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A Novel Approach for Ganglioside Structural Analysis Based on Electrospray Multiple-Stage Mass Spectrometry

Alina D. Zamfir,[1,2] Željka Vukelić,[3] Andrea Schneider,[4] Eugen Sisu,[5] Nicolae Dinca,[2] and Arnd Ingendoh[4]

[1]Mass Spectrometry Division, National Institute for Research and Development in Electrochemistry and Condensed Matter Research, Timisoara, Romania; [2]Department of Chemistry and Biology, "Aurel Vlaicu" University of Arad, Arad, Romania; [3]Department of Chemistry and Biochemistry, Faculty of Medicine, University of Zagreb, Zagreb, Croatia; [4]Bruker Daltonik GmbH, Bremen, Germany; [5]Department of Biochemistry, University of Medicine and Pharmacy "Victor Babes," Timisoara, Romania, and Chemistry Institute of Academy, Timisoara, Romania

A powerful method for detailed structural analysis based on electrospray ionization high-capacity ion-trap multiple-stage mass spectrometry (MS) is for the first time introduced in glycolipidomics. The method was optimized for accurate structural elucidation of human brain gangliosides and specifically applied to normal adult human hippocampus-associated structures. The multiple-stage MS experiments reported here allowed for a complete structural characterization of the oligosaccharide moiety of a GM1 ganglioside species. This was achieved by elucidating the sequence and identification of the GM1a structural isomer from the sialic acid attachment site at the neutral oligosaccharide chain. Moreover, the determination of the d18:1/18:0 sphingoid base/fatty acid composition of the ceramide moiety could be confirmed by this method. The novel protocol developed here proves high potential for rapid, reliable, and reproducible investigation of complex lipid-linked carbohydrates such as polysialylated gangliosides or species carrying some other groups that easily cleave off.

Key Words: electrospray, high-capacity ion-trap mass spectrometry, glycolipidomics, brain gangliosides, structural analysis.

Gangliosides, a large group of sialylated glycosphingolipids, are primarily plasma-membrane components, enriched within the functional membrane units—microdomains, which participate in cell-to-cell recognition/communication, cell adhesion, and cell signaling.[1–3] Both abundance and structural diversity of ganglioside species are the highest in the brains of mammals.[4] They are considered as biomarkers of human brain development, aging, and certain diseases.[5–7] Their diagnostic/therapeutic potential is the current focus of research, because of numerous effects they exhibit in vivo and in vitro.[8,9] As the most extensively tested, the monosialo-ganglioside form GM1 exhibiting such actions is on the list of pharmacotherapeutic drugs for treatment of Alzheimer's disease (AD).[10,11] The hippocampus, a morphoanatomically and functionally highly specific brain region located inside the temporal lobe, playing an essential role in learning and memory formation, as well as in regulation of sexual and emotional behavior,[12] is usually one of the first regions affected by neurodegenerative processes occurring in AD.[13] Due to specific physicochemical properties and their plasma-membrane localization, gangliosides fulfill the task of neuromodulators in connection with calcium, and thus contribute to memory formation.[14] As recently reported, GM1 may protect neurogenesis in the aging brain.[15] Also, a treatment with gangliosides could reverse lead-induced impairments of synaptic plasticity of the hippocampus in rats and might be effective in attenuating cognitive deficits induced by lead.[16] Moreover, gangliosides also occur in the nuclear envelope, where GM1 influences Ca^{2+} flux by activation

Address correspondence and reprint requests to: Prof. Dr. Alina D. Zamfir, Department of Chemistry and Biology, Aurel Vlaicu University of Arad, Revolutiei Blvd. 77, 310130-Arad, Romania (phone: +40-256-494413; fax: +40-256-204698; email: zamfir@uav.ro).

of a Na^+/Ca^{2+} exchanger located in the inner membrane of the nuclear envelope. The tightly associated GM1/exchanger complex was shown to exert a cytoprotective role in neurons and other cell types, as absence of this nuclear complex rendered cells vulnerable to apoptosis.[17] Ganglioside GD1b also acts as an apoptosis suppressor,[18] while GD3 induces apoptosis in different cell types,[19] and GM3 acts as a mediator and a modulator of neuronal cell death in vivo and in vitro.[20]

So far, a human brain region–specific ganglioside composition, as well as the composition changes in correlation with brain development, aging, and AD-caused neurodegeneration have been documented based on thin-layer chromatographic, immunochemical, and immunocytochemical evidence.[21–25] However, our recent mass spectrometric studies[26–30] demonstrated that brain region-specific ganglioside patterns contain a much larger number of individual species than previously reported, and a considerable number of them, especially various isomers/isobars, are still not unambiguously structurally identified. Detailed structural determination of individual species, particularly distinguishing of various isomers, requires even more selective and thorough structural analysis.

Current sophisticated electrospray ionization (ESI) mass spectrometry (MS)-based techniques, characterized by high sensitivity, reproducibility, resolution, and accuracy, offer a solid platform to develop fast and sensitive strategies for detailed investigation of brain gangliosides. Such strategies, using nanoESI and/or chip ESI in combination with either quadrupole time-of-flight (QTOF) MS,[27–29] off-line capillary electrophoresis (CE) and QTOF MS,[31] or Fourier transform ion cyclotron resonance tandem MS[30] were established by us during the last few years and for the first time introduced in brain ganglioside research.

Recently, we developed a fully automated chip-based nanoESI–QTOF tandem MS approach for systematic comparative compositional and structural investigation of gangliosides from human fetal and adult hippocampus.[32] The hippocampus was chosen because this brain region is among the first ones affected in AD and related disorders. Our chip ESI-MS method allowed a detailed profiling of the hippocampus-associated species, a high reproducibility of the comparative analyses, and reliable MS/MS structural characterization, though in many cases not completely diagnostic. Thus, in the case of parent ions associated with several isomers/isobars, for the unequivocal structural identification we realized the necessity of a novel protocol for additional sequencing of the fragment ions obtained by MS/MS.

In the last few years, the continuous development of MS systems providing multiple-stage fragmentation of selected precursor ions considerably increased the potentials of MS for detailed structural elucidation and broadened its applicability in biomedical research. The latest generation of MS instrumentation, equipped with nano-electrospray sources in combination with high-capacity ion storage trap (HCT) analyzer and multiple-stage sequencing, is particularly capable of providing an exhaustive structural identification, which no other MS technique currently offers.

In this context, as a part of our effort to improve MS methodology for structural elucidation of glycoconjugates, and implement novel, high-performance MS techniques in glycomics,[33–37] we report here upon the development of the first protocol for systematic and thorough structural investigation of gangliosides based on multiple-stage MS fragmentation. The approach was optimized to provide determination of ganglioside structural diversity, including isomers. The method efficiency was tested on a native mixture of gangliosides purified from normal human hippocampus. Multiple-stage MS provides ultrafast and efficient dissociation, reproducibility, sensitivity, and accurate structural data, offering a reliable characterization of human brain–associated monosialotetraose ganglioside structure(s) and the first clearcut identification of the GM1a positional isomer's presence in the normal adult human hippocampus.

MATERIALS AND METHODS

Chemicals

All reagents used in this study were of analytical grade. Methanol and chloroform were obtained from Merck (Darmstadt, Germany) and used without further purification. Distilled and deionized water was obtained from Mili-Q water systems Millipore, Bedford, MA.

Ganglioside Sample

In this study, a native ganglioside mixture from a 20-y-old adult hippocampus (denoted AH20) was investigated. The adult hippocampus originated from a healthy subject deceased in a traffic accident; according to morphoanatomical and histopathological examination,[26] the tissue exhibited no pathological signs. The tissue was obtained from the Department of Forensic Medicine, Faculty of Medicine, University of Zagreb, Croatia. Permission for experiments with human tissue for scientific purposes was obtained from the Ethical Commission of the Zagreb Medical Faculty. All ethical rules of the World Medical Association Declaration of Helsinki (revised Edinburgh 2000) were obeyed.

The tissue sample was weighed and stored at –20°C. The native mixture of gangliosides was extracted and

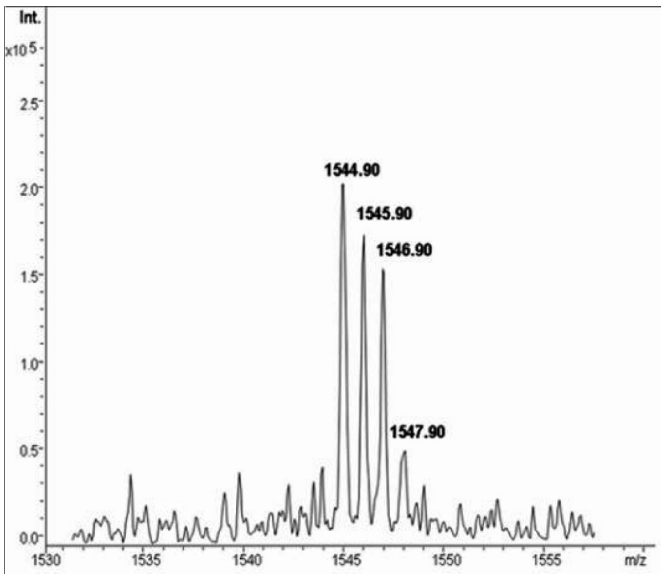

FIGURE 1

The GM1 precursor ion detected as singly charged at *m/z* 1544.90 in MS^1 (zoomed area within *m/z* 1530–1560).

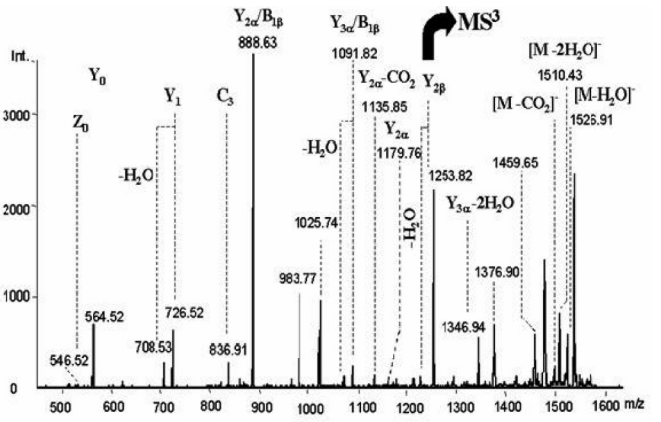

FIGURE 2

Negative ESI HCT MS^2 of the singly charged ion at *m/z* 1544.90 corresponding to the monosialotetraose (d18:1/18:0) ganglioside species detected in MS^1 mode. The ion assignment is in accordance with the published recommendations.[41,42]

purified in our laboratories as described in detail previously.[26] The sample was dried in a Speed Vac SPD 111V system (Savant, Düsseldorf, Germany). The stock solution of the sample of approximately 1 mg/mL was prepared by dissolving the dried material in methanol of analytical grade, and stored at –20 °C prior to analysis. For ESI HCT multiple-stage MS analysis, the stock solution was diluted in methanol to yield a concentration of approximately 10 pmol/μL (as calculated using an average molar mass of the gangliosides of 2000 g/mol).

Mass Spectrometry

Mass spectrometry was performed on a High Capacity Ion Trap Plus (HCTplus) mass spectrometer from Bruker Daltonics, Bremen, Germany. The HCT mass spectrometer is interfaced to a PC running the Compass 1.1. integrated software package, which includes the Hystar 3.1.52 module for instrument control and spectrum acquisition, and EsquireControl 5.3.11 and DataAnalysis 3.146 modules for storing the ion chromatograms and processing the MS data. The sample was infused into MS by on-line syringe pump electrospray at a constant flow rate of 1 μL/min. Nitrogen at 6 L/min flow rate and 300°C temperature was employed for desolvation and as a nebulizer gas at 10 psi. The HCT instrument was set to operate in the negative ion mode, which was shown to be advantageous for MS analysis of native, underivatized gangliosides.[26–31] All spectra were acquired in the mass range 100–2200 *m/z*, with a scan speed of 8100 *m/z* per second. Tandem mass spectrometry was performed by collision-induced dissociation (CID) using He as the collision gas. The precursor ion was selected within an isolation width of 2 u. The multiple stage sequencing up to MS^4 was carried out using a fragmentation amplitude of 1.0 V, ramped from 30 to 200% within 40 msec for each single spectrum and a fragmentation cutoff default of 27% of the precursor ion *m/z*.

RESULTS AND DISCUSSION

The AH20 ganglioside sample, dissolved in pure methanol to a concentration of approximately 10 pmol/μL, was infused into the HCT mass spectrometer via negative-ion mode ESI. In order to develop a reliable protocol for stepwise ganglioside sequencing by multiple-stage MS, the ion detected in the MS^1 screening mode as singly charged at *m/z* 1544.90 was isolated (Figure 1) and submitted to the first stage of fragmentation analysis in the MS^2 mode. According to the theoretical mass calculation, the ion at *m/z* 1544.90 corresponds to four possible brain-associated monosialotetraose-Cer (d18:1/18:0) isomers/isobars, GM1a, GM1b, nLM1, and/or LM1. For the determination of the oligosaccharide and ceramide compositions as well as for collecting specific data upon sialic acid localization leading to isomer identification, the MS^2 conditions were optimized to provide as many informative sequence ions as possible. The ESI HCT MS^2 of the ion at *m/z* 1544.90 is presented in Figure 2. Inspection of the spectrum depicted in Figure 2 shows that the cleavage of the glycosidic linkages resulted mostly in Y-type ion formation accompanied by two B-type fragments. The fragmentation pathway is depicted in Figure 3. Due to the highest structural selectivity, the presence of the following singly charged fragment ions is of the highest structural relevance: the ion at *m/z* 1179.76, corresponding to the NeuAc-Gal-Glc-Cer

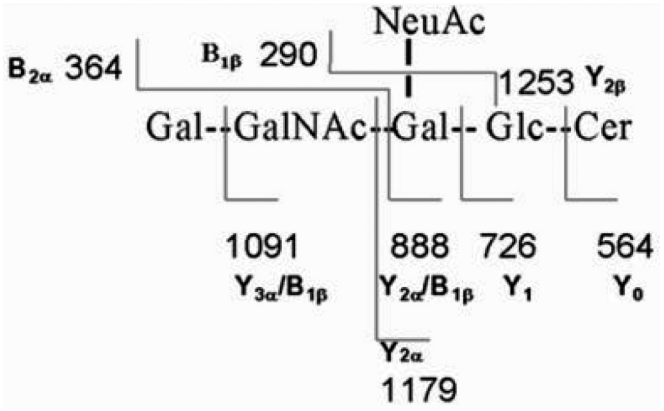

FIGURE 3

The fragmentation pathway of the singly charged ion at *m/z* 1544.90 in the MS^2 mode. The fragmentation data correspond to the GM1a isomer. Therefore, the ion assignment is adjusted to the GM1a structure (the NeuAc linked to the inner Gal). The ion assignment is in accordance with the published recommendations.[41,42]

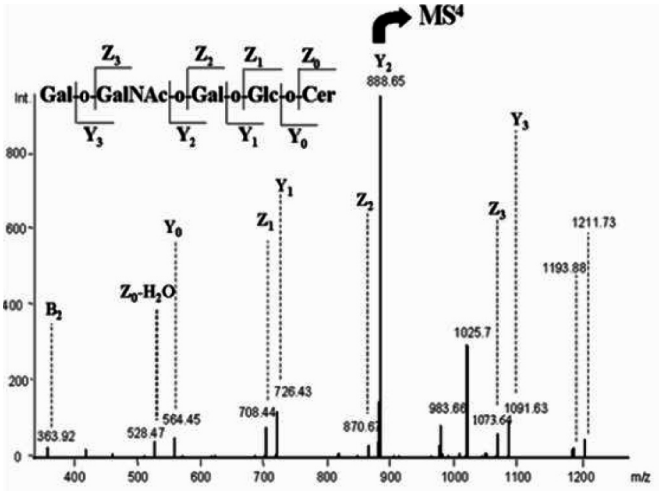

FIGURE 4

Negative ESI HCT MS^3 of the singly charged ion at *m/z* 1253.82 corresponding to the desialylated monosialotetraose (d18:1/18:0) fragment ion detected in the MS^2 mode. Inset: the fragmentation pathway in the MS^3 mode. The ion assignment is in accordance with the published recommendations.[41,42]

(d18:1/18:0) sequence, and its counterpart formed after neutral loss of CO_2 at *m/z* 1135.85. Both ions are indubitable diagnostics for the localization of the NeuAc moiety at the inner Gal residue of the monosialotetraose-Cer (d18:1/18:0) oligosaccharide backbone. These data give hard evidence of the presence of the GM1a structural isomer in the AH20 mixture, as probably the most abundant monosialotetraose-Cer form, which is in accordance with our previously collected data[26–27] showing that the GM1a is the major monosialylated ganglioside in the human brain.

For the next stage of fragmentation, the singly charged ion appearing in the MS^2 mode at *m/z* 1253.82, corresponding, according to mass calculation, to the desialylated monosialotetraose-Cer (d18:1/18:0) fragment, was isolated and further submitted to fragmentation in the MS^3 experiment. The spectrum is presented in Figure 4 along with the scheme of the ion fragmentation in this dissociation stage. Interestingly, the sequencing conditions employed in the MS^3 induced generation of the complete set of Y- and Z-type fragment ions, from which the $GalGalNAcGalGlcCer^-$ composition of this ion could be accurately deduced. An important aspect resulting from this experiment is the reduced dehydration of the fragment ions in the MS^3 mode. Thus, the neutral loss of water was practically observed only for the Z_0 fragment. As shown in Figure 3, the most abundant ion in this spectrum occurred at *m/z* 888.65 and was assigned to the Y_2 fragment ion, having the composition $GalGlcCer^-$.

To complete the protocol for stepwise sequencing of gangliosides ensuring their detailed structural identification, the ion at *m/z* 888.65 was further isolated and submitted to MS^4 experiment. The MS^4 spectrum is displayed in Figure 5 along with the scheme of the ion fragmentation as an inset. Similarly to the MS^3 experiment, the MS^4 generated the entire series of Y- and Z-type fragment ions. Interestingly, the formation of the Z_0-H_2O ion arising from the ceramide moiety upon the Z-type cleavage was again favored.

Besides the full characterization of the oligosaccharide motif of the GM1 species and identification of the GM1a structural isomer, the multiple-stage MS fragmentation provided data on the d18:1/18:0 constitution of the ceramide, which is directly supported by the fragment ions Y_0, Z_0, and Z_0-H_20 occurring in all spectra at the nominal *m/z* 564, 546, and 528, respectively.

CONCLUSIONS

This study was designed to assess the potential of the (-) ESI HCT multiple-stage MS screening and sequencing of lipid-linked carbohydrates, which are known to require special conditions for ionization/detection and fragmentation in tandem MS experiments. In this regard, an ESI HCT multiple-stage MS approach was developed and optimized for ganglioside analysis, and applied to a native mixture purified from normal adult hippocampus. To the best of our knowledge, this is the first mass spectrometric investigation of lipid-linked carbohydrates by multiple-stage sequencing.

We found that detailed identification of the ganglioside structure at high sensitivity may be achieved by stepwise CID fragmentation, inducing up to four sequencing

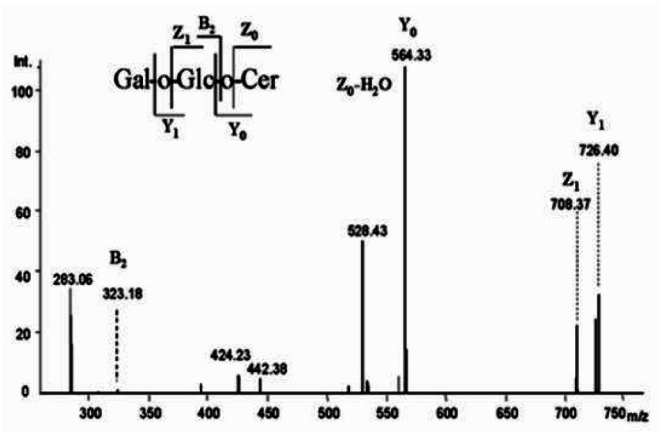

FIGURE 5

Negative ESI HCT MS^4 of the singly charged ion at *m/z* 888.65, corresponding to the Gal-Glc-Cer fragment detected in the MS^3 mode. Inset: the fragmentation pathway in the MS^4 mode. The ion assignment is in accordance with the published recommendations. [41,42]

events. As shown in Figures 3 and 4, an elevated signal-to-noise ratio was obtained even for advanced dissociation stages, proving the gain in sensitivity provided by the new generation of ion-trap mass spectrometers of higher ion-storage capacity. Moreover, all MS stages, from MS^1 screening to the MS^4 fragmentation, were obtained in one and the same experiment, in a high-throughput protocol within only 6 min of signal acquisition. The constant flow rate of approximately 1 μL/min provided by the online ESI infusion therefore yielded only 6 μL volume consumption for the entire experiment. Consequently, for generating one MS^1 and three tandem mass spectra, about 60 pmols of sample was used.

The data presented here clearly indicate that this novel method is of general applicability in glycolipid analysis, and we consider it a valuable platform for future studies related to ganglioside biomarker discovery and their *de novo* sequencing and identification. For this reason, further efforts will be focused toward investigations of fragmentation mechanisms under low-energy CID multiple-stage MS in order to improve protocols for discrimination of isomeric/isobaric species in complex mixtures without the need for prior separation.

ABBREVIATIONS

Gangliosides and their precursor glycosphingolipids are abbreviated according to the system of L. Svennerholm [38] and the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature[39,40] as follows: LacCer, Galβ4Glcβ1Cer; Gg_3Cer, GalNAcβ4Galβ4Glcβ1Cer; Gg_4Cer, Galβ3GalNAcβ4Galβ4Glcβ1Cer; nLc_4Cer, Galβ4GlcNAcβ3Galβ4Glcβ1Cer; GM3, II^3-α-Neu5Ac LacCer; GD3, II^3-α-$(Neu5Ac)_2$-LacCer; GT3, II^3-α-$(Neu5Ac)_3$-LacCer; GM2, II^3-α-Neu5Ac-Gg_3Cer; GD2, II^3-α-$(Neu5Ac)_2$-Gg_3Cer; GM1a, II^3-α-Neu5Ac-Gg_4Cer; GM1b, IV^3-α-Neu5Ac-Gg_4Cer; GD1a, IV^3-α-Neu5Ac,II^3-α-Neu5Ac-Gg_4Cer; GD1b, II^3-α-$(Neu5Ac)_2$-Gg_4Cer; GT1b, IV^3-α-Neu5Ac,II^3-α-$(Neu5Ac)_2$-Gg_4Cer; GQ1b, IV^3-α-$(Neu5Ac)_2$,II^3-α-$(Neu5Ac)_2$-Gg_4Cer; 3'-nLM1 or nLM1, IV^3-α-Neu5Ac-nLc_4Cer; nLD1, disialo-nLc_4Cer, IV^3-α-$(Neu5Ac)_2$-nLc_4Cer; LM1, IV^3-α-Neu5Ac-Lc_4Cer.

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