Chapter 3

Sphingolipid Analysis by High Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS)

Jacek Bielawski,* Jason S. Pierce, Justin Snider, Barbara Rembiesa, Zdzislaw M. Szulc and Alicja Bielawska

Abstract

Sphingolipid (SPL) metabolism (Fig. 1) serves a key role in the complex mechanisms regulating cellular stress responses to environment. Several SPL metabolites, especially ceramide (Cer), sphingosine (Sph) and sphingosine1-phosphate (S1P) act as key bioactive molecules governing cell growth and programmed cell death (Fig. 2). Perturbations in sphingolipids of one type may enhance or interfere with the action of another. To monitor changes in SPL composition therefore, reliable analytical methods are necessary.

Here we present the liquid chromatography tandem mass spectrometry (LC-MS/MS) approach for simultaneous qualitative and quantitative monitoring of SPL components (classes and molecular species) in biological material as an effective tool to study sphingolipid signaling events. The LC-MS/MS methodology is the only available technique that provides high specificity and sensitivity, along with a wealth of structural identification information.

Introduction

Although sphingolipids (SPLs) have long been thought to function exclusively as structural constituents of the plasma membranes, in the past two decades research into the sphingolipids has progressed along two areas. First, SPLs have been shown to influence membrane structure, where they have been proposed to exist in clusters and form microdomains containing cholesterol at the plasma membrane, the so-called "lipid rafts".[1] These lipid microdomains are thought to function as platforms for effective signal transduction and correct protein sorting. Second, many SPLs have been shown to act as both first and second messengers, as well as bioactive mediators, in a variety of signaling pathways. Thus, the SPL metabolites—ceramide (Cer), ceramide-1-phosphate (Cer1P), sphingosine (Sph) and sphingosine 1-phosphate (S1P)—have emerged as a new class of lipid biomodulators for various cell functions and through participation in and influencing of multiple signaling pathways.[2-5] During the last 25 years there has been a dramatic increase in the studying of sphingolipid signaling in many patho-biological disorders but only recently new tools and approaches became available to examine these processes out, such as highly sensitive mass

*Corresponding Author: Jacek Bielawski—Department of Biochemistry and Molecular Biology, Medical University of South Carolina, 173 Ashley Ave., P.O. Box 250509, Charleston, South Carolina 29425, USA. Email: bielawsj@musc.edu

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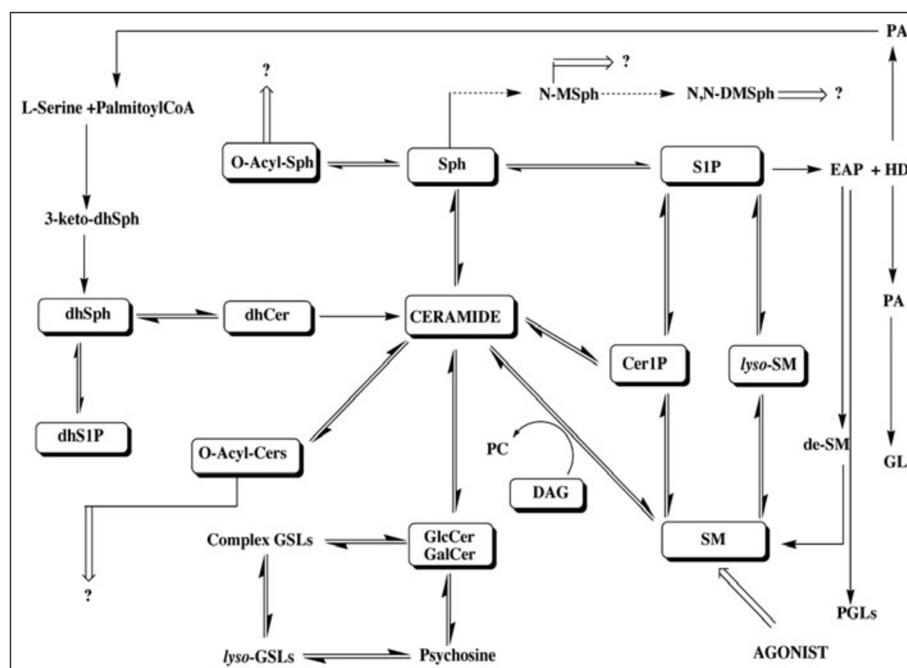

Figure 1. Sphingolipid biosynthesis and metabolic pathways; metabolomic profiling of sphingolipids. Abbreviations used in the figure: 3-keto-dhSph, 3-keto-dihydrosphingosine; dhSph, dihydrosphingosine; dhS1P, dihydrosphingosine 1-phosphate; dhCer, dihydroceramide; Sph, sphingosine; S1P, sphingosine 1-phosphate; Cer1P, ceramide-1-phosphate; SM, sphingomyelin; *lyso*-SM, lyso-sphingomyelin; DAG, diacylglycerol; O-Acyl-Sph. O-acyl-sphingosine, O-Acyl-Cer, O-acyl-ceramide; N-Me-Sph, N-methyl-sphingosine; N.N-DMSph, N,N-dimethyl-sphingosine; PA, palmitic acid; EAP, ethanolamine phosphate; HD, hexadecenal; GL, glycerolipids; de-SM, demethylated sphingomyelin; PGLs, phosphoglycerolipids; GlcCer, glucosylceramide; GalCer, galactosylceramide; GSLs, glycosphingolipids.

spectrometry methods for sphingolipid analysis. This chapter describes the high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) for sphingolipid analysis in biological samples.

Sphingolipids: Structure and Composition

Prevalent complex SPLs: phosphosphingolipids (PSLs) and glycosphingolipids (GSLs) are found in all eukaryotes, some prokaryotes and viruses, mainly as components of the plasma membrane and related organelles. SPLs constitute about 30% of the total lipid of plasma membranes.

SPLs constitute one of the most structurally diversified classes of amphipathic lipids abundant in all living organisms. Variations in the nature of the head group attached to the primary hydroxyl group (carbohydrates, phosphocholine, phosphate or phosphoinositol), N-acyl group and sphingoid base (SB) backbone result in a great number of chemically distinct SPLs, where Sph, sphinganine (dhSph) or phytosphingosine (phytoSph) are the core structural moieties. Thousands of natural complex SPLs have been isolated based on almost 60 distinct species of sphingoid bases, although most of them are very minor components. SBs, the backbone of all SPLs, encompass a wide array of (2S, 3R, 4E)-2-amino-1,3-dihydroxyalkenes (Sphs), (2S, 3R)-2-amino-1,3-dihydroxy-alkanes (dhSphs) and (2S, 3S, 4R)-2-amino-1,3,4-trihydroxyalkanes (phytoSphs) with alkyl chain lengths from 14 to 22 carbon atoms and variations in the number and

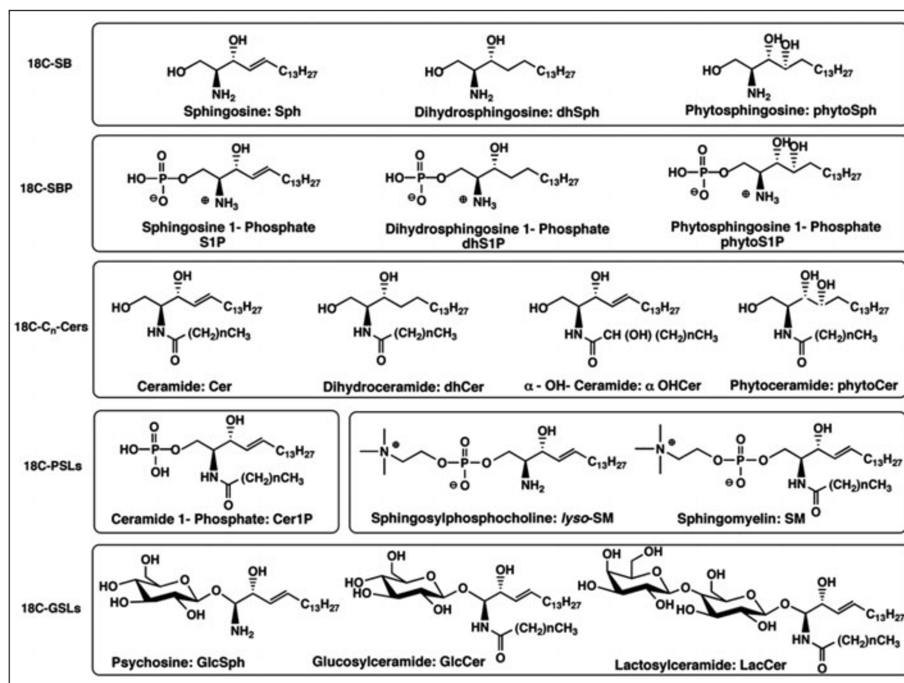

Figure 2. Natural sphingolipids are a highly heterogenous system related to the sphingoid bases and derivatization made on the amino- and hydroxy-functions. Structures shown in this figure represent derivatives of 18C-SB (sphingoid bases containing C18-backbone chain) indicating SPLs containing 2-amino-1,3-dihydroxy-octadecene-4E, 2-amino-1,3-dihydroxy-octadecane and 2-amino-1,3,4-tri-hydroxy-octadecane. General structures, nomenclature and abbreviations for SPLs are cited and described in this presentation. Cn -indicates the chain length of N-acyl part of SPLs.

position of the double bonds, hydroxyl groups and branching methyl groups. Mammalian SPLs are predominantly composed of 2-amino-1,3-dihydroxyoctadecene (18CSph, abbreviated here as Sph) and 2-amino-1,3-dihydroxyoctadecane (18CdhSph, abbreviated here as dhSph) (Fig. 2). Yeast and plant SBs are mainly composed of 2-amino-1,3,4-trihydroxyoctadecane (18CphytoSph), 18CdhSph and their eicosa-homologs (20CphytoSph and 20CdhSph). Additionally, some SPLs' SBs may contain a double bond in position 8 or have double bonds in positions 4 and 8 or/and have a methyl group in position 9 of the sphingosine backbone (which can be found in plant and fungi SPLs).

Ceramides are N-acyl-derivatives of SBs. Combinations of different SBs with different fatty acids (including their hydroxy-analogs) generates a huge variety of Cers, dhCers and phytoCers.

These basic SPLs are modified at the 1-hydroxyl group to: (i) phosphates (e.g., S1P and Cer1P), (ii) phosphocholine-analogs (e.g., sphingomyelin, SM and lysosphingomyelin, *lyso*-SM) and (iii) glucosyl- and galactosyl-analogs (e.g., glucosylceramide, GlcCer and galactosylceramide, GalCer, known as cerebrosides and their *lyso*-form: psychosine). Members of the latter group also serve as precursors to hundreds of different species of complex GSPLs.

SPLs constitute the second major category of polar lipids and for example they represent approximately 5-10% of total lipid mass in mammalian brain (6-8). Abnormal SPL metabolism could lead to their accumulation and deposition in multiple tissues, especially neural tissues, that results in potentially severe clinical manifestations, known as the sphingolipidoses.[6]

The structural diversity of SPLs dictates that every step in analysis of these natural products must be carefully evaluated.

LC-MS Methods for Detection and Analysis of Bioactive Sphingolipids

Technological advances in lipid detection, analysis and quantitation have played a key role in promoting the development of the sphingolipid research field. Traditional lipid analytical methods, such as thin-layer chromatography, are hampered by limited sensitivity, selectivity and resolution. Metabolic labeling using lipid precursors (such as serine or palmitic acid) have been wildly used for selective labeling of certain classes of lipids, which are then typically separated using thin-layer chromatography and visualized by autoradiography. However, this method is affected by incorporation of radioactive substrates and this does not always reflect the primary lipid contents in cells. Furthermore, thin-layer chromatography has low resolution and low sensitivity; thus it is difficult to identify the subspecies of individual SPLs.

The development of advanced mass-spectrometry-based methodologies has allowed the simultaneous assessment of several SPL subgroups as well as the probing of individual molecular subspecies such as various chain-length Cers. To understand the physiological function of sphingolipid metabolites, it has become important to know the metabolic change of particular SPLs and their individual subspecies from one sample. Here, we review methods for simultaneous analysis of SPLs using liquid chromatography tandem mass spectrometry techniques.

Lipidomic Approach

The term "lipidomics" has recently emerged[9-13] in relation to genomics and proteomics. Thus, lipidomics can be defined as the full characterization of lipid molecules in the studied biological material "Sphingolipidomics" will define the field of sphingolipids.

A variety of sample preparation, ionization modes and instrumental designs have been developed to analyze particular SPL classes by MS technology.[14] Design for this methodology has been based on the fact that different SPL subclasses dissociate into structurally distinctive patterns corresponding to their sphingoid bases, N-acyl chains and polar headgroups.[15-25] Recent advances in electrospray ionization (ESI) have provided a new approach to successfully examine total SPL components in crude lipid extracts.[19,22,23,25] Electrospray ionization (ESI) methodology allows generation of intact molecular ions of molecules from solution, delivered by direct infusion or by coupling high performance liquid chromatography (HPLC) column directly to the mass spectrometer. Further improvements in instrumentation, such as the triple quadruple with robust ion sources, fast scanning mass analyzers and reduced chemical noise (mainly in MS/MS technique) allow the identification and quantitation of SPLs with great sensitivity (sub-picomol detection limit) in a highly reproducible manner. SPL identification is accomplished by tandem mass spectrometry (MS/MS) with precursor ion scans to distinguish various molecular species in crude lipid extract by taking advantage of the unique molecular decomposition pattern[19,25] for each SPL class (Fig. 3). SPL quantitation is performed by using positive ionization and multiple reaction monitoring (MRM) in conjunction with HPLC separation.[25] Liquid chromatography/tandem mass spectrometry (LC-MS/MS) is the only technology available that provides structural specificity, quantitative precision and relatively high-throughput for analysis of complex SPLs in small samples.

Sample Preparation

The extraction process is one of the most important steps in pretreatment of solid (cell pellets, tissue) and liquid (plasma, serum, whole blood, biological fluids) samples.

Chloroform: methanol 2:1 (v/v) extraction, developed in 1956 for fish tissue,[26] further improved in 1959 by Bligh and Dyer,[27] became a golden standard procedure, known as the "Bligh & Dyer" (B & D) method and it is still commonly used for lipid extraction from all biological matrices. It involves a two step extraction employing chloroform: methanol: water at well-defined ratios of 1:2:0.8 and 2:2:1.8, respectively. According to the originators, the upper (methanol: water) phase contains all the "nonlipid" substances, while most lipids remain in the lower (chloroform) phase. This virtually unchanged procedure is commonly applied to most SPL sample preparation, regardless of the analytical procedure subsequently used e.g., TLC, HPLC

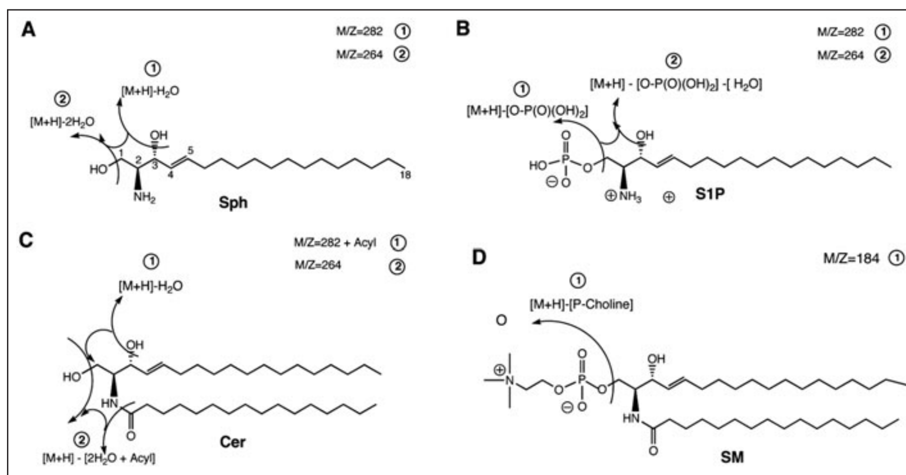

Figure 3. Typical MS/MS fragmentation patterns of sphingosine (A), sphingosine 1-phosphate (B), ceramide (C) and sphingomyelin (D). The specific common fragment ion of m/z = 264 (2, panel C) for Cers and m/z = 184 (1, panel D) for SM are used in the Parent Ion Scan experiments for determination of molecular species composition prior to quantitative analysis with the MRM experiments.

or MS, although the extraction efficiency, particularly for the most polar SPL components as S1P or *lyso*-SM seems to be questionable.

Over time, some modifications to the B & D have been incorporated in the isolation of SPLs, mostly intended to remove the bulk of the major co-extracted components, especially the glycerolipids, by subjecting the initial chloroform extract to a mild alkaline hydrolysis that cleaves ester linkage.[25,28-36] However, 1-O-acyl-ceramides (O-AcylCer) and related compounds[37] will also be hydrolyzed, thus artificially increasing the level of Cers. Comparison of the Cer level calculated from lipid extracts that were prepared with and without the base hydrolysis step can provide important data about the level of O-AcylCers. Our results showed some (20-40%) increase in Cers after this treatment. Nevertheless, this simple approach is recommended in the preparation of samples for SM analysis to allow elimination of phosphatidylcholine (PC) from the lipid extract, which may interfere with SM determination, even at highly specific LC-MS/MS analysis, due to close masses and fragmentation pattern.

Several attempts to further separate the initial total lipid extracts into particular lipid classes by a set of solid phase extraction (SPE) cartridges[38-40] proved to be very time consuming and not reproducible. Moreover, it may not be necessary when selective LC/MS technology is employed for analysis.

We developed[41] a one-phase extraction, using ethyl acetate: iso-propanol: water system at 60:30:10; (v/v/v) and 85:15:0 (v/v/v) for cell pellets and tissue homogenates and aqueous samples, respectively. The protocol describes lipid extraction under a safe and neutral condition to avoid destruction of the parent "soft" SPLs (e.g., SPLs containing O-acyl group), assuring efficient and quantitative extraction of the SB-1Ps from biological material since the latter are notoriously difficult to recover quantitatively.[39,42]

Readers interested in developing and/or improving existing methods of sample preparation are referred to an excellent review by McDowall.[43]

Analysis of Intact Sphingolipids by Mass Spectrometry

Mass spectrometry is a powerful detection technique that enables separation and characterization of compounds according to their mass-to-charge ratio (m/z). Its essential components include

a sample inlet, ion source, mass analyzer, detector and data handling system. The combination of sensitivity, selectivity, speed and ability to provide invaluable structural information makes MS an ideal method for analysis of intact lipid molecular species.

The interest in the analysis of lipids in general especially SBs, SB1Ps, Cers, Cer1Ps, Glc/GalCers and SMs has continued to evolve due to the importance of these molecules in various biological transformations. SPL molecular species exist in nature as a complex mixture of closely related components which differ in the fatty acid chain length, degree on unsaturation and hydroxylation. These species differ greatly in their chemical and biological properties. Various analytical methods have been employed to separate and analyze individual species from intact (underivatized) form, out of which ESI/MS is the method of choice providing the following advantages:[44]

- Elimination of time consuming derivatization steps
- Making possible the study and follow-up of biosynthesis, metabolism, turnover and transport of the molecular species
- Protect possible rearrangement of the fatty acid chain during derivatization.

The sample introduction can be either by direct infusion or through preceding separation devices such as liquid chromatography (LC).

Mechanism of Electrospray Ionization Mass Spectrometry (ESI/MS)

ESI/MS, invented in the 1960s, was put into practice by Fenn et al.[45,46] It involves transformation of ions from the liquid to the gas phase. It is a method that operates at atmospheric pressure and ambient temperature. Initially, a solution containing the analytes of interest is introduced to the ESI ion source through capillary tubing. The narrow orifice at the end of the capillary and the dynamic forces facilitate formation of sprayed small droplets in the ionization chamber. Application of electric potential (approximately 2-5 kV) causes ionization, consequently the droplets carry a net charge. The charged droplets are then directed into the mass analyzer by the applied electric field. The applied potential may be positive or negative depending on physicochemical properties of the analytes. Passing through the ionization chamber, the droplets dissolve and this effect dramatically increases the columbic forces between the ions. Once this force exceeds the surface tension of the solvent, the droplets explode to form a fine mist of smaller droplets. This cycle is repeated until molecular ions are generated prior to their entrance into the mass analyzer.[47]

The soft ionization can generate lipid molecular ions without causing extensive fragmentation.[18,25,48-50]

MS Scan Modes

A number of mass analyzers are available, e.g., quadruple, ion trap, time of flight, ion cyclotron resonance, or sector instruments, which separate charged molecules in vacuum depending on their m/z ratio.

In so called full scan (FS) mode, a spectrum of primary, mostly molecular, ions is identified. This is the least specific mode with low sensitivity and it is mostly used for a rough assessment of major components of biological material when no or very limited information about SPL composition exist. However, interferences from other compounds present can either suppress ionization or cause a high chemical noise making such detection of SPL virtually impossible.

Moreover, the mass analyzers can also be used for fragmentation, predominantly in the triple quadruple instrument. In this instrument, the middle (Q2) field free quadruple either focuses and transmits all ions, or can be used as a collision cell for controlled fragmentation, called collision induced dissociation (CID). As results of a collision with an inert gas, introduced into the collision cell, the internal energy of the ions increases through conversion of kinetic energy breaking out specific bonds, depending on the collision energy applied.[51-52] The fragment ions are then analyzed in the second mass analyzer (third quadruple Q3). The choice of collision gas, its pressure and particularly the applied collision polarization and energy, affect the degree of fragmentation.

When a single quadruple instrument is used, partial fragmentation can be induced in the source by elevating the cone-to-skimmer potential difference. Protonated molecules desorbed from the

ESI droplets are accelerated between the cone and skimmer, undergoing CID upon collision with residual carrier gas molecules.[52]

Initial "soft" ionization of extracts prepared from biological samples results in numerous SPL molecular ions either positive $(M + H)^+$ or negative $(M\text{-}H)^-$. When the precursor ion fragments, it generates secondary (called daughter) distinctive pattern of ions related to the head group, SBs and fatty acids. This provides a wealth of structural information, enabling identification of SPLs in particular biological material. The positive ionization fragmentation can be enforced by incorporation of alkali metal ions $(M + Me)^+$ where Me = Li^+, Na^+, K^+, Rb^+, or Cs^+.[18,53] In addition to structural information, tandem MS provides a higher sensitivity, specificity and greatly reduced chemical background, thanks to very selected mode of monitored masses.

Specific Scan Modes for MS/MS Instrumentation

Product Ion Scan

In product ion scan, the first mass analyzer (Q1) allows a single ion with a set m/z value to pass and this is then further fragmented by CID in the second quadruple (Q2), the secondary (daughter) ions are then scanned over a defined mass range by the third quadruple (Q3) and passed to the detector. The relative abundance of the product ions depends on the dissociation dynamic; therefore, changing the CID collision energy, a fragmentation pattern is observed which is specific for each SPL class of compounds.

Neutral Loss (NL)

In a neutral loss scan, Q3 is offset from Q1 by fixed m/z, corresponding to specific neutral loss, e.g., 18 Dalton for loss of a water molecule. Both Q1 and Q3 scan over specified ranges of m/z values. In this mode, the detector records only those precursor ions that decompose, losing the specified neutral fragment. This type of MS experiments highly decreases chemical noise and is very helpful in identification of unknown SPLs.

Precursor Ion Scan (PI)

In a PI scan mode, the Q3 is set to pass specific m/z value, characteristic of a defined secondary ion. The Q1 scans across m/z range, recording only those primary ions which decompose to the specified product ion of interest. This highly specific scan mode eliminates or at least greatly reduces chemical noise and it constitutes a very useful identification tool since each class of SPLs yields at least one common product ion. Thus, setting Q3 to this specific daughter ion and scanning Q1 over the expected parent ion mass ranges, a spectrum of molecular species for an unknown biological sample may be identified.

Multiple Reaction Monitoring (MRM)

In a MRM experiment, the Q1 is set to pass specific precursor ion m/z and Q3 specific daughter ion m/z only.

This makes the MRM the most specific and sensitive MS/MS experiment allowing the analysis of even very minor components of a complex mixture with great precision and sensitivity. Such experiment practically eliminates chemical noise, thus makes it an ideal tool for quantitative analyses, particularly if coupled with HPLC physical separation. Multiple mass transitions, specific for particular compound, may be monitored sequentially; therefore, a large number of compounds may be analyzed together. Optimization of CID parameters for each compound of interest results in best sensitivity and specificity.

Sphingolipid Identification

Due to the complexity of sphingolipids, which usually constitute minor components of a crude lipid extracts, identification of individual molecular species is necessary before attempting any quantitative determination. This task can only be achieved with application of MS, particularly with precursor ion scan (PI) experiments. Although direct infusion full scan MS have been

attempted,[30,36] reliable results may be obtained only for negative mode, in which a limited number of SPLs, such as free fatty acids, are ionized. In full scan positive mode, high chemical background makes any identification virtually impossible.

Qualitative analysis of SPLs from crude extracts is best accomplished by analysis of their unique molecular decomposition products using a PI scan of common fragment ions, characteristic for the particular class of SPLs (Fig. 3).[32,33,48] Readers are directed to the comprehensive studies on fragmentation patterns for mammalian and yeast SPLs, presented by Sullard[18] and Shevchenko,[54] respectively. Briefly, for mammalian SPLs, the m/z 264 and m/z 266 are the common fragment ions used for identification of Sph and its saturated counterpart, dhSph derivatives, respectively.

Considering the complexity of SPL composition, as well as the presence of many other lipid related compounds in biological material extracts, it is advisable to confirm initial identification, derived from PI scan, in order to avoid false identification. This may be accomplished by other, more compound-specific MS experiments, such as product ion scan of the newly identified molecular ion, or in a MRM experiment, with mass transition unique for the particular molecule e.g., single or double dehydration for Cers or NL of sugar moiety for GlcCers.

Ionization conditions and collision energy are optimized for individual molecular species to achieve maximum sensitivity and quantitative accuracy. SPLs composition has to be established for every new matrix.

Cer, Cer1P and GlcCers's molecular species (C18-SB) is established by the Precursor Ion scan, performed for the common Product Ion (m/z) 264.2 and 266.1 for Sph and dhSph derivatives, respectively at the high collision energy (35-55 eV), operating in positive ionization mode (Fig. 3). A representative sample extract is infused directly into ESI source and it is then scanned for molecular ions of the potential SPLs. Further confirmation of identity is achieved through MRM analysis with "soft" fragmentation (15-30 eV). Running sample through the HPLC system also confirms a reasonable retention time. Only SPLs that satisfy identification criteria in both analyses should be considered truly present in the sample.

SM and dhSM molecular species (18C-SB). Identification of the SM and dhSM components is performed similarly, employing common Product Ion (m/z 183.9) at 40 eV collision energy (Fig. 3).

Note: It is important to optimize the ionization conditions for each class of SPLs and collision energy for each individual molecular subspecies to be applied for quantitative MRM analysis.

HPLC-MS/MS Methodology

High Performance Liquid Chromatography (HPLC) is often employed for the separation of intact lipid molecules using various detectors. SPLs lack chromophores that would have enabled direct specific spectrophotometric detection. Some attempts have been made with UV[55-59] and evaporative light scattering (ELSD).[60-62] Both detectors, however, lack specificity and impose additional limitations. With UV detection, it is very difficult to select a working mobile phase (MB) since underivatized SPLs absorb close to the 200-210 nm range, depending upon the degree of unsaturation of the FA moiety and most of the commonly used solvents strongly absorb in this region.[59,63]

In the ELSD detector, the HPLC column effluent is evaporated, leaving the solute components as fine droplets, which are illuminated by laser and the scattered light is measured. This is an indiscriminatory detection since any compound that does not evaporate may be detected.[63]

An alternative technique that overcomes most of the above problems and provides both compound specificity and quantitative sensitivity is the use of HPLC coupled with ESI/MS. It is one of most powerful technologies for analysis of intact polar lipid molecules. The physical separation power of HPLC into either various lipid subclasses and/or individual molecular species within the class, together with MS highly selective detection, makes possible simultaneous determination of either protonated or deprotonated molecules, providing also invaluable structural information.[64]

Both Normal (NP) and Reverse Phase (RP) HPLC have been employed and it is important to select a solvent system that renders chromatographic resolution and ESI/MS compatibility to achieve maximum sensitivity.[18,25,32,33,41,65-67]

Recently, the most powerful technique applied for SPL molecular species is the HPLC-MS/MS instrumentation with the MRM scanning mode where each target analyte is uniquely identified by the Precursor-Product ion mass transition and the specific retention time.

Quantitation

Quantitation of SPLs in biological extracts has been a least developed segment of the HPLC/MS/MS analysis due to very limited supply of commercially available individual standards. Only laboratories that have access to custom made synthetic standards[41] were able to set up a reliable comprehensive quantitative protocols for various SPL classes. Recently, however, Avanti Polar Lipids Inc. (Alabaster, AL) and Matreya Inc. (Pleasant Gap, PA) significantly increased their offer of synthetic standards, so those major difficulties can be gradually overcome.

To achieve reliable quantitation of all molecular species, calibration curves should be generated for as many representative components of SPL as possible, due to diversified MS responses, as reflected by calibration curve slopes (Fig. 4).

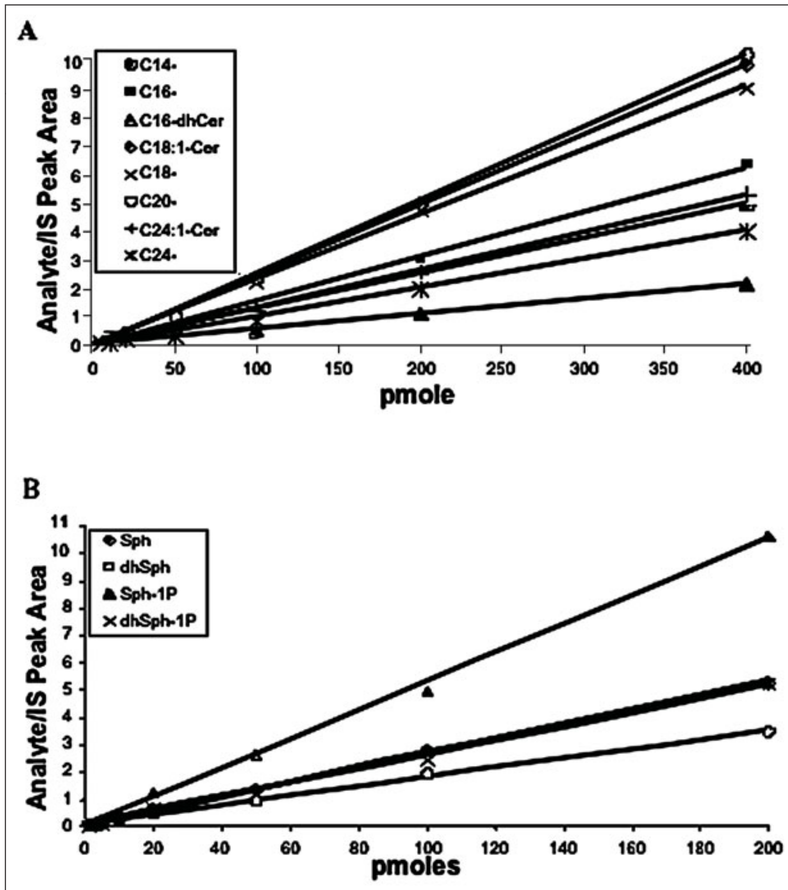

Figure 4. Calibration curves of sphingoid bases and ceramides. The MS response varies for molecular species even within the particular SPL class, as indicated by the calibration curve slops; therefore, individual calibrations are generated for as many target analytes as possible. Linear instrument response (R^2 value of .99) is obtained for the typical calibration ranges: 1.0-400.0 pmoles for SBs and SB 1Ps (lower panel), as well as for all ceramide species, Cn-Cers (upper panel).

Selection of Internal Standards (ISs)

Selection of a representative set of internal standards, which serve as a reference for both identification and quantitation, is critical for the analysis of a complex mixture of SPLs. Internal standards should be as close as possible to the target analytes, presenting similar MS fragmentation pattern as well as physicochemical properties reflected by similar solubility, extraction efficiency and mobile-stationary phase relationship during the HPLC separation. The best IS would be a compound which is chemically identical to target analyte labeled with a stable isotope, usually ^{2}H or ^{13}C. However, considering the large number of SPL molecular species, such approach is impractical; therefore, some compromise has to be applied. Often one IS per SPL class is used, mostly a sphingolipid with unnatural, usually lower, number of carbon atoms in the FA moiety.[18,25,31-34,66,67]

Bielawski et al.[41] have introduced ISs for particular SPL classes synthesized from C17- SB as the closest "unnatural" sphingoid base to the natural C18-SB counterpart. This selection gives a confidence that physicochemical properties such as the elution order and mass fragmentation pattern accurately reflect natural SPLs, but are not present in the analyzed sample. Moreover, since they are introduced to the samples prior to extraction, incomplete extraction efficiencies are compensated for, rendering quantitation of the target SPLs more precise.

Quantitative Calibration

Generating a calibration mechanism for each target SPL in a class greatly improves the quality of the obtained quantitative results. However, due to the limited availability of authentic standards (see above), calibration for, as many as is practical, representative standards should be generated, so that calibration devised for the synthetic standard can be also used for few structurally closely related analytes.

Sometimes[18,25,31-34,48,66,67] quantitation is performed using one IS as a single point calibration. This is not a very good practice since it assumes equal MS response to all molecular species in the class. Unfortunately, MS responses varies widely, depending on both structural features (number of carbon atoms, branching, unsaturation etc.) as well as mobile phase composition which changes over time, particularly when gradient elution is employed.

Based on the above considerations, we have adopted the following approach to SPL quantitative analysis.[41] In this approach, quantitative analyses of SPLs are based on the eight-point calibration curves generated for each target analyte. The synthetic standards along with a set of ISs are spiked into an artificial matrix, then subjected to the identical extraction procedures as the test samples and then analyzed by the HPLC-MS/MS system operating in positive MRM mode, employing HPLC with gradient elution. Peaks for the target analytes and IS are recorded and processed using the instrument software system. Plotting the analyte/IS peak area ratios against analyte concentration generates the analyte-specific calibration curves. Any SPL for which no standard is available is quantitated using the calibration curve of the closest counterpart.

Data Handling

Results from the MS analysis represent the mass level of particular SPLs (in pmols) per total sample used for lipid extract preparation and quantitative analysis. In general, treatment with exogenous agents causes changes in SPL levels and compositions. For the final data presentation, MS results should be normalized to some stable parameters (which are considered not affected by that particular treatment). Total protein (mg), or phospholipid contents Pi (nmol) present in the Bligh & Dyer extract,[27] which corresponds to the amount of the biological material used for MS analysis, can be used as the normalization parameters.[29,68,69] Also normalization to the total cell number is used.[70] Final results should be shown as changes in the relation to the control (% control). From our experience, data normalized to the protein or to the Pi (shown as % of the control) are not exactly the same. It is critical that once the user selects the normalization parameter, it carry it out consequently throughout the total study for consistency of the generated quantitative results.

Alternative Methodology

A variety of different techniques (mostly radio-labeling, HPLC analysis of fluorescent analogs and enzymatic methods) in addition to MS methodology are used for SPLs measurement. Up to now, the enzymatic method employing diacylglycerol kinase and (^{32}P) ATP has been the most commonly used procedure for total sphingolipids quantitation in the range of 25 pmols to 2 nmols.[69] Cellular SBs are most often analyzed by the HPLC technique developed for their fluorescent derivatives.[71] Cellular SB-1Ps are analyzed via their derivatization to (^{3}H) C2-ceramide phosphate, by an enzymatic method (employs alkaline phosphatase), followed by action of recombinant sphingosine kinase and (^{32}P ATP)) after TLC separation of S1P from the cellular Sph, or by employing HPLC analysis of OPA-derivatised S1P.[72-76] These procedures require less expensive equipment than mass spectrometry but are not as informative. SM may be determined by several different approaches including TLC analysis, GC analysis of silylated derivatives and MS techniques.[49,50,68,70] Total Cer and SM can be determined following hydrolysis and analysis of the liberated and derivatised SBs by means of HPLC[71,74] and the liberated fatty acids by means of GC[77] or GC/MS.[78]

Conclusion

This chapter describes quantitative analysis of virtually all compounds involved in sphingolipid metabolism and turnover (signaling) such as sphingoid bases, sphingoid base-1-phosphates, lysosphingolipids, ceramides, ceramide-1-phosphates, sphingomyelin and cerebrocides. The major emphasis was put on the most versatile LC/MS technology, that provides a wealth of structural information and specificity, essential in developing reliable analytical protocols, due to complexity of "sphingolipidome".

The LC/MS/MS methods have been successfully applied to a large number of different mammalian, and yeast cell lines, as well as various tissue samples, that typically contain many different sphingolipid subspecies, but constitute only a small fraction of crude lipid extracts.

So far there are not very good, mass spectrometry based analyses for some part of "sphingolipidome", namely complex gangliosides e.g GD3, due to lack of authentic, synthetic standards. Once this problem will overcome new analytical methodology will follow.

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