Mass and relative elution time profiling: two-dimensional analysis of sphingolipids in Alzheimer’s disease brains

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INTRODUCTION

The sphingolipids are a diverse family of structural and signalling lipids that have a broad range of functions in normal physiology and pathology [1,2]. Altered sphingolipid metabolism has been implicated in the pathology of cancer [2], cardiovascular diseases [3], Type II diabetes [4], and neurodegenerative diseases such as Parkinson’s disease [5] and AD (Alzheimer’s disease) [6]. The common constituent of all sphingolipids is the sphingoid base, which in mammals is most commonly 18-carbon sphingosine or dihydrosphingosine. A family of six ceramide synthases catalyse the transfer of fatty acids to the amine group of sphingosine or dihydrosphingosine, forming the central sphingolipid metabolite ceramide (Figure 1a). The fatty acids vary in length from 14 to 26 carbons, may contain one or more double bonds, and may be hydroxylated. These variations create significant structural and biophysical heterogeneity within ceramides. Addition of different headgroups to the primary hydroxy group of ceramides gives rise to the different classes of sphingolipids, which are all built on a ceramide lipid ’backbone’.

Ceramide is itself a signalling molecule that is frequently associated with execution of apoptosis [2], but also plays a role in establishing epithelial cell polarity [7] and the formation of cell–cell junctions [8]. The abundant plasma membrane lipid SM (sphingomyelin) is formed by the transfer of a phosphocholine headgroup on to ceramide (Figure 1a). The interaction of SM with cholesterol gives rise to lipid rafts, specialized regions of the plasma membrane where cell-signalling platforms are formed. Alternatively, glucose, galactose or phosphate headgroups may be transferred on to ceramide, giving rise to lipid mediators with distinct functions. GalCer (galactosylceramide) may be sulfated (forming sulfatide), and both of these lipids are major constituents of myelin. Accordingly, GalCer synthesis is critical for normal brain function [9,10].

The aim of this research project was to develop a 2D (two-dimensional) sphingolipid profiling approach, in which the lipids are separated and identified on the basis of both mass and hydrophobicity. Sphingolipids in biological samples are usually quantified using LC (liquid chromatography)-MS/MS (tandem MS) in general, a defined number of lipid metabolites are quantified by running the mass spectrometer in MRM (Multiple Reaction Monitoring) mode, in which a specific precursor and product ion mass are used to identify each metabolite of interest, which is quantified relative to external standards [11,12]. However, MRM assumes prior knowledge about which lipids are present in a given extract. A more objective profiling approach can be achieved by scanning for precursor ions within a defined mass range, while monitoring for product ions that are characteristic of a particular lipid backbone, e.g. sphingosine (m/z 264) or dihydrosphingosine (m/z 266) [12]. Lipids identified using this approach may then be quantified more accurately in a follow-up MRM experiment. However, assigning a molecular structure to a

Abbreviations used: AD, Alzheimer’s disease; CV, coefficient of variation; 2D, two-dimensional; GalCer, galactosylceramide; GluCer, glucosylceramide; HFA, hydroxylated fatty acid; LC, liquid chromatography; MDMS, multi-dimensional MS; MRM, Multiple Reaction Monitoring; MS/MS, tandem MS; NFA, normal (non-hydroxylated) fatty acid; PMI, post-mortem interval; RT, retention time; SM, sphingomyelin.

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given lipid based only on its precursor and product masses is not always feasible, because a given mass can often be assigned to two or more biologically relevant structures.

Incorporating a LC step prior to MS/MS improves sensitivity by removing interfering ions, separating out different lipid components in the cell or tissue extract, and concentrating each lipid species into a narrow elution time window. In theory, the incorporation of an LC column prior to MS can also provide biochemical information that helps to definitively assign a structure to a given precursor and product mass. However, LC is not used in mass scanning/profiling experiments, first because column RT (retention time) is not informative unless referenced to structurally defined standards; and secondly because in reverse-phase chromatography of lipids with the same headgroup, differences in mass conferred by different acyl chain lengths have a large impact on RT. To effectively use RT in the characterization of unknown lipids from a lipid profiling experiment, we first needed to develop a method in which RT is made independent of N-acyl chain length, and therefore mass. To do this we adapted and modified a method that was used for separation of hydrocarbons [13, 14] and fatty acid methyl esters [15, 16].

We describe in the present paper an LC × MS/MS approach (as distinct from an LC-MS/MS approach) for characterization of sphingolipids in complex biological extracts that we refer to as MRET (mass and relative elution time) profiling. After excluding the influence of mass on RT for any given class of sphingolipid, the resulting 2D plots can be used to predict the number and position of double bonds within the lipid tails, and the presence of functional groups such as hydroxy groups, without any need for chemical derivatization of the lipids or the use of high-resolution mass analysers. We describe how this approach can be used to collate the MS output of brain tissue extracts from twelve different human subjects, forming 2D plots of the SM, ceramide and GalCer content that also incorporate a semi-quantitative measure of the relative abundance for each distinct metabolite. These plots are an informative and versatile way to visualize complex lipidomic data sets. Importantly, we show how this approach allows us to distinguish between GalCer species that are structurally and biochemically different, but cannot be distinguished on the basis of precursor and product ion mass.

**EXPERIMENTAL**

**LC and MS**

LC-MS/MS was performed on a Thermo TSQ triple quadrupole mass spectrometer, operating in positive ion mode, coupled to a 3 × 150 mm Agilent XDB-C8 column (5 μm pore size) on an Accela UPLC system (Thermo). In both methods, lipids were separated using a binary gradient program, at a flow rate of 0.5 ml/min: mobile phase A was 0.2% formic acid/2 mM ammonium formate in water; and mobile phase B
was 0.2% formic acid/1 mM ammonium formate in methanol. The HPLC gradient for separation of ceramide and GalCer was as follows: 0 min, 20:80 A/B; 2 min, 15:85 A/B; 3 min, 10:90 A/B; 4 min, 1:99 A/B; 6 min, 0/100 A/B; 18 min 0:100 A/B; and 20 min, 20:80 A/B. C18 chromatography was performed on a 2×150 mm Phenomenex C18 column (3 μm pore size). The gradient employed was: 0 min, 10:90 A/B; 2 min, 0/100 A/B; 2 min, 10:90 A/B; 3 min, 1:99 A/B; 5 min, 0/100 A/B; 18 min, 0:100 A/B; and 20 min, 20:80 A/B. 

Ceramide and GalCer were analysed simultaneously by precursor ion scanning over the mass range 450–1050 Da, for three different GalCer standards: d18:1/12:0, RT = 6.88 and 6.88 min; d18:1/18:0, RT = 8.42 and 8.36 min; and d18:1/24:1, RT = 10.39 and 10.33 min. For lipid detection in MRM mode, the TSQ was set to monitor a list of d18:1/12:0, RT = 6.88 and 6.88 min; d18:1/18:0, RT = 8.42 and 8.36 min; and d18:1/24:1, RT = 10.39 and 10.33 min. For lipid detection in MRM mode, the TSQ was set to monitor a list of events as shown in Table 1, each with a scan time of 0.06 s. Note that mass accuracy for the Quantum TSQ is ±0.5 Da.

High-mass-accuracy analysis of GalCer species was performed using an LTQXL Orbitrap Mass Spectrometer operating in positive (electrospray) mode, connected to an Accela LC system (Thermo Scientific). The gradient program, at a flow rate of 0.4 ml/min, was as follows: 0 min, 20:80 A/B; 2.5 min, 15:85 A/B; 3.8 min, 10:90 A/B; 5 min, 1:99 A/B; 6.5 min 0:100 A/B; 30 min 0:100 A/B; and 32 min 20:80 A/B. Mass spectra were recorded in Fourier transform full scan mode, mass range 80–1100 Da and mass resolution 60000 (m/Δm). The alignment process is based on sample comparison, peak lists generated from each sample were further compiled to generate a list of peaks with corresponding abundance for each selected reaction monitoring transition events across the chromatogram were cull within the most intense peak within each mass and RT window. To determine the abundance of a peak within a sample, the selected ion chromatogram is computed with a mass window of ±0.25 m/z and smoothed using the Savitzky–Golay filter [21] before the area under the chromatogram was calculated. This procedure results in a final list of peaks and their corresponding abundance for each selected reaction monitoring event. For MRM experiments, the precursor mass was used directly from each monitored event.

Lipid extraction from human brain tissue

Human brain tissues were obtained from the Sydney Brain Bank and the New South Wales Tissue Resource Centre. Ethics approval for the current study was from the University of Wollongong Human Research Ethics Committee (HE10/327). Tissue samples were obtained from brains of six AD patients, clinically and pathologically defined using NIA (National Institute on Aging)-Reagan criteria, and sex- and gender-matched controls [17]. These 12 samples have been used in another unrelated study [18]. Clinical information for each case, including gender, age at death, PMI (post-mortem interval) and clinical cause of death is provided in Table 2. Frozen brain tissue from the hippocampus and cerebellum was pulverized over dry ice and stored at −80°C until required for analysis.

Lipids were extracted from approximately 10 mg of frozen brain tissue, using a modification of the single-phase extraction method described by Bielawski et al [19]. A 1.5 ml mixture of ethyl acetate/propan-2-ol/water (30:10:60, by vol.) (solution A), together with 1.5 ml of chloroform/methanol (1:2) (solution B), was added to the frozen brain tissue in a 15 ml glass tube. A 50 μl cocktail of internal standards was added, comprising 5 μM each of ceramide(d18:1/17:0), SM(d18:1/12:0) and GalCer(d18:1/12:0). The brain tissue was further crushed with a dounce homogenizer, sonicated for 2 h in a Unisons FXP10M water bath and incubated, with occasional sonication, for 10 h at 35°C to extract sphingolipids [12]. The extract was centrifuged for 10 min at 3700 g (at 25°C), and the supernatant was transferred to a clean glass tube. The residue was re-extracted with 1.5 ml of solution A as described above, then 1 ml of chloroform/methanol (2:1). The supernatants from each extraction step were combined, divided into two parts, then dried down in a Thermo SC210 SpeedVac. One half of the extract was reconstituted in 200 μl of HPLC mobile phase (80:20 A/B), vortex mixed thoroughly, and centrifuged at 3700 g for 5 min (at 25°C). The supernatant was transferred to an HPLC vial with a 200 μl glass insert, and stored at −20°C until LC-MS/MS analysis of ceramide and GalCer/GluCer (glucosylceramide) content. The other half of the extract was further processed for SM analysis, exactly as described previously [19]. Note that, for SM preparation, an alkaline hydrolysis step is included to remove phosphatidylcholine, which is highly abundant and may interfere with SM analysis. Analytical (HPLC) grade solvents were purchased from Merck. All lipid standards were purchased from Avanti Polar Lipids, except for SM(d18:1/22:0), which was from Matreya LLC.

Data extraction and collation from multiple samples

LC-MS/MS data from each sample was converted into the mzXML file format using ReAdW (version 4.3.1) [20] with default parameters. The results were then processed using in-house software: mass spectra were de-isotoped based on the detection of estimated mass-dependent isotope profiles. In this process, the intensity of all isotopic peaks is combined into a single monoisotopic peak. Furthermore, water loss events (−18 ± 0.25 m/z) were also removed from each spectrum. Peaks were then detected in each spectrum within the LC-MS/MS run. We defined a peak as ions with an intensity of greater than 2.5% of the base peak and with at least 10 000 ion counts. To eliminate redundancy, all peaks with a similar mass (±0.25 m/z) and RT (±0.5 min) arising from the same transition events across the chromatogram were cull within the most intense peak within each mass and RT window. To determine the abundance of a peak within a sample, the selected ion chromatogram is computed with a mass window of ±0.25 m/z and smoothed using the Savitzky–Golay filter [21] before the area under the chromatogram was calculated. This procedure results in a final list of peaks and their corresponding abundance for each selected reaction monitoring event. For MRM experiments, the precursor mass was used directly from each monitored event.

For sample comparison, peak lists generated from each sample were further compiled to generate a list of peaks with aligned mass and RTs. The alignment process is based on the use of mass and RT windows (% m/z and ±0.5 min respectively). For the SM analysis, the RT window was increased to ±1.0 min to accommodate broader elution peaks, and to account for chromatographic variation between analyses which were performed over several days. We did not find it necessary to apply sophisticated non-linear alignment procedures [22].

This software tool is implemented using C++ and is available to the research community as a web resource, located at http://www.cancerresearch.unsw.edu.au/crcweb.nsf/page/lipidms. Source code will be made available to non-commercial users upon request.

Data processing for 2D plots and lipid quantification

The RT versus m/z data for d18:1/12:0, 14:0, 16:0, 18:0, 20:0, 22:0 and 24:0 ceramide standards was fitted to a centred second-order polynomial equation, given by:

\[ y = ax^2 + bx + c \]

where \( a \), \( b \), and \( c \) are coefficients determined by the software tool.
Table 1 Abundance of individual ceramide and GalCer species in human hippocampus extracts, as determined by MRM

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Precursor mz</th>
<th>Abundance in control hippocampus</th>
<th>Abundance in AD hippocampus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cer(d18:1/16:0)</td>
<td>538.6</td>
<td>0.30 ± 0.15</td>
<td>0.52 ± 0.23</td>
</tr>
<tr>
<td>Cer(d18:1/18:0)</td>
<td>564.6</td>
<td>6.44 ± 2.46</td>
<td>6.43 ± 4.31</td>
</tr>
<tr>
<td>Cer(d18:1/20:0)</td>
<td>594.7</td>
<td>0.12 ± 0.033</td>
<td>0.18 ± 0.10</td>
</tr>
<tr>
<td>Cer(d18:1/22:0)</td>
<td>622.7</td>
<td>0.10 ± 0.040</td>
<td>0.098 ± 0.022</td>
</tr>
<tr>
<td>Cer(d18:1/23:1)</td>
<td>634.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cer(d18:1/23:2)</td>
<td>636.7</td>
<td>0.097 ± 0.042</td>
<td>0.060 ± 0.022</td>
</tr>
<tr>
<td>Cer(d18:1/24:1)</td>
<td>648.8</td>
<td>2.81 ± 1.11</td>
<td>1.52 ± 0.83</td>
</tr>
<tr>
<td>Cer(d18:1/24:0)</td>
<td>650.8</td>
<td>0.27 ± 0.14</td>
<td>0.15 ± 0.068</td>
</tr>
<tr>
<td>Cer(d18:1/25:1)</td>
<td>662.8</td>
<td>0.55 ± 0.27</td>
<td>0.23 ± 0.15*</td>
</tr>
<tr>
<td>Cer(d18:1/25:0)</td>
<td>664.8</td>
<td>0.043 ± 0.026</td>
<td>0.015 ± 0.009*</td>
</tr>
<tr>
<td>HFA-Cer(d18:1/16:0)</td>
<td>700.7</td>
<td>3.00 ± 1.62</td>
<td>2.98 ± 1.31</td>
</tr>
<tr>
<td>GalCer(d18:1/16:0)</td>
<td>726.7</td>
<td>184.5 ± 89.5</td>
<td>106.2 ± 120.9</td>
</tr>
<tr>
<td>GalCer(d18:1/18:0)</td>
<td>728.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HFA-GalCer(d18:1/18:1)</td>
<td>742.7</td>
<td>7.38 ± 3.25</td>
<td>5.98 ± 3.68</td>
</tr>
<tr>
<td>GalCer(d18:1/18:0)</td>
<td>744.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HFA-GalCer(d18:1/18:0)</td>
<td>754.7</td>
<td>10.97 ± 3.73</td>
<td>7.16 ± 8.59</td>
</tr>
<tr>
<td>HFA-GalCer(d18:1/18:0)</td>
<td>756.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HFA-GalCer(d18:1/20:1)</td>
<td>770.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HFA-GalCer(d18:1/20:0)</td>
<td>772.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GalCer(d18:1/22:1)</td>
<td>767.7</td>
<td>6.87 ± 2.73</td>
<td>4.06 ± 4.65</td>
</tr>
<tr>
<td>GalCer(d18:1/22:0)</td>
<td>784.7</td>
<td>39.95 ± 13.83</td>
<td>26.82 ± 24.85</td>
</tr>
<tr>
<td>GalCer(d18:1/23:1)</td>
<td>796.7</td>
<td>22.91 ± 10.24</td>
<td>11.51 ± 12.36</td>
</tr>
<tr>
<td>HFA-GalCer(d18:1/22:1)</td>
<td>798.7</td>
<td>64.11 ± 20.91</td>
<td>40.89 ± 27.20</td>
</tr>
<tr>
<td>HFA-GalCer(d18:1/22:0)</td>
<td>800.7</td>
<td>23.48 ± 9.99</td>
<td>28.21 ± 9.06</td>
</tr>
<tr>
<td>HFA-GalCer(d18:1/24:1)</td>
<td>810.7</td>
<td>685.3 ± 291.1</td>
<td>387.9 ± 366.6</td>
</tr>
<tr>
<td>HFA-GalCer(d18:1/24:0)</td>
<td>812.7</td>
<td>247.5 ± 85.6</td>
<td>155.8 ± 106.8</td>
</tr>
<tr>
<td>HFA-GalCer(d18:1/23:1)</td>
<td>812.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HFA-GalCer(d18:1/23:0)</td>
<td>814.7</td>
<td>34.06 ± 18.47</td>
<td>47.20 ± 18.74</td>
</tr>
<tr>
<td>HFA-GalCer(d18:1/25:1)</td>
<td>824.8</td>
<td>202.4 ± 84.7</td>
<td>122.1 ± 89.7</td>
</tr>
<tr>
<td>HFA-GalCer(d18:1/25:0)</td>
<td>826.8</td>
<td>85.77 ± 28.99</td>
<td>56.96 ± 43.49</td>
</tr>
<tr>
<td>HFA-GalCer(d18:1/24:1)</td>
<td>826.8</td>
<td>168.1 ± 70.0</td>
<td>142.6 ± 50.1</td>
</tr>
<tr>
<td>HFA-GalCer(d18:1/24:0)</td>
<td>828.8</td>
<td>53.80 ± 28.07</td>
<td>67.57 ± 33.13</td>
</tr>
<tr>
<td>GalCer(d18:1/26:1)</td>
<td>838.8</td>
<td>208.5 ± 57.9</td>
<td>126.5 ± 66.1*</td>
</tr>
<tr>
<td>GalCer(d18:1/26:0)</td>
<td>840.8</td>
<td>30.21 ± 7.70</td>
<td>20.02 ± 17.30</td>
</tr>
<tr>
<td>GalCer(d18:1/24:1)</td>
<td>840.8</td>
<td>19.81 ± 12.87</td>
<td>22.42 ± 16.82</td>
</tr>
<tr>
<td>HFA-GalCer(d18:1/25:1)</td>
<td>842.8</td>
<td>17.70 ± 8.28</td>
<td>21.7 ± 8.54</td>
</tr>
<tr>
<td>HFA-GalCer(d18:1/25:0)</td>
<td>854.8</td>
<td>21.55 ± 10.65</td>
<td>40.33 ± 23.28</td>
</tr>
<tr>
<td>HFA-GalCer(d18:1/26:1)</td>
<td>856.8</td>
<td>9.57 ± 3.38</td>
<td>8.50 ± 3.79</td>
</tr>
<tr>
<td>Total NFA-GalCer</td>
<td>1792.7</td>
<td>1392 ± 613</td>
<td>1062 ± 860</td>
</tr>
<tr>
<td>Total HFA-GalCer</td>
<td>332.5 ± 28.07</td>
<td>393.7 ± 135.5</td>
<td></td>
</tr>
<tr>
<td>Total NFA + HFA-GalCer</td>
<td>2145.8</td>
<td>1462 ± 888</td>
<td></td>
</tr>
</tbody>
</table>

\[
y = 9.423 + 0.0353(x - 566.5) + 0.000133(x - 566.5)^2
\]

using GraphPad PRISM \((R^2 = 0.9994)\). For Cer chromatography, the relationship between \(m/z\) and RT was also quadratic \((R^2 = 0.9987)\), given by:

\[
y = 9.610 + 0.09921(x - 576.7) + 0.0005569(x - 576.7)^2
\]

This equation was then used to derive a theoretical RT for all \(m/z\) values. The theoretical RT was subtracted from the observed RT, yielding the relative RT (which is approximately 0 for all points used to fit the curve). There were insufficient commercially available GalCer and SM standards to derive the RT versus \(m/z\) relationship. We therefore extracted the \(m/z\) and RT for GalCer and SM bearing saturated N-acyl chains with an even number of carbons ranging from 16 to 26 in length (i.e. d18:1/16:0 through to d18:1/26:0), in the collated hippocampus extracts. The RT values were compared with those observed with the commercially available standards (d18:1/16:0 and d18:1/18:0 for GalCer; d18:1/16:0, d18:1/22:0 and d18:1/24:0 for SM), to confirm that the RT values were accurate to within 0.1 min. The six data points obtained from the hippocampus extracts were then fitted to a centred second-order polynomial \((R^2 = 0.9992\) and 0.9993, for GalCer and SM respectively) and relative RT values were obtained by fitting all observed \(m/z\) values to the equation and subtracting the resultant theoretical RT from

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the observed RT, as described above. Peak areas for each lipid identified were expressed as ratios to the relevant internal standard [SM(d18:1/12:0), ceramide(d18:1/17:0) or GalCer(d18:1/12:0)], and normalized for the amount of starting brain tissue. For peaks identified by the \( m/z \) 264 product ion, the data was normalized separately to both ceramide and GalCer internal standards, yielding two distinct intensity plots.

Ceramide and GalCer content (in pmol/mg of tissue) was calculated from standard curves constructed with available synthetic standards. In the case of GalCer, standards for GalCer(d18:1/12:0) and GalCer(d18:1/24:0) were used to quantify long-chain (C\( _{16} \)–C\( _{20} \)) and very-long-chain (C\( _{22} \)–C\( _{26} \)) GalCer respectively.

Statistical analysis

Two-tailed Student's \( t \) tests were used to compare lipid levels or ratios between normal and AD hippocampus, and between normal hippocampus and normal cerebellum. Equal variances were not assumed.

RESULTS

MRET profiling approach

In the present study we use the LipidMAPS (http://www.lipidmaps.org) nomenclature system for sphingolipids, represented as ‘lipid class(length:double bonds of sphingoid base chain/length:double bonds of N-acyl chain)’, e.g. SM(d18:1/24:0) refers to SM with an 18-carbon sphingosine backbone with one double bond, and a 24-carbon N-acyl chain with no double bonds (Figure 1a).

We aimed to create a system that facilitates the use of column RT, as well as precursor and product masses, for characterizing the sphingolipid content of complex biological extracts. To define a relationship between mass and RT, we characterized the sphingolipid content of complex biological extracts.

The ion with \( m/z \) 264 is produced by sphingolipids with a d18:1 sphingosine backbone, whereas the ion with \( m/z \) 184 is the choline headgroup of SM. The LC-MS/MS data was processed as described in the Experimental section to generate a summary table showing \( m/z \) and RT values for all the distinct peaks in a set of samples, and the corresponding peak area (i.e. ion abundance) for that particular metabolite in each individual sample.

The principle SM, ceramide and GalCer components in the cerebellum and hippocampus extracts are shown in Figure 2, in which the mean peak area (\( n = 6 \)) relative to other lipids is represented by the size of the bubble. A colour gradient is used to show abundance in AD, relative to control brain extracts. Lipids with no double bonds in their N-acyl chain, but differing in the number of N-acyl chain carbons align horizontally with a relative RT of approximately 0. Lipids with an identical number of carbons, but differing in the number of acyl chain double bonds aligned vertically. Ceramides with 2-hydroxy group in their N-acyl chain, referred to as HFA (hydroxylated fatty acid) ceramides, are less hydrophobic and consequently have a more negative RT relative to that expected for a NFA [normal (i.e. non-hydroxylated) fatty acid] ceramide of equivalent mass. We next sought to determine whether this approach would aid in the identification and characterization of sphingolipids in a complex biological extract.

Table 2  Patient brain samples used in the present study

<table>
<thead>
<tr>
<th>ID number</th>
<th>Age (years)</th>
<th>Gender</th>
<th>PMI (h)</th>
<th>Cause of death</th>
<th>Disease duration (years)</th>
<th>Braak NFT stage [38]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>85</td>
<td>Female</td>
<td>23</td>
<td>Pneumonia</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>79</td>
<td>Male</td>
<td>8</td>
<td>Pulmonary embolism</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>93</td>
<td>Female</td>
<td>21</td>
<td>Cardiac failure</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>78</td>
<td>Female</td>
<td>11</td>
<td>Respiratory failure</td>
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the identity of these products as SM species with a sphingosine backbone, we verified that both 184 (phosphatidylcholine) and 264 (sphingosine backbone) \textit{m/z} fragments were produced at the same column RT in a single sample run, after fragmentation of the \textit{m/z} 731.6, 813.7 and 815.7 [SM(d18:1/18:0), SM(d18:1/24:1) and SM(d18:1/24:0)] precursor ions (collision energy 35 eV). Similarly, GalCer species with C24:1, C25:1 and C26:1 N-acyl chains (horizontal row at $-1.0$ to $-1.1$ min) align vertically with their C24:0, C25:0 and C26:0 counterparts (Figure 2c).

We identified two horizontal series of lipids whose mass corresponds to GalCer with a hydroxylated fatty acid (i.e. HFA-GalCer; $+16$ Da), and 0 or 1 double bonds in their N-acyl chain, indicated by long arrows in Figure 2(c). These lipids cluster in terms of \textit{m/z} and RT with other GalCer species, but as would be expected for HFA-GalCer, they elute from the C column earlier than their NFA counterparts, with relative RTs of $-1.1$ to $-1.4$ (C22:0, C23:0, C24:0, C25:0 and C26:0 N-acyl chains) and $-1.9$ to $-2.4$ (C23:1, C24:1, C25:1 and C26:1 N-acyl chains), providing important evidence to confirm our prediction of their structure based on mass. Note that the shifted RTs of these ions relative to their NFA counterparts also confirms that they are not oxidized GalCers, created during electrospray ionization. As expected, we observed no mass corresponding to the hydroxylated (\textit{m/z} $+16$) version of the GalCer(d18:1/12:0) internal standard. HFA lipids were observed only in GalCer, reflecting the strong preference of the enzyme UDP-galactose ceramide galactosyltransferase for HFA-ceramide substrates [26–28].

The importance of using relative RT for lipid identification is emphasized by the presence of two distinct peaks for \textit{m/z} 826.7 indicated by arrowheads in Figure 2(c). Using the online metabolomic search tool METLIN, this metabolite is identified as GalCer(d18:1/25:0). In accordance with that prediction, one of the peaks with \textit{m/z} 826.7 has a relative RT of $-0.07$ and sits on the y axis. However, the second peak has a relative RT of $-2.17$, and based on its position on the 2D plot, we predict that this ion is HFA-GalCer(d18:1/24:1). It aligns horizontally with 840.7 and 854.7 Da ions, which correspond by mass to HFA-GalCer with C25:1 and C26:1 N-acyl chains. Because GalCer(d18:1/25:0) and HFA-GalCer(d18:1/24:1) are separated in mass by only 0.04 Da and generate the same product ion (\textit{m/z} 264), they would probably have been misconstrued as the same lipid, using any mass spectrometer other than a very high-mass-accuracy instrument. To further verify that these are indeed distinct GalCer species, we checked their mass and relative elution pattern on...
Values are means ± S.E.M. (n = 6). P values were derived from unpaired two-tailed Student’s t tests.

Figure 3  Decreased GalCer and increased GalCer hydroxylation in AD hippocampus

(a) Total GalCer peak area is expressed relative to total SM peak area in each of the brain samples. Horizontal bars represent the mean (n = 6). (b) Total HFA-GalCer peak area, as a proportion of total (HFA-GalCer + NFA-GalCer) peak area, in each brain sample. (c) HFA-GalCer peak area, as a proportion of total (HFA-GalCer + NFA-GalCer) peak area, for each different N-acyl chain length. Values are means ± S.E.M. (n = 6). P values were derived from unpaired two-tailed Student’s t tests.

Figure 4(a) shows the total ion chromatogram for a single hippocampus sample, together with extracted ion chromatograms for the events with a precursor ion m/z 826.7 or 840.7. As described above, the two peaks observed for the m/z 826.7 ion are NFA-GalCer(d18:1/25:0) and HFA-GalCer(d18:1/24:1), which are separated in mass by only 0.04 Da. There are no synthetic or purified standards for these lipids, so they can only be differentiated either through further fragmentation studies, or on the basis of their RT relative to known standards, as illustrated in Figure 4(b). Similarly, two of the peaks observed in the m/z 840.7 event (Figure 4a) may be identified, based on their relative RT, as NFA-GalCer(d18:1/26:0) and HFA-GalCer(d18:1/25:1). The third middle peak is a heavy isotope of NFA-GalCer(d18:1/26:1), which is removed by de-isotoping the data.

As was observed with precursor ion scanning, there was a reduction in the NFA-GalCer content of AD hippocampus, compared with the controls (Figure 4b and Table 1). All NFA-GalCers were reduced in abundance, in AD compared with control hippocampus samples, and there were statistically significant reductions in a number of very long chain ceramides (Table 1). In contrast, HFA-GalCer content did not decrease. Rather, there was a slight increase in total HFA-GalCer content, and calculation of the HFA-GalCer content as a proportion of total GalCer confirmed the statistical significance of this difference between control and AD subjects (Figure 4c).

Applicability of the relative elution time method to C₁₈ chromatography

To demonstrate the portability of our method to other column types, we ran our mixture of ceramide and dihydroceramide standards and one of the hippocampus extracts on a C₁₈ column, which are commonly used in sphingolipid analyses. As expected, the quadratic relationship between column RT and mass was also seen with this column (eluting under isocratic conditions with 100 % methanol) (Figures 5a and 5b). The same (quadratic) relationship between RT and mass is observed when the ceramides are eluted with 90 % propan-2-ol/10 % water (results not shown). The pattern of brain GalCers eluting from the C₁₈ column was the same as that observed with a C₈ column: RT for GalCer species with a sphingosine base and a saturated N-acyl chain (i.e. d18:1/N:0) followed a quadratic relationship with respect to mass, and the NFA-GalCer and HFA-GalCer species form distinct rows on a plot of relative RT versus m/z (Figure 5c). There was slightly better resolution of HFA- from NFA-GalCers on the C₈ column,
and we would recommend using a C₈ rather than a C₁₈ column to resolve diacyl sphingolipids due to the shorter chromatography times required.

**DISCUSSION**

The lipid profiling approach that we describe in the present paper represents a significant improvement over current methods because the physicochemical characteristics of the lipids facilitate their identification. These characteristics may be critically important in resolving structurally different lipids that have almost identical mass and are within the same broad functional class, as in the example we present with NFA-GalCer(d18:1/25:0) and HFA-GalCer(d18:1/24:1). Approaches that employ the physicochemical characteristics of lipids, including MDMS (multi-dimensional MS)-based shotgun lipidomics [29] and TLC-MALDI (matrix-assisted laser-desorption ionization) [30], have been employed to separate different classes of lipids before MS analysis, but not to separate lipids within the same structural class. In fact, shotgun approaches such as MDMS are unable to resolve HFA- and NFA-GalCers that have an almost identical mass [31].

The relative RT method that we describe in the present paper greatly aids in the identification of lipids within the same structural class and in determining whether a particular metabolite belongs to a particular lipid class. We note that current published protocols for comprehensive sphingolipidomic analysis do not incorporate monitoring of HFA-GalCer [11,12]. Although we employed a high-mass-accuracy Orbitrap instrument to verify our method, the use of these instruments is inherently more complex and they cannot be set to perform precursor ion scans with defined product ions or MRM.

In addition to improving the accuracy of lipid structure characterization, the resultant 2D plots greatly improve visualization of the data, when compared with a tabulated list. A recent review identified visualization of data as one of the key issues in sphingolipidomic systems analysis [1]. To illustrate this point, a 2D map of the MS intensity output using precursor ion scanning over the m/z range 680–900 (product ion m/z 264), from a single cerebellum sample before and after RT transformation is shown in Supplementary
Mass and relative elution time profiling of sphingolipids

Figure 5 Application of the relative elution time method with a C18 chromatography column
(a and b) Elution pattern for a mixture of ceramide standards eluted from a C18 column under isocratic conditions (100% methanol). (a) shows absolute RT, whereas (b) shows relative RT. Using a C18 column, the relationship between m/z and RT fits a quadratic equation. (c) Relative RT versus m/z plot of the GalCer content in a control cerebellum extract, using a C18 chromatography column. Cer, ceramide; dhCer, dihydroceramide.

Figure S1 (at http://www.BiochemJ.org/bj/438/bj4380165add.htm). Plotting distinct LC peaks (as in Figure 2) rather than the raw intensity data (Supplementary Figure S1) improves the visual appearance of the data and makes it much simpler to present the mean of multiple samples, thereby improving our capacity to draw meaningful inferences from the data. Using our method, we could readily visualize the GalCer content of brain samples taken from AD patients and normal controls. Synthetic or purified standards are not available for most of the GalCer species shown in Figures 2 and 4, which makes the unambiguous assignment of lipid structure based on the most commonly employed MS1 or MS2 approaches impossible. More detailed analyses of the fragmentation pattern of individual lipids generally reveals the structure, but this is not always the case, and this approach often necessitates chemical derivatization of lipids prior to MS analysis or the use of more reactive collision gases [32]. Using our approach, chemical derivatization is not necessary and important biochemical information can be obtained for many lipids simultaneously.

It is generally accepted that precursor ion scanning, because of its reduced duty cycle, is less quantitative than MRM approaches [33]. However, precursor ion scans such as we have used in the present study are an important tool for establishing the principal lipids of interest for subsequent MRM experiments, and are a valid approach for establishing trends in the data. We found that the trends observed using precursor ion scanning – a reduction in total GalCer content and an increase in HFA-GalCer content – were reproduced in MRM mode which, as expected, yielded a greater linear range for absolute quantification. Although ceramide 1-phosphates were not abundant in the brain tissues analysed in the present study, we have found that the precursor ion scan with product ion m/z 264 used in our study is also sufficient to detect gross changes in ceramide 1-phosphate content in cultured cells. We have been unable to detect the principle dihydroceramide species using precursor ion scanning coupled to a product ion m/z of 266, due to their low abundance and their relatively poor fragmentation to the m/z 266 backbone ion (compared with the m/z 264 ion characteristic of sphingosine). However, we have found that sphingolipids bearing a sphinga-2,4-diene backbone can readily be identified with a precursor scan coupled to a product ion with m/z 262 (Supplementary Figure S1c) [34].

The present study is the first to demonstrate an increase in the ratio of HFA-GalCer/NFA-GalCer in the brains of AD patients. This change was accompanied by an overall decline in hippocampal GalCer content, although not statistically significant in the present study. The loss of GalCer in AD is in agreement with older literature employing TLC techniques [35,36]. These changes were observed in the hippocampus but not the cerebellum, in accordance with the pathology of AD: hippocampal atrophy associated with a pronounced loss of pyramidal neurons is a defining feature of AD, whereas the cerebellum is much less affected [37,38]. More recent MS analysis has shown a decline in sulfatide content, in the absence of any decline in GalCer content, in various regions of the cerebral cortex of AD subjects [39]. We note that these authors did not examine changes in the hippocampus, whereas we did not examine any frontal or temporal cortex tissue. In future studies, we aim to investigate the changes to these myelin sphingolipids using a much larger cohort of patient samples derived from different brain regions.

We found HFA-GalCer to comprise approximately 50% of total GalCer in the cerebellum, and 20% in the hippocampus of control subjects. Previous studies have reported that HFA-GalCer comprises approximately half of total GalCer in myelin [40,41]. Although very-long-chain ceramides (those with >22-carbon N-acyl chains) declined in abundance in AD hippocampus,
we observed an increase in the only detectible HFA-ceramide d18:1/24:0 (Table 1). In precursor scanning mode, HFA-Cer(d18:1/24:0) was detected in all six AD hippocampus samples, but only one of the control hippocampus samples. These results suggest increased fatty acid hydroxylase activity in AD. Barrier et al. [42] have recently reported an increase in the ratio of HFA-ceramide/NFA-ceramide in the cerebral cortex, in a mouse model of AD (APPswe/PS1 Ki). Interestingly, the increased ratio was only observed in female mice. Our human cohort is too small to draw firm conclusions on sex-related differences (see Table 2), but we note that, when separated on the basis of gender, the HFA/NFA GalCer ratio was significantly different between AD and control females (P = 0.0006), but not males.

In summary, a rapidly growing body of literature is now highlighting the importance of lipid metabolites to cell signalling and pathophysiological processes. We present a new method that improves the combined use of LC and MS to create a 2D analysis of sphingolipids in a complex biological extract. The data transform that we apply can in theory be applied to any chromatography in which the separation of different lipids within the same functional class is partially dependent on the length of the lipid acyl chains. The increased capacity of our approach to objectively profile lipid content is illustrated with the new findings we describe regarding changes in the sphingolipid content in the brains of AD patients. Our approach can be applied to other classes of lipids, and its application in lipidomics will improve the discovery process.

AUTHOR CONTRIBUTION
Leila Hejazi initiated the idea of LCxMS/MS, and contributed to method development, experimental work and data analysis. Jason Wong contributed to method development, and extracted and collated peak information from the LC-MS/MS raw data files. Danni Cheng prepared human brain samples. Nicholas Proschogo contributed data analysis and interpretation. Anthony Don contributed to method development and study design, performed experimental work and data analysis. Jason Wong contributed to method development, and extracted and collated peak information from the LC-MS/MS raw data files. Danni Cheng prepared human brain samples. Nicholas Proschogo contributed data analysis and interpretation. Anthony Don contributed to method development and study design, performed experimental work and data analysis. Jason Wong contributed to method development and study design, performed experimental work and data analysis, and wrote the manuscript, with contributions from Leila Hejazi, Jason Wong, Diako Ebrahimi and Brett Garner.

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REFERENCES

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SUPPLEMENTARY ONLINE DATA

Mass and relative elution time profiling: two-dimensional analysis of sphingolipids in Alzheimer’s disease brains

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Figure S1 RT versus m/z map of intensity data

Data shown are from a single control cerebellum sample before (a) and after (b) data transformation, as described in the Experimental section of the main text. (c) TICs for precursor ion scan over m/z range 455–1050, coupled to defined product ions with m/z 262 (sphinga-2,4-diene long-chain base) and 264 (sphingosine long-chain base); extracted ion chromatograms for precursor ions with m/z 826.2–827.2 (showing two distinct peaks with m/z 826.7) and 840.2–841.2 (showing two distinct peaks with m/z 840.7), coupled to 264 m/z product ion. Chromatograms in (c) are derived from the same sample as shown in (a) and (b).

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