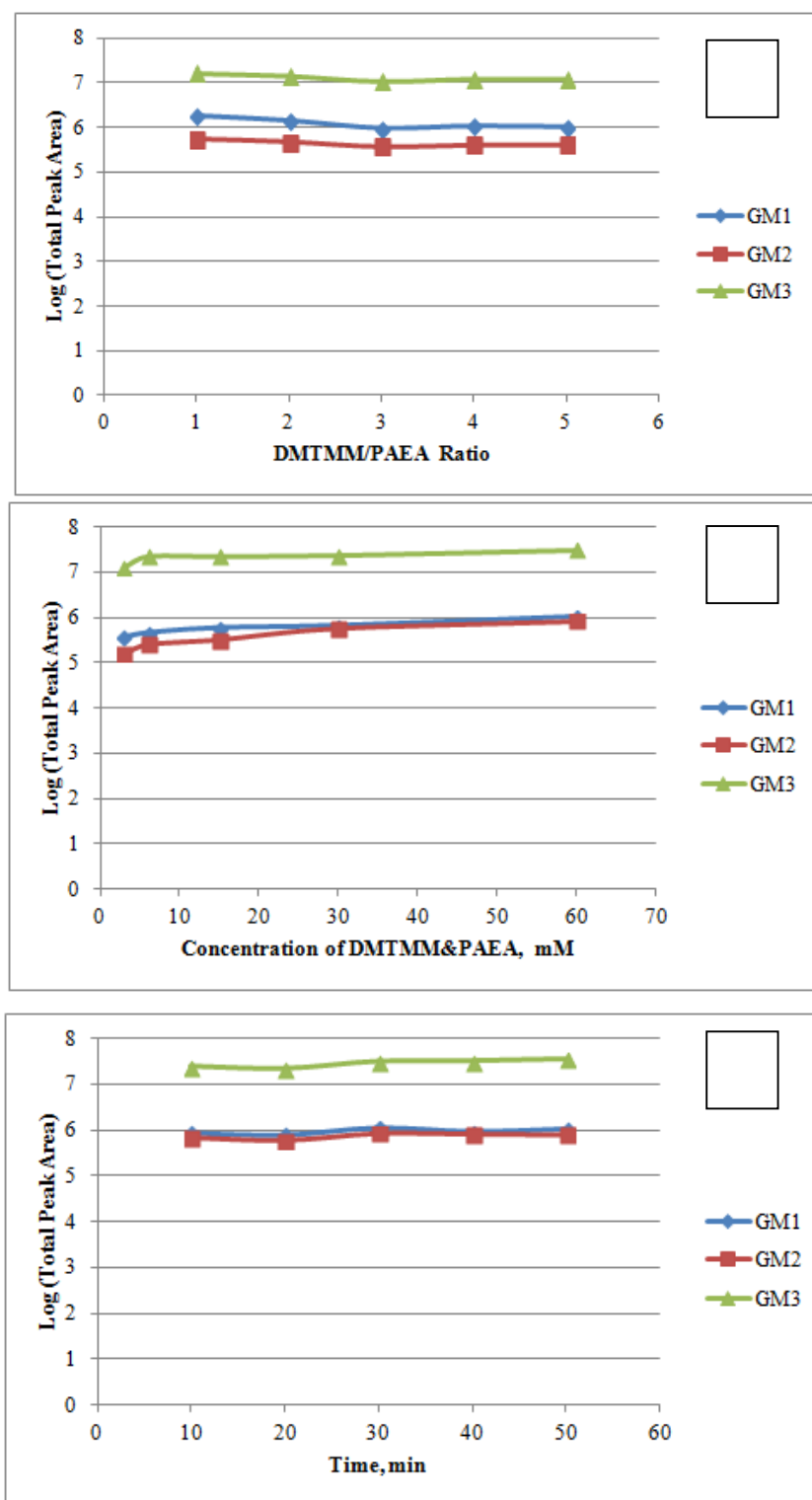
Fig.13 Optimization of reaction conditions

The conditions were optimized based on (A) reagent ratio, (B) reagent concentration, and (C) reaction time.
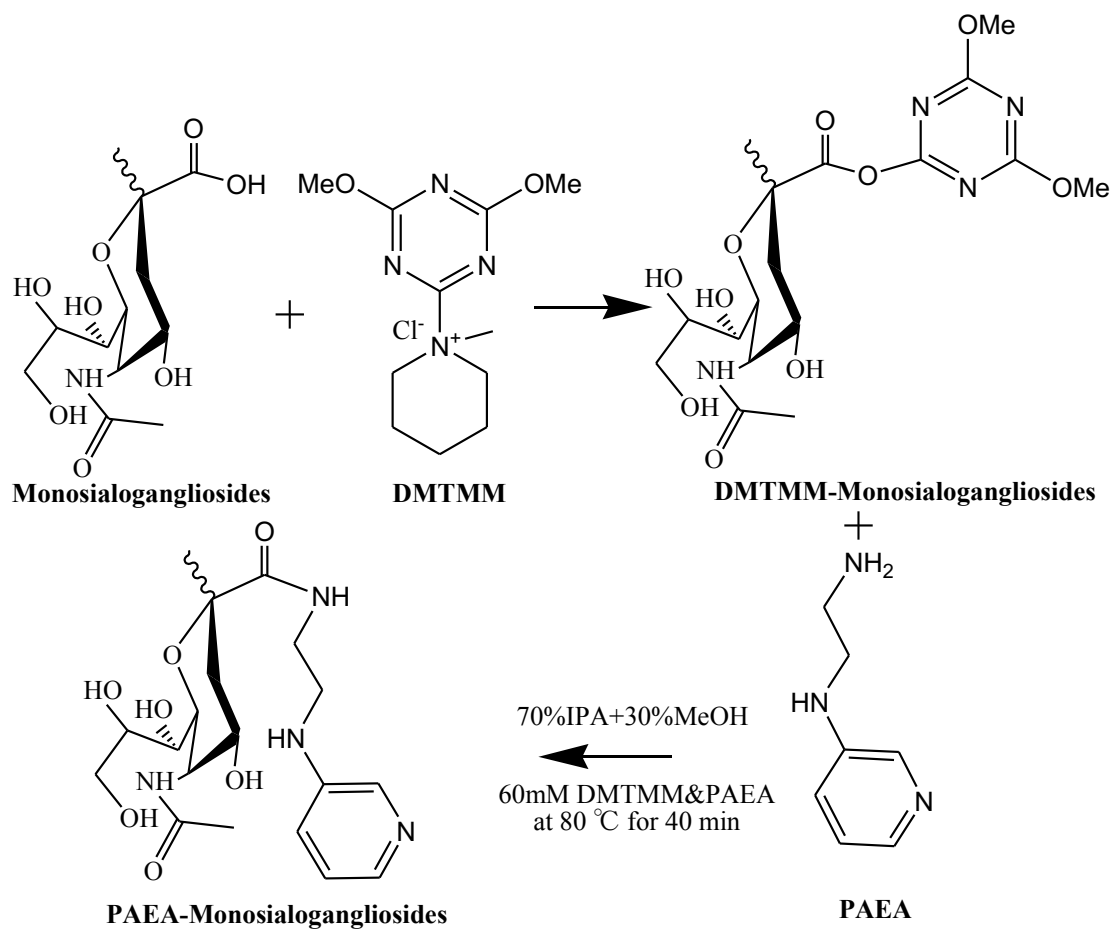
Fig.14 Elaborated reaction scheme for PAEA&DMTMM-based derivatization

3.3.2 Mass Spec Conditions

The representative product ion mass spectra of d18:1-20:0 components for GM1, GM2, and GM3 were shown in Fig. 3.6. As illustrated by their MS/MS profiles, monosialogangliosides appeared to share certain degree of similarities in their fragmentation patterns, including common fragments m/z = 411.2 and 573.2, corresponding to fragments [PAEA-Neu5Ac+H]$^+$ and [PAEA-Neu5Ac-Gla+H]$^+$, respectively, confirming the desired position has been preferentially derivatized. In addition, the universal fragment m/z=411.2 was observed to be the most abundant among any other fragments present in the MS/MS profiles of PAEA-monosialogangliosides.

After evaluating the quality of different possible precursor → daughter ion pairs for quantitation, we found that the following MRM transitions provided the optimal quantitative performance in terms of detection sensitivity and specificity: [PAEA-GM1+2H]$^{2+}$ → 411.2 for GM1, [PAEA-GM2+2H]$^{2+}$ → 411.2 for GM2, and [PAEA-GM3+2H]$^{2+}$ → 411.2 for GM3. Moreover, transitions [PAEA-GM1+2H]$^{2+}$ → 573.2, [PAEA-GM2+2H]$^{2+}$→ 573.2, and [PAEA-GM3+2H]$^{2+}$ → 573.2 were also monitored for quality assurance. The monitored MRM transitions for quantitation have been listed in Table 10. The fragmentation mechanisms for GM1, GM2, and GM3 have been interpreted and elaborated in Fig.15.

In addition, since sialic acid residues are well known to be fragile during electrospray ionization, in-source loss of sialic acid residues has been the primary concern associated with the analysis of sialic acid-containing molecules employing ESI-MS. Therefore, in order to determine the degree of in-source sialic acid loss during ESI, we

carried out a series of experiments by individually injecting the derivatized standards into the LC and implementing MS full scan on Q3 under our experimental conditions. Based on the MS spectra we obtained at the retention times of our analytes (data not shown), the amount of PAEA-monosialogangliosides that underwent in-source loss was found to be insignificant during the ionization process.
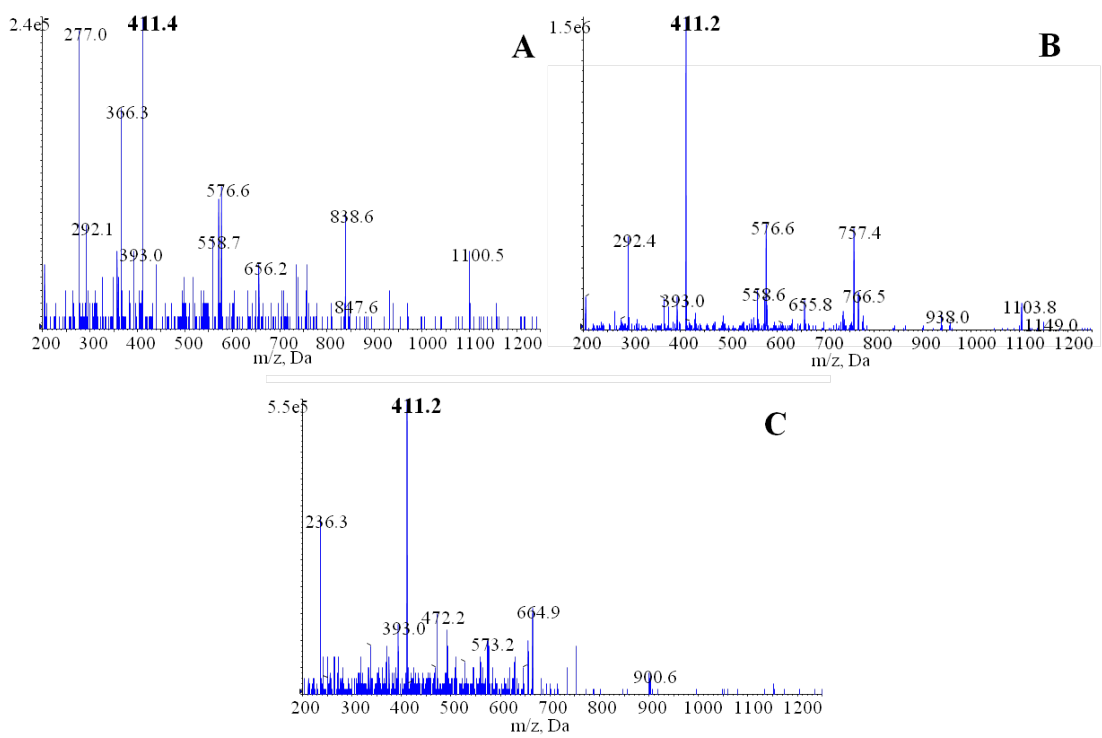
Fig.15 The product ion (MS/MS) spectra for d18:1-20:0 components of

PAEA-monosialogangliosides

MS/MS spectra for PAEA-GM1 (A), PAEA-GM2 (B), and PAEA-GM3 (C)

Fragmentation

[PAEA-GMx+2H$^+$]$^{2+}$

R = C$_n$H$_{2n\pm1}$, n = 15~24

PAEA-GM1, R$_1$=

PAEA-GM2, R$_1$=

PAEA-GM3, R$_1$=H

m/z=411.2
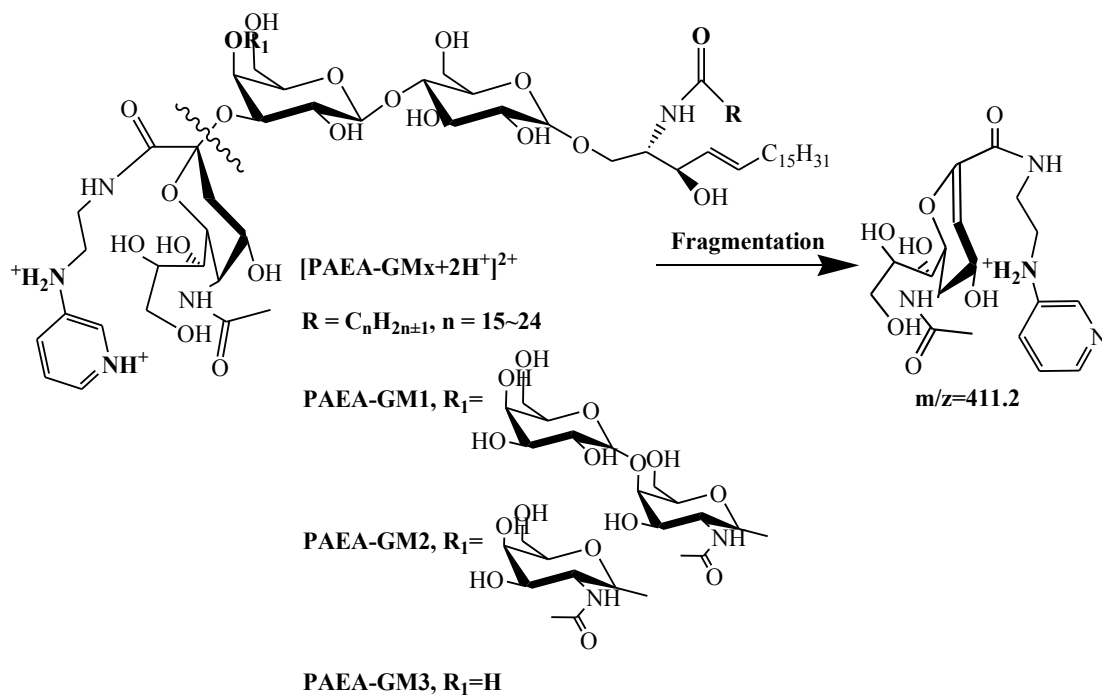
Fig.16 Proposed fragmentation mechanism for PAEA-monosialogangliosides

3.3.3 HPLC Conditions

Due to the chemical complexity of monosialoganglioside species, an appropriate chromatographic separation with one component being resolved and separated from another is essential to avoid the competition for ionization between co-eluates and to eliminate potential ionization suppression from matrix interferences during analysis. Based on our preliminary study, the analytes generally showed good resolution on separation but appeared to be overwhelmingly retentive on the C18 stationary phase. This was probably due to their elevated hydrophobicity after derivatization, which not only widened the chromatographic peak of individual components, but also prolonged the analysis time. In comparison, the C8 stationary phase with lower hydrophobicity was observed to retain the analytes less vigorously, enabling sharper chromatographic peaks and a shorter LC run without sacrificing the separation resolution. The addition of acetonitrile to mobile phase B significantly reduced the retention of the analytes on the C8 stationary phase and effectively shortened the chromatographic run in comparison with methanol only, leading to better peak shape and faster analysis. On the other hand, the certain portion of methanol was necessary to maintain the analytes to be solubilized in the mobile phase during the analysis. Therefore, the ratio of acetonitrile to methanol was optimized at 6 to 4 for the best chromatographic performance. Under our optimized LC conditions, different monosialoganglioside components were spread in a chromatographic window from 4.5 to 8 min. Chromatograms for PAEA-GM3 are shown in Fig.17.
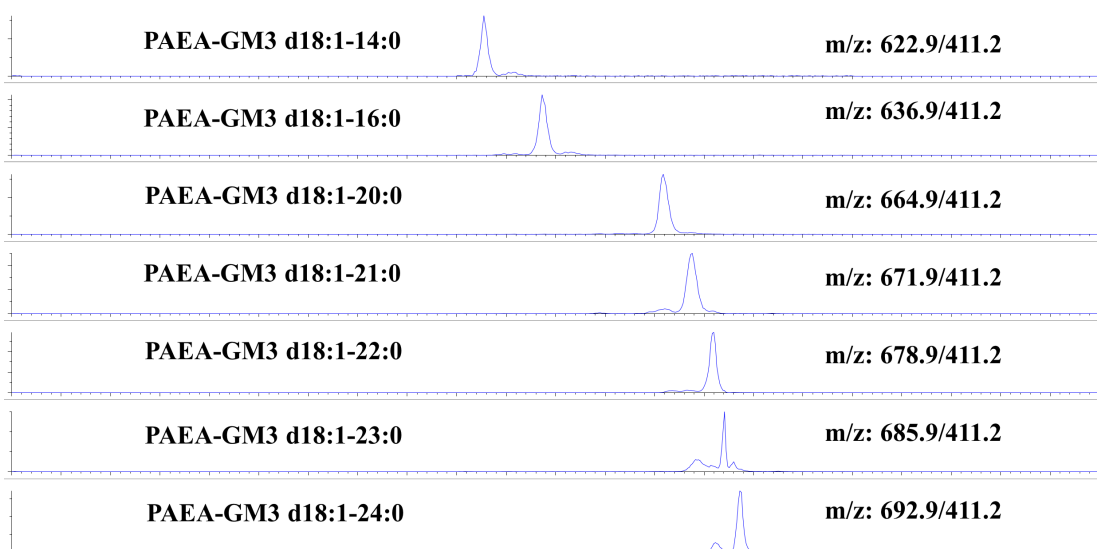
Fig.17 MRM chromatograms for different components from PAEA-GM3

It is worth noting that the addition of 1% formic acid to the mobile phase substantially enhanced the peak intensities from all MRM transitions by multiple times in positive ESI. This experimental phenomenon could be reasonably explained by the increased proton donating capability of the mobile phase upon the introduction of formic acid, allowing the dual-proton acceptance of PAEA-monosialoganglioside to be achieved at an elevated level in positive ESI. Expectedly, the reinforcement in MS response was observed to be less significant by the addition of ammonium formate and ammonium acetate in comparison with formic acid. This was due to their inability to further lower the pH of the mobile phase below the level that has been achieved by formic acid.

In addition, the addition of 1% formic acid into the mobile phase considerably reduced the buildup of derivatizing reagents on the column solid phase. This was due to enhanced ionization of leftover DMTMM&PAEA, thereby decreasing their retention on the stationary phase, resulting in the consistency and reproducibility of separation performance from the column over time.

3.3.4 LLOQ, Precision and Accuracy

The LLOQ, defined as S/N near or above 10, for each analyte was determined by analyzing samples prepared from 5 individual lots of plasma spiked with their corresponding deuterium-labeled siblings. The LLOQs were 10, 10, and 80 ng/mL for GM1, GM2, and GM3, respectively, demonstrating the specificity and sensitivity of this assay for the accurate determination of monosialogangliosides in human plasma.

The precision and accuracy for intra-assay and inter-assay measurements were assessed by analyzing QC standards prepared at three different concentrations in five replicates. As shown in Table 10, the coefficient of variation (CV %) and relative error (RE %) for intra- and inter-assay measurements were within 9% and 11%, respectively. In addition, the precision for intra-assay and inter-assay measurements in the plasma matrix was also evaluated by analyzing samples prepared from five individual lots of high and low plasma QCs from unaffected adults and GSD patients on a single day and five different days, respectively. As shown in Table 11, the CV% from both high and low plasma QC measurements were found to be below 15% as required by the FDA guidelines for bioanalytical method validation [13].

Table 10 Intra- and inter-assay precision and accuracy for the measurement of QC standards (n=5)

Species	QC	Spiked Conc (ng/ml)	Intra-assay			Inter-assay		
			Mean ± SD (ng/ml)	Precision (CV %)	Accuracy (R.E. %)	Mean ± SD (ng/ml)	Precision (CV %)	Accuracy (R.E. %)
GM1	Low	25.0	27.7±0.90	3.0	11	27.3±1.7	6.0	9.0
	Mid	$2.50×10^2$	$(2.63±0.18)×10^2$	7.0	6.0	$(2.74±0.15)×10^2$	5.0	10
	High	$1.60×10^3$	$(1.43±0.13)×10^3$	9.0	-11	$(1.58±0.040)×10^3$	3.0	-1.0
GM2	Low	25.0	23.4±1.9	8.0	-6.0	25.0±0.60	2.0	0
	Mid	$2.50×10^2$	$(2.35±0.099)×10^2$	4.0	-6.0	$(2.42±0.081)×10^2$	3.0	-3.0
	High	$1.60×10^3$	$(1.62±0.040)×10^3$	2.0	1.0	$(1.67±0.11)×10^3$	7.0	4.0
GM3	Low	$2.00×10^2$	$(2.09±0.14)×10^2$	7.0	5.0	$(2.11±0.083)×10^2$	4.0	5.0
	Mid	$2.00×10^3$	$(2.06±0.080)×10^3$	4.0	3.0	$(2.06±0.060)×10^3$	3.0	3.0
	High	$1.28×10^4$	$(1.26±0.050)×10^4$	4.0	-2.0	$(1.26±0.060)×10^4$	5.0	-3.0

Table 11 Intra- and inter-assay precision for the measurement of plasma QCs (n=5)

Species	QC	Intra-assay		Inter-assay	
		Mean±SD (ng/ml)	Precision (CV %)	Mean±SD (ng/ml)	Precision (CV %)
GM1	Low	987±59	6	879±118	13
	High	962±89	9	920±112	12
GM2	Low	ND	N/A	ND	N/A
	High	308±12	4	302±33	11
GM3	Low	54.4±3.0	5	46.7±6.6	14
	High	$(9.05\pm0.58)\times10^3$	6	$(9.65\pm1.41)\times10^3$	15

Note:

1. Due to the nature of GSD and reverse phase LC, no ideal low plasma QC was currently available for GM1

2. Each of low plasma QC was prepared from 20ɭ L of patient plasma spiked with 10ɭ L of IS work solution

3. Each of high plasma QC was prepared in a similar manner except the plasma was sample from unaffected adult

3.3.5 Stability and Extraction Recovery

Since stabilities of gangliosides in plasma under various storage conditions had already been evaluated by our previous studies [12], only the autosampler storage stability of PAEA-monosialogangliosides was evaluated in this study by analyzing samples prepared from five individual lots of plasma from GSD patients spiked with deuterium-labeled standards at QC concentrations before and after overnight storage at 4 ℃. The stability values for the three monosialogangliosides were about 100% under such conditions as shown in Table 12, indicating no significant loss of any analytes under the storage condition of autosampler.

The extraction recoveries for all monosialogangliosides were assessed by comparably analyzing samples prepared from five individual lots of plasma from GSD patients spiked with deuterium-labeled standards at QC concentrations in both pre- and post-extraction stages. As shown in Table 12, about 80~100% of analytes were recovered from the plasma matrix at the studied concentrations, indicating the effectiveness of our optimized sample extraction procedure.

Table 12 The stability of PAEA-monosialogangliosides and extraction recovery of

monosialogangliosides from plasma samples at QC concentrations (n=5)

Species	QC	Spiked Concentration (ng/ml)	Autosampler Stability Mean ± SD (%)	CV (%)	Extraction Recovery Mean ± SD (%)	CV (%)
GM1	Low	25.0	103±7.0	7.0	94.0±7.0	8.0
	Mid	2.50×10^2	101±8.0	8.0	84.0±3.0	4.0
	High	1.60×10^3	102±3.0	3.0	80.0±2.0	3.0
GM2	Low	25.0	104±13	12	84.0±3.0	4.0
	Mid	2.50×10^2	107±3.0	3.0	83.0±2.0	3.0
	High	1.60×10^3	100±3.0	3.0	81.0±6.0	7.0
GM3	Low	2.00×10^2	100±10	10	102±4.0	4.0
	Mid	2.00×10^3	103±5.0	5.0	83.0±8.0	10
	High	1.28×10^4	100±4.0	4.0	80.0±6.0	8.0

3.3.6 Method Application

The validated method was successfully applied to measure the levels of monosialogangliosides in plasma samples from different specimens, including unaffected adults, GSD heterozygous carriers, and GSD patients, during the ongoing clinical study. As shown in Table 13, the plasma levels of monosialogangliosides from 31 normal adults were $(1.35\pm0.39) \times 10^3$, $(4.71\pm1.8) \times 10^2$, and $(9.93\pm2.9) \times 10^3$ ng/mL for GM1, GM2, and GM3, respectively. In comparison, averaged monosialoganglioside levels in plasma samples from 12 heterozygous carriers were $(7.12\pm2.4) \times 10^2$, $(2.38\pm1.0) \times 10^2$, and $(6.23\pm1.9) \times 10^3$ ng/mL for GM1, GM2, and GM3, respectively, slightly lower than those unaffected subjects, which is consistent with results reported by previous studies [14].

In comparison with plasma concentrations from measurements on unaffected adults and GSD patients by the diagnostic method established in chapter 2, the measured values of GM3 in plasma samples from unaffected adults determined by the new method $(9.93\pm2.9$ μg/mL$)$ was found to be comparable with the previous results $(9.72\pm3.68$ μg/mL$)$, whereas significant variation was noticed from the measured values of GM2 on unaffected adults between two different methods (0.47 ± 0.18 versus 2.37 ± 0.79 μg/mL), which could be possibly attributed to the physiological variance on GM2 abundance in blood circulations of people from disparate populations.

Interestingly, as a result of the enhanced sensitivity produced by the chemical derivatization of monosialogangliosides with DMTMM&PAEA, we detected a significant amount of GM3 in the plasma of GSD patients with the newly developed method. As shown in Table 3.5, the level of GM3 from 12 of 14 GSD patients (2 patients remained

immeasurable) averaged $(1.13\pm0.33)\times10^2$ ng/mL, while the level of GM2 remained undetectable. Although the amount of GM3 in the plasma of the GSD patients was small, accounting for approximately 1% of plasma abundance of GM3 in the unaffected adults, its presence was unequivocal. Since GM3 synthase is the key enzyme involving the initial stages of ganglioside biosynthesis, GSD would theoretically result in a complete absence of GM3 and all downstream biosynthetic derivatives in these patients [14-16]. Given that a fair amount of GM3 was detected in the most affected patients, the question raised from the result is where the GM3 comes from. It is possible that the detected GM3 amount may be ingested from milk or food or generated from some unidentified ganglioside biosynthetic pathways in humans. Apparently the origin of the GM3 in the plasma from GSD patients remains elusive and is currently under investigation in our laboratory.

3.4 Conclusion

This study detailed the development and validation of a sensitive LC-MS/MS method for the determination of monosialogangliosides GM1, GM2, and GM3 in human plasma via PAEA&DMTMM-based chemical derivatization by using $^2D_3$-GM1, $^2D_3$-GM2, and $^2D_3$-GM3 as ISs. In brief, the standards and ISs were rapidly extracted from plasma using protein precipitation, cleaned up through liquid-liquid extraction, and derivatized under optimized conditions prior to LC-MS/MS analysis. This method employed the UPLC system in gradient elution for chromatographic separation and Multiple Reaction Monitoring (MRM) for mass spectrometric detection, which has been successfully applied

to the measurement of ganglioside levels in plasma samples from GSD patients, carriers, and normal human subjects. Regarding its high specificity, sensitivity, and throughput, this method could be applicable for therapeutic evaluation and clinical diagnosis of other ganglioside-related neurodegenerative disorders in the near future.

Table 13 Determination of monosialoganglioside levels in plasma samples from

different specimens

Subject	Total	GM1 Mean±SD (ng/mL)	GM2 Mean±SD (ng/mL)	GM3 Mean±SD (ng/mL)
Normal Subject	31	$(1.35\pm0.39)\times10^3$	$(4.71\pm1.8)\times10^2$	$(9.93\pm2.9)\times10^3$
GSD Carrier	12	$(7.12\pm2.4)\times10^2$	$(2.38\pm1.0)\times10^2$	$(6.23\pm1.9)\times10^3$
GSD Patient	14	$(9.81\pm3.9)\times10^2$	NM	$(1.13\pm0.33)\times10^2$

Note:

1. NM-Not Measurable
2. Plasma levels of GM2&GM3 below LLOQ were determined by using 20 uL plasma sample in conjunction with 10 uL calibrator as described in **Section 2.2.4, Plasma Sample Preparation**.

3.5 Future Direction

In this study, we have developed a novel chemical derivatization-based UPLC/MS/MS method for quantification of monosialogangliosides in human plasma with superior sensitivity and specificity. Upon the introduction of DMTMM & PAEA derivatization method, virtually their first application in quantitative analysis, we have successfully enhanced the MS sensitivities of monosialogangliosides by 15~20 times compared to other reported methods [12,17], which serves as a very significant breakthrough in the field of ganglioside analysis on LC-ESI-MS. More importantly, by taking advantages from significantly increased sensitivities from monosialogangliosides upon derivatized, we have, in fact the first time, tentatively found the presence of endogenous GM3 in the plasma circulation of patient with GSD and promisingly confirmed this exciting finding by repetitively analyzing plasma samples from many different GSD patients, suggesting that this finding might be acting as a milestone or even foundation for future studies related to GSD. Furthermore, with the support from full validation data under FDA guideline for bioanalytical method development, this standardized method will be quite versatile in fulfilling diverse demands from clinical laboratory and answering questions that remain elusive for other ganglioside-related clinical diseases in terms of its high sensitivity, specificity, and throughput.

However, there are still a few aspects for further improvement. Firstly, considering the PAEA-ganglioside derivatives are preferably analyzed in ESI positive under acid conditions, whereas disialo- and trisialogangliosides are more easily ionized in ESI negative under basic conditions, making them practically incompatible with each other

for the single-run-analysis. One possible means to circumvent this problem to chemically convert mono-, di-, and trisialogangliosides into their corresponding PAEA derivatives at comparable degree through a one-pot reaction. Unfortunately, due to the bulky size of the pyramiding ring from PAEA, spatial hindrance chemically prevents the DMTMM intermediate of both disialo-and trisialogangliosides from undergoing the dual coupling with PAEA and makes the formation of di-PAEA- and tri-PAEA-ganglioside derivative extremely unfavorable by this reaction as shown by our previous study, which generates a great bottleneck for their MS analysis. After numerous trial-and-error studies, we have discovered that the one-pot derivatization of mono-, di-, and trisialogangliosides becomes feasible only when a structural analog of PAEA with diminished chemical size is used in place of PAEA for the amidation reaction, suggesting improved reactivity of multisialogangliosides is directly associated with reduced intensity of spatial hindrance their DMTMM intermediates experience in the course of the coupling reaction (data not shown).

In addition, based on chemical structures of different gangliosides, alpha-carboxylic acid group at the sialic acid residue Neu5Ac of carbohydrate moiety might have been the only possible target that can undergo chemical derivatization. Unfortunately, since carboxylic acid groups are ubiquitously presented on numerous compounds existing in the biological fluid, the chemical selectivity of reactions targeting carboxylic acid group has become a very critical challenge for their practical application. In this study, although the solvent system of the reaction has been optimized to reduce the amount of potential interferences introduced by the plasma matrix and excess quantity of

DMTMM&PAEA reagents has been used to maximize the reaction yield, decent degree of reduction on the reaction yield has still been observed following in-matrix derivatization. This matrix intervention problem becomes even more significant when plasma quantity is scaled up for larger-scale analysis. In addition, as the quantities of derivatizing chemicals are enlarged in line with the increased matrix intervention from up-scaled plasma sample, certain degree of column overload has been observed to cause peak widening and tailing from the experiments. As a result, liquid-liquid extraction method might not be a suitable option in handling plasma samples with increased scale. Thus, extraction methods with greater robustness, such as solid phase extraction, would be needed to process and clean up larger scale plasma sample for the analysis in terms of the elimination of matrix interferences.

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CHAPTER IV

CLINICAL STUDIES ON PATIENTS WITH GANGLIOSIDE GM3 SYNTHASE

DEFICIENCY

4.1 Introduction to Ganglioside GM3 Synthase Deficiency and G500 Therapy

GM3 synthase is essentially a ubiquitously expressed protein that is particularly abundant in the central nervous system of vertebrates. The primary function of this protein is to catalyze the enzymatic transferring of sialic acid residue Neu5Ac onto the substrate, lactosylceramide, through the α-2, 3-glycosdic linkage. In human, it is a protein encoded by SIAT9/ST3Gal5 gene which is located at chromosome 2p11.2 and composed by 14 exons that encode 419 amino acid residues. This protein is characterized by the presence of three very distinguished regions on its primary structure, a single transmembrane region spanning across the mammalian cell membrane, a long sialyl motif, and a short sialyl motif, all of which are found to be essential in order for the protein to exert its normal enzymatic function.

Ganglioside GM3 Synthase Deficiency (GSD) is an autosomal recessive disorder

prevalently identified in the Amish population around the U.S. The primary pathogenesis of this unique disorder is etiologically attributed to the inborn-onset metabolic disruption on the biosynthetic pathway of gangliosides associated with a homozygous loss-of-function mutation occurred on one of the vital enzymes, GM3 synthase, for the de-novo biosynthesis of a- and b-series gangliosides. Based on the nature of autosomal recessive inherited disorder, a heterozygous carrier of this mutation in general does not have any clinical manifestation as an unaffected individual differing only in the level of GM3 within the circulations which appears to be slightly (20~30%) lower than the unaffected individual. In contrast to heterozygous carrier, the homozygous patient of this mutation suffers severe clinical disease as the endogenous biosynthetic GM3 as well as the GM3-derived downstream more complex ganglioside a- and b-series have been completely depleted from their physiological systems by the genetic defect (Fig.18) [1-4].

The homozygous loss-of-function mutation accounted for this unique disorder has been discovered by Simpson et al. to be a single base substitution pertaining to position 694 from cytidine to thymine causing a DNA codon shifting from "CGA" arginine codon to "TGA" stop codon. Furthermore, the mutation has been mapped on the exon 8 of GM3 synthase genome, which is located right between the long and short sialyl motifs, leading to the unsuccessful translation of short sialyl motif and thus the truncated primary sequence in the mutant protein [5].

A number of studies have documented substantial evidence to demonstrate that both long and short sialyl motifs possess a highly conserved cysteine residue to generate an inter-motif disulfide linkage that has been found to be essential for the enzyme to preserve its

catalytic capability. As the formation of inter-motif disulfide bond was intentionally disrupted by substituting the invariant cysteine residue to other amino acid residues with site-direct mutagenesis, the ability of the enzyme to transfer and attach sialic acid residue Neu5Ac onto the substrate became completely abolished, which sheds a new light not only on elucidating the essential role of that cysteine residue in preserving the integrity of the whole enzymatic machinery to allow it to perform its regular catalytic duty, but also on facilitating our understanding on the development of disease-related symptoms in terms of the disease etiology [6].
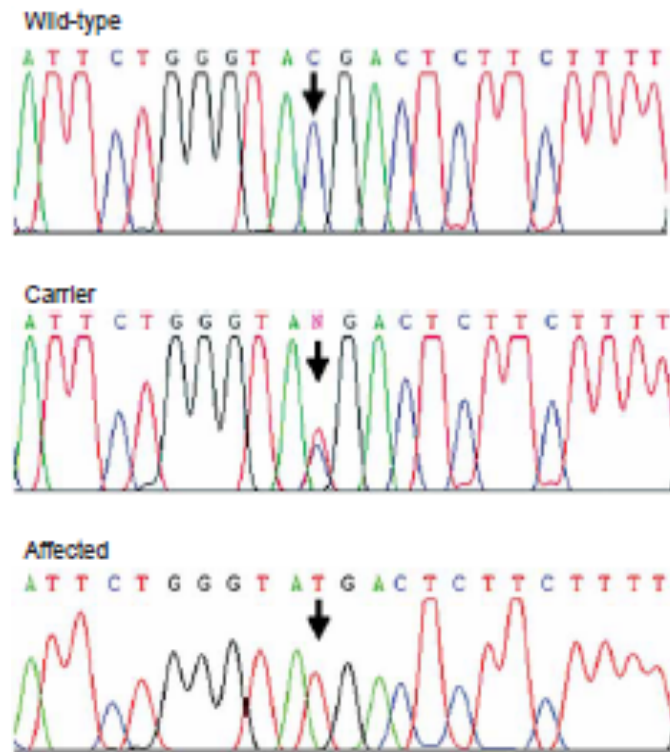
Fig. 18 Genotyping results from unaffected adult, GSD carrier, and GSD patient [5].

As gangliosides are glycosphingolipids playing crucial roles for the development and maturation of central nervous system, the lack of gangliosides in human is extremely devastating. For children affected by GSD, they would typically develop symptoms like severe irritability, failure to thrive, profound developmental delay, and retractable seizure before 6 months of age. By early childhood, the majority of affected patients will start having cortical blindness, choreoathetosis, flaccid weakness, seizure, profound physical and mental retardation, and become completely caregiver dependent. For years, due to the practical limitations in adequately in-vivo modeling this neurological disorder, particularly in mice, as mice might have a completely different ganglioside metabolic pathway from human, the GM3 synthase knockout mice have been discovered to have essentially normal phenotypes [7]. So, the effective treatment on this devastating condition remains highly undeveloped and the progress on its discovery has been significantly slowed.

However, we have always believed that this is a very treatable neurological condition from the time we identified it as we noticed that the affected children appear to be fairly normal for approximately eight weeks after their birth, which clues us to speculate that the affected children might have acquired sufficient amount of gangliosides from maternal circulation before their birth to make the symptoms remain undeveloped during the early age after birth and then gradually develop related symptoms after the endogenous gangliosides have eventually been depleted. Based on this rationale, it becomes rather plausible that if we can replace the endogenous gangliosides in deficient caused by this genetic defect in those affected kids with continuous supplement of exogenous gangliosides from handful sources, we might be able to correct their pathological stigmas and partially or restore their normal

ganglioside biosynthetic pathway, which might eventually lead to a potential cure.

So, in the past few years, we have been searching extensively for the available sources of supplemental gangliosides that are suitable and affordable for our intended clinical trial. Initially, the animal tissues with relatively more abundant ganglioside content appeared to be the most enriched and accessible natural source we could find for the study. Therefore, three affected newborns were continuously given with a pork brain formula once they were diagnosed with DNA mutation analysis soon after birth. This formula essentially provided approximately 10 mg GM3 per day, with incremental amounts given along with their weight gain. Incredibly, all infants being treated showed no early signs or symptoms of the disease except hypotonia when they were fed on the pork brain formula, although the effects seemed to be not as much dramatic as the similar therapy was conducted on the older children. Unfortunately, all young children involved in the study started to develop an allergic reaction against the pork brain formula by 9 months of age, thus this pork brain formula-based therapy had to be discontinued on those affected kids from that point due to the complexity of brain tissue matrix.

In the course of searching for new supplements, we identified a highly-concentrated and appropriately formulated dairy ganglioside product, called G500, manufactured by Fonterra in New Zealand. This G500 product is consisted of high levels of GM3 and GD3 purified from natural sources, which has been formulated and commercialized into a form of integral infant formula to make it suitable for direct oral administration on daily basis with minimal restrictions. This formula has been successfully used as a nutritional supplement in normal infants, which showed almost no signs of triggering immunological

responses on studied infants at given dosage in the previous study [8]. With the development of this newer generation of GM3&GD3-enriched infant formula, the continuous supplementation of highly-pure and -concentrated gangliosides on those affected kids has been made practical and feasible for treating this neurological disorder from the long term perspective. Furthermore, by developing a collaborative partnership with this company, we have been able to obtain sufficient amount of G500 product at no financial cost and fulfill the large-scale need on this G500 product from this project for the subsequent clinical study.

Based on the promising preliminary results we have observed in the younger children fed with pig brain formula, with continuous oral G500 supplementation on daily basis, we first expect that the orally administered gangliosides from G500 may be adequately absorbed and taken up into the circulation of affected patients, and utilized by their metabolic pathway. In addition, the gangliosides provided by G500 are also expected to be delivered across the blood-brain barrier, used by the brain of affected patients, and compensate for metabolically deficient gangliosides in their physiological system. Finally, we hypothesize that if the absorption and utilization of administered GM3 is a reversible pathological process, some of the manifestations and symptoms associated with this neurological disorder might get partially or even completely relieved by introducing adequate amounts of GM3 into their body, particularly into the CNS, which may potentially lead to a normal life in these children when the exogenous GM3 in adequate quantity was given before they started to develop the related symptoms.

4.2 Materials and Methods

4.2.1 Overall Approaches

The study design is fairly straightforward. We orally administered an adequate amount of GM3 and GD3 gangliosides from G500 to the children with GM3 synthase deficiency and assessed them clinically, and monitored them biochemically, particularly focusing on bioavailability and metabolism of gangliosides, and the gangliosides' therapeutic effects on the intellectual disability in this condition. Since there is no endogenous ganglioside synthesized in these patients, the study design is overly simplified in many ways. For instance, if a detectable level of gangliosides is found in the patients' plasma and cerebrospinal fluid, our main hypotheses, that the orally administered gangliosides may be adequately absorbed, taken up into the circulation, and cross the blood-brain barrier, are confirmed. Further studies will be focusing on clinical assessments of these patients to determine if the orally delivered gangliosides are therapeutic.

4.2.2 Quantitative Analysis on Gangliosides by LC/MS/MS

As our study is designed to determine the bioavailability and metabolism of administered gangliosides provided by G500 in the affected patients, we are in an urgent need to have a reliable and sensitive analytical assay on hand in order to periodically monitor and quantitative determine the levels of administered gangliosides to be absorbed by the

physiological circulations of studied patients. As a result, the established UPLC/MS/MS method from chapter 3 will be used in combination with the chemical derivatization protocol as the primary means for implementing the intended study on collected plasma samples.

In addition, as an alternative means to confirm the positive existence of GSD disease on newborns screened and diagnosed by DNA mutation analysis, the UPLC/MS/MS method described in chapter 2 will be serving as the secondary confirmatory technique to rapidly assure the positive existence of targeted mutation by quantitatively looking at the levels of endogenous GM2, GM3, GD2, and GD3 in plasma samples collected from diagnosed patients prior to embarking on the G500 therapy.

4.2.3 Subjects of the Study

5 patients with GM3 synthase deficiency aged from birth to 26 years old were recruited into this study and divided into two separate groups, control (n=2) and treated (n=3) group. We plan to include as many younger patients as possible, particularly the patients under 2 years old, as the shorter time period of exposure to this condition in the younger patients might cause less brain damage, thus may lead to more dramatic and positive clinical outcomes. Since contraception is against the Amish religious belief, we expect more newborns with GM3 synthase deficiency will be coming in into these affected families, who are going to be receiving an immediate and continuous treatment with G500 and thus hopefully preventing the disease progress to a level at which the normal life can be brought back once again in these new patients.

4.2.4 Assessments

For this study, each patient is expected to receive oral ganglioside administration for at least 12 months. Formal clinical evaluations and ganglioside measurements are performed periodically before and during the study. The comprehensive clinical evaluations performed include: 1) a comprehensive examination with a list of clinical parameters for each patient by the PI and a pediatric neurologist (the pediatric neurologist will be single-blinded as the participation information is not available to him); 2) developmental assessments, including Battelle Developmental Inventory (2nd edition) by the developmental psychologist.

4.2.5 Dosage and Ganglioside Monitoring

The amount of the gangliosides to be administrated remains to be determined. We estimate that 120 mg dietary ganglioside may be needed to have 2.4 mg (2%) reaching to the brain. This amount can be delivered through 10 ml of Ganglioside 500 (G500), which contain 50 mg GM3, 60 mg GD3 and trace amounts of other gangliosides. We expect that the needs for gangliosides might be bigger at the start as the patients are in complete depletion status for GM3 and its downstream derivatives. We will closely monitor the plasma ganglioside level and increase the dosage as needed until the plasma GM3 level becomes reasonably stable and close to the level of the low range of normal heterozygous controls.

4.2.6 Data Analysis

Since there is no endogenous ganglioside synthesized in these patients, the data analysis is fairly easy and straightforward. In comparison to the levels of gangliosides in patients' plasma and cerebrospinal fluid at the pre-dosed stage, if detectable or increased levels of gangliosides are found in their biological circulations after being dosed with G500, our hypotheses are confirmed. Further data analysis will focus on the comparison of clinical assessments of these patients before and during the study to determine if the orally delivered gangliosides are therapeutic.

4.3 Results

4.3.1 Assessment on Physical Development

Owing to the infantile-onset trait of GSD on patients affected by the homozygous mutation, they in general begin to develop symptoms like severe irritability, failure to thrive, profound developmental delay, and retractable seizure before 6 months of age and more deteriorating symptoms like cortical blindness, choreoathetosis, flaccid weakness, seizure, and profound physical and mental retardation by early childhood. So, the inborn-onset symptoms associated with retarded and dysfunctional development on a variety of physical parameters, such as body weight, body length, and head circumference, could not only act as a direct and straightforward indicator for the severity of the disease progression on affected children, but also able to serve as a characteristic marker for the early-on evaluation of

delivery strategy as well as therapeutic effectiveness of G500 formula to the administered patients during the ongoing clinical trial.

From the standpoint of evaluating the progression of disease severity in terms of the degree of improvements on the physical development, the virtual values of a number of physical parameters, including body weight, body length, and head circumference, have been acquired in 6-month window along the ages of affected children to obtain the growth trend and tendency regarding their physical development in comparison with the unaffected children. As shown in Fig.19, compared to the unaffected kids, the typical physical development of patients with GSD initially appears to be fairly normal in the first few weeks after birth, starts to develop moderate delaying symptoms in next a couple of months, and becomes progressively severe in terms of retardation associated with their increasing age, which consistently matches the expected clinical outcome of GSD with age-dependent and stage-specific severity as described previously.
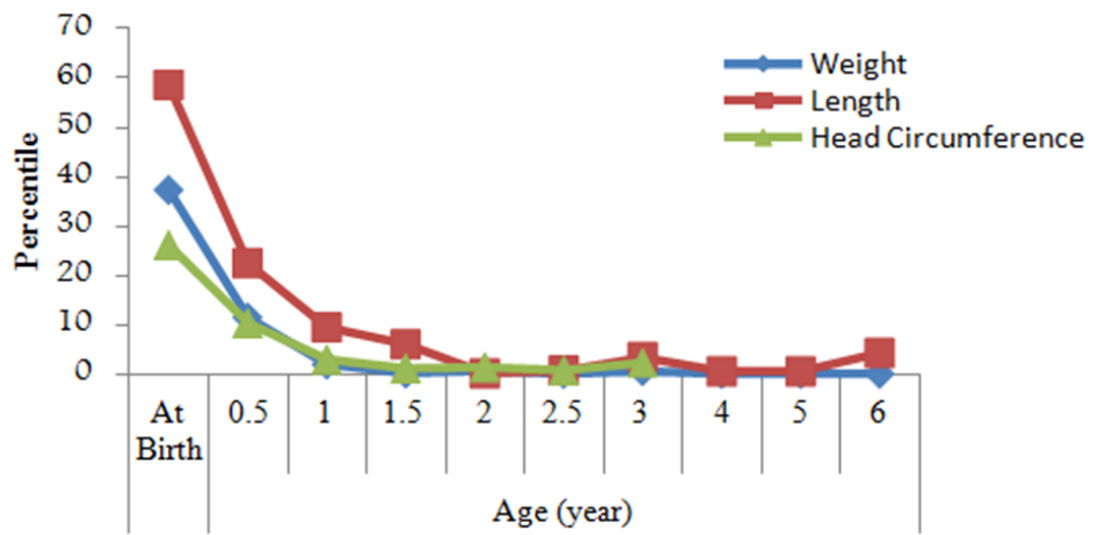
Fig.19 The disease progression in relation to the patient age

For the purpose of evaluating the delivery strategy as well as the therapeutic effectiveness of the G500 formula, the actual values of physical parameters, including body weight, body length, and head circumference, have also been acquired on 3-month basis from affected children in two separate groups, control and treated group, along their ages to establish the pattern of overall physical development compared to unaffected kids in response to the G500 therapy. As illustrated by Fig.20, the individual from control group (A) with no access to G500 treatment exhibited significant deviations on the growth patterns from the normal growth curves in all studied aspects, especially for growths after 6-month post-birth, which can be similarly predicted from we have seen previously from other untreated patients. However, based on the growth curves we have obtained from individuals (B&C) in G500-dosed group, compared to the control group without receiving the G500 treatment, they showed approximately 20~30% improvements on their physical developments and being more mathematically correlated towards the growth pattern of normal individuals regarding almost every single aspect in response to G500 therapy.
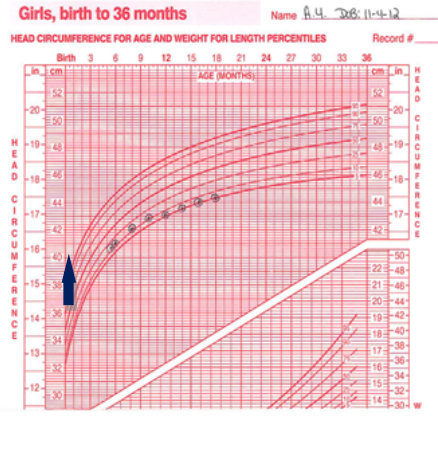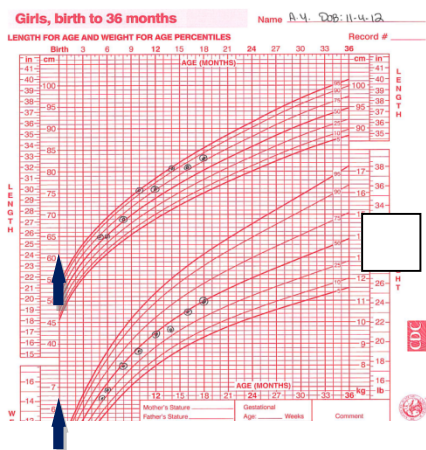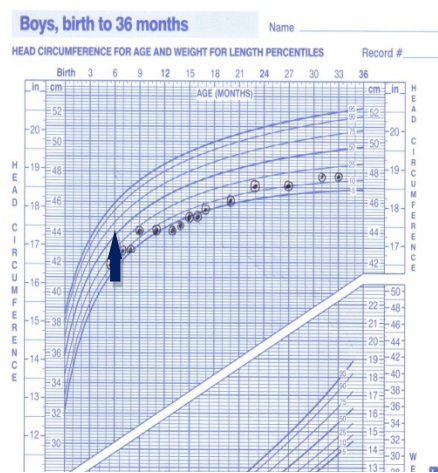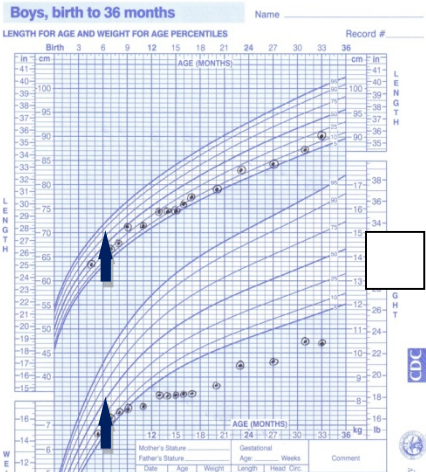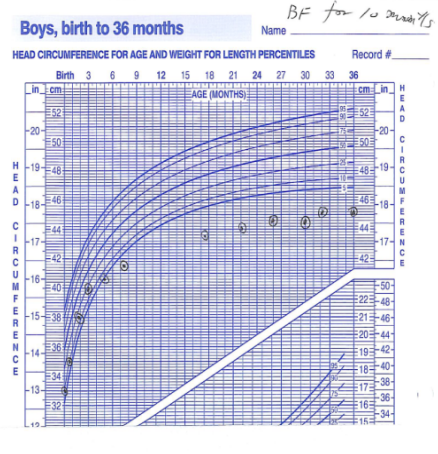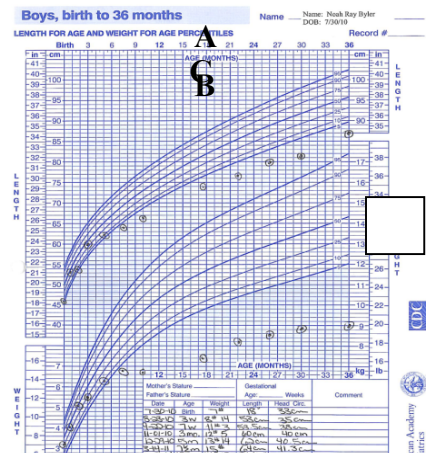
Fig.20 Growth charts for individual patients

Results of physical examination for (A) patient with GSD; (B) patient with GSD on G500; (C) patient with GSD on G500, and the arrow bars are indications of specific time points at which the therapy was initially started.

4.3.2 LC/MS/MS Analysis

Due to the ease of sample handling and collection, we have been continuously analyzing plasma samples collected from different patients at various time points to monitor the possible changes on ganglioside levels within the blood circulation of GSD patients before/after the dosage of G500. As we previously speculated, if the administered GM3 and GD3 from G500 indeed gets absorbed and utilized by the physiological system of patient to reactivate the compromised biosynthetic pathway of downstream GM3-related derivatives, the blood system would be the primary circulation that those absorbed gangliosides rely upon to get spread out and delivered across the entire physiological system of the patient. Therefore, the levels of gangliosides in the blood circulation following the G500 treatment are expected to be continuously increased as the therapy goes on and on, which is also consider as another important indicator of the successful administration and absorption of G500-derived gangliosides during the clinical study.
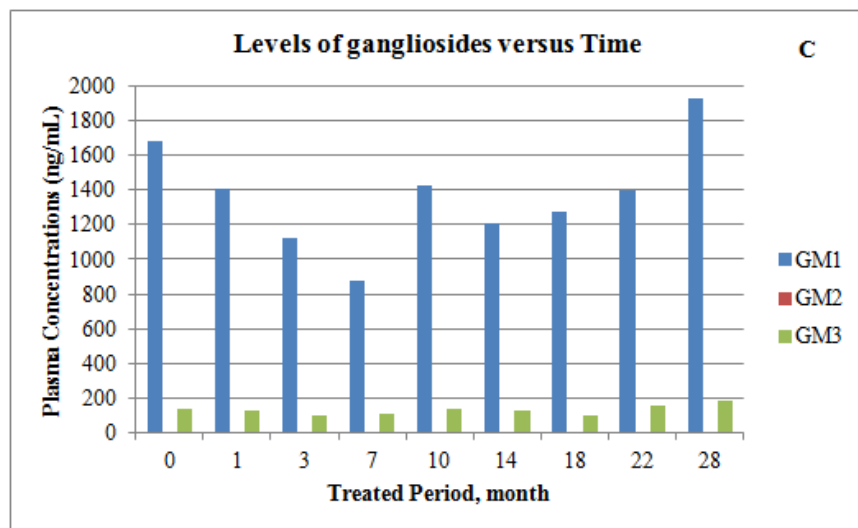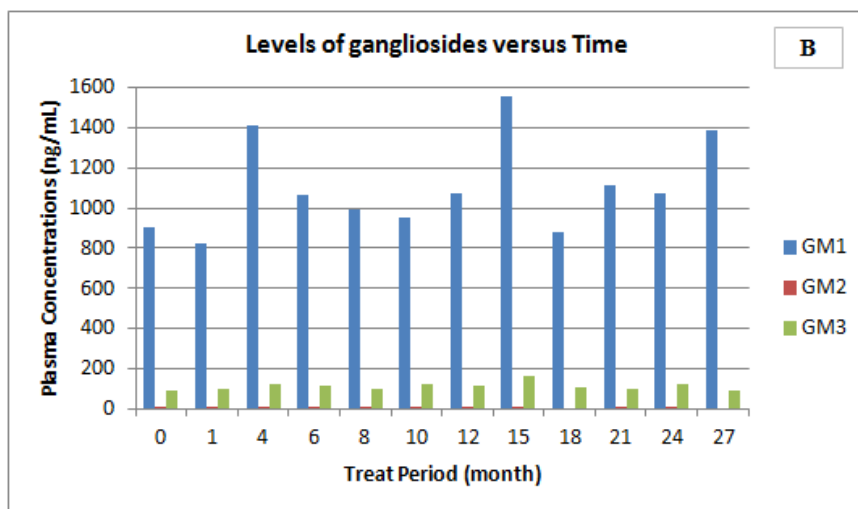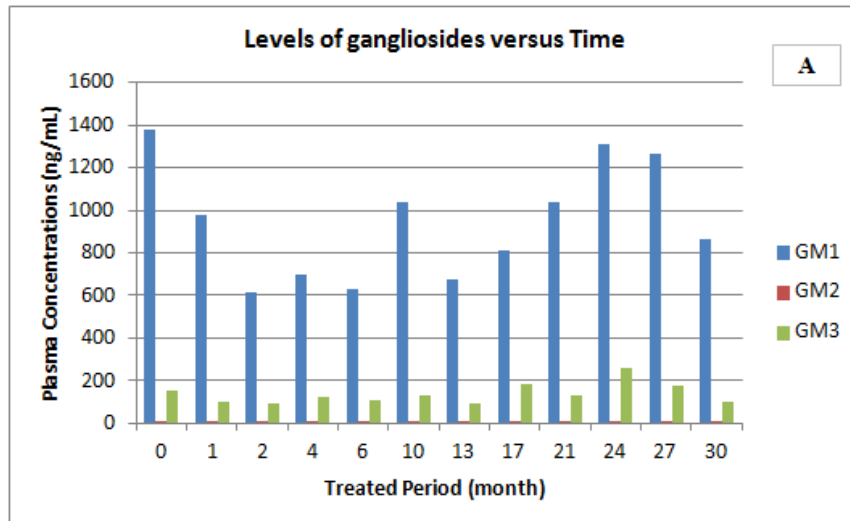
Fig.21 Levels of circulating monosialogangliosides in the plasma samples of GSD patients determined by LC/MS/MS

Practically, the analysis on plasma samples collected from patients based on a regular 3/4-month interval has been carried out by employing the LC/MS/MS method established in chapter 3, and a portion of the quantification results have been shown individually in Fig.21. As can be seen from Fig.21, the patients currently undergoing treatment generally show irregularly fluctuated pattern on the level of circulating GM3 from 80~200 ng/ml in their plasma samples in response to the progression of G500 therapy, while the level of circulating GM2 remained consistently undetectable throughout the entire study, which is contradicted against our hypothesis that as the changes on circulating GM3 level in plasma turned out to be insignificant in comparison with the pre-dosed plasma level and inconsistent with what has been expected from longterm continuous exposure of orally administered G500 under normal physiological conditions, possibly accounting for the absence of circulating GM2 in the patient's plasma sample with insufficient synthetic precursor, GM3.

Moreover, regarding the circulating GM1 whose level represents the combination of total concentration from two predominant co-existing subspecies, GM1a and GM1b, the concentration values presented here on GM1 are relatively less informative than GM2 and GM3 levels for this particular study as GM1a itself belongs to the family of GM3-derived downstream derivative, whereas endogenous GM1b is mainly synthesized by a different precursor through a GM3-independent pathway. Based on the disease etiology as well as the biosynthetic pathways they are involved, GM1a is expected to undergo premature biosynthetic termination and possess consistent behavior with circulating GM2 to remain completely absent from patient plasma samples during the analysis. However, the

biosynthesis of GM1b is expected to be highly up-regulated in order to relieve the accumulation of lactosylceramide in the body by fluxing the accumulated lactosylceramide through the pathway.

4.3.3 Assessment on Intelligential Development

Based on the disease pathology described by previous studies [1-4], one of the typical symptoms closely associated with the progression of GSD is the profound mental development retardation, a common and characteristic clinical manifestation to the majority of neurodegenerative disorders affecting the maturation of central nervous system (CNS), which is a critical step in maintaining the development of intelligence and brain functions at regular pace. In the absence of endogenous GM3 as well as its downstream complex metabolites, all of which are indispensable molecular valves ascertaining the CNS is appropriately matured and regulated, development of normal intelligence and brain functions have been demonstrated to be severely compromised under this particular physiological condition. From this regard, the extent to which the intelligential development has been virtually improved before/after G500 therapy could certainly be used as one of the primary clinical indicators to establish the logical correlation between the therapeutic effectiveness of administered G500 and progression of GSD.

The results of intelligential development assessment from two affected patients have been summarized and shown in Fig.22. In Fig.22, parameters related to intelligential development have been determined in parallel studies between the G500-treated and

untreated patients before/after the intervention at comparable timelines, and the measured values have been normalized to standard levels to evaluate the potential impact of administered G500 in terms of clinically improving their intelligential activities along the therapeutic intervention. As illustrated by Fig 4.5, in comparison with untreated patient who in general possesses minimally developed or even highly undeveloped physical behaviors pertaining to his/her intelligential development at the age of 24-month, pretreated patient overall displays more progressive trends of development on intelligence except for fine motor at the age of 26-month. However, as the pretreated patient embarking on the therapeutic intervention, significant degree of behavioral improvements have been observed in aspects like self care and self conception, whereas minor improved or even reduced levels of intelligential activities have been observed in other studied criteria on treated patient compared to the untreated one at the age of 42- and 41-month, respectively.

A

B
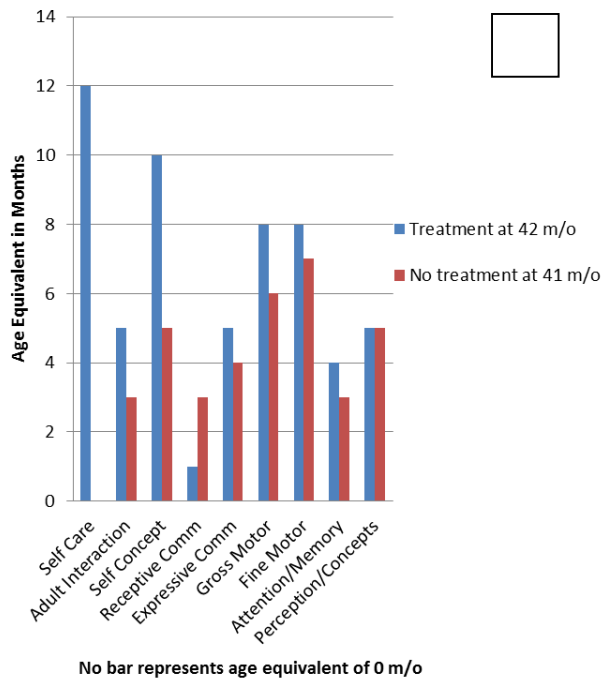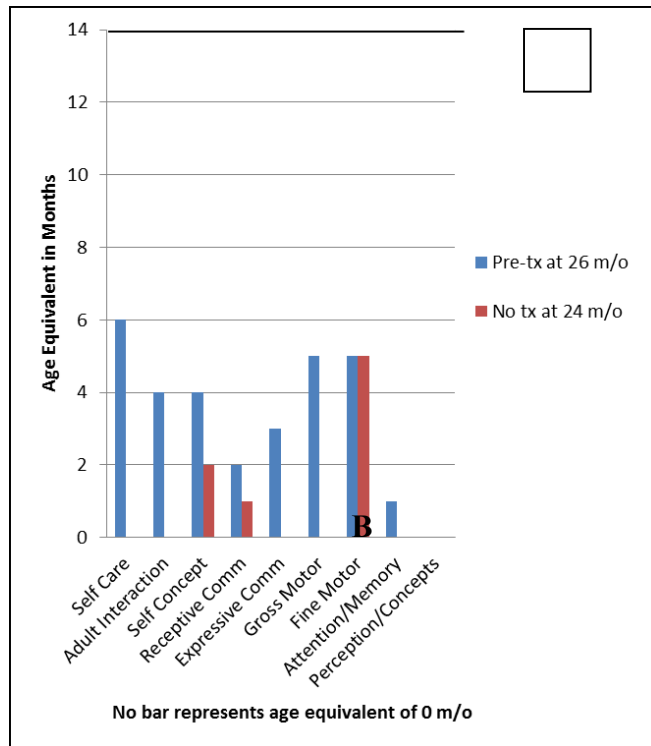
Fig.22 Comparison on intelligential development assessment between one control and one treated patients before(A)/after(B) the therapeutic intervention

4.4 Discussion

4.4.1 Assessment on Physical Development

Based on Fig.19, one very interesting observation to note is that children affected by GSD generally have comparably normal clinical behavior and developmental trend at the time of birth, which could be primarily ascribed to their continuous absorption of exogenous a- and b-gangliosides via maternal circulation during embryogenesis due to their incapability of endogenously synthesizing a- and b-gangliosides on their own, confirming part of our hypothesis that the homozygous affected babies receive the major source of supplemental gangliosides from their heterozygous mothers for being able to maintain the regular pace of their development at the pre-natal stage. However, as the maternal provision on supplemental gangliosides has been physically terminated from them after birth, their circulating a- and b-gangliosides start to undergo gradual depletion from their bodies, which is usually accompanied by the gradual development of typical early signs of neurodegenerative disorders, particularly seriously delayed physical development. Moreover, the developmental retardation directly corresponding to the disease progression becomes increasingly severe as their demand in obtaining molecules of GM3 and its downstream derivatives gets progressively urgent with increasing age, which is also complied with our previous predictions on the further development of other complex symptoms associated with this clinical condition, suggesting that the severity of physically developmental retardation is essentially a good clinical marker for the evaluation of age-dependent and stage-specific progression of this unique syndrome.

In Fig.20 (A), a typical progressive developmental stagnation pattern following age-dependent manner has also been described in terms of three variables, body weight, body length, and head circumference, from a 3-year old boy affected by GSD. As shown by the growth charts from Fig.20 (A), the physical growth of this boy appears to be fairly normal in the first 6 months after birth, falls out from the reference ranges of normal growth in following months, and continues to stay degenerately deviated from the normal curves for the rest of the studied period, which is consistent with the result from Fig.19.

However, in the presence of G500 therapy, another 3-year old boy affected by GSD who starts off the initial G500 intervention at the age of 12-month, as described in Fig.20 (B), has undergone significant improvements on his physical development to boost his falling growth curves back into the reference ranges in terms of body weight and head circumference after the existing gangliosides received from maternal circulation have been thoroughly depleted from his body, suggesting that those exogenous gangliosides provided by G500 formula have somehow been absorbed and utilized by his physiological system to replace the missing gangliosides from the metabolic pathways, reactivate the metabolism and biosynthesis of a- and b-ganglioside series, and thus facilitate his physical development from the early stage, which closely matches a portion of our hypothesis that the orally administered gangliosides from G500 could be absorbed by the patient's physiological circulations and utilized by the biosynthetic and metabolic pathways to make more complex downstream derivatives.

Likewise, based on the growth curves from another 18-month old girl affected by GSD, who embarks on the G500 intervention at the age of 6-month, as described in Fig.20

(C), she has displayed a full spectrum of significantly improved growth curves well above the minimal cutoff of the reference ranges. More importantly, compared to the previous 36-month old boy, she has showed even more significant improvements on her physical development in all studied aspects when the intervention was initiated 6-month earlier, suggesting that affected children with younger age might receive less serious damage to their body from the metabolic disruption and depletion as they suffer shorter exposure to ganglioside deficiency in terms of timeframe and thus are more sensitive and responsive to the G500 intervention than the older ones. Namely, the earlier those affected children begin to receive the G500 intervention, the better chance they stand of possibly getting more effectively treated by the G500 therapy, confirming some of our hypothesis on treatment implementing strategy.

4.4.2 LC/MS/MS Analysis

In Fig.21, quantitative results from three patients with GSD receiving G500 therapy have been selected to represent the entire treated group and demonstrate the potential changes on monosialoganglioside levels in patient's plasma circulation in response to the daily G500 administration. Based on the plasma concentration levels of monosialogangliosides presented in Fig.21 (A), the circulating GM3 level unexpectedly vibrates up and down along the baseline value in a very irregular pattern in response to the G500 intervention, whereas the circulating GM2 level remains constantly undetectable during the analysis, suggesting the possibilities of either inefficient absorption of exogenous GM3

and GD3 from G500 by blood circulation through oral administration or the rapid flux-through of absorbed GM3 and GD3 on the metabolic intermediates by the over-active enzymes for the biosynthesis of complex ganglioside molecules at the terminal of the pathway. In comparison with circulating GM2&3 levels, level of circulating GM1 seems to be not quite indicative as this concentration value represents essentially the sum of concentrations from two co-existing positional isomers, GM1a and GM1b, both of which are essentially GM1 gangliosides with the sialic acid residue being placed at disparate positions and appear to have completely opposite behaviors in the course of G500 treatment and disease progression, leading to unpredictable consequence based on alterations on total GM1 concentration during the current study.

Likewise, based on quantification results we have observed from patients in Fig.21 (B) & (C), the levels of GM1&2&3 have been found to possess similar quantitative behaviors in the plasma circulation of other patients as we have seen previously, which could be potentially used as a representation to gain the general picture on patient's overall response to the ongoing G500 intervention implemented through oral administration at the blood circulation level. However, due to the possible actions of either aforementioned reasons or even the combination of both, from this study, we failed to quantitatively observe the outcome of major increases on the levels of metabolic intermediates along the pathway in the blood circulation of patients dosed with G500 as we expected, which might require a more robust analytical method that is capable of including more downstream derivative species onto the quantification list and further identify the actual cause that is responsible for this undesirable outcome. Unfortunately, owing to the practical limitations we have encountered

from the analytical instrumentation as well as the methodology, the method with the ability to simultaneously quantify a full panel of analytes of our particular interest from the metabolic pathway is still undergoing initial methodological design on the early phase.

4.4.3 Assessment on Intelligential Development

Based on the concepts of fundamental psychology, Battelle Developmental Inventory is defined as the measurement of developmental milestones allowing adaptations for specific disabilities, including cognitive skills, adaptive skills, motor skills, communication skills, and personal-social development, whereas Vineland Adaptive Behavior Scale is described as the measurement of adaptive behaviors associated with the ability to cope with environmental changes, including fundamental socialization skills, communication skills, basic motor skills, and daily living adeptness. So, based on these psychological definitions, a combination of Battelle Developmental Inventory-2 and Vineland Adaptive Behavior Scale-2 have been selected as the primary criteria to evaluate the potential improved level of intelligential development on G500-treated patients versus the G500-untreated ones under this particular circumstance.

In Fig.22 (A), the baseline values of intelligence-associated psychological behaviors have been evaluated on two studied patients prior to the commencement of G500 therapeutic intervention, and it has been revealed that the 26-month old patient displaying more significantly aggressive pattern of intelligence-related behavioral development then the 24-month old patient in the majority of studied aspects except for fine motor whose level are

equivalently developed in both studied subjects, implying the possibility of age-dependent baseline shifting between studied patients on the measuring values in relation to intelligential development, which might act as a possible influential factor affecting the data quality of following studies.

In Fig.22 (B), with the continual administration of G500 to one of the studied patients, in comparison with the untreated patient, the extent to which the psychological activities associated with the intelligential development are being virtually improved have been observed to be significant for self care and self concept, suggesting the gangliosides absorbed by the patients' physiological system and utilized by their metabolic pathways from administered G500 might have been therapeutic in terms of facilitating the intelligential developments on self-related behaviors. However, only minor/none degree of behavioral improvements have been observed for aspects like adult interaction, expressive communication, fine motor, attention/memory, and perception/concepts, which might be caused by shifting on the baseline values at the pretreated stage. Surprisingly, the treated patient has been found to possess diminished level of receptive communication development compared to the untreated one, which is not consistent with the psychological patterns determined by other intelligential behaviors.

4.5 Conclusion

In the present work, we proposed a therapeutic intervention strategy based on oral administration of exogenous gangliosides to cure an inherited neurological disorder,

Ganglioside GM3 Synthase Deficiency, and established a systematic approach to biochemically evaluate the clinical behaviors of studied patients as well as the therapeutic effectiveness of the proposed therapy from many different aspects. From the clinical perspective, compared to untreated patients suffering severe physical and mental developmental stagnation, treated patients displayed significantly improved physical development and moderately improved psychological behavior as the treatment progresses, implying the ultimate uptake and utilization of the administered gangliosides by their physiological systems to replace the missing gangliosides from their metabolic pathways. From the analytical perspective, the plasma levels of circulating monosialogangliosides determined by LC/MS/MS from treated patients showed inconsistent patterns of fluctuation compared to baseline values determined from the pre-treated stage, suggesting the possibility of rapid metabolism of exogenous gangliosides might have undergone to downstream derivatives following their physiological absorption.

By principle, plasma level of free GM3 acts as a direct reflection on the abundance of GM3 being integrated onto the cell membrane lipid bilayer as a physiological equilibrium must be existing between the unbound GM3 presenting in the blood circulation and bound GM3 incorporated to the cell membrane. In the complete absence of endogenous unbound GM3 in patient blood circulation in response to enzymatic dysfunction on GM3 synthase, the level of bound GM3 being integrated into the glycolipid-enriched microdomain is expected to be equivalently absent from the cell membrane. Additionally, as GM3 serves as a universal and indispensable synthetic precursor for the de-novo biosynthesis of a- and b-ganglioside series, its endogenous absence usually causes the premature biosynthetic

termination of all GM3-derived downstream metabolites, especially those more complex gangliosides such as GD1a, GD1b, GT1a, and GT1b, all of which are fine molecular regulators in modulating cell proliferation, differentiation, migration, and adhesion during the maturation and maintenance of physiological and psychological systems, leading to the substantial alternations on the composition and organization of lipid-anchoring scaffold within the lipid raft and thus the significant disruptions on the ordinary functionalities of glycolipid-enriched microdomain in orienting the incorporated cell surface receptor tyrosine kinases towards the incoming ligands for transmitting the extracellular biological signaling into the cytoplasmic machinery. Based on previous studies, although it has been revealed that increased GM3 level in general up-regulates the inhibitory effects on the dimerization and autophosphorylation of the majority of receptor tyrosine kinases, whereas the increased level of its downstream derivatives, including GD1a, GD1b, GT1a, and GT1b, has also been found to be able to effectively activate those receptors by inducing the dimerization of the extracellular ligand-binding domain and the subsequent autophosphorylation of the cytoplasmic tyrosine kinase domain at low concentration or complete absence of specific ligands, suggesting distinctive regulatory roles of various ganglioside species. As a result, the overall impact of the metabolic disruptions by genetic defects, as demonstrated in the case of GSD patients, on balancing act between the molecular interactions of GM3 and its downstream more complex metabolites with individual receptor tyrosine kinases seems to be the primary driving force accounted for the pathogenesis of GSD as well as the eventual development of severe symptoms associated with the disease, which could be better illustrated by analyzing results obtained from clinical studies in conjunction with discoveries

reported by other studies as following.

Recently, previous study has demonstrated that GD1a, one of the major downstream metabolites of GM3 from the biosynthetic pathway of a-series ganglioside that are expected to present in the blood circulation of patients undergoing G500 treatment, is capable of stimulating the proliferation of normal human dermal fibroblasts by promoting the EGFR dimerization through a ligand-independent mechanism and hence enhancing the autophosphorylation of the cytoplasmic tyrosine kinase domain of EGFR, leading to the subsequent activation of Ras and Raf, which in turn causes the EGFR-mediated activation of mitogen-activated protein kinase (MAPK) pathway [9]. Moreover, alternative study has also discovered that GD1a facilitates the differentiation of human mesenchymal stem cells to osteoblasts primarily by stimulating the autophosphorylation of cytoplasmic tyrosine kinase domains from extracellular signal-regulated kinases (ERK), mitogen-activated protein kinase (MAPK), and epidermal growth factor receptor (EGFR). In the presence of decreased level of GD1a through knockdown of ST3Gal II, the enzyme that catalyzes the synthesis of GD1a from GM1, by shRNA transfection, inhibitory effects caused by reduced phosphorylation of ERK, MAPK, and EGFR has been observed during the differentiation [10], implying one of the potential molecular mechanisms by which the physical developments of treated patients are clinically improved by acquiring certain amounts of circulating GD1a through the metabolism of absorbed exogenous GM3 and stimulating the activity of EGFR and its downstream signaling cascades upon its interaction with GD1a.

In addition, it has been revealed that the over-expression of gangliosides, especially GD1b and GT1b, significantly enhances the proliferation of a rat

pheochromocytoma cell line (PC12) by transfecting the cell line with ganglioside GD3 synthase gene. The increased levels of GD1b and GT1b, both of which are major downstream metabolites of GM3 from the biosynthetic pathway of b-series gangliosides that are expected to present in the blood circulations of treated patients, stimulate the proliferative event by continuously phosphorylating the tropomyosin receptor kinase A (TrkA) and constitutively inducing a conformational change of TrkA to form a active dimer, leading to elevated activation of the downstream ERK1/2 and PI3K/Akt pathways even in the absence of nerve growth factor (NGF) [11], suggesting an alternative molecular mechanism by which the significant improvements on physical development of treated patients in comparison with control patients are accomplished partially through the cell proliferation stimulated by triggering an continual dimerization of Trkt and activation of ERK1/2 and PI3K/Akt pathways with elevated levels of GD1b and GT1b.

Furthermore, mounting evidence has also illustrated that the hepatocyte growth factor receptor c-Met displayed a ganglioside-dependent manner of activation in breast cancer cells by expressing the GD3 synthase in MDA-MB-231 breast cancer cells to induce the cell surface accumulation of b- and c-series gangliosides, including GD3, GD2, and GT3, all of which are practically accessible to the patients receiving ongoing oral administration of G500. Among these complex gangliosides, GD2 was found to be extensively implicated in the activation of c-Met as well as the subsequent activation of MEK/ERK and PI3K/Akt signaling pathways, leading to enhanced cell migration and proliferation, which is further confirmed with the competition assays by competitively blocking the oligosaccharide moiety of GD2 from interacting with the c-Met using anti-GD2 mAb and thus inhibiting the

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phosphorylation of c-Met. Comprehensively, markedly reduced levels of GD2 biosynthesis and c-Met phosphorylation and the reverse proliferative phenotypes were observed as the GD2 synthase gene was silenced by siRNA transfection [12-14], indicating the potential regulatory role of GD2 in facilitating the physical and mental development of treated patients.

Finally, it has been evident that ganglioside enrichment in human umbilical vein vascular endothelial cells (HUVEC) can sensitize the vascular endothelial growth factor receptor (VEGFR) by inducing the VEGFR dimerization and auto phosphorylation at sub-threshold level of VEGF, causing the subsequent activation of VEGFR/PKC/MAPK and VEGFR/PI3K/MAPK pathways, which in turn leads to the markedly potentiated cell proliferation and migration [15]. In parallel studies, pre-incubation of HUVEC with exogenous GD1a prior to the addition of VEGF has also been found to substantially up-regulate the VEGF-induced cell proliferation and migration [16], shedding a new light on elucidating the underlying mechanism by which the clinical symptoms of GSD on treated patients are being gradually relieved in response to the G500 therapeutic intervention strategy.

Overall, this oral administration-based therapeutic intervention strategy demonstrates certain potentials in terms of curing this infantile-onset clinical condition, especially for affected newborns that typically have short exposure to the premature ganglioside biosynthetic termination and endogenous depletion. Since this is the first study to use dairy-originated gangliosides to treat a metabolic defect and assess the bioavailability and metabolism of orally administered gangliosides in humans, we believe that this therapeutic strategy might serve as a breakthrough for treating this devastating condition. Moreover, as

gangliosides' neurotrophic and neuritogenic effects have already been demonstrated in various neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease, the therapeutic implications of this study might be much broader. Considering the gangliosides in our study are obtained from milk and given orally, the therapy could fall into the category of nutritional supplementation, which might dramatically accelerate its implications in all ganglioside-related neurological conditions. Undoubtedly, this study sheds a new light on fundamentally understanding the physiological and pathological functions of gangliosides in human brain.

4.6 Future Direction

In this study, we implemented a clinical study in attempts to treat an inherited metabolic disorder associated with ganglioside GM3 biosynthesis by using a therapeutic intervention strategy based on oral administration and established a corresponding assessment system to biochemically evaluate the clinical response of studied patients as well as the therapeutic effectiveness of the therapy. Based on the results we collected from the clinical trial, the treated patients in general displayed positive feedbacks in terms of relieving the severity of the developing symptoms from the clinical standpoint, demonstrating the potential of this therapeutic intervention strategy in treating this particular condition. However, from the analytical standpoint, the concentration values of several major metabolic intermediates on the pathway in the plasma circulation of studied patients showed irregular variation around the baseline value in response to the therapy progression, signifying that minor

quantity of administered molecules might have been obtained from the absorption event during delivery and the rapid influx and efflux of absorbed molecules through the whole metabolic pathway, both of which could be attributed to inefficient delivery and inconsistent absorption pattern of administered molecule in relation to the complexity of oral administration. As a result, the conditions of oral administration associated have to be further optimized to improve the delivery as well as absorption efficiency and thus increase the bioavailability of the administered molecule for being able to observe more confident, promising, and convincing clinical outcomes from the ongoing clinical trial.

By principle, oral administration is usually well-known as the most complicated drug delivery system with the most unpredictable outcome as the orally administered molecule might be subject to all possible kinds of physiological modifications and degradations associated with digest system prior to its entry into the blood circulation and ultimate reachout to the targeted site for interaction. Based on our discussion on gangliosides' molecular structure from the previous chapter, the only possible site available for covalent modification on their structures is the protruding carboxylic acid group on the sialic acid residue Neu5Ac, which might be subjected to modifications like esterification, decarboxylation, and amidation catalyzed by various enzymes as they are traveling through the different physiological system. Therefore, the co-administration of synthetic carboxyl group-containing structural analog, GM4 for instance, in combination with G500 formula is necessitated to substitute administered gangliosides from undergoing possible structural modifications and thus preserve them to proceed into the circulations in their original structure, enabling them to further participate into the metabolic pathway and exert their

normal biological functions to compensate for the physiological damage caused by longterm GM3 deficiency.

In addition, as gangliosides are featured by carbohydrate moieties made up with monosaccharide building blocks through either α-1, 3- or α-1, 4-glycosidic linkage except for Neu5Ac whose linkages are usually α-2, 3- or α-2, 4-glycosidic bond, stomach who has the capability of secreting the gastric acid and reducing the PH to as low as 2 during the digestion could potentially act as the primary organ facilitating the degradation of administered gangliosides and breaking them down into individual monosaccharide units before they are absorbed by the circulations due to the fact that glycosidic bonds are extremely sensitive and fragile to hydrolysis catalyzed by high concentration of acids. Hence, in order to preserve administered gangliosides to enter the circulations in their biological active form, the secretion of gastric acid by the stomach has to be properly inhibited by synthetic inhibitors specifically targeting the gastric acid secretion pathway to minimize their possible degradation while the oral administration is undertaken. Currently, two main classes of gastric acid secretion inhibitors have been made available to the market, histamine H2-receptor antagonists and proton pump inhibitors, both of which are FDA approved drugs to treat disorders associated with gastric acid secretion. Mechanistically, the histamine H2-receptor antagonists act by reversibly blocking the action of histamine to its receptor, a stimuli released by neighboring mucosal cells to stimulate the secretion of gastric acid in anticipation of a meal or when the food enters the stomach, which is capable of moderately elevating the pH value in the stomach by approximately one unit. While the proton pump inhibitors act by inactivating the final step in acid secretion - the transportation of proton ions

from the parietal cells to the lumen of the gastric glands, which has a more potent and longer lasting effect on acid secretion than the histamine H2-receptor antagonists, allowing larger amount of administered gangliosides to survive through gastric digestion and longer time window for the subsequent absorption to take place in the intestine. Expectedly, with the co-administered proton pump inhibitors to reduce the secretion of gastric acid, more exogenous gangliosides from G500 would get thoroughly preserved through the gastric digestion for later absorption, and thus their bioavailability in the blood circulation would be increased significantly.

Lastly, since gangliosides are predominantly localized at the outer leaflet of cell plasma membrane in a special configuration where the hydrophobic ceramide portion is embedded inward into the plasma membrane for stable anchoring and the hydrophilic carbohydrate moiety is protruding outward into the extracellular matrix for accessible interaction, their carbohydrate moieties are usually conferred with pretty high degree of protein-binding affinity via non-covalent interactions like hydrogen bonding and electrostatic interaction, which possibly prohibits them from being taken up by the tissues or cells around the circulations as the huge size of ganglioside-protein complex makes it stand little chance of penetrating through and being settled down onto the cell membrane. More importantly, the dissociation of this ganglioside-protein complex could be an unfavorable kinetic process, which impedes the release of gangliosides into their free molecular forms and requires the application of a carrier vehicle to create an isolated protein-free environment to physically prevent the interaction from happening between gangliosides and proteins. Therefore, the enriched gangliosides purified from cow or bovine milk are ought to be formulated into a

protein-free lyophilized powder and encapsulated into carrier vehicle along with proton pump inhibitors as well as carboxyl group-containing structural analogs for administration to increase the efficiency of preservation and reduce the occurrence of undesirable bindings/interactions with other proteins during the absorption.

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