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Nano-LC–MS/MS for the quantitation of ceramides in mice cerebrospinal fluid using minimal sample volume

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ABSTRACT

A new nano-liquid chromatography–ESI–MS/MS method has been developed for the sensitive quantitation of C8:0, C16:0, C18:0, C18:1, C20:0, C24:1 and C24:0 ceramide in cerebrospinal fluid of mice using minimal sample volume. Volumes of 2 μL CSF were undertaken a simple, fast extraction procedure involving protein precipitation with methanol and dilution. Ceramides were separated by nano-liquid chromatography with a reversed phase C8 column and detected with a triple quadrupole mass spectrometer. C17:0 ceramide was used as internal standard. The method has been validated in terms of linearity, lower limit of quantitation, precision, accuracy and autosampler stability. Calibration curves covered a range of 2.25–120 pg/μL for most ceramides (7.5–120 pg/μL for C24:0 ceramide). The lower limits of quantitation determined for C8:0, C16:0, C18:1, C18:0, C20:0 and C24:1 ceramide were 0.225 pg on column (2.25 pg/μL) and that for C24:0 ceramide 0.750 pg on column (7.5 pg/μL). Intra- and interday precision and accuracy values, expressed as relative standard deviation and relative error, respectively, were lower than 15% in all cases. Autosampler stability for calibration standards and CSF samples was proven for at least 24 h for all analytes. The suitability of the method has been demonstrated by quantifying the analytes, except the non-endogenous C8:0 ceramide, in cerebrospinal fluid samples of 12 mice. Calculated concentrations ranged from 3 to 120 pg/μL in cerebrospinal fluid for all analytes, except for C24:0 ceramide, which could not be detected in any of the analyzed samples.

1. Introduction

Ceramides are a class of sphingolipids involved in many biological processes, such as signal transduction, stress response and apoptosis [1]. Moreover, they are involved in membrane structure formation [2,3]. Chemically, ceramides are composed of the amino alcohol sphingosine and a fatty acid. Fig. 1 shows the general structure of these lipid molecules. It is well known that physiological levels of ceramides can be altered in various diseases, including metabolic disorders such as diabetes [4,5] and arteriosclerosis [6]. Haus et al. reported increased levels of C18:0, C20:0, C24:1 and total ceramides in plasma of type 2 diabetes patients and proposed that they are mediators of insulin resistance [4]. Increased levels of ceramides were also found by Satoi et al. in the cerebrospinal fluid (CSF) of Alzheimer's disease patients [7]. An increased level of C18:0 and C24:0 ceramide was also found by Cutler et al. in membranes of brain tissue samples of Alzheimer's disease patients. The extent of the increase was positively correlated with disease severity, but it is not entirely known what role ceramides play in the pathogenesis of the disease [8,9]. Moreover, ceramides play a role in cancer [10], skin diseases, like psoriasis or atopic dermatitis [2] and inflammatory processes, like multiple sclerosis and cystic fibrosis [11–13]. Schiffmann et al. recently found increased concentrations of C16:0 ceramide in the cerebrospinal fluid of multiple sclerosis patients [13]. In cystic fibrosis, different types of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene cause reduced mucociliary clearance, chronic bacterial airway infection and inflammation [11]. Teichgräber et al. described ceramide accumulation in CFTR-knockout mice [12]. Furthermore, increased levels of long-chain ceramides (C16:0–C20:0) in lower airway epithelium of patients with cystic fibrosis are associated with infection susceptibility and apoptotic cell death [11]. Short chain, cell-permeable ceramides, like C8:0 ceramide, do not occur physiologically but have been administered in studies to mimic the effects of endogenous ceramides [14]. For this reason, C8:0 ceramide was also included in the validation of the nano-LC–MS/MS method.

In order to better understand the pathomechanisms of diseases in which ceramides are involved, exact and reliable methods for ceramide quantitation are absolutely essential. Nowadays, mass spectrometry is the method of choice for quantitating ceramides and other sphingolipids in biological samples as it enables the determination of large numbers of analytes with the highest sensivity [15,16]. Consequently, many mass spectrometric

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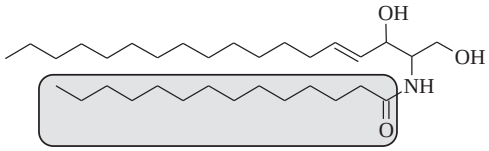

Fig. 1. General structure of ceramide using the example of C16:0 ceramide. The amino alcohol sphingosine is bound to the gray highlighted palmitic acid. Variation in the length of the fatty acid carbon chain leads to different ceramides.

methods have already been described for various biological matrices [2,17–26]. Bui et al. analyzed more than 39 sphingolipids extracted from human plasma in a single run with lower limits of quantitation (LLOQ) for several ceramides in the range of 0.06–0.26 ng/mL [26]. Kasumov et al. developed and validated a method for quantifying 7 ceramide species from human plasma and tissue samples with LLOQ in the range of 0.01–0.50 ng/mL [20]. These two exemplary methods already provided a very good sensivity but the required sample amount ranged between 25 μL plasma [26] and 50 μL plasma (or 7–15 mg tissue) [20], respectively. Injection volumes for both methods were 25 μL. In our case, the challenge was to quantify ceramides in the cerebrospinal fluid (CSF) of mice. As only 2 μL of cerebrospinal fluid (CSF) could be obtained from the mice and be used for quantitation, we were not able to quantify any ceramide species with the routine method we employ in our lab to quantify ceramide species in plasma (20 μL) and tissue samples (data not shown). The aim of the present work was to develop and validate a highly sensitive analytical method for quantitation of ceramide species when very low sample volumes are available, such as for mice CSF. Due to the necessity of high sensitivity and chromatographic separation to reduce matrix effects, nano-liquid chromatography (nano-LC) coupled to tandem mass spectrometry (MS/MS) has been chosen.

Nano-LC was first applied by Karlsson and Novotny in 1988 using a capillary column with an inner diameter of 44 μm [27]. Today the nano-LC technique is combined with various detector systems like UV or MS and is mainly applied in the proteomic field, as well as in pharmaceutical, environmental and enantiomeric analysis [28–32]. So far, only a few methods using nano-LC coupled to ESI–MS/MS have been reported for quantitation of small molecules. These include arrays for histamine, chlorogenic acid, mirtazapine, betalactam antibiotics and substances of environmental interest [33–37]. Farnawah et al. used nano-ESI–MS/MS for structural characterization of skin ceramides [2]. To our knowledge, no other method has been published for quantifying ceramides or any other class of sphingolipids by means of nano-LC–ESI–MS/MS.

2. Materials and methods

2.1. Materials

The following ceramide standards were purchased from Avanti Polar Lipids (Alabaster, AL, USA): C8:0 ceramide (N-octanoyl-D-*erythro*-sphingosine), C16:0 ceramide (N-palmitoyl-D-*erythro*-sphingosine), C17:0 ceramide (N-hepta-decanoyl-D-*erythro*-sphingosine), C18:1 ceramide (N-oleoyl-D-*erythro*-sphingosine), C18:0 ceramide (N-stearoyl-D-*erythro*-sphingosine), C20:0 ceramide (N-arachidoyl-D-*erythro*-sphingosine), C24:1 ceramide (N-nervonoyl-D-*erythro*-sphingosine) and C24:0 ceramide (N-lignoceroyl-D-*erythro*-sphingosine). Methanol and water (LC–MS grade) were obtained from Roth (Karlsruhe, Germany). Acetone (HPLC grade) was purchased from AppliChem (Darmstadt, Germany), formic acid (pro analysis) from VWR (Darmstadt, Germany) and ammonium formate (pro analysis) from Sigma-Aldrich (Steinheim, Germany). Phosphate buffered saline (PBS) was self-made by solving 400 mg NaCl, 10 mg KCl, 57.5 mg Na_2HPO_4 and 10 mg KH_2PO_4 in 50 mL water (LC–MS grade).

2.2. Instrumentation

Standard and CSF samples were analyzed by an Eksigent nano-LC 2D Ultra system (AB Sciex, Darmstadt, Germany) equipped with an AS-2 autosampler and a 10 μL sample loop. The autosampler was tempered at 4 °C. The LC-system was connected to a hybrid triple quadrupole – ion trap mass spectrometer 5500 QTRAP (AB Sciex, Darmstadt, Germany). Analyte ions were generated by nanospray ionization using a Silica Pico Tip emitter (10 μm tip; New Objective, Woburn, MA, USA).

Samples were centrifuged in an Eppendorf microcentrifuge 5424 (Eppendorf, Wessling-Berzdorf, Germany). Chromatographic mobile phases were degassed by sonication (USR 90H ultrasonic bath, VWR, Darmstadt, Germany).

2.3. Standard preparation

Standard solutions were prepared on ice. A standard mixture of 50 ng/mL was prepared by mixing 50 μL of every ceramide stock solution (1 μg/mL in methanol) with 650 μL methanol. This mixture was diluted with methanol to obtain various calibration standard solutions. 50 μL of every calibration standard solution were mixed with 2 μL PBS to simulate CSF and 8 μL C17:0 ceramide (20 ng/mL in methanol) as internal standard in polypropylene tubes. The mixture was vortexed for 2 min and centrifuged at 20,238g for 3.5 min. 40 μL of the supernatant of each sample were transferred to a new tube and solvent was evaporated under nitrogen at 45 °C. Samples were reconstituted in 40 μL solvent A (methanol–water, 80:20 (v/v), containing 10 mmol/L ammonium formate and 0.1% formic acid) and centrifuged again at 20,238g for 30 s.

2.4. Sample extraction

In all experiments, ethics guidelines for investigations in conscious animals were obeyed and the experiments were approved by the local Ethics Committee for Animal Research. Eight- to ten-week-old female C57BL/6 mice, weighing 18–20 g, were used, obtained from Harlan Laboratories (Horst, Netherlands). The area under the occipital bone from euthanized mice was surgically exposed. The cisterna magna was punctured with a 30G needle which was attached to a 1 mL syringe over a 0.012-inch siliconized tubing and 2–5 μL of CSF were extracted by slow retraction of the syringe.

Sample extraction was done on ice. CSF samples were prepared by mixing 2 μL of CSF with 50 μL methanol and 8 μL of the internal standard C17:0 ceramide (20 ng/mL in methanol). The extraction procedure was the same as that mentioned above. Every sample was injected twice.

2.5. Chromatographic conditions

Chromatography was performed under gradient conditions using a reversed-phase C8 column (75 μm inner diameter, length 15 cm, particle size 5 μm) packed in-house. 3 μL of every sample were injected and directly loaded via a 10 μL sample loop into the analytical column. The analytes were eluted at 350 nL/min with solvent A (methanol–water, 80:20 (v/v), containing 10 mmol/L ammonium formate and 0.1% formic acid) and solvent B (methanol–acetone, 80:20 (v/v), containing 10 mmol/L ammonium formate and 0.1% formic acid). The gradient started with 90% B for 1 min, was linearly increased for 2 min to 100% B and was maintained there for 9 min. Then a fast change to 90% B was

made within 0.5 min and the column was reconditioned with the initial conditions for 3.5 min. The overall runtime was 16 min. All solvent mixtures were sonicated for degassing before use.

The mass spectrometer was operated in positive multiple reaction monitoring mode (MRM) with all quadrupoles running at unit resolution. Operating conditions were set as follows: curtain gas: 20 psi; collision gas: 6 psi; nanospray voltage: 2500 V; interface heater temperature: 200 °C; ion source gas 1:20 psi; entrance potential: 10 V; declustering potential: 150 V.

Collision energy (CE) and collision cell exit potential (CXP) were optimized manually for every precursor-to-product ion transition by infusing standard solutions of 10 ng/mL in acetonitrile–methanol, 80:20 (v/v) into the mass spectrometer (flow rate: 0.5 μL/min). Fragmentation of ceramides has already been described [20,38]. All ceramides showed an intensive fragment at 264.4 resulting from the backbone sphingosine after loss of water. These fragments were used for ceramide quantitation with CE and CXP set as follows: 426.4→264.4 (CE 35 V, CXP 20 V) for C8:0 ceramide, 538.5→264.4 (39 V, 10 V) for C16:0 ceramide, 564.5→264.4 (36 V, 25 V) for C18:1 ceramide, 566.4→264.4 (36 V, 20 V) for C18:0 ceramide, 594.5→264.4 (42 V, 15 V) for C20:0 ceramide, 648.5→264.4 (44 V, 18 V) for C24:1 ceramide, 650.4→264.4 (44 V, 18 V) for C24:0 ceramide and 552.4→264.4 (35 V, 20 V) for the internal standard C17:0 ceramide. Qualifier transitions were 426.4→408.4 for C8:0 ceramide, 538.5→520.4 for C16:0 ceramide, 564.5→546.4 for C18:1 ceramide, 566.4→548.2 for C18:0 ceramide, 594.5→576.2 for C20:0 ceramide, 648.5→630.4 for C24:1 ceramide, 650.4→632.3 for C24:0 ceramide and 552.4→534.4 for the internal standard C17:0 ceramide. For all transitions, a dwell time of 50 ms was set.

Data were processed using Analyst Software version 1.5 (AB Sciex, Darmstadt, Germany).

2.6. Quantitation

Ceramide species were quantified using calibration curves and C17:0 as internal standard. Every integrated analyte peak area was divided by the integrated internal standard peak area of the same run in order to correct errors like sample loss during the extraction procedure, injection errors, matrix effects etc. Calibration curves were constructed by plotting the corrected analyte areas versus the corresponding nominal analyte concentrations and performing a linear regression with $1/x$ weighting.

2.7. Method validation

To demonstrate suitability of the method, validation according to the FDA guidelines [39], in terms of linearity, autosampler stability, precision, accuracy and LLOQ was performed.

For determining linearity, 6 calibration curves of every analyte were generated as described above. The curves contained a blank sample (PBS+methanol), a zero sample (PBS+internal standard +methanol) and 9 different concentrated non-zero (PBS+methanol +corresponding standard in methanol) samples (7 non-zero samples for C24:0 ceramide) in the range of 2.25 pg/μL (7.5 pg/μL for C24:0 ceramide)–120 pg/μL. Concentrations of the non-zero samples were 2.25, 3, 7.5, 15, 22.5, 30, 45, 60 and 120 pg/μL. The weighting factor was $1/x$.

The standard concentration with a signal to noise ratio of 10 or higher was set as LLOQ. This value was validated in terms of precision and accuracy.

Precision and accuracy were ascertained with three different concentration levels covering the range of the calibration curve. Concentrations were the LLOQ (2.25 pg/μL for all analytes and 7.5 pg/μL for C24:0 ceramide), 24 pg/μL and 96 pg/μL (80% of the highest standard of the calibration curve). Each concentration was prepared three times. The measurement was repeated on two other days with freshly prepared samples. The intraday precision was evaluated by calculating the relative standard deviation (RSD) of the three samples. Interday precision was expressed as total imprecision s_T according to [40]. For better comparability to the RSD, s_T-values were divided by the mean value (MV) of all 9 measurements for every concentration. Accuracy was measured by the relative error term (RE). RE is described by the formula [(calculated concentration – nominal concentration)/nominal concentration]100%.

Stability of samples in the autosampler at 4 °C was tested by measuring ceramide levels repeatedly at the start and after 24, 48 and 72 h for which the same three standard concentrations as for precision and accuracy were used. For every time point the corrected analyte peak areas were divided by the corrected areas determined at the start time. Analytes were considered stable when this ratio was in between 85 and 115%. To test the suitability of the internal standard and the influence of CSF matrix on it, the peak areas of the analytes without internal standard correction were evaluated. Additionally, effects of matrix on the autosampler stability were investigated. Two CSF samples were prepared and stored for 72 h in the autosampler at 4 °C. Ceramide concentrations were measured at the start time and every 12 h.

2.8. Matrix effects and standard addition approach

CSF is a protein-poor fluid and little matrix effects are expected. However, to evaluate the influence of CSF in the determination of the analytes and the suitability of PBS as alternative matrix for calibration purpose, the standard addition procedure was carried out.

CSF samples from 15 different mice were pooled (only small volumes of 3–5 μL) can be obtained from one mouse. For every ceramide, a calibration curve containing one zero sample and 6 non-zero samples was constructed. The non-zero samples were generated by spiking 6 samples of 2 μL CSF each with 50 μL of 6 differently concentrated calibration standard solutions. A zero sample was made of 2 μL CSF and 50 μL methanol. 8 μL of the internal standard were added to all samples and extraction was done. Linear calibration curves were constructed for all analytes as described above. The nominal concentrations of the zero sample were set at zero. The calibration curves were extrapolated to the negative intercept on the x-axis ($y=0$), corresponding to the amount of the analyte in the zero sample. This value was calculated as the ratio of the intercept on the y-axis and the slope. Besides, the concentrations of ceramides in the zero sample were determined with a calibration curve extracted in PBS and the calculated concentrations using internal standard and standard addition approaches were compared.

In order to investigate recovery in CSF, the ceramide concentrations in blank and spiked CSF samples were determined. 2 μL of CSF samples were spiked with 50 μl of a calibration standard solution, resulting in an increase of the concentration of 15 pg/μL all analytes. The concentrations determined in the blank sample were subtracted from the total concentrations found in the spiked sample and the results compared to the theoretical results of 15 pg/μL for all analytes.

Furthermore, suitability of C17:0 ceramide as internal standard in CSF was assayed by comparing the chromatographic peak areas of the IS in 10 calibration standard samples (PBS) and the peak areas in 10 extracted samples (CSF).

3. Results

3.1. Linearity and calibration range

For every ceramide species, 6 linear calibration curves were constructed with $1/x$ as a weighting factor. Table 1 shows means

Table 1
Mean values and standard deviations (SD) of slopes, intercepts and regression coefficients (r) of 6 different calibration curves obtained from spiked PBS samples.

Ceramide	Slope ± SD	Intercept ± SD	r ± SD
C8:0	0.246 ± 0.0305	1.87E-03 ± 7.21E-03	0.9977 ± 0.0011
C16:0	0.281 ± 0.0392	1.14E-02 ± 6.24E-03	0.9989 ± 0.0004
C18:1	0.302 ± 0.0525	4.33E-03 ± 2.98E-03	0.9987 ± 0.0007
C18:0	0.256 ± 0.0397	3.77E-03 ± 1.63E-03	0.9989 ± 0.0004
C20:0	0.149 ± 0.0167	2.71E-03 ± 2.59E-03	0.9989 ± 0.0004
C24:1	0.127 ± 0.0054	1.65E-03 ± 3.40E-03	0.9974 ± 0.0013
C24:0	0.0318 ± 0.0062	1.01E-03 ± 2.48E-03	0.9982 ± 0.0008

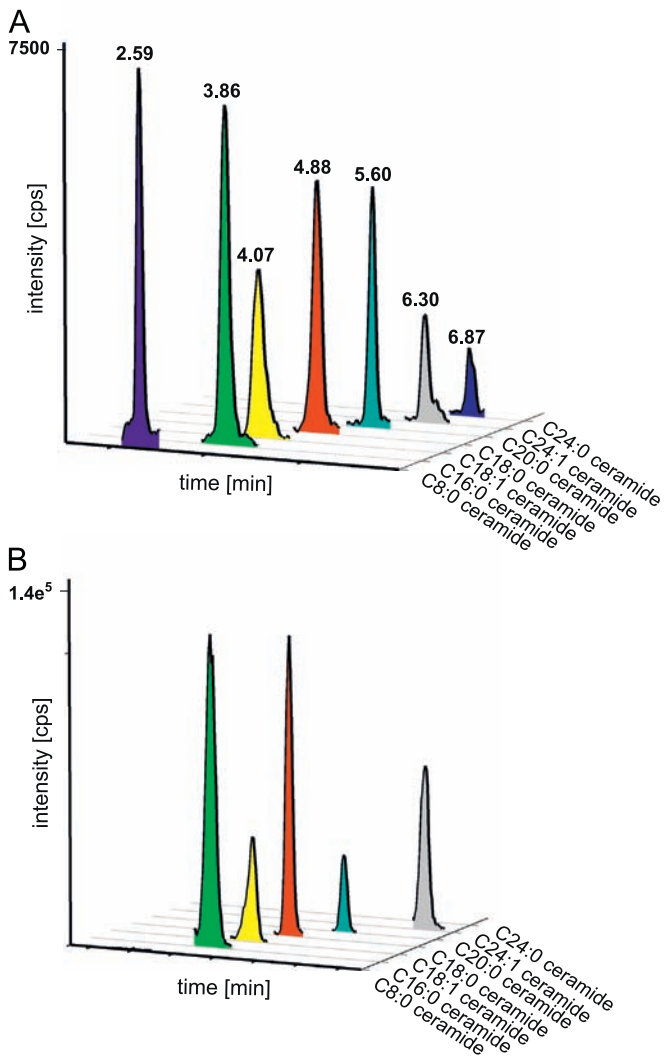

Fig.2. Chromatograms of a standard mixture of C8:0, C16:0, C18:1, C18:0, C20:0, C24:1 and C24:0 ceramide at the concentration levels of LLOQ (A) and of ceramides determined in one CSF sample (B).

and standard deviations of slopes, intercepts and regression coefficients of the calibration curves for each analyte.

3.2. Lower limit of quantitation

For C8:0, C16:0, C18:1, C18:0, C20:0 and C24:1 ceramide, the concentration of 0.225 pg on column (2.25 pg/μL cerebrospinal fluid) could be set as the LLOQ. For C24:0 ceramide, the LLOQ was 0.750 pg on column (7.5 pg/μL cerebrospinal fluid). These levels were chosen on the basis of a signal to noise ratio equal or higher than 10 and interday accuracy (RE) and interday precision (s_T/MV) less than 20%. All s_T/MV values for these concentrations were lower than 9%, the RE was always lower than 11%. Fig. 2A shows a chromatogram of ceramide standards at the LLOQ concentration (top).

3.3. Precision and accuracy

Precision and accuracy values for all analytes at the tested concentrations were lower than 15%. The RE values expressing intraday accuracy ranged from 6.58% to 10.89% (2.25 pg/μL), 4.04% to 13.38% (24 pg/μL) and 1.56% to 2.81% (96 pg/μL) for C8:0 ceramide; 2.49% to 4.53%, 3.92% to 10.75% and 1.98% to 5.52% for C16:0 ceramide; 3.38% to 4.27%, 3.04% to 6.96% and 3.75% to 5.73% for C18:1 ceramide; 2.36% to 6.53%, 3.25% to 7.33% and 1.46% to 2.08% for C18:0 ceramide; 3.69% to 6.18%, 4.38% to 5.04% and 1.56% to 3.96% for C20:0 ceramide; 1.47% to 5.47%, 2.83% to 5.46% and 4.79% to 7.29% for C24:1 ceramide and 1.47% to 7.02% (7.5 pg/μL), 2.50% to 11.83% and 5.10% to 8.54% for C24:0 ceramide. To express intraday precision, RSD was used. Values ranged from 1.39% to 2.60% (2.25 pg/μL), 0.22% to 9.43% (24 pg/μL) and 1.79% to 4.05% (96 pg/μL) for C8:0 ceramide; 0.71% to 4.09%, 2.93% to 6.12% and 2.70% to 5.50% for C16:0 ceramide; 3.35% to 5.44%, 2.32% to 4.99% and 3.28% to 5.84% for C18:1 ceramide; 2.02% to 3.36%, 3.02% to 8.16% and 1.59% to 2.32% for C18:0 ceramide; 1.10% to 6.42%, 3.67% to 5.08% and 2.15% to 3.02% for C24:1 ceramide and 1.46% to 7.14% (7.5 pg/μL), 0.90% to 11.75% and 5.12% to 8.55% for C24:0 ceramide. Detailed results for interday precision and accuracy are shown in Table 2.

3.4. Autosampler stability

Table 3 gives an overview of the collected stability data. For the standard samples, the results demonstrate that all measured concentrations of C16:0, C18:0 and C18:1 ceramide were stable at 4 °C in the autosampler for 72 h. C8:0 and C24:1 ceramide could be considered stable for 48 h, while after 72 h, the highest concentration of C24:1 ceramide and the lowest concentration of

Table 2
Interday accuracy and precision values of the analytes for the three studied concentrations, measured at three different days (n=3). RE=relative error, s_T=total imprecision, MV=mean value.

Ceramide	Concentration [pg/μL]	Interday accuracy RE (mean ± SD) [%]	Interday precision		
			s_T [pg/μL]	MV [pg/μL]	s_T/MV [%]
C8:0	2.25	8.33 ± 2.27	0.21	2.16	9.81
	24	8.44 ± 4.69	2.03	25.41	7.98
	96	2.40 ± 0.72	3.14	96.7	3.24
C16:0	2.25	3.27 ± 1.10	0.11	2.26	4.87
	24	6.46 ± 3.74	1.79	25.08	7.15
	96	3.30 ± 1.94	4.1	96.57	4
C18:1	2.25	3.97 ± 0.51	0.1	2.31	4.35
	24	4.56 ± 0.39	1.56	24.17	6.45
	96	4.69 ± 0.99	5.33	94.03	4.39
C18:0	2.25	4.19 ± 2.13	0.13	2.28	5.62
	24	5.65 ± 2.14	1.51	24.7	6.13
	96	1.84 ± 0.33	2.24	96.77	2.28
C20:0	2.25	5.14 ± 1.30	0.15	2.26	6.78
	24	4.99 ± 0.59	1.55	24.46	6.34
	96	2.40 ± 1.35	3.62	95.63	2.48
C24:1	2.25	3.48 ± 2.00	0.08	2.19	3.73
	24	3.92 ± 1.37	0.51	23.06	2.15
	96	5.94 ± 1.26	5.63	91.7	6.1
C24:0	7.5	1.59 ± 1.16	0.36	7.23	4.96
	24	7.88 ± 6.17	2.56	23.3	10.99
	96	7.40 ± 1.99	8.62	94.23	9.14

Table 3
Autosampler stability at 4 °C of standard solutions extracted in PBS ($n=3$) and of two different samples. C8:0 was not present in samples and C18:0 was under LLOQ. Area t_x=analyte peak area t_x /IS peak area t_x.

Ceramide	Concentration [pg/μL]	t_1=24 h |(100 – area t_1/area t_0)| ± SD [%]	t_2=48 h |(100 – area t_2/area t_0)| ± SD [%]	t_3=72 h |(100 – area t_3/area t_0)| ± SD [%]
C8:0	2.25	6.10 ± 4.49	9.12 ± 3.25	32.6 ± 8.46
	24	6.25 ± 2.19	4.21 ± 2.97	5.60 ± 4.36
	96	5.69 ± 6.50	3.44 ± 2.37	8.41 ± 3.73
C16:0	2.25	3.95 ± 3.02	4.85 ± 3.64	4.25 ± 3.23
	24	2.47 ± 2.75	2.54 ± 1.68	8.17 ± 2.99
	96	1.80 ± 0.77	3.34 ± 0.57	7.47 ± 1.24
Sample 1	2.82	9.79	11.7	159.6
Sample 2	36.6	0.82	9.02	50.8
C18:1	2.25	4.15 ± 1.76	5.70 ± 2.59	2.79 ± 1.92
	24	3.79 ± 2.23	4.28 ± 2.98	2.96 ± 0.96
	96	0.83 ± 0.81	2.24 ± 0.84	3.96 ± 2.97
C18:0	2.25	6.45 ± 3.04	1.85 ± 1.79	6.03 ± 2.51
	24	11.4 ± 1.93	4.44 ± 1.17	4.64 ± 3.76
	96	3.77 ± 2.25	3.07 ± 0.84	11.7 ± 1.16
Sample 1	52.2	2.87	10.3	21.3
Sample 2	> HLOQ			
C20:0	2.25	5.14 ± 2.74	16.3 ± 2.03	12.3 ± 15.6
	24	9.75 ± 2.65	3.58 ± 4.44	7.82 ± 2.51
	96	1.52 ± 1.74	5.17 ± 4.03	17.6 ± 4.28
Sample 1	< LLOQ			
Sample 2	19.08	9.91	61	–
C24:1	2.25	8.81 ± 4.73	4.27 ± 3.74	11.3 ± 2.39
	24	11.0 ± 2.59	2.52 ± 1.37	4.02 ± 3.72
	96	7.81 ± 6.60	4.76 ± 3.51	12.1 ± 9.75
Sample 1	4.81	11.7	54.9	–
Sample 2	56.7	11.6	54.1	
C24:0	7.5	5.79 ± 4.42	33.2 ± 19.2	55.2 ± 16.5
	24	8.52 ± 1.04	40.6 ± 5.83	55.1 ± 6.22
	96	8.02 ± 3.27	39.4 ± 2.21	60.1 ± 8.75
Sample 1	< LLOQ			
Sample 2	51.9	11	conc. < LLOQ	–

C8:0 ceramide were out of the range. For C20:0 and C24:0 ceramide, stability could be guaranteed for 24 h for all concentrations.

For the CSF samples, stability data slightly differ from those obtained in PBS. C8:0 ceramide was not present in CSF samples and C18:1 ceramide was under LLOQ. C16:0, C18:0, C24:0 and C24:1 ceramide were stable for at least 24 h whereas C16:0 and C18:0 ceramide could be determined correctly for 48 h. This indicates that the analysis of ceramides in CSF has to be performed as close as possible to sample preparation.

If the analyte peak areas are directly compared (without IS correction), it is clearly shown that the analytes are not stable. In CSF samples, the analytes' peak areas after 12 h in the refrigerated autosampler are minor than 85% of the chromatographic peak areas at time zero. In PBS samples, the effect is weaker but still obvious. The use of C17:0 ceramide as internal standards allows compensating this effect for several hours. For example, concentrations of C16:0 ceramide close to the LLOQ value can be determined correctly for only 24 h when analyte peak areas are used instead of at least 72 h when area ratios analyte/C17:0 ceramide as internal standard are used. C17:0 ceramide undergoes a similar degradation process as the analytes and therefore is suitable to determine the studied ceramides in CSF samples. However, depending on the analyte, we advise not to quantitate samples stored in the autosampler for more than 72 h.

3.5. Matrix effects and standard addition approach

The standard addition experiment showed good conformance of the calculated concentrations using the negative x-intercepts of the standard addition curve in CSF and the calibration curve using PBS. Results are shown in Table 4. We can conclude that PBS is an adequate alternative matrix for CSF.

Table 4
Ceramide concentrations calculated in one CSF sample using the standard addition approach (A) and the internal standard calibration in PBS (B). The accuracy was calculated as A/B*100%.

Ceramide	A. Concentration in CSF [pg/μL]	B. Concentration in CSF [pg/μL]	Accuracy [%]
C16:0	3.72	3.30	88.61
C18:1	0.72	< LLOQ	–
C18:0	48.1	46.5	96.67
C20:0	3.25	3.03	93.13
C24:1	6.71	6.06	90.38
C24:0	13.0	11.9	91.47

The peak areas for C17:0 ceramide are about the same in extracted PBS samples and in extracted CSF samples. The mean value of the area counts in PBS is 4.61E+05 ± 1.87E+04 compared to 4.22E+05 ± 1.90E+04 in CSF. As a result, there are no noticeable matrix effects on the internal standard which can be attributed to CSF matrix, confirming the suitability of the internal standard.

The influence of the matrix on autosampler stability has been discussed above.

3.6. Application of the method

The method has been applied to the quantitation of ceramides, except for the non-endogenous C8:0 ceramide, in 12 CSF samples of differently treated mice. Every sample was extracted as described in Section 2.4 and injected twice. RSD values for both runs of every sample were always below 3%, showing good precision for the quantitation of ceramides in 2 μL CSF. C24:0 ceramide could not be detected in any of the samples. In Fig. 2B, a chromatogram corresponding to one CSF sample is shown (bottom). The calculated concentration ranges for the analytes were: C16:0 ceramide: 8–120 pg/μL, C18:0 ceramide: 6–120 pg/μL, C18:1 ceramide: 5–35 pg/μL, C20:0 ceramide: 3–40 pg/μL and C24:1 ceramide: 3–50 pg/μL. Fig. 3 shows the distribution of the ceramide concentrations in the CSF samples obtained from differently treated mice using a box plot. Regrettably, there is no information in the literature about the ceramide concentrations in mice CSF and therefore the here presented results could not be compared. However, the validation of the method and the successful quantitation of the studied ceramides in CSF samples indicate the suitability of the developed analytical procedure.

4. Discussion

The new nano-LC–ESI–MS/MS method described here is an innovative application combining high sensivity and low volume sample preparation. It allows the quantitation of ceramides from a sample volume of 2 μL and provides a very low LLOQ. The LLOQ values ranged from 0.225 pg on column for C8:0, C16:0, C18:1, C18:0, C20:0 and C24:1 ceramide to 0.750 pg on column for C24:0 ceramide. To our knowledge, the sensivity of our method is the highest yet described in the literature at the moment. The suitability of the new method was demonstrated by the validation procedure and by applying it to the determination of ceramides in CSF samples from mice.

Cerebrospinal fluid is a useful matrix to study the role of ceramides and other sphingolipids in neurological and inflammatory diseases like Alzheimer's disease or multiple sclerosis [7,13]. Understanding the role of these lipid biomarkers in inflammatory processes could help to better understanding the causes and progress of several

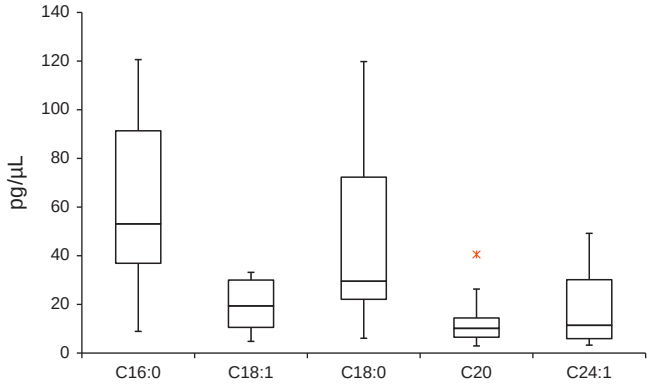

Fig. 3. Box plot of ceramide concentrations [pg/µL] determined in 12 CSF samples from differently treated mice. The top and the bottom of the box represent the first and the third quartile while the median is shown as the band inside the box. The ends of the whiskers are set at 1.5* interquartile range (IQR) above the third quartile and 1.5* IQR below the first quartile maximum but end at the last data point included in this range. Minimum or maximum values outside this range are shown as outliers.

diseases and to improve the medical treatment and also reduce the progression of the illness. Nano-LC has been the method of choice because of the low sample volumes available. Other methodologies described in the bibliography [20,22,26] require sample amounts from 25 to 100 µL. By using sample volumes of 2 µL, a reduction in sample amount of 92–98% is achieved. On the one hand, this reduction allows the application of the method when only low sample volumes are available, as in case of CSF. On the other hand, being sparing with the sample is very useful because the remaining sample can be used for other analytical procedures. As a consequence, the number of analytes that can be determined in one sample is enhanced, leading to a gain in knowledge.

In our method, we used nanospray ionization in the positive ionization mode. In that case, analyte ions are generated by protonation. The modifier formic acid works as a proton donor and thus, improves ionization efficiency. It was used it in combination with ammonium formate which was essential for a stable nanospray and improved chromatographic separation. During the method development, we got the best results with the described concentrations of modifiers. The sample preparation is very fast, cheap and easy. After dilution with methanol, the samples are centrifuged and 40 µL of the supernatant are transferred to a new tube. This step allows removing particles which could plug the nano-LC system. The reconstitution volume of 40 µL allows repeated measurements several times, as the injection volume is only 3 µL. Ceramides are endogenous lipids also present in CSF. Therefore, alternative matrices with similar characteristics to the real matrix but free from the analytes must be used. Because of this, due to the low contain of protein in CSF and the electrolyte concentrations similar to blood plasma [41], PBS was used as alternative to construct the calibration curves for the analytical methodology described here. The suitability of PBS as alternative matrix to CSF has been demonstrated by comparing the calculated concentrations of the analytes in CSF samples by using the standard addition approach and the internal standard (in PBS) approach. As well, any influence of CSF on the recovery of the analytes or internal standard during sample pre-treatment could be discharged.

Up to now stable isotope-labeled analoges for each of the studied ceramides are not commercially available. This complicates the ESI–MS/MS analysis as the ion signal of an analyte can be enhanced or suppressed by matrix substances which would be adjusted by an isotope-labeled internal standard [42]. In our case, matrix effects were minimized by dilution of the samples 1:30 during the analytical preparation. Suitability of C17:0 ceramide as internal standard in CSF was shown by comparing its chromatographic peak areas in extracted PBS and CSF samples. These areas were similar. As shown, the use of C17:0 ceramide compensates the degradation of the analytes in the autosampler for several hours. Nevertheless, analysis of ceramides in CSF should be done directly after sample preparation and the extracted samples should not be stored for more than 24 h in the autosampler. All in all, deuterated internal standards could simplify the analysis and further improve precision and accuracy values.

The method has been applied to 7 analytes but will be further developed with the aim of including more ceramide and sphingolipid species. Nano-LC–MS/MS is likely to be a helpful tool in future investigations on the role of ceramides and sphingolipids in neurological and inflammatory diseases.

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