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Opinion Article on Lipidomics: Inherent challenges of lipidomic analysis of sphingolipids

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Abstract

A challenge for sphingolipidomic analysis is the vast number of subspecies, including a large number of isomers--a complication that was even appreciated by the original discoverer of sphingolipids J. L. W. Thudichum (*The Chemistry of the Brain*, p. x, 1884): "In the course of my researches many unforeseen complications arose, prominent amongst which were those caused by the occurrence of chemical principles having the same atomic or elementary composition, but differing in other chemical, or in physical properties, varieties producing the phenomenon which in chemistry is termed isomerism." Therefore, it is essential to choose the appropriate method(s) for the goal of the analysis, to know the assumptions and limitations of method(s) used, and to temper interpretation of the data accordingly.

Introduction

Sphingolipids are defined by having a sphingoid base backbone to which an amide-linked fatty acid is often added to form "ceramides" and/or polar headgroups to make simple (e.g., sphingosine 1-phosphate) or complex sphingolipids such as sphingomyelins, gangliosides, sulfatides and other subspecies [1, 2]. The lipid and headgroup moieties of mammals each have over a hundred structural variants, so the number of discrete chemical entities is very large.

Lipidomic analysis of sphingolipids

Extraction conditions must take into account that sphingolipids include some of the most hydrophobic compounds made by mammals (1-deoxyceramides) [3] as well as others (e.g., some gangliosides) that are so water-soluble that they prefer the aqueous phase of organic solvent extraction systems [4]. To complicate matters further, the amounts differ by many orders of magnitude. Therefore, no protocol extracts all subspecies with uniformly high yields, but quite a few methods are effective with subcategories (for examples, [5] and [6]). Some extraction protocols for general lipidomic analyses also work well for the included

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subspecies [7, 8]. Recoveries must be assessed for each application using internal standards for every subcategory, including representatives of different sphingoid bases and amide-linked fatty acids [9].

Mass spectrometry (MS) is usually employed for analysis of the sphingolipids using “untargeted” protocols (where the goal is to survey a wide spectrum of ions to look for noticeable differences between two biological samples) [10] or focused methods, such as tandem MS where data is collected for precursor-product pairs for known sphingolipids [11]. Localization of some subspecies is also possible by tissue-imaging MS [11].

Complex glycosphingolipids pose additional challenges because multiple rounds fragmentation (MSⁿ) is required to achieve step-by-step cleavage of the glycosidic bonds and ceramide backbones [11, 12]. Glycan headgroups are also sometimes determined by MS after removal of the ceramide, which has the advantage of providing information about the location of the glycan linkages [13]. Other approaches, such as ion mobility MS [14, 15], provide information about some hard to distinguish sphingolipids. All of these methods involve assumptions that simplify the analysis but can compromise the results unless important factors are kept in mind, as will be described in the next section.

For data analysis, the online resources developed by LIPID MAPS (www.lipidmaps.org), are particularly useful and have been explained in several published reports [16–20]. Other sources of information include the American Oil Chemistry Society (AOCS) Lipid Library (lipidlibrary.aocs.org/), Cyberlipid (<http://www.cyberlipid.org>) and LipidBank (lipidbank.jp/); for glycans, the Consortium for Functional Glycomics (<http://www.functionalglycomics.org>) (now part of the National Center for Functional Glycomics, <http://ncfg.hms.harvard.edu>) and KEGG Glycan (<http://www.genome.jp/kegg/glycan/>).

Current limitations and future challenges for sphingolipidomic analysis

Fundamental problems for sphingolipidomic analysis are the vast number of compounds and the large number of isomers, as noted by the original discoverer of sphingolipids, J. L. W. Thudichum (see abstract). The lipid backbone is relatively easy to determine by tandem MS because one can follow fragments that are distinctive for the sphingoid base and thereby deduce the ceramide moiety (albeit, with assumptions about the position of double bonds, extra hydroxyl-groups and branching methyl groups, when present). However, if only MS is performed, or the fragment ion that is followed does not define the ceramide backbone, then only the sum of carbons and double bonds is known with certainty.

This occurs fairly frequently in analysis of sphingomyelins, which form abundant (M + H)⁺ ions that undergo collision induced dissociation to a headgroup ion of *m/z* 184.4; therefore, they are easy to scan or analyze by the precursor/product pairs (which is typically done after treatment with mild base to eliminate interfering phosphatidylcholines). The figure shows a typical scan for precursors of *m/z* 184.4 and the interpretations most often made for several of the ions (in bold). A few have been selected for expansion in call-outs that give the results obtained with human plasma [21] by a tandem MS method that can distinguish the sphingoid base and fatty acyls [5]. Note that the correspondence between the assumed

composition and the analytical result is fairly close in some cases (e.g., ~100% for d18:0/C16:0 and 96% d18:1/C16:0), but for others, very different. The ceramide with precursor m/z 701.8 that would often be interpreted as d18:1/C16:1 was only 7% of this isomer and primarily (85%) of the isomer with a sphingadiene (d18:2) backbone and C16:0 fatty acid (likewise, the ceramide with m/z 729.8 that is usually interpreted as being d18:1/C18:1 is 32% of that isomer and 53% of d18:2/C18:0). In some cases, there is also small percentage of the shorter chain length sphingoid base (d16:1), which has been known to exist for a long time, but has only recently gotten attention [22]. A nomenclature that uses number of carbons and double bonds for the lipid moieties has been proposed for cases where the individual components has not been defined [23].

The isomer problem is multiplied for the glycosphingolipids because many of the glycans have the same formula (e.g., glucose and galactose) and polysaccharides can have linkages at multiple positions and sometimes with options for either α - or β -glycosidic bonds. This is particularly problematic for “shotgun” lipidomic studies unless they are combined with chromatography or other techniques that help overcome some of these technical limitations [24].

In addition to the known unknowns, there are also the unknown unknowns (to borrow a well worn expression). A surprising number of new variants have been found in recent years, such as glucosylceramides with α - rather than the more prevalent β -glycosidic linkage [25], backbone “ceramides” lacking the 1-hydroxyl group [26] or having a fatty acyl attached to the 1-hydroxyl [27], and mammalian sphingolipids with polyunsaturated very-long-chain fatty acids [28]. Thus, the sphingolipidome is not only amazingly large, it is expanding.

Concluding remarks

The large number of sphingolipids and the high degree of structural variation in the lipid backbones and headgroups precludes their analysis in a truly “omic” manner (i.e., including all molecular subspecies) by any technology currently available, but this has not prevented many important discoveries. It is essential to be mindful of the compounds in the sphingolipidome, to choose the appropriate method(s) for the goals of the analysis, to know the assumptions and limitations of method(s) used, and to temper interpretation of the data accordingly.

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Highlights

- The sphingolipidome is difficult to analyze due to its large size and complexity.
- Methods are available for a large number of species, including the most of the known “signaling” metabolites
- Additional methods are needed for analysis of isobaric species and isomers of many kinds.

Figure 1.

A typical precursor ion spectrum of m/z 184.4 with examples of the isomeric sphingomyelins that have been found in human plasma. A lipid extract from human plasma was treated with mild base to remove the phosphoglycerolipids then infused into an ABI QTrap 4000 in positive ionization mode to obtain the spectrum. The compositions of the sphingomyelin isomers were separately determined [5] and representative findings [21] are shown in the call-outs for five of the precursor ions. The bold labels are the molecular subspecies that are assigned to the data collected at the indicated m/z in most lipidomic studies; the % in the call-outs are the relative amounts of the isomers that were found. The following abbreviations are used: d18:0, 18-carbon chain-length sphinganine; d18:1, 18-carbon chain-length sphingosine; d18:2, 18-carbon chain-length sphingadiene; d16:1 and d19:1, other chain length sphingosines; C16 to 24 represent the chain lengths of the amide-linked fatty acids (“:0” and “:1” represents zero and one double bond, respectively); the Σ provides the total number of carbons and double bonds for the N-acyl-sphingoid base.