Journal of Chromatography B, 877 (2009) 2976–2982

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Normal phase liquid chromatography coupled to quadrupole time of flight atmospheric pressure chemical ionization mass spectrometry for separation, detection and mass spectrometric profiling of neutral sphingolipids and cholesterol

Hany Farwanah[a], Jennifer Wirtz[a], Thomas Kolter[a], Klaus Raith[b,1], Reinhard H.H. Neubert[b,1], Konrad Sandhoff[a,*]

[a] *LiMES – Life and Medical Sciences Institute, Membrane Biology and Lipid Biochemistry Unit, c/o Kekulé-Institut für Organische Chemie und Biochemie, University of Bonn, Gerhard-Domagk Str. 1, D-53121 Bonn, Germany*
[b] *Institute of Pharmacy, Martin Luther University Halle-Wittenberg, Wolfgang-Langenbeck Str. 4, D-06120 Halle (Saale), Germany*

ARTICLE INFO

Article history:
Received 1 May 2009
Accepted 7 July 2009
Available online 14 July 2009

Keywords:
Lipidomics
Sphingolipidoses
Neutral sphingolipids
Cholesterol
HPTLC
HPLC/MS
APCI-MS
Q-TOF

ABSTRACT

Many lipidomic approaches focus on investigating aspects of sphingolipid metabolism. Special emphasis is put on neutral sphingolipids and cholesterol and their interaction. Such an interest is attributed to the fact that those lipids are altered in a series of serious disorders including various sphingolipidoses. High performance thin-layer chromatography (HPTLC) has become a widely used technique for lipid analysis. However, mass spectrometric profiling is irreplaceable for gaining an overview about the various molecular species within a lipid class. In this work we have developed a sensitive method based on a gradient normal phase high performance liquid chromatography (HPLC) coupled to quadrupole time of flight (QTOF) atmospheric pressure chemical ionization mass spectrometry (APCI-MS) in positive mode, which for the first time enables separation, on-line detection, and mass spectrometric profiling of multiple neutral sphingolipids including ceramide, glucosylceramide, lactosylceramide, globotriaosylceramide, globotetraosylceramide, sphingomyelin as well as cholesterol within less than 15 min. An important advantage of the presented HPLC/APCI-MS approach is that the separation pattern emulates the one obtained by an optimized HPTLC method with a multiple stage development. Thus, the lipid classes previously separated and quantified by HPTLC can be easily screened regarding their mass spectrometric profiles by HPLC/APCI-MS. In addition, the selected ionization conditions enable in-source fragmentation providing useful structural information. The methods (HPLC/APCI-MS and the optimized HPTLC) were applied for the analysis of the mentioned lipids in human fibroblasts. This approach is aimed basically at investigators who perform studies based on genetic modifications or treatment with pharmacological agents leading to changes in the biochemical pathways of neutral sphingolipids and cholesterol. In addition, it can be of interest for research on disorders related to impairments of sphingolipid metabolism.

1. Introduction

Sphingolipids (SLs) are not only structural components of cellular membranes, but also bioactive compounds with crucial biological functions in the areas of signal transduction, cell growth, cell–cell recognition, and cell–cell adhesion [1–4].

Ceramide (Cer) is the backbone of most sphingolipids and consists of a sphingoid base (long chain base, LCB) linked to a fatty acid (FA) moiety by an amide-bond. Structural variations in the fatty acid part are due to differences in chain length, hydroxylation, and the presence of double bonds. The same variations can be present in the LCB with (2*S*, 3*R*, 4*E*)2-amino-octadec-4-en-1,3-diol being the most abundant species in mammals [5,6].

Glucosylceramide (GlcCer), lactosylceramide (LacCer), globotriaosylceramide (Gb3), and globotetraosylceramide (Gb4) are derivatives of ceramide (Fig. 1) with one to four hexosyl residues. In all these compounds, a β-glycosidically linked D-glucose (Glc) moiety represents the first sugar linked to the 1-hydroxyl group of ceramide. The second hexose residue is β-1,4-D-galactose (Gal). Within the globo-series glycosphingolipids (GSLs), α-1,4-D-galactose and β-1,3-*N*-acetyl-D-galactosamine (GalNAc) are linked to LacCer forming Gb3 and Gb4, respectively. In case of sphingomyelin (SM), the 1-hydroxyl group of ceramide is connected to

* Corresponding author. Tel.: +49 228 735346; fax: +49 228 737778.
E-mail address: sandhoff@uni-bonn.de (K. Sandhoff).
[1] Tel.: +49 345 5525215; fax: +49 345 5527292.

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.07.008

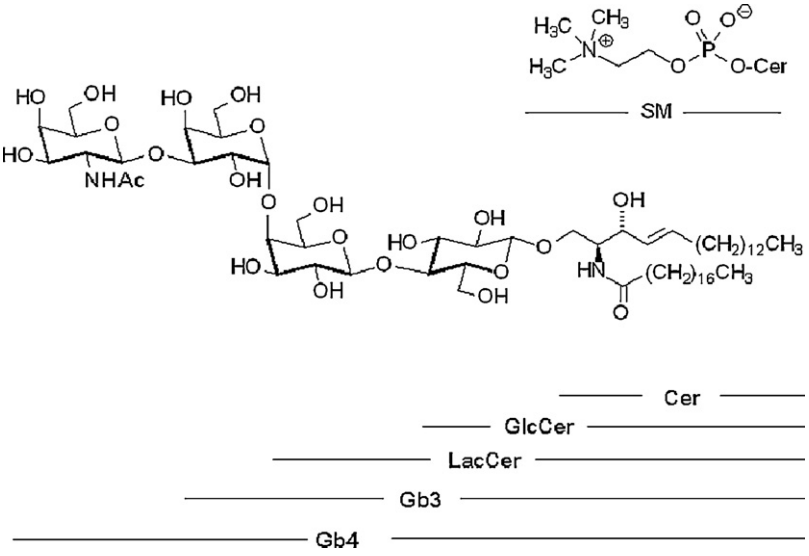

Fig. 1. Structures of the neutral SLs Cer, GlcCer, LacCer, Gb3, Gb4, and SM.

phosphorylcholine. The absence of a net charge distinguishes these neutral sphingolipids from the acidic ones, which bear one or more sialic acid or sulfate residues, and from positively charged sphingoid bases.

In living cells, the constitutive degradation of GSLs and SM takes place in the endosomes and lysosomes [7]. It requires the presence of water-soluble exohydrolases and lipid binding proteins, especially the sphingolipid activator proteins (SAPs). Inherited deficiencies of exohydrolases or SAPs can lead to the accumulation of GSLs resulting in sphingolipidoses with dramatic clinical manifestations in human patients [8]. Inherited human diseases resulting from the lysosomal accumulation of neutral sphingolipids or cholesterol (Chol) are Farber disease (Cer storage), Gaucher disease (GlcCer storage), Fabry disease (Gb3 storage), Niemann–Pick disease, types A and B (primary storage of SM), and type C (primary storage of Chol) [8]. In addition to acidic GSLs, Gb4 also accumulates in Sandhoff disease. Moreover, neutral SLs have been reported to be altered in many other disorders such as cancer, metabolic syndrome, insulin resistance, cystic fibrosis, and Alzheimer disease [9–12]. In this context, many studies based on genetic engineering or treatment with pharmacological agents have been performed to investigate different aspects of the biochemical pathway related to the turnover of neutral SLs as well as to their interplay with cholesterol (Chol) [13–18]. In this respect, analysis of SLs and GSLs in combination with Chol from cells and tissues by means of modern analytical tools is essential.

For many decades, HPTLC has been the method of choice for the analysis of lipids isolated from cells, tissues, and organs. This is due to its easy handling, robustness, low costs, and the ability to analyze different lipid classes from many samples simultaneously. However, the structural information obtained by HPTLC remains limited unless the analysis is complemented by mass spectrometry (MS).

Various mass spectrometric approaches have been developed for the analysis of SLs [5,6,19–30]. However, previous methods did not cover all interesting neutral SLs, or/and required laborious, as well as time consuming, steps. More details in this regard are given in the Section 4.

In this work we present a gradient HPLC/APCI-MS method allowing separation, detection, and mass spectrometric profiling of Cer, GlcCer, LacCer, Gb3, Gb4, SM, and Chol in less than 15 min. In addition, it provides valuable structural information about the separated sphingolipid classes by in-source fragmentation. Taking into consideration that HPTLC is widely used by many investigators for lipid analysis, we optimized the HPLC/APCI-MS method to yield a separation profile similar to the one obtained by an improved HPTLC protocol.

2. Chemicals and reagents

All lipid standards including Cer(d18:1(4E)/19:0), GlcCer(bovine), LacCer (d18:1(4E)/16:0), Gb3(d18:1(4E)/23:0), Gb4(bovine), and SM(bovine) were purchased from Biotrend (Cologne, Germany). Cholesterol (Chol) was obtained from Sigma (Taufkirchen, Germany). The lipid designation was made according to the updated lipid classification system of LIPID MAPS [31].

Silica gel HPTLC plates (Kieselgel 60; 20 cm × 10 cm) were supplied by Merck (Darmstadt, Germany). Solvents for extraction, HPTLC, and HPLC/MS were of HPLC grade and were obtained from Baker (Deventer, The Netherlands) or Merck (Darmstadt, Germany).

2.1. Cell culture

Culture medium (Dulbecco's modified Eagle's medium, DMEM) and Fetal calf serum were obtained from Sigma (Taufkirchen, Germany). Trypsin was supplied by Invitrogen (Karlsruhe, Germany). Plastic culture flasks were from Falcon (Heidelberg, Germany). All others chemicals were of analytical grade and obtained from Sigma (Taufkirchen, Germany).

Human fibroblast cells were cultured in 175 cm^2 flasks in DMEM, supplemented with 10% heat-inactivated fetal calf serum, and the antibiotics streptomycin (100 mg/L) and penicillin (100 U/L). Cells were incubated at 37 °C in a 5% CO_2 atmosphere, and the medium was renewed every 48 h. After reaching a confluent state, cells were kept quiescent for 2 days, washed with PBS, and harvested by incubation with 0.25% trypsin/EDTA for 5 min. The cell suspension was pelleted by centrifugation at 300 × *g* for 10 min at 4 °C. After removing the supernatant, cells were washed twice with PBS and stored at −20 °C.

2.2. Lipid extraction

Before extraction, cell pellets were suspended in water (0.8 mL) and were homogenized in an ultrasound bath. For isolation of lipids, 1 mL chloroform and 2 mL methanol were added to this suspension. After vortexing, a homogenous liquid phase was formed, and

the suspended cells were extracted in a water bath at 40 °C over night. The extraction procedure was repeated three times and the supernatants were pooled. After removing cell debris by centrifugation, the solvents were evaporated under a stream of nitrogen. Afterwards, alkaline hydrolysis and subsequent neutralization were performed similar to a previous protocol [5], which ends with a phase separation into an aqueous and a chloroform phase. Briefly, the lipid extract was dissolved in 2 mL chloroform, methanol (1:1; v/v). To this solution 150 μl 1 M KOH was added. After vortexing, the solution was incubated for 2 h at 40 °C in a water bath. After cooling to room temperature, 6 μl of acetic acid was added for neutralization. A solvent ratio of 1.8 water:2 chloroform:4 methanol (v/v/v) led to a phase separation. The upper layer was removed. The chloroform phase was taken, and the solvent was evaporated. The resulting lipid extract was re-dissolved in a mixture of chloroform and methanol (2:1; v/v).

2.3. HPTLC separation of neutral sphingolipids and cholesterol

Fibroblast and standard samples were applied automatically on HPTLC-plates using a Linomat® 4 system (CAMAG, Berlin, Germany). The plates were developed twice to a distance of 8 cm using a solvent mixture consisting of chloroform, methanol, and acetic acid (70:30:8; v/v/v). Afterwards, a third development step to the top of the plate using only chloroform was carried out.

After drying, lipid bands were visualized by treatment of the plates with a solution of 10% $CuSO_4$ and 8% H_3PO_4 (w/v) and heating the plates to 180 °C for 15 min. Then, the plates were scanned by a densitometer TLC 3 Scanner (CAMAG, Berlin, Germany). The measurements were made in reflectance mode at a wavelength of 595 nm. Quantitative results were related to calibration curves of corresponding lipid standards. Calculations of the molar amounts were based on a Cer average molecular weight of 600 Da. Accordingly, the addition of the head group to this molecular weight gives the molecular weight of the corresponding SL class. For example LacCer contains 2 hexoses. Thus, the molecular weight here would be $600 + (2 \times 162) = 924$ Da. The HPTLC experiments were performed in triplicate.

2.4. Separation and mass spectrometric profiling of neutral sphingolipids and cholesterol using normal phase HPLC/APCI-MS

The used HPLC system was an Alliance separation module 2695 (Waters, Eschborn, Germany) equipped with an autosampler. A LichroCart column (125 mm × 4 mm, 5 μm particle size) filled with Si 60 Lichrospher particles (Merck, Darmstadt, Germany) was utilized and the separation was carried out using a gradient from A: chloroform, to B: chloroform, n-propanol, acetic acid (80:20:2; v/v/v) in 3 min and then from B to C: ethanol, water, acetic acid (100:25:2.5; v/v/v) in 15 min with a flow rate of 1 mL/min. The injected amount of the standard lipids was always 480 ng each (0.53 nmol for each sphingolipid by an overall average molecular weight of 907 Da).

In case of the fibroblast sample the total injected sphingolipid amount (as determined by densitometry) was 4.9 ± 0.3 nmol. The amount of total cholesterol was 5.8 ± 0.2 nmol.

The HPLC system was coupled to a Q-TOF 2 mass spectrometer (Micromass, Manchester, UK, now Waters Inc.) equipped with an atmospheric pressure chemical ionization probe (APCI). The APCI heater was adjusted to 500 °C and the heated block to 150 °C. In addition, sample cone and APCI pin voltage were set to 45 V and 10 V, respectively. Unless otherwise stated, full scans were recorded between m/z 250 and m/z 1500 in a positive ion mode. The quadrupole mass filter was adjusted to less than one unit resolution to enhance detection sensitivity.

Control of separation module and mass spectrometer as well as data acquisition and processing were carried out using the MassLynx software, version 3.5 (Waters, Eschborn, Germany).

The run-to-run reproducibility of the method within a day was investigated by performing triplicate HPLC/APCI-MS experiments and determining the retention times of the separated lipids from the total ion chromatogram. The retention times of the separated lipid peaks showed an average variation of ± 0.2 min. The day-to-day variation was in the same range.

3. Results

3.1. HPTLC separation of fibroblast neutral sphingolipids and cholesterol

As shown in Fig. 2, Chol, Cer, GlcCer, LacCer, Gb3, Gb4, and SM could be separated in the standard mixture (lane 1) as well as in the lipid extract derived from fibroblasts (lane 2). In addition, contaminants resulting after the alkaline hydrolysis of the glycerolipids in the fibroblast sample have been dragged to the top of the plate, where they cannot disturb the further analysis.

We have developed a protocol enabling the separation of the above-mentioned lipids that comprise a wide polarity range, in one experiment. This was possible by the sequential development of the plates with chloroform, methanol, acetic acid (70:30:8; v/v/v,

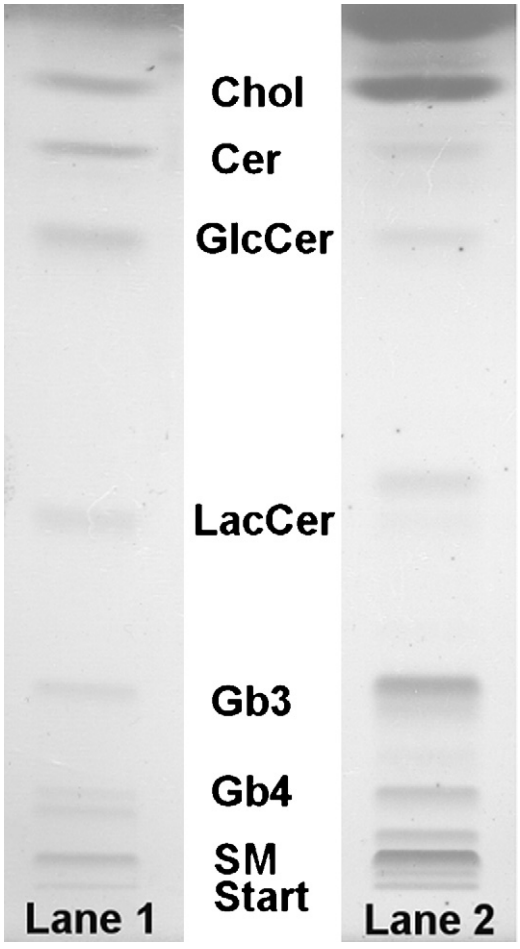

Fig. 2. HPTLC Separation of neutral SLs and Chol. Lane 1: standard lipids (amount: 0.8 μg each). Lane 2: neutral SLs and Chol derived from cultured human fibroblasts (amount: 11.3 μg sphingolipids and 5.6 μg cholesterol). The lipid band assignments between lane 1 and lane 2 apply for both lanes. Slight differences of the R_f values among some of the separated lipid bands in lane 1 and the corresponding ones in lane 2 are mainly due to different chain lengths.

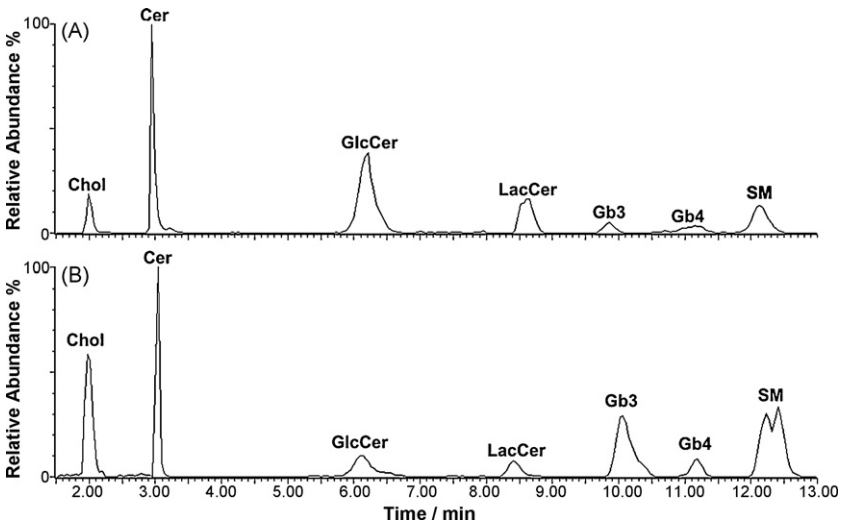

Fig. 3. (A) TIC (total ion chromatogram) in full scan mode (m/z 250–1500) of a standard lipid mix consisting of Cer, GlcCer, LacCer, Gb3, Gb4, SM, and cholesterol. B: TIC in full scan mode (m/z 250–1500) of cholesterol, Cer, GlcCer, LacCer, Gb3, Gb4, and SM in wild type fibroblasts.

twice), and then with 100% chloroform. Serial multiple developments of HPTLC plates with solvent systems of decreasing polarity have been previously shown to be suitable for separating complex biological lipid mixtures such as skin ceramides [32].

The development was carried out in a CAMAG horizontal chamber, which was saturated with chloroform, methanol, acetic acid (70:30:8; v/v/v) during the whole experiment. The first two steps were introduced to allow the separation of SLs, while the last step was set up for the separation of cholesterol and contaminants resulting after the alkaline analysis.

The assignment of lipid class structures to the separated bands (lane 2) was initially based on comparison of their R_f values with those of commercially available standards (lane 1), and then confirmed by HPLC/APCI-MS. The split into double-bands by some lipids either in the standard or in the fibroblast sample lane results mainly from structural differences of the FA moiety within the separated SLs.

The visualized lipid bands were densitometrically scanned and quantified using calibration curves of the corresponding standard lipids. According to the quantified data and based on corresponding average molecular weights, SM, Gb4, Gb3, LacCer, GlcCer, and Cer accounted for $44 \pm 3\%$, $8 \pm 0.7\%$, $33 \pm 3\%$, $9 \pm 0.6\%$, $2 \pm 0.2\%$, and $4 \pm 0.5\%$ of total neutral sphingolipids, respectively. It has to be mentioned here that the sphingolipid profile of fibroblasts has been previously found to depend on variations in cell culture conditions [33].

3.2. Separation and detection of neutral sphingolipids and cholesterol using normal phase HPLC/APCI-MS

Fig. 3A shows the separation of standard (neutral) SLs and cholesterol using the HPLC/APCI-MS method. The elution occurred according to the polarity of the head groups. The larger the carbohydrate moiety, the later the sphingolipids elute. The separation pattern mimics the one obtained by HPTLC (compared to lane 1 in Fig. 2). This similarity is due to the fact that the stationary phase in both cases consists of silica gel.

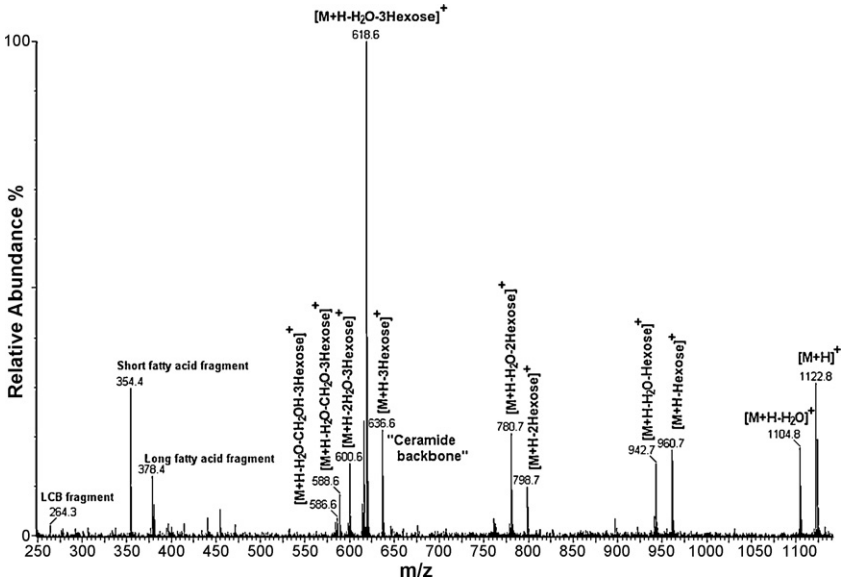

Fig. 4. Full scan mass spectrum (m/z 250–1500) in positive mode of authentic Gb3 (d18:1(4E)/23:0), corresponding to the Gb3 peak in Fig. 3A. Sample cone and APCI pin voltage were adjusted to 45 V and 10 V, respectively. The fragmentation took place in the ionization source.

The first part of the gradient (A and B in 3 min) enabled the separation of cholesterol from the SLs. Otherwise, this separation was not possible. The cholesterol peak corresponds to molecular ion $[M+H-H_2O]^+$ with m/z of 369.3.

The use of chloroform in the solvent mixture was required for successful separation. Replacement of chloroform by n-hexane or acetonitrile led to extensions in the retention times particularly of SM (data not shown). In this context, it is worth mentioning that the presence of water in the gradient at the used percentage is of significance for eluting SM properly. The detection limit depends on the lipids to be investigated and lies between 0.05 nmol and 0.1 nmol. Cer showed the lowest detection limit among the SLs. The highest ones were those of Gb4 and Gb3. In addition, it is obvious that the detection limit of cholesterol is higher than that of the SLs, which may result from the reduced stability of the cholesterol molecular ion $[M+H-H_2O]^+$. However, it has to be taken into account that the total cholesterol level in cells and tissues is usually relatively high, so that it can be easily detected.

The HPLC/APCI-MS method has been applied for the separation of neutral SLs and total cholesterol in human fibroblasts (Fig. 3B). As described in the methods part, the amounts of the applied SLs and cholesterol were determined by densitometry and accounted for 4.9 ± 0.3 nmol and 5.8 ± 0.2 nmol, respectively.

To avoid contamination of the spectrometer, the eluate of the first 1.5 min had to be discarded. During this time, contaminants resulting after the alkaline hydrolysis eluted from the column. In a previous publication it has been mentioned that fatty acids resulting after an enzymatic or alkaline treatment of glycerol-based neutral lipids and phospholipids in a GSL-containing mixture can disturb a direct MS detection of neutral GSL components [19].

Base line separations between all neutral SLs could be achieved. The less abundant metabolites such as GlcCer are a limiting factor for the fibroblast sample amount, which need to be injected in order to obtain suitable spectra of all lipids of interest.

3.3. *Mass spectrometric profiling and structural characterization of authentic and fibroblasts Gb3 by means of HPLC/APCI-MS as an example for neutral sphingolipids*

In Fig. 4, a full scan spectrum of the authentic standard Gb3(d18(4E)/23:0) is shown. The used conditions lead (due to in-source fragmentation) to the formation of fragments characteristic for the three building blocks of the investigated (glyco) sphingolipid, namely the carbohydrate chain, the FA and the LCB. The m/z values of 1122.8 and 1104.8 correspond to $[M+H]^+$ and $[M+H-H_2O]^+$, respectively. The sequential neutral loss (NL) of the 3 hexoses (m/z: 162 each) leads to the formation of the molecular ions 960.8 and 942.8, 798.7 and 780.7 as well as 636.6 and 618.6. This pattern is here characteristic for Gb3. The fragment with the m/z value 636.6 represents the Cer molecular ion (ceramide backbone), from which a sequential NL of $2H_2O$ molecules results in the formation of the ions at m/z 618.6 and 600.6. The NL of either formaldehyde (m/z: 30) or methanol (m/z: 32) out of the molecular ion 618.6 leads to the formation of the fragments 588.6 and 586.6, respectively. Most notably, the Cer part dissociates in fragments characteristic for the FA as well as for the LCB moiety. Accordingly, the well-known fragment at m/z 264.3 originates from a C18 LCB. The most abundant fragments belonging to the FA moiety are those at m/z 354.4 and 378.4. These typical fragments have been previously referred to as "short" and "long fatty acid" fragments [20]. It has to be mentioned here, that increasing the cone voltage leads to an increase in the intensity of the LCB and the FA fragments (data not shown). Fig. 5 shows the suggested structures of the most relevant fragments from Gb3(d18(E)/23:0), which are related to the Cer part of the molecule. Hsu et al. have published detailed works about the fragmentation mechanisms of neutral SLs using LiCl [34,35]. SLs

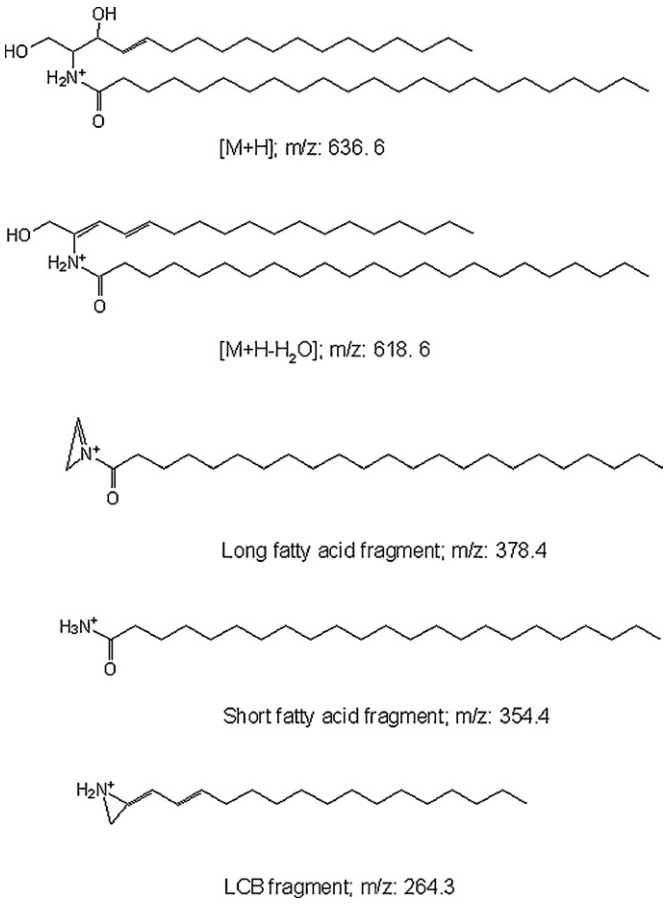

Fig. 5. Proposed structures of the FA and the LCB fragments of the Cer(d18:1(4E)/23:0) molecular species.

are likely to show different fragment patterns in the presence of Li^+-ions. Hence, our suggested fragments are similar to those suggested by Byrdwell et al. who analyzed sphingomyelin by HPLC/APCI-MS [21].

In Fig. 6 a full scan mass spectrum of Gb3 derived from fibroblasts (corresponding to the Gb3 peak in Fig. 3B) is depicted. The fragmentation pattern here (due to in-source fragmentation) is similar to that found by the authentic Gb3 standard. In view of that, the abundance of the LCB molecular ion at m/z of 264.3 in combination with the FA fragment pairs (long and short FA fragment) 392.4 and 368.4, 390.4 and 366.4, 364.4 and 340.4 and 280.3 and 256.3 indicates the abundance of the Cer backbones Cer(d18:1/24:0), Cer(d18:1/24:1), Cer(d18:1/22:0), and Cer(d18:1/16:0), respectively. Besides the retention time, the sequential NL of 3 hexoses beginning with the parent ions $[M+H]^+$ and $[M+H-H_2O]^+$ with m/z values at 1136.8, 1134.8, 1108.8, 1024.7, and at 1118.8, 1116.8, 1090.8, 1006.7 confirms the identity of the sphingolipid species to be globotriaosylceramides. The mentioned sequential NL (NL of m/z: 162) leads to the occurrence of 3 fragment series corresponding to the molecular ions $[M+H-Hexose]^+$ and $[M+H-H_2O-Hexose]^+$ (974.8, 972.8, 946.7, 862.6 and 956.7, 954.7, 928.7, 844.6), $[M+H-2Hexose]^+$ and $[M+H-H_2O-2Hexose]^+$ (812.7, 810.7, 784.7, 700.6 and 794.7, 792.7, 766.7, 682.6) as well as $[M+H-3Hexose]^+$ and $[M+H-H_2O-3Hexose]^+$ (650.6, 648.6, 622.6, 538.5 and 632.6, 630.6, 604.6, 520.5). Accordingly, the detected species correspond to the molecular ions of Gb3(d18:1(4E)/24:0), Gb3(d18:1(4E)/24:1), Gb3(d18:1(4E)/22:0), and Gb3(d18:1(4E)/16:0).

The mass spectra of Gb3 represent examples of the fragmentation pattern and the mass spectrometric profiling of the other separated SLs (data not shown). The NL of the head group compo-

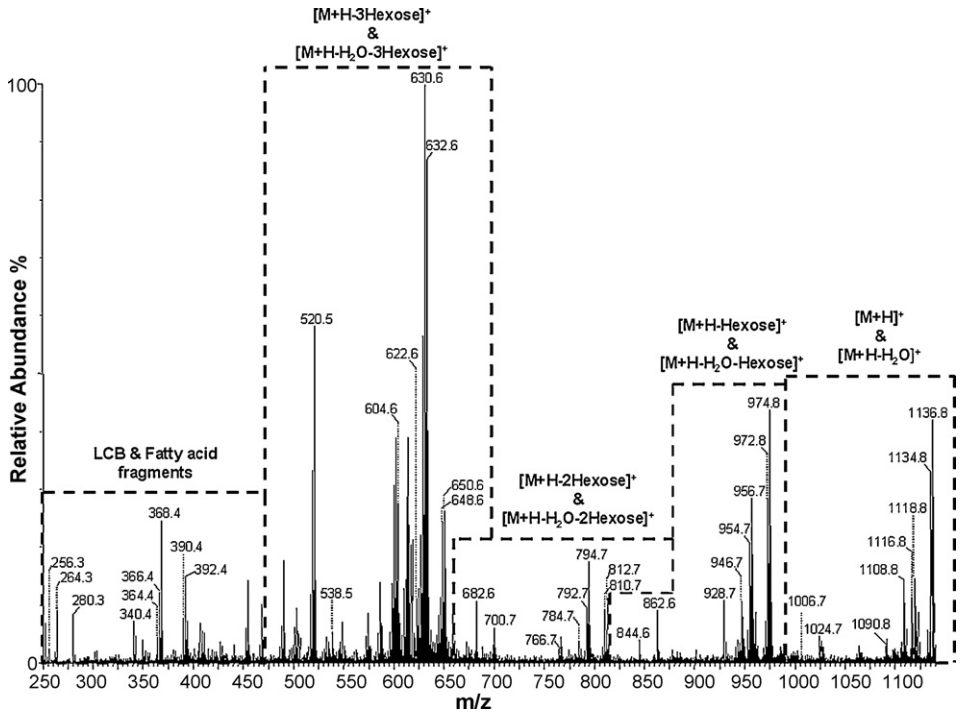

Fig. 6. Mass spectrum in full scan mode (m/z 250–1500) of Gb3 species in wild type fibroblasts, corresponding to Gb3 peak in Fig. 3B. Sample cone and APCI pin voltage were set to 45 V and 10 V, respectively. The fragmentation was an in-source one.

nents yields fragments related to the Cer building blocks. GlcCer shows a NL (m/z: 162) of one hexose whereas LacCer displays a sequential NL (m/z: 162) of two hexoses. Gb4 shows a sequential NL of GalNAc (m/z: 203) and three hexoses (NL: m/z 162). The most abundant peaks in case of SM are the molecular ions $[M+H-183]^+$. Under the used conditions, the molecular ions $[M+H]^+$ of SM exhibit a low intensity.

4. Discussion

Currently, two main strategies for MS analysis of (sphingo) lipids can be distinguished. The first one consists of analyzing lipids out of crude extracts, mainly by means of ESI/MS and ESI/MS/MS usually without previous chromatographic separation [22,36–40]. This approach, known as shotgun lipidomics, is characterized by its simplicity and high throughput potential. However, lipid extracts comprise a complex mixture of different compounds, which display different ionization behaviors. Herein, signal suppression effects may occur and some species can be hardly analyzed. For example, alkaline methanolysis has been reported to be advantageous for the shotgun analysis of low-abundance SLs [36].

The second advance is based on chromatographic separation of lipids prior to mass spectrometry, either in an on-line, or in an offline system. In this regard, many successful methods based on HPLC/ESI-MS, HPLC/ESI-MS/MS, HPLC/APPI-MS, and TLC/MALDI-MS have been reported [5,6,19–21,24–30,41–48]. However, limitations in the protocols include considering only some of the neutral GSLs, no combination with Chol, long retention times, or laborious sample preparation and complex instrumentation. For example, the analysis of Cer, GlcCer, LacCer and SM using HPLC/ESI-MS/MS in the multiple reaction monitoring (MRM) mode has been reported [5,6,28,44]. However, neither Gb3 nor Gb4 was ever considered there. In addition, the MRM mode enabled identification and quantification of the separated SLs based on the structural characteristics of the LCBs. Accordingly, only those SLs, which possess certain LCBs would be detected. Others may be overseen. Very recently, nano-HPLC/ESI-MS methods allowing separation, detection, and structural elucidation of GlcCer, LacCer, Gb3 and Gb4 have been published [19]. This approach showed a distinguished sensitivity. However, long retention times were a considerable limitation. In addition, only the analysis of neutral GSLs but not that of Cer or SM was the objective of that study.

APPI-MS coupled to graphitic carbon HPLC has been applied to the Fabry disease [26]. Here, in-source fragmentation has been utilized to gain structural information about accumulated Gb3.

Methods based on hyphenation of TLC and MALDI-MS are offline approaches [25,45]. Moreover, they are laborious and time consuming.

Due to a series of advantages, APCI-MS is frequently used for the analysis of many lipid classes [20,21,29,41,42,44–46]. For example, APCI-MS does not depend largely on the nature of the solvent used and has a lesser tendency than ESI-MS of building adducts. In this work, we took advantage of these features, and developed a new HPLC/APCI-MS method enabling separation, detection, and mass spectrometric profiling of multiple neutral SLs in combination with Chol without the need for any derivatisation steps for Chol. The separation pattern here was designed to resemble that obtained by a HPTLC method with an optimized solvent system. This may be advantageous since HPTLC represents a widely distributed means for the analysis of lipids. The working range, which is in the same range as that of previous HPLC/ESI-MS methods [28], as well as the relatively short time of analysis are notable characteristics for the applicability of the method. In-source fragmentation is usually considered as a disadvantage of APCI-MS. However, we could use this effect to obtain an overview on the structures of the neutral SL species without the need of further fragmentation steps. As the developed HPLC/APCI-MS method was designed to enable qualitative and semi quantitative comparisons between the mass spectrometric profiles of different biological samples, no absolute quantifications were conducted.

Although a comprehensive determination of each single species within each separated lipid class was beyond the scope of this work, we think that a software, which enables an automatic identification of the investigated species based on the nature of the formed

fragments, their intensities, and the retention times would be very helpful.

5. Outlook

The presented HPLC/APCI-MS method is supposed to open the door for simultaneous analysis of SLs, Chol, and other non-polar lipids, which is the goal of many lipidomics investigations. In addition, we think that this method can be useful for comparative studies between cells, tissues, and other biological samples. Computational algorithms that are based on statistical comparison of the relative intensities of the detected species can provide a powerful tool for a semi-quantitative evaluation of the mass spectra. Such approaches have been recently published [49–54].

Acknowledgments

The authors would like to thank Christine Oeste, Andrea Raths, and Martina Domgörgen for technical assistance. The financial support by the Deutsche Forschungsgemeinschaft (SFB 645) and the European Community (7th framework program "LipidomicNet", proposal No. 202272) is gratefully acknowledged.

References

[1] N. Bartke, Y.A. Hannun, J. Lipid Res. 50 (Suppl.) (2009) S91.
[2] R.K. Yu, Y. Nakatani, M. Yanagisawa, J. Lipid Res. 50 (Suppl.) (2009) S440.
[3] A. Regina Todeschini, S.I. Hakomori, Biochim. Biophys. Acta 1780 (2008) 421.
[4] S.I. Hakomori, Biochim. Biophys. Acta 1780 (2008) 325.
[5] M.C. Sullards, A.H. Merrill Jr., Sci. STKE 2001 (2001) PL1.
[6] A.H. Merrill Jr., M.C. Sullards, J.C. Allegood, S. Kelly, E. Wang, Methods 36 (2005) 207.
[7] T. Kolter, K. Sandhoff, Annu. Rev. Cell Dev. Biol. 21 (2005) 81.
[8] T. Kolter, K. Sandhoff, Biochim. Biophys. Acta 1758 (2006) 2057.
[9] S. Koybasi, C.E. Senkal, K. Sundararaj, S. Spassieva, J. Bielawski, W. Osta, T.A. Day, J.C. Jiang, S.M. Jazwinski, Y.A. Hannun, L.M. Obeid, B. Ogretmen, J. Biol. Chem. 279 (2004) 44311.
[10] W.L. Holland, S.A. Summers, Endocr. Rev. 29 (2008) 381.
[11] T.S. Worgall, Curr. Opin. Clin. Nutr. Metab. Care 12 (2009) 105.
[12] N. Marks, M.J. Berg, M. Saito, Brain Res. 1191 (2008) 136.
[13] M.F. De Rosa, D. Sillence, C. Ackerley, C. Lingwood, J. Biol. Chem. 279 (2004) 7867.
[14] J. Matsuda, A. Yoneshige, K. Suzuki, J. Neurochem. 103 (Suppl. 1) (2007) 32.
[15] L.G. Rodrigues, M.J. Ferraz, D. Rodrigues, M. Pais-Vieira, D. Lima, R.O. Brady, M.M. Sousa, M.C. Sa-Miranda, Neurobiol. Dis. 33 (2009) 48.
[16] G.B. Sinclair, G. Jevon, K.E. Colobong, D.R. Randall, F.Y. Choy, L.A. Clarke, Mol. Genet. Metab. 90 (2007) 148.
[17] B. Garner, D.A. Priestman, R. Stocker, D.J. Harvey, T.D. Butters, F.M. Platt, J. Lipid Res. 43 (2002) 205.
[18] T.S. Park, R.L. Panek, S.B. Mueller, J.C. Hanselman, W.S. Rosebury, A.W. Robertson, E.K. Kindt, R. Homan, S.K. Karathanasis, M.D. Rekhter, Circulation 110 (2004) 3465.
[19] S. Kirsch, M. Zarei, M. Cindric, J. Muthing, L. Bindila, J. Peter-Katalinic, Anal. Chem. 80 (2008) 4711.
[20] W.C. Byrdwell, R.H. Perry, J. Chromatogr. A 1133 (2006) 149.
[21] W.C. Byrdwell, R.H. Perry, J. Chromatogr. A 1146 (2007) 164.
[22] X. Han, H. Cheng, J. Lipid Res. 46 (2005) 163.
[23] X. Han, R.W. Gross, J. Lipid Res. 44 (2003) 1071.
[24] U. Sommer, H. Herscovitz, F.K. Welty, C.E. Costello, J. Lipid Res. 47 (2006) 804.
[25] D. Touboul, S. Roy, D.P. Germain, A. Baillet, F. Brion, P. Prognon, P. Chaminade, O. Laprevote, Anal. Bioanal. Chem. 382 (2005) 1209.
[26] A. Delobel, S. Roy, D. Touboul, K. Gaudin, D.P. Germain, A. Baillet, F. Brion, P. Prognon, P. Chaminade, O. Laprevote, J. Mass Spectrom. 41 (2006) 50.
[27] S. Roy, A. Delobel, K. Gaudin, D. Touboul, D.P. Germain, A. Baillet, P. Prognon, O. Laprevote, P. Chaminade, J. Chromatogr. A 1117 (2006) 154.
[28] R.L. Shaner, J.C. Allegood, H. Park, E. Wang, S. Kelly, C.A. Haynes, M.C. Sullards, A.H. Merrill Jr., J. Lipid Res. 50 (2009) 1692.
[29] B.J. Pettus, M. Baes, M. Busman, Y.A. Hannun, P.P. Van Veldhoven, Rapid Commun. Mass Spectrom. 18 (2004) 1569.
[30] J. Bielawski, Z.M. Szulc, Y.A. Hannun, A. Bielawska, Methods 39 (2006) 82.
[31] E. Fahy, S. Subramaniam, R.C. Murphy, M. Nishijima, C.R. Raetz, T. Shimizu, F. Spener, G. van Meer, M.J. Wakelam, E.A. Dennis, J. Lipid Res. 50 (Suppl.) (2009) S9.
[32] H. Farwanah, R. Neubert, S. Zellmer, K. Raith, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 780 (2002) 443.
[33] Z. Vukelic, S. Kalanj-Bognar, Glycoconj. J. 18 (2001) 429.
[34] F.F. Hsu, J. Turk, M.E. Stewart, D.T. Downing, J. Am. Soc. Mass Spectrom. 13 (2002) 680.
[35] F.F. Hsu, J. Turk, J. Am. Soc. Mass Spectrom. 12 (2001) 61.
[36] X. Jiang, H. Cheng, K. Yang, R.W. Gross, X. Han, Anal. Biochem. 371 (2007) 135.
[37] X. Han, R.W. Gross, Expert Rev. Proteomics 2 (2005) 253.
[38] X. Han, R.W. Gross, Mass Spectrom. Rev. 24 (2005) 367.
[39] C.S. Ejsing, J.L. Sampaio, V. Surendranath, E. Duchoslav, K. Ekroos, R.W. Klemm, K. Simons, A. Shevchenko, Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 2136.
[40] D. Schwudke, G. Liebisch, R. Herzog, G. Schmitz, A. Shevchenko, Methods Enzymol. 433 (2007) 175.
[41] B.J. Pettus, B.J. Kroesen, Z.M. Szulc, A. Bielawska, J. Bielawski, Y.A. Hannun, M. Busman, Rapid Commun. Mass Spectrom. 18 (2004) 577.
[42] B.J. Pettus, A. Bielawska, B.J. Kroesen, P.D. Moeller, Z.M. Szulc, Y.A. Hannun, M. Busman, Rapid Commun. Mass Spectrom. 17 (2003) 1203.
[43] M. Valsecchi, L. Mauri, R. Casellato, S. Prioni, N. Loberto, A. Prinetti, V. Chigorno, S. Sonnino, J. Lipid Res. 48 (2007) 417.
[44] W.C. Byrdwell, Rapid Commun. Mass Spectrom 12 (1998) 256.
[45] W.C. Byrdwell, Front. Biosci 13 (2008) 100.
[46] M. Lisa, M. Holcapek, J. Chromatogr. A 1198–1199 (2008) 115.
[47] M.C. Sullards, J.C. Allegood, S. Kelly, E. Wang, C.A. Haynes, H. Park, Y. Chen, A.H. Merrill Jr., Methods Enzymol. 432 (2007) 83.
[48] K. Nakamura, Y. Suzuki, N. Goto-Inoue, C. Yoshida-Noro, A. Suzuki, Anal. Chem. 78 (2006) 5736.
[49] S. Milne, P. Ivanova, J. Forrester, H. Alex Brown, Methods 39 (2006) 92.
[50] J.S. Forrester, S.B. Milne, P.T. Ivanova, H.A. Brown, Mol. Pharmacol. 65 (2004) 813.
[51] P.T. Ivanova, S.B. Milne, J.S. Forrester, H.A. Brown, Mol. Interv. 4 (2004) 86.
[52] M. Sysi-Aho, M. Katajamaa, L. Yetukuri, M. Oresic, BMC Bioinform. 8 (2007) 93.
[53] K.H. Pietilainen, M. Sysi-Aho, A. Rissanen, T. Seppanen-Laakso, H. Yki-Jarvinen, J. Kaprio, M. Oresic, PLoS ONE 2 (2007) e218.
[54] M. Katajamaa, M. Oresic, BMC Bioinform. 6 (2005) 179.