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methods

High-throughput analysis of sulfatides in cerebrospinal fluid using automated extraction and UPLC-MS/MS

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Abstract Sulfatides (STs) are a group of glycosphingolipids that are highly expressed in brain. Due to their importance for normal brain function and their potential involvement in neurological diseases, development of accurate and sensitive methods for their determination is needed. Here we describe a high-throughput oriented and quantitative method for the determination of STs in cerebrospinal fluid (CSF). The STs were extracted using a fully automated liquid/liquid extraction method and quantified using ultra-performance liquid chromatography coupled to tandem mass spectrometry. With the high sensitivity of the developed method, quantification of 20 ST species from only 100 µl of CSF was performed. Validation of the method showed that the STs were extracted with high recovery (90%) and could be determined with low inter- and intra-day variation. Our method was applied to a patient cohort of subjects with an Alzheimer's disease biomarker profile. Although the total ST levels were unaltered compared with an age-matched control group, we show that the ratio of hydroxylated/nonhydroxylated STs was increased in the patient cohort. In conclusion, we believe that the fast, sensitive, and accurate method described in this study is a powerful new tool for the determination of STs in clinical as well as preclinical settings.—Blomqvist, M., J. Borén, H. Zetterberg, K. Blennow, J-E. Månsson, and M. Ståhlman. **High-throughput analysis of sulfatides in cerebrospinal fluid using automated extraction and UPLC-MS/MS.** ***J. Lipid Res.*** **2017.** 58: **1482–1489.**

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Sulfatides (STs; 3′-*O*-sulfogalactosylceramide) are glycosphingolipids consisting of a hydrophobic moiety called ceramide (sphingoid base and a fatty acid) and a sulfated galactose. STs are, together with galactosylceramides, the most typical myelin lipids, where the long-chain fatty acid moieties C24:0 and C24:1 are expressed in particular (1, 2). In addition to myelin, STs have been shown to be expressed in subpopulations of neurons and astrocytes in the gray matter of the brain (3, 4). STs and galactosylceramides are required for the stability and maintenance of the myelin sheet (5), whereas the STs found in other brain regions suggest more diverse functions of these glycosphingolipids (6). Furthermore, STs have been suggested to participate in other cellular processes, such as protein trafficking, cell adhesion, and aggregation and immune responses, among others (7). STs are also present in many other organs, including kidney, liver, heart, intestine, muscle, and pancreas (8), although in minor amounts compared with brain, except in kidney.

Findings indicate that the release of STs from myelin is associated with the development of CNS diseases and that STs in cerebrospinal fluid (CSF) might function as a marker of demyelination, myelin damage, and myelin turnover (9–13). Changes in CSF and brain ST levels have also been observed in other neurological disorders, such as early Alzheimer's disease (AD) (14–16). Furthermore, the absence of hydroxylated forms of ST in CSF could be used as a diagnostic tool for fatty acid 2-hydroxylase deficiency (17). These examples accentuate the need for a robust and sensitive method for quantification of STs in CSF.

In previous studies, CSF ST measurements have usually been performed by TLC with monoclonal antibody

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Abbreviations: AD, Alzheimer's disease; BUME, butanol:methanol; CID, collision-induced dissociation; CSF, cerebrospinal fluid; MRM, multiple reaction monitoring; NFL, neurofilament light protein; ST, sulfatide; UPLC, ultra-performance liquid chromatography.

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detection (18), which has a limited capacity in sample throughput and also requires large volumes of CSF. Furthermore, the ST lipid class consists of many molecular species with different fatty acids attached to the sphingoid base and TLC cannot distinguish between the different species, which might give additional information about disease progress.

Even though methods for total ST quantification (e.g., TLC) have been available for more than 50 years, detailed information regarding the molecular species has only recently been possible as a result of important advances in MS technology. In the last 15 years several methods have been described using TOF-secondary ion MS (19), MALDI-TOF MS (20, 21), HPLC-MS (22–26), and direct infusion MS (15, 27) for measuring ST species in different biological matrices. Quantitative and qualitative analyses of STs by LC-MS/MS have emerged as a powerful tool in research (28) and also in clinical diagnostics (29, 30). However, there are only a few studies, mainly performed by Han and coworkers, describing the quantification of STs in CSF (14, 15).

In this article, we describe a high-throughput oriented and sensitive method for measuring STs in CSF. The method is based on automated liquid-liquid extraction of small volumes of CSF using the chloroform-free butanol:methanol (BUME) method (31) and detection and quantification using ultra-performance liquid chromatography (UPLC) coupled to MS/MS. Using the described method, we determined whether the ST levels in the CSF of adults were in line with previously reported control values and the method was further applied to a cohort of patients having an AD biomarker profile. Furthermore, the usage of STs as a CSF biomarker was also discussed.

METHODS

Standard solutions and internal standards

Due to the limited numbers of commercially available standards, native STs were used as standards for quantification. The native STs were isolated from pig brain, as described previously (32), and the purity was >95% as determined by TLC and MS. For determination of individual ST species levels, a known amount of total STs, as determined by sphingosine base measurement (33), was separated using the developed UPLC method. The amount of individual ST species was then determined from the relative response (area) of the individual ST species. See the Results for further information.

Standard curves were made from a stock solution containing 10 μmol/l of native ST dissolved in chloroform:methanol (1:1; v/v). The standard curve was extracted together with the samples.

STs were synthesized as described previously (34). ST with C19:0 fatty acid (ST 19:0) was used as an internal standard and ST 16:0, ST 24:0, and ST 24:1 were used to investigate the response of individual ST species. The synthesized isoforms were free of detectable contaminations, as determined by TLC and MS, revealing a purity of >99%.

CSF biomarker analysis

The CSF samples were de-identified leftover samples from clinical routine at the Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden. The samples were designated as having an AD biomarker profile based on the cutoffs total tau >350 ng/l, phosphorylated tau >70 pg/ml, and Aβ42 <530 ng/l (all three biomarkers had to be abnormal) that are >90% specific for AD (35), while control samples had normal levels of all three CSF biomarkers. This procedure followed the Swedish Biobank law and was approved by the Ethical Review Board at the University of Gothenburg. Pooled CSF from control samples was used for method development and the validation procedure.

CSF AD biomarkers were analyzed using INNOTEST ELISAs (Fujirebio, Ghent, Belgium) by board-certified laboratory technicians according to protocols accredited by the Swedish Board for Accreditation and Conformity Assessment (SWEDAC). Neurofilament light protein (NFL) was measured using the NF-Light ELISA from UmanDiagnostics (Umeå, Sweden). Serum and CSF albumin concentrations were measured using immunonephelometry on a Beckman Immage immunochemistry system (Beckman Instruments, Beckman Coulter, Brea, CA). The albumin ratio was calculated as CSF albumin (milligrams per liter)/serum albumin (grams per liter) and was used as a measure of the blood-brain barrier function.

In order to determine the normal levels of STs in CSF, at adult age, and compare them to previous reports (15, 18), a cohort of 25 healthy subjects between the ages of 28 and 68 years were analyzed (11 male and 14 female, 50 ± 14 years).

ST extraction from CSF

The extraction of STs from CSF was performed in 1.2 ml glass tubes in the 96-well format on a BRAVO robot (Agilent Technologies, Santa, Clara, CA) using the fully automated BUME method (31). Briefly, 300 μl of butanol:methanol (3:1; v/v) (BUME solution) containing 15 nmol/l (4.5 pmol) of internal standard (ST 19:0) were added to the samples. After integrated mixing, 300 μl of heptane:ethyl acetate (3:1; v/v) and 300 μl of acetic acid (1%) were added and the samples were mixed thoroughly using repeated aspiration/dispensing. After spontaneous phase separation, the upper fractions containing the STs were transferred to a new tube and the bottom fractions were washed twice with heptane:ethyl acetate (3:1; v/v). The pooled upper fractions were evaporated under a stream of nitrogen and reconstituted in 100 μl chloroform:methanol:water (3:6:2; v/v/v) for UPLC-MS/MS analysis.

UPLC-MS/MS conditions

The STs were injected (5 μl) using a HTC-xt PAL (CTC Analytics, Zwingen, Switzerland) and separated on a Kinetex C18 column (2.1 × 100 mm, 1.7 μ) (Phenomenex, Torrance, CA) kept at 60°C. The mobile phases, which were pumped through the column at 400 μl/min using a quaternary 1290 Infinity UPLC pump (Agilent Technologies), consisted of water:acetonitrile:formic acid (70:30:0.1; v/v/v) as mobile phase A and acetonitrile:isopropanol:formic acid (50:50:0.1; v/v/v) as phase B. Separation was performed using gradient elution starting at 65% B. After 0.5 min of isocratic elution, the B phase was increased to 100% during 5 min and then held at 100% for 2 min. The gradient was returned to 65% B and held for 2.5 min, giving a total runtime of 10 min. ST detection was made using negative electrospray on a QTRAP 5500 (Sciex, Concord, Canada). The data were collected using scheduled multiple reaction monitoring (MRM) algorithm with m/z 96.9 (sulfate fragment) as product ion for all ST species (for all transitions and mass spectrometric parameters see supplemental data). In total, 20 ST species, including hydroxylated species, were monitored and quantified against the external calibration curve.

Method validation

ST extraction recovery. In order to evaluate the extraction recovery, ST 19:0 (4.5 pmol) was added before or after extraction of

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100 μl CSF (n = 5 for each). The recovery was then determined by comparing the ST 19:0 signals from the extracted samples with the unextracted samples. To validate that all ST species were extracted equally, the profiles of individual ST species from an extracted and an unextracted native ST mixture were also compared.

Precision. Inter- and intra-day precision for the determination of total and individual STs was estimated by quantifying STs in a CSF pool at five different time points (n = 10 at every time point) during 30 days.

Linearity and LOQ. The linearity of the method was tested by making serial dilutions (1:3) of ST 19:0 in CSF. The ST 19:0 concentration range was 1,200–0.02 nmol/l and from this the LOQ was estimated as the lowest concentration for which the signal-to-noise ratio was >10. Three serial dilutions were made and all measurements were made in triplicate.

ST stability during storage and freeze-thaw cycles. To investigate the stability of the STs during storage, a CSF pool was stored at −80°C for 1 year. The stability of the STs during freeze-thaw cycles was tested by thawing and refreezing aliquots (n = 3) one to five times.

Statistics

Correlation analysis was performed using the Pearson correlation coefficient. Statistical significance between groups was evaluated using the unpaired two-tailed Student's *t*-test. A *P* value below 0.05 was considered significant.

RESULTS

UPLC-MS/MS development

For optimization of MS conditions, a solution containing C19:0 ST was infused into the mass spectrometer and ionized in both positive and negative mode. In positive mode, the most abundant fragment was the $[M+H]^+$ ion, which produced the *m/z* 264 as the most abundant product ion under collision-induced dissociation (CID) (see supplemental Fig. S1A). The most abundant ion in negative mode was the deprotonated ST, $[M-H]^-$, which gave rise to a very strong fragment at *m/z* 96.9 originating from the sulfate fragment under CID (see supplemental Fig. S1B). To obtain optimal fragmentation, a collision energy of −150 eV was used (for detailed MRM transitions and instrument parameters see supplemental Tables S1, S2). In order to investigate whether there was any difference in response between ST species with different fatty acids, an equimolar solution (1 μmol/l) of three different STs (C16:0, C24:0, and C24:1) was infused into the mass spectrometer. A comparison of the MRM traces during the infusion showed that all three tested ST species were detected with similar response (supplemental Fig. S2A). UPLC-MS/MS analysis of the same ST mixture also showed a similar response of the three tested ST species (supplemental Fig. S2B), indicating that the difference in mobile phase composition during elution did not affect ionization efficiency. Using isopropanol as a mobile phase can be associated with high backpressures. For this reason, a column temperature of 60°C was used, which reduced the backpressure to below 500 bar. The relatively high column temperature also had a positive effect on peak shape.

Using the developed UPLC method, all monitored STs eluted between 3 and 6 min (**Fig. 1**). By using the integrated scheduled MRM algorithm, individual ST species were monitored during a limited time window, which enabled a minimum of 12 data points across all monitored peaks. As illustrated in Fig. 1, a good separation was needed for accurate determination and quantification of ST. In the MRM trace for ST 26:0, three peaks eluted close together. The first one was ST 25:1-OH, which had almost the same mass and the same MRM transition. The middle peak was from the +2 isotope of ST 26:1 and the last peak was ST 26:0. The reason that there were only two peaks in the ST 25:1-OH trace, which was exactly the same transitions as for ST 26:0, was because of the MRM scheduling algorithm. The identities of the peaks eluting from the chromatogram were determined by analyzing the native ST mixture in both negative and positive mode.

Method validation

Using the BUME method, up to 96 samples can be automatically extracted in less than 1 h and our results show that the extraction efficiency was 90%. Furthermore, comparing the profile of individual ST species before and after the extraction of a native sulfide standard mixture showed that all the different species were extracted with similar efficiencies (supplemental Fig. S3).

A serial dilution of ST 19:0 in CSF showed that the method was linear between 0.1 and 200 nmol/l with an r^2 value of 0.999. The LOQ was determined to be 0.1 nmol/l (supplemental Fig. S4), while the intra- and inter-day precision was determined to be ⩽10% for all ST species except for ST 18:0, ST 18:0-OH, and ST 26:0, which had an inter-day precision of 12, 17, and 27%, respectively (**Table 1**). Importantly, the validation also showed that the measured ratio between hydroxylated/nonhydroxylated STs could also be determined with high precision.

No significant changes in ST concentrations were observed during five freeze-thaw cycles (the coefficient of variation for the five determinations of total sulfatides was 1.5%). Furthermore, quantification of STs in a CSF pool stored at −80 for 1 year gave similar results (data not shown). Taken together, these results show that STs are stable in CSF, both during long-term storage and during freeze-thaw cycles.

CSF ST levels in control individuals

The method was first applied to a cohort of "healthy" individuals (28–68 years of age, n = 25). The concentration of STs in CSF was 71 ± 18 nmol/l (mean ± SD, range 33–101 nmol/l). No age-related correlation could be found in this cohort (supplemental Fig. S5).

CSF ST levels in patients with an AD biomarker profile

The total amount of ST in CSF from individuals with an AD biomarker profile was not significantly different from control individuals (**Fig. 2A**, $P = 0.90$). However, comparing

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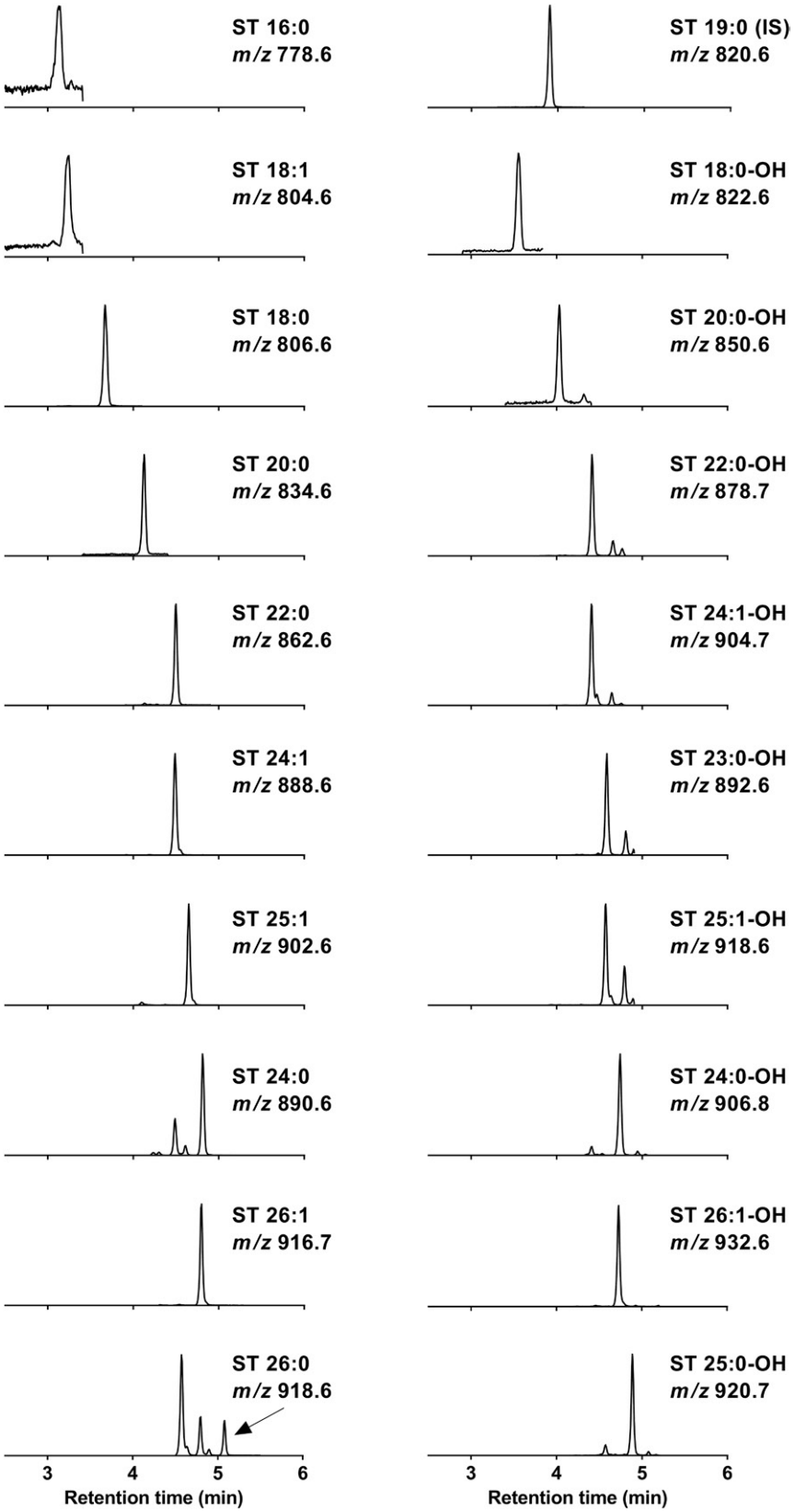

Fig. 1. Extracted ion chromatograms of individual ST species identified from a native ST mix separated using the described method. As can be seen in the different MRM traces, the separation is important for accurate quantification. For example ST 26:0 and ST 25:1-OH have the same MRM transition, but are clearly separated using the C18 column. In addition, without sufficient separation the M+2 isotope from one ST species would contribute to the signal of another ST species (e.g., the M+2 isotope of ST 24:1 gives a peak in the ST 24:0 trace). All STs are annotated with name and parent ion mass. In all ion chromatograms, with the exception of ST 26:0, the largest peak corresponds to the annotated ST.

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TABLE 1. Intra- and inter-day variation for the quantification of STs in a CSF pool

	Day 1		Day 2		Day 3		Day 4		Day 5		Inter-day Variation	
Concentration (nmol/l)	Average	CV (%)	Average	CV (%)	Average	CV (%)	Average	CV (%)	Average	CV (%)	Average	CV (%)
ST 24:1	27.5	3.7	28.7	3.8	24.6	3.7	30.3	4.2	32.0	2.9	28.6	9.9
ST 18:0	10.3	6.1	10.3	2.3	7.5	3.1	9.1	2.4	9.7	1.8	9.4	12.4
ST 24:0	6.6	4.3	7.7	3.2	7.9	4.4	7.8	3.3	8.4	4.2	7.7	8.6
ST 18:1	7.8	3.2	7.4	2.9	6.9	1.9	6.8	2.7	7.0	2.1	7.2	5.9
ST 24:1-OH	4.7	3.4	4.7	5.3	4.7	7.9	5.0	4.1	5.2	5.2	4.9	4.5
ST 22:0	3.8	3.4	3.7	3.8	3.5	4.8	3.8	5.6	4.3	3.3	3.8	7.3
ST 22:0-OH	3.0	4.0	2.9	4.3	3.0	5.2	3.1	2.1	3.6	3.4	3.1	9.4
ST 20:0	3.0	2.2	2.8	3.3	2.6	5.2	2.8	5.2	2.8	3.5	2.8	4.5
ST 16:0	2.7	3.2	2.5	4.3	2.4	3.7	2.2	3.1	2.4	2.5	2.4	7.3
ST 25:1	2.2	4.2	2.3	4.8	2.3	4.6	2.6	3.6	2.5	3.5	2.4	6.5
ST 23:0-OH	2.3	4.1	2.4	2.9	2.3	4.9	2.4	5.6	2.6	3.9	2.4	4.8
ST 24:0-OH	2.3	4.5	2.5	1.8	2.4	6.3	2.4	5.1	2.4	2.9	2.4	3.0
ST 20:0-OH	2.5	1.7	2.3	4.1	2.2	4.6	2.3	3.4	2.4	2.8	2.3	6.1
ST 18:0-OH	2.6	2.1	1.6	3.0	2.2	4.6	2.4	2.2	2.4	5.1	2.2	17.4
ST 26:1	1.4	3.7	1.4	4.4	1.7	3.8	1.6	6.3	1.6	3.9	1.6	7.9
ST 25:1-OH	0.5	6.8	0.5	6.9	0.6	7.5	0.6	3.3	0.5	5.0	0.5	5.3
ST 26:1-OH	0.4	7.4	0.4	4.0	0.4	5.9	0.4	8.7	0.4	8.3	0.4	10.3
ST 26:0	0.3	4.1	0.3	4.6	0.3	3.7	0.5	10.2	0.3	5.2	0.3	27.4
ST 25:0-OH	0.3	6.0	0.3	4.0	0.3	7.8	0.3	7.9	0.3	10.5	0.3	8.3
Sum	84.4	2.0	84.6	1.8	77.6	2.1	86.4	2.3	90.7	1.4	84.8	5.6
ST/ST-OH ratio	3.5	1.2	3.8	2.1	3.3	3.1	3.6	1.7	3.6	1.9	3.6	5.1

Ten samples were measured at five different time points,

the ratio of hydroxylated species (HFA) to nonhydroxylated species (NFA) of ST, a significant increase of the HFA/NFA ratio was detected in the CSF from patients with an AD biomarker profile compared with control samples (Fig. 2B, $P = 0.001$).

Correlation of ST levels to CSF-NFL and albumin ratio

NFL is a sensitive CSF biomarker for neuronal injury and, more specifically, myelin damage. In our material, there was no correlation of ST with NFL levels either in AD patients or in controls (**Fig. 3A**; $P = 0.35$ and 0.92, respectively). On the other hand, a correlation was observed between CSF ST levels and the albumin ratio (CSF/serum) in both patients with an AD biomarker profile and controls (Fig. 3B, $P = 0.001$ for both).

DISCUSSION

In this study, we describe a method for quantification of ST species in CSF. By using the robot-assisted BUME method, samples were automatically extracted in the 96-well format in less than 1 h and, without any further cleanup, directly analyzed using UPLC-MS/MS. Because of the high sensitivity of the method, only 100 µl of CSF were required. We then used the method for determining the level of STs in a cohort of subjects with an AD biomarker profile and showed that, even though the total concentration of STs was unchanged, the ratio of hydroxylated/nonhydroxylated species was increased in the AD subjects compared with the control group.

Lipid extraction can be a time-consuming process and is often associated with manual work using chloroform-based solvents (36, 37). However, with the development of pipetting robots, it is now possible to automate these processes and we have recently described and validated the BUME method for extraction of a broad range of lipids, including sphingolipids such as ceramides and glucosylceramides (31). Here we show that this method can also be used for STs. A limitation of the current extraction method, which is performed in 1.2 ml glass tubes, is that it can only handle a maximum of 100 µl of CSF. To guarantee accurate ST

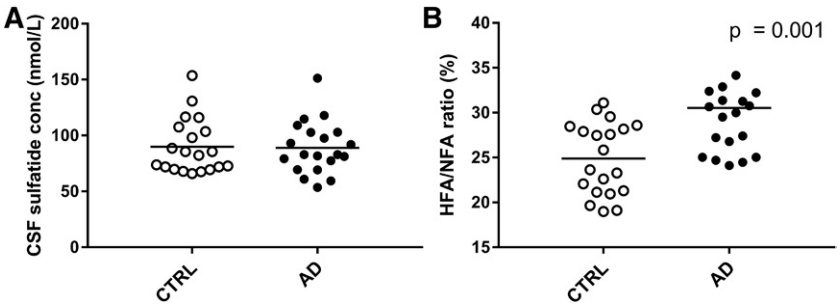

Fig. 2. A: No significant difference was observed in total ST CSF concentration between patients with an AD biomarker profile and controls (CTRL). B: The ratio between hydroxylated/nonhydroxylated STs (HFA/NFA ratio) was significantly higher in patients with an AD biomarker profile compared with the controls.

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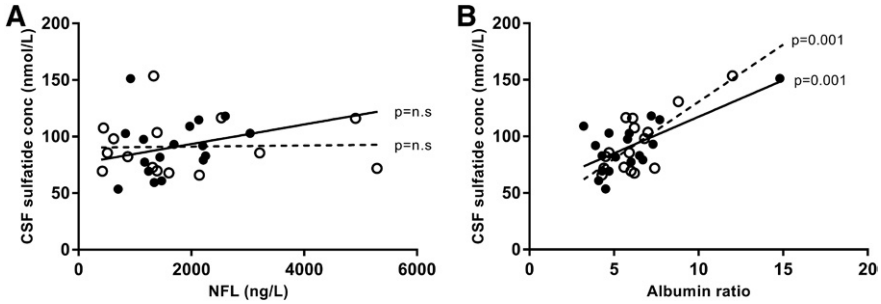

Fig. 3. The CSF ST levels in control subjects (open circles, dashed line) and subjects with an AD biomarker profile (closed circles, solid line) were correlated against the CSF-NFL concentrations and albumin ratios (CSF/serum). For NFL, no significant correlation was observed (A). However, a significant correlation was observed between CSF ST concentration and the albumin ratio (B).

recoveries from larger CSF volumes, the extraction procedure needs to be scaled up. However, the extraction procedure does not require a robot and can also be performed manually.

Although there are methods of analyzing STs in positive mode using the *m/z* 264 fragment (25, 38), we chose, like most other published methods, to perform the analysis in negative mode where all the detected ST species gave rise to a very strong signal for the sulfate fragment at *m/z* 96.9. A drawback of analyzing STs in negative mode is that no fatty acid-dependent fragment is formed during CID. In positive mode, a fatty acid-dependent fragment is formed and this can potentially be used for structural confirmation (see supplemental Fig. S1). However, because of the low intensity of this fragment, it was not possible to accurately detect it when analyzing sulfated species from 100 µl of CSF. However, analysis in positive mode was used during method development for identification and confirmation.

In our hands, performing the analysis in negative mode was beneficial because the fragmentation efficiency was similar for all STs, independent of the fatty acid constituent. Furthermore the fragmentation efficiency was optimal at a very high collision energy (−150 eV), which was beneficial because it reduced background noise and LOQ for the method. Because no one, to the best of our knowledge, has reported an LOQ for STs in CSF, comparisons with other methods should be made with caution. Han and coworkers have analyzed STs in CSF, but have not reported the concentration of individual STs (14, 39). However, for reliable measurement, 0.5–1.0 ml of CSF was needed, indicating a lower LOQ, which can in part be explained by the use of direct infusion MS. For plasma LOQ, values between 5 and 50 nmol/l have been reported (23, 26, 28, 38). In this report, we chose to only focus on CSF as matrix, but we believe that the method could also be applied to other matrices. However, this will require further investigation.

Similar to many other groups, we used reversed-phase chromatography for the separation of ST species prior to MS detection (23, 24, 26, 28, 38). Using this approach, the separation of individual species was mainly based on the difference in fatty acid moieties attached to the sphingosine backbone. This was very different compared with HILIC methods where the separation is more dependent on the polar head groups (in this case the galactosyl sulfate) (22). As a result, using HILIC methods or direct infusion methods (15, 27), all ST species are ionized and detected simultaneously in the mass spectrometer. Although this might have some beneficial effects, such as fast run times and similar ionization environment for all ST species, these methods are also associated with some drawbacks. For example, using HILIC or direct infusion methods, ST 26:0 (monoisotopic mass 919.6782) and ST 25:1-OH (monoisotopic mass 919.6418) are very difficult to distinguish from each other without a high-resolution mass spectrometer. Furthermore, our initial tests using HILIC methodology showed reduced sensitivity compared with the described reversed-phase method, mainly due to massive ion suppression effects caused by salts coeluting with the STs. Because salts might compromise ionization efficiency, we excluded salts from our method and only used formic acid as an additive to the mobile phases.

For optimal quantification using UPLC-MS/MS, two standards (a reference substance and an endogenously uncommon or isotopically labeled internal standard) for each measured component are needed. However, due to the great diversity of lipids, it is often impossible and extremely expensive to attain these for all the investigated lipid species. One commonly used alternative is to make standard curves from commercially available standards and quantify ST species that lack attainable standards to the one closest in retention time or structure (26). However, because of the similar fragmentation pattern of the STs in negative mode, with the sulfate group as common fragment, all ST species have similar ionization efficiencies and, therefore, similar responses. This means that quantification can be made against one reference substance or even the internal standard with reasonable accuracy (23). In this method, we used a mixture of native STs isolated from bovine brain as a standard for quantification and one synthesized ST with the unusual fatty acid, 19:0, as internal standard. The benefit of using this mixture for quantification, instead of using one or several commercially available standards, is that individual calibration curves could be made for each component, which facilitated data evaluation. Furthermore, the use of a native ST mixture also functioned as a quality check because inter-day variations of chromatographic behavior, such as retention times and signal intensities, could be monitored. Using our new procedure, we determined

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the total ST concentration [71 ± 18 nmol/l (mean ± SD)] in a control cohort (age range 28–68 years). The result was in line with previous data from both TLC-ELISA [reference range of 100 ± 46 nmol/l CSF (mean ± SD), 25–80 years of age] (18) and mass spectrometric determinations (14).

Earlier studies by Han and colleagues have shown ST depletion in patients with mild cognitive impairment due to AD, suggesting ST to be a biomarker for early clinical stages of AD (14–16). Furthermore, Han (40) demonstrated that ApoE is involved in mediating ST depletion; an effect that might be influenced by the AD-associated *APOE* ε2/ε3/ε4 polymorphism. Herein, ST levels were determined in a cohort of patients with an AD biomarker profile and, in contrast to a previous study (14), no difference was found in the concentration of STs in our patient cohort as compared with age-matched controls. The patients included in this study were selected by their pathological levels of AD biomarkers (CSF Aβ42, T-tau, and P-tau) using cut-off values that were 90% sensitive and specific for AD (35). We found, however, a significantly increased ratio of hydroxylated/nonhydroxylated STs in the patient cohort compared with the age-matched controls. Using MALDI-IMS, Yuki et al. (41) showed that STs in gray matter had a higher proportion of hydroxylated species than in white matter, but they found no difference between control and AD brains. Our finding might still, in some way, reflect the gray matter pathology in AD.

STs are mainly synthesized by oligodendrocytes and myelin is the major source of STs in the CNS. As a consequence of the brain localization, STs in CSF have been suggested to be a potential biomarker for white matter disease (9, 18, 42, 43). However, the origin and transport forms of STs in CSF are not yet fully elucidated. Harrington et al. (44) showed that nanostructures (30–200 nm) are naturally occurring nano-sized particles in CSF of both healthy individuals and patients with different brain diseases. The shedding and occurrence of these nanostructures in CSF might not generally represent the total membrane content in the brain, but more likely might represent the accessibility to the CSF. STs may well be associated with these nanostructures, considering their hydrophobic nature, but this remains to be elucidated.

The contribution of STs originating from the circulation must also be taken into consideration. The albumin ratio (CSF albumin/serum albumin) is a biomarker used to evaluate the blood-brain or blood-CSF barriers (45). The positive correlation between CSF STs and the albumin ratio found in our study may suggest a contribution from blood that is contrary to an earlier report (9). These authors concluded that only a minor portion of CSF STs were derived from blood based on their finding of expected CSF/plasma lipid concentration ratios of different lipid classes. This assumption was further supported by the enrichment of C18:0 ST in CSF. In plasma, the ratio between C16:0 and C18:0 ST is 16:1 (23); while in CSF, the same ratio is 1:4 (present study). Alternatively, the increased CSF/serum albumin ratio in AD patients has been associated with leukoaraiosis, or white matter lesions (46), that may increase CSF ST levels.

Another CSF biochemical marker, NFL, is used as a biomarker of neuronal degeneration. It has been assumed that increased NFL levels reflect destruction of myelinated axons (47). We found no correlation between STs and NFL in either our control cohort or in the patient material. Thus, our results suggest that CSF ST is a poor biomarker for demyelination, but might still be useful to identify changes in the ceramide composition, e.g., fatty acid 2-hydroxylase disorders (17) and pathological brain ST accumulation (32).

To conclude, in this study, we have developed an automated and high-throughput oriented method for measuring STs in a small volume of CSF. We then used this method to quantify CSF STs in a patient cohort with an AD biomarker profile. Even though, when compared with a control group, no differences could be observed in the total amount of STs, we observed a higher ratio of hydroxylated/nonhydroxylated STs in patients with an AD biomarker profile. Due to the high degree of automation and low sample volume, we believe that the developed method could be of great use in basic research and in a clinical setting.

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