Quantitative Analysis of the Molecular Species of Monosialogangliosides by Continuous-flow Fast-atom Bombardment Mass Spectrometry

S. Chen, G. Pieraccini and G. Moneti
† Fidia Research Laboratories, 35031 Abano Terme, Italy
and Mass Spectrometry Centre, Department of Pharmacology, Florence University, 50134 Florence, Italy

SPONSOR REFEREE: Dr Sergio Daolio, CNR, Corso Stati Uniti 4, Padova, Italy

Negative-ion continuous-flow fast-atom bombardment mass spectrometry was evaluated as a means for the quantitative analysis of N-acetyleneuraminyl-galactosyl-glucosyl-ceramide (NeuAc-GM₃) and N-acetylgalactosaminyl-(N-acetyleneuraminyl)galactosyl-glucosyl-ceramide (NeuAc-GM₂). This study was carried out on a 7070-EQ mass spectrometer (VG Analytical, Manchester, UK) using a home-made continuous-flow fast-atom bombardment probe with a mixture of methanol + water + triethanolamine (70:27:3, v/v/v) as the mobile phase. Utilizing 100 ng of acetyl-lysogalactosyl-N-acetylgalactosaminyl-(N-acetyleneuraminyl)galactosyl-glucosyl-ceramide (acetyl-lysoGM₃) as an internal standard, standard curves for NeuAc-GM₃ d18:1-18:0 and NeuAc-GM₂ d18:1-18:0 were found to be linear over the range 5-250 ng, with associated correlation coefficients of 0.990-0.997. The lower limit of detection was found to be 2.5 ng. Satisfactory results could also be obtained when the calibration curves were derived from the deprotonated molecular ions of a mixture of the NeuAc-GM₃ and NeuAc-GM₂ classes. Using this approach, quantitative determination of NeuAc-GM₃ d18:1-16:0 from rat adrenal gland was performed using N-acetylenearminic acid assay as a test control. We found 278±36 ng of this species in 1 mg of tissue (three replicate experiments). The procedure represents a sensitive method for the quantitation of monosialogangliosides and its capability to give molecular species information.

Gangliosides are sialic acid containing glycosphingolipids and are present in the cell membranes of various tissues that have characteristic differences in their ganglioside composition.¹ Gangliosides on cell surface membranes have been reported to undergo significant changes in their chemical composition and metabolism in association with oncogenic transformation and differentiation.²⁻⁴ Structural diversity of gangliosides found in normal and tumor cells occurs not only in the sialic acid and oligosaccharide moieties but also in the ceramide residue.⁵⁻⁷ N-acetylenearminyl-galactosyl-glucosyl-ceramide (NeuAc-GM₃) and N-acetylgalactosaminyl-(N-acetyleneuraminyl)-galactosyl-glucosyl-ceramide (NeuAc-GM₂) are the two common tumor-associated monosialogangliosides⁷⁻¹⁰ (their structures are shown below). The detailed knowledge of their structure, including the molecular species, is essential to an understanding of their roles in oncogenesis. For this purpose, it is necessary to develop less laborious and more sensitive methods for quantitative analysis of the molecular species of NeuAc-GM₃ and NeuAc-GM₂, from small amounts of experimental material.

Thin-layer chromatography is widely employed for the isolation and quantitation of gangliosides,¹¹ since it is relatively simple and economical. However, it fails to provide information about the ganglioside molecular species. Reversed-phase high-performance liquid chromatography is powerful in the separation of ganglioside molecular species.¹² This procedure involves the prior isolation by normal-phase chromatography¹³,¹⁴ (high-performance liquid chromatography or thin-layer chromatography), of the ganglioside fraction based on the presence of N-acetylenearminic acid and saccharide groups. However it is possible to lose some components of interest during the isolation procedure. The sensitivity of detecting intact gangliosides utilizing ultraviolet absorbance (195-205 nm) is insufficient for the quantities of material dealt with.

Mass spectrometry has proved to be effective in the identification of the molecular species of gangliosides¹⁵ and continuous-flow fast-atom bombardment (CF-FAB) mass spectrometry¹⁶ is an appropriate technique for the rapid analysis of complex biological samples. In this study, negative-ion CF-FAB mass spectrometry has been evaluated as a method for the quantitative analysis of the molecular species of NeuAc-GM₃.
QUANTITATIVE ANALYSIS BY CF–FAB MASS SPECTROMETRY

Figure 1. Negative-ion CF-FAB mass spectrum of acetyl-lysoG₃ (internal standard; 100 ng).

and NeuAc-G₃, using acetyl-lysoagalactosyl-N-acetylgalactosaminy1-(N-acetylneuraminyl)galactosyl-glucosyl-ceramide (acetyl-lysoG₃) as an internal standard (refer to Fig. 1). The use of this approach to quantitatively determine NeuAc-G₃, molecular species from rat adrenal gland is described.

MATERIALS AND METHODS

6 Sprague Dawley rats (6–8 weeks old; 450–500 g each) were killed under ether anaesthesia, and their adrenal glands (12 pieces) were withdrawn. 6 pieces of the glands were used for the N-acetylneuraminic acid assay; 3 individual pieces for the negative-ion CF-FAB quantitation and the remainder for qualitative analysis. NeuAc-G₃, and G₃, from bovine brain, NeuAc-G₃, from human Gaucher spleen and acetyl-lysoG₃ (internal standard) prepared from bovine brain monosialoganglioside G₃l7 were obtained from our laboratories. Quantitative analysis was carried out on a 7070-EQ mass spectrometer (VG Analytical, Manchester, UK) equipped with a standard FAB ion source, FAB gun and a PDP-8 computer system. The CF-FAB system consisted of a home-made probe, Rheodyne 7250 injector with a 0.5 µL internal loop (Rheodyne, CA, USA) and a fused-silica capillary column (1 m; 50 µm ID). Methanol + water + triethanolamine (70:27:3) was used as the mobile phase and pumped by a Kontron Micropump (Kontron Instruments, Milan, Italy) at a flow rate of 2–3 µL/min. A 7–8 keV beam of xenon atoms was used as the fast-atom beam. The mass resolution of the instrument was set to approximately 1500.

The percentage distribution of the molecular species of G₃, and G₃, used in this study was found13 by reversed-phase high-performance liquid chromatography on a RP-8 column (5 µm particle size, 46 × 250 mm; HPLC Technology, Cheshire, UK). The major molecular species are NeuAc-G₃, d18:1–18:0 in the bovine brain G₃ (85%), NeuAc-G₃, d18:1–18:0 in the bovine brain G₃, (64%) and NeuAc-G₃, d18:1–16:0 in the human Gaucher spleen (87%), respectively. Standard calibration curves were prepared by adding known amounts of the G₃, the G₃, as well as the G₃ + G₃, to a constant amount of the acetyl-lysoG₃.

Ion signals from deprotonated molecules were acquired using the continuous scan mode at 10 s/decade from m/z 1050–1450. Absolute peak intensities were used for the quantitative analysis. The samples were dissolved in the mobile phase. The total lipids were extracted three times from the gland material using chloroform + methanol (1:1, v/v). For CF-FAB quantitation an internal standard was added to each sample before extraction. The gangliosides were dried under a stream of nitrogen and were purified from the total lipid extracts by a three-component solvent system18 consisting of diisopropyl ether + 1-butanol + 50 mM aqueous NaCl (6:4:5; v/v/v). Finally, the samples were desalted with a Microconcentrator (Centricon™ 10, Danvers, MA, USA). Silica thin-layer chromatography (TLC) was used for the identification of rat adrenal gland ganglioside classes. The TLC plate (Merck, Darmstadt, Germany) was developed in chloroform + methanol + 0.25% CaCl₂ (60:40:9; v/v/v) and the gangliosides were detected following treatment with resorcinol + HCl reagent.19 The N-acetylneuraminic acid in the rat adrenal gland gangliosides was quantitatively determined by the method reported by Svennerholm.20 The fatty acids in the sample were analysed by gas chromatography21, and the major fatty-acid methyl ester peaks were identified by comparison with a standard mixture (methyl palmitate, 16:0;...
methyl stearate, 18:0; methyl eicosanoate, 20:0; methyl tricosanoate, 23:0; methyl tetrascanoate, 24:0 (Fluka, Buchs, Switzerland) and docosanoic acid methyl ester, 22:0 (Sigma, St Louis, MO, USA)) on the basis of their retention times.

RESULTS AND DISCUSSION
Assessment of the CF-FAB method
Figure 1 shows the negative-ion CF-FAB mass spectrum of acetyl-lysoG₃₄. Abundant ions from deprotonated molecules are observed at m/z 1349 and 1321, corresponding to the two molecular species, the major one being acetyl-lysoG₃₄ d20:1 (m/z 1349; internal standard. Standard curves were prepared with either d18:1-18:0; NeuAc-G₄ d18:1-18:0; NeuAc-G₃ d18:1-18:0 or a couple of the species together (NeuAc-G₄ d18:1-16:0 + d18:1-18:0; NeuAc-G₃ d18:1-16:0 + NeuAc-G₃ d18:1-18:0; NeuAc-G₃ d18:1-18:0 + NeuAc-G₃ d18:1-18:0) and were linear over the ranges of 5–250 ng, with associated correlation coefficients of 0.990 to 0.997. The lower limit of detection was about 2.5 ng for NeuAc-G₄ (signal-to-noise ratio >4). Figure 2 shows the negative-ion CF-FAB mass spectrum of NeuAc-G₄ d18:1-18:0 + NeuAc-G₄ d18:1-18:0 with the internal standard (a) and their standard curves (b).

Figure 2. (a) Negative-ion CF-FAB mass spectrum of bovine brain G₄ + GM₁ + acetyl-lysoG₄. Ions at m/z: 1179, 1382 and 1349 relate to NeuAc-G₄ d18:1-18:0, NeuAc-G₄ d18:1-18:0 and acetyl-lysoG₄ d20:1 (100 ng of acetyl-lysoG₄), and (b) their standard curves. Correlation coefficient = 0.990 for NeuAc-G₄ d18:0-18:1; Correlation coefficient = 0.996 for NeuAc-G₄ d18:0-18:1. Error bars are smaller than symbols if not shown.

Figure 3. (a) Negative-ion CF-FAB mass spectrum of the rat adrenal gland gangliosides (h16:0 corresponding to hydroxy-palmitate; the sample was purified by liquid-liquid extraction; see the Materials and Methods); (b) the calibration curve of NeuAc-G₄ d18:1-16:0 that was used. The linear equation obtained was y = 0.00178x + 0.181 with a correlation coefficient of 0.995. Error bars are smaller than symbols if not shown; (c) quantitation of the rat adrenal gland NeuAc-G₄, d18:1-16:0 by negative-ion CF-FAB mass spectrometry. Absolute intensity of the deprotonated molecules (m/z 1151 and 1349) was used for the quantitation.

To our knowledge, this is the first study of G₄ and GM₁ molecular species quantitation by negative-ion CF-FAB mass spectrometry. The advantages of using acetyl-lysoG₄ as the internal standard are: (i) it is absent in biological material; (ii) deprotonated ion signals appear between the common mass ranges of NeuAc-G₄ species (m/z 1150–1280 from d18:1-24:0 up to d18:1-24:0) and NeuAc-G₄ species (m/z 1350–1470 from d18:1-16:0 up to d18:1-24:0); and (iii) it has an identical behaviour with other monosialogang-
Quantitative analysis by CF–FAB mass spectrometry

Table 1. NeuAc-GM<sub>1</sub> d18:1–16:0 content in rat adrenal gland (ng in 1 mg material (wet weight))

<table>
<thead>
<tr>
<th>NANA</th>
<th>Present results</th>
<th>Reported data&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NANA&lt;sup&gt;b&lt;/sup&gt; assay</th>
<th>NANA&lt;sup&gt;b&lt;/sup&gt; assay CF-FABMS&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
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<tr>
<td>NeuAc-GM&lt;sub&gt;1&lt;/sub&gt;, d18:1–16:0 (51.3%)</td>
<td>198</td>
<td>147</td>
<td>573</td>
<td>294</td>
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<sup>a</sup>See Ref. 22.
<sup>b</sup>NANA: N-acetylaceuraminic acid.
<sup>c</sup>Three replicate experiments.

Arita et al.,<sup>22</sup> reported the components of gangliosides in rat adrenal gland where NeuAc-GM<sub>1</sub> (94%) was the major class. But information regarding the molecular species was not mentioned. After extraction and purification, the gland gangliosides were analysed by CF-FAB mass spectrometry. Figure 3(a) shows the negative-ion CF-FAB mass spectrum of the sample. Abundant ions from deprotonated molecules correspond to NeuAc-GM<sub>1</sub> species. They are d18:1–16:0 (m/z 1151); d18:0–h16:0 (m/z 1167; h16:0 corresponding to hydroxypalmitate); d18:1–20:0 (m/z 1207); d18:1–22:0 (m/z 1235); d18:1–23:0 (m/z 1249); d18:1–24:0 (m/z 1263) and d18:1–25:0 (m/z 1277). No significant peak at m/z 1179, related to d18:1–18:0, was observed in the spectrum. Gas chromatographic analysis of the fatty acid methyl esters supports these identifications, and NeuAc-GM<sub>1</sub>, d18:1–16:0 (51.3%) is the major molecular species in adrenal NeuAc-GM<sub>1</sub>. Using the appropriate standard curve (Fig. 3(b)) we quantitatively determined this species (Fig. 3(c)) and found 278 ± 36 ng in 1 mg of gland tissue. This value is very close to that obtained by N-acetylaceuraminic acid assay (294 ng in 1 mg of the material). The differences between the published data and the present result (Table 1) could be due to the diversity of the biological materials analysed and the procedures used for sample preparation. In our experiment, column chromatography was not used. This avoided losing gangliosides in the isolation procedure.

In conclusion, quantitation of the molecular species of NeuAc-GM<sub>1</sub>, by negative-ion CF-FAB mass spectrometry with acetyl-hysoG<sub>M</sub> as an internal standard offers several advantages: (i) it appears to be hundreds of times more sensitive than the N-acetylaceuraminic acid assay and five times more sensitive than static FAB, so it would be suitable for the analysis of smaller amounts of sample; (ii) it is based on the direct intensity measurement of the deprotonated molecules of the molecular species of NeuAc-GM<sub>1</sub> of interest, thus it is more specific than the N-acetylaceuraminic acid assay and thin-layer chromatographic detection; and (iii) the use of analogue internal standard improves the accuracy of the quantitation.

REFERENCES