Journal of Chromatography B, 878 (2010) 1098–1102

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Liquid chromatography–tandem mass spectrometry assay for the quantification of free and total sialic acid in human cerebrospinal fluid

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ARTICLE INFO

Article history:
Received 22 December 2009
Accepted 11 March 2010
Available online 18 March 2010

Keywords:
Sialic acid
Cerebrospinal fluid
High-performance liquid chromatography–tandem mass spectrometry

ABSTRACT

Background: Analysis of sialic acid (SA) metabolites in cerebrospinal fluid (CSF) is important for clinical diagnosis. In the present study, a high-performance liquid chromatography–tandem mass spectrometry (HPLC/MS/MS) method for free sialic acid (FSA) and total sialic acid (TSA) in human CSF was validated.
Methods: The method utilized a simple sample-preparation procedure of protein precipitation for FSA and acid hydrolysis for TSA. Negative electrospray ionisation was used to monitor the transitions m/z 308.2 → 87.0 (SA) and m/z 311.2 → 90.0 ($^{13}C_3$-SA). Conjugated sialic acid (CSA) was calculated by subtracting FSA from TSA. We established reference intervals for FSA, TSA and CSA in CSF in 217 control subjects. The method has been applied to patients' samples with known differences in SA metabolites like meningitis (n = 6), brain tumour (n = 2), leukaemia (n = 5), and Salla disease (n = 1).
Results: Limit of detection (LOD) was 0.54 μM for FSA and 0.45 μM for TSA. Intra- and inter-assay variation for FSA (21.8 μM) were 4.8% (n = 10) and 10.4% (n = 40) respectively. Intra- and inter-assay variation for TSA (35.6 μM) were 9.7% (n = 10) and 12.8% (n = 40) respectively. Tested patients showed values of TSA above established reference value.
Conclusion: The validated method allows sensitive and specific measurement of SA metabolites in CSF and can be applied for clinical diagnoses.

1. Introduction

Sialic acid (SA) (N-acetyl neuraminic acid) is a nine carbon monosaccharide that is present at the non-reducing terminus of glycoproteins and glycolipids. In addition, SA is also present in its free form (FSA). SA is widely distributed throughout human tissues and is found in several fluids including serum, urine, saliva, breast milk and CSF. Endogenous synthesis of SA takes place in the cytoplasm of all tissues from N-acetylglucosamine or N-acetyl-D-mannosamine precursors. SA is present in humans, while N-glycolylneuraminic acid is common in many animal species [1] but not found in humans except in the case of a particular cancer [2]. There are numerous physiological and pathological processes in which SA is involved (for review see [3,4]). Disturbances in SA metabolism (either due to genetic error or at the post-translational level), may interfere with its normal physiological functions and analyses of SA metabolites is useful.

About sixty genes are involved in SA biology [5]. There are genetic disorders involving transport (Salla disease; intralysosomal accumulation of FSA) or overproduction (sialuria) of sialic acid. On the other hand, changes in SA metabolism are seen in many pathological states (e.g. infections and inflammations, endocrinological, cardiovascular, neurological, nephrological).

Increased concentration of SA in CSF has been described in bacterial meningitis compared to tubercular meningitis [6]. The exact mechanism for increased FSA in bacterial meningitis is not known. Pneumococci have been shown to elaborate a neuraminidase. This enzyme is secreted during the logarithmic phase of growth and cleaves terminal molecules of SA from CSF proteins, gangliosides, and the glycoproteins of neurons and other central nervous system tissues. The non-significant differences of CSF free SA in tubercular meningitis are explained due to lack of neuraminidase enzyme in tubercle bacilli. The amount of SA in CSF may also be a good

Abbreviations: SA, sialic acid; CSF, cerebrospinal fluid; FSA, free sialic acid; TSA, total sialic acid; CSA, conjugated sialic acid; LOD, limit of detection; IS, internal standard; QC's, quality control samples; ESI, electrospray ionisation; LOQ, limit of quantification.

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doi:10.1016/j.jchromb.2010.03.020

indicator of central nervous system leukaemia [7] and for brain tumours [8]. Changes in the metabolism of SA by tumour cells are characterized by aberrant glycosylation processes by tumour cells resulting in larger amounts of SA on their surface compared with normal cells. Sialoproteins are then released into the surrounding environment primarily by shedding or cell lysis. Recently, a novel form of cerebellar ataxia with increased FSA in CSF was reported [9], FSA in CSF was analyzed using ^{1}H NMR spectroscopy. In these five patients, SA content was normal in urine, plasma and cultured fibroblasts. Since the abnormal concentration of FSA was only restricted to CSF in this group of patients, it is essential to measure this metabolite in the right body fluid. This new identified group of patients extends the range of human diseases involving SA metabolism in CSF.

In human CSF, the determination of SA has been carried out by HPLC with fluorimetric detection [10,11], the photometric resorcinol reaction or thiobarbiturate (Warren's) method [12,13]. These are relatively insensitive and lack specificity. ^{1}H NMR spectroscopy which has also been used [9], but can never be applied in a routine diagnostic setting and require CSF volumes of approximately 1 ml, a considerable amount when diagnostic procedures need to be applied to newborns. Liquid chromatography–tandem mass spectrometry (HPLC/MS/MS) has the advantage of improved sensitivity, selectivity and the advantage of ease of operation compared with the more elaborate procedures. The objectives of this study were to develop and validate a simple HPLC/MS/MS method for the determination of FSA and TSA in CSF. The developed method was used to establish reference intervals for free, total and the calculated conjugated SA covering 0.1–85.9 years of age. Central nervous system inflammation may reduce the value of the present assay because leukocytes can produce large amounts of SA and the presence of elevated leukocyte count have to be taken in account.

CSF samples from patients with a known disease like meningitis (n = 6), brain tumour (n = 2), leukaemia (n = 5) and Salla disease (n = 1) were analyzed.

2. Experimental

2.1. Reagents

N-acetylneuraminic acid (sialic acid, SA), 1,2,3-$^{13}C_3$ N-acetyl-D-neuraminic acid (internal standard, IS), ammonium formate and methanol were obtained from Sigma–Aldrich Chemie (Steinheim, Germany). Sodium chloride 0.9% was purchased from Fresenius Kabi ('s Hertogenbosch, The Netherlands). For HPLC/MS/MS analysis formic acid and sulphuric acid were purchased from Merck (Darmstadt, Germany). Acetonitrile was obtained from Rathburn Chemicals (Walkerburn, Scotland).

2.2. Cerebrospinal fluid (CSF) samples

For metabolic profiling, CSF from lumbar puncture was collected in six fractions (0.5, 1, 1, 1.5, 1–2 and 2 ml respectively) and put on ice immediately, before storing at −20 °C. From three patients two different fractions (2 and 6) were analyzed for SA. For investigations other than metabolic profiling (most to exclude meningitis), CSF was not subdivided.

As a positive control, CSF was obtained from patients with meningitis (n = 6), patients with leukaemia (n = 5), patients with brain tumour (n = 2), and Salla disease (n = 1). The diagnosis of Salla disease has been confirmed earlier by increased FSA excretion in urine, accumulation of FSA in cultured fibroblasts and mutations in the *SLC17A5* gene. Cell count data and protein content were normal for all CSF specimens.

Control values of SA were derived from CSF samples with normal cell count and protein content (n = 217, 134 males, 83 females) from individuals between 0.1 and 85.9 years were used to establish control values.

2.3. Sample preparation for free sialic acid (FSA)

For analysis of FSA, 15 μL IS (170 μmol/L) was added to 75 μL CSF followed by protein precipitation with 300 μL methanol. After mixing and centrifugation (16,060 × g for 5 min) supernatant was dried under a stream of nitrogen at 40 °C. The residue was redissolved in 90 μL Milli-Q water.

2.4. Sample preparation for total sialic acid (TSA)

For analysis of TSA, 15 μL IS (170 μmol/L) was added to 15 μL CSF followed by addition of 60 μL sulphuric acid (63 mmol/L). Samples were placed by 80 °C for 1 h to obtain all CSA in free form (TSA). After cooling, samples were analyzed.

2.5. Calibration curve

A six-point calibration curve with concentrations till 69 μmol/L SA was prepared in Milli-Q water. Before analyzing these standards 15 μL IS (170 μmol/L) was mixed with 75 μL standard.

2.6. Quality control samples (QC's)

CSF from different individuals was pooled and two pools were used as quality controls (QC 1: FSA = 23 μM and TSA = 44 μM and QC 2: FSA = 44 μM and TSA = 88 μM).

2.7. Mass spectrometry

Chromatographic and mass spectrometric conditions on the HPLC/MS/MS system were used as described before [14] (Table 1) with some slight modifications to improve sensitivity. In short, injection volume was increased from 10 to 35 μL and dwell time analyzing channels of SA and IS was changed from 0.4 to 0.6 s.

Mass transitions m/z 308.2 → 87.0 (SA) and m/z 311.2 → 90.0 (IS) were measured (Fig. 1). MassLynx software (v 4.1; Waters) was used to control the instrument and for data acquisition.

2.8. Method validation

QC 1 and QC 2 were used for method validation. Precision (intra- and inter-assay variation), accuracy, sensitivity (limit of detection (LOD) and limit of quantification (LOQ)), linearity, recovery, matrix effect, short-term stability and a freeze–thaw experiment were performed to validate FSA as well as TSA.

Intra-assay variation was studied by analyzing FSA and TSA in QC 1 and QC 2 (n = 10) and inter-assay variation of FSA and TSA was studied by analyzing QC 1 and QC 2 (n = 40, twenty-one runs).

Accuracy was tested by spiking SA (34, 136 and 339 μM SA respectively) to three different CSF samples.

LOD and LOQ were determined for FSA and TSA, as the amount for which signal-to-noise ratios (S/N) were higher than 3 and 10 respectively by using both QC samples.

Linearity was evaluated using a twelve points high range calibration curve (range 13 till 12,804 μmol/L SA).

Recovery was tested by addition SA to CSF at two levels (8.6 and 34.4 μM) before and after protein precipitation for FSA and before and after hydrolysis for TSA. Matrix effect was determined by comparing area of $^{13}C_3$ labelled SA (IS) in standards of calibration curve (prepared in Milli-Q water) with area of IS in CSF samples in three different series.

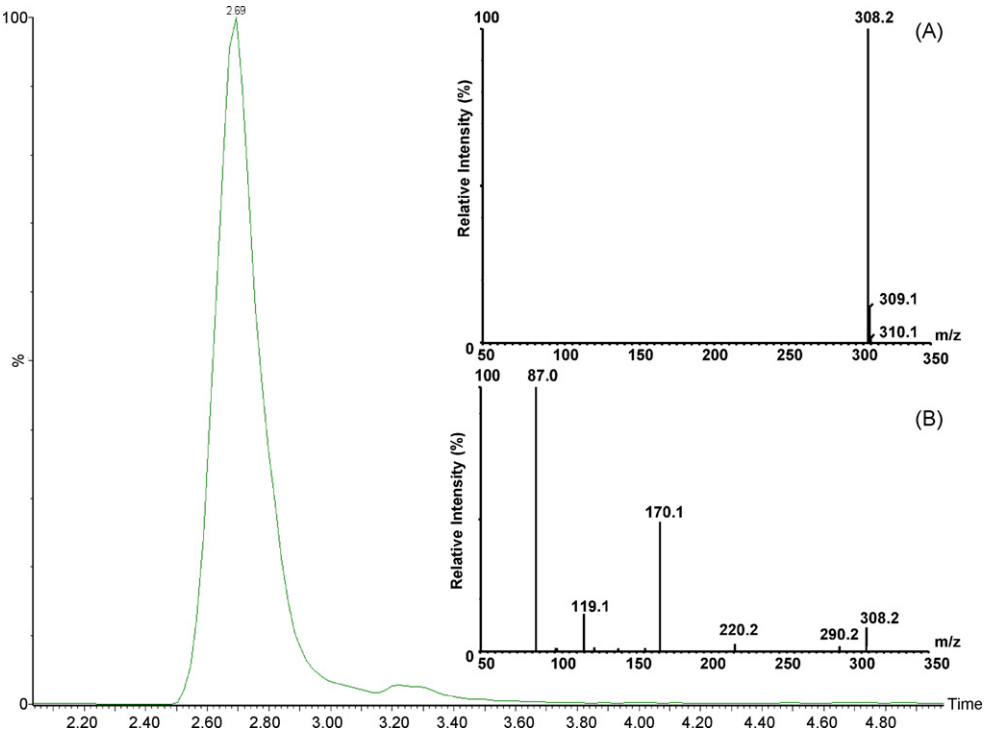

Fig. 1. MRM chromatogram of SA (m/z 308.2 > 87.0) in CSF (21 μmol/L) with parent scan (A) and daughter scan of m/z 308.2 (B) of SA.

Short-term stability in prepared samples was tested by measuring FSA and TSA in prepared calibration curve together with prepared QC's (1 and 2) at 5 time points during 15 days.

Both QC samples were used to test the stability of FSA and TSA by 1, 3, 5 and 10 freeze–thaw cycles (n = 3).

2.9. Calculation and statistical analysis

Analyse-it (Microsoft, v 2.12) was used to establish reference values and check normal distribution within control samples and to check linearity of FSA. CSA was calculated by subtracting FSA from TSA.

3. Results

3.1. Method validation

With the HPLC/MS/MS instrument settings as described before, intra- and inter-assay variation of FSA was 4.8% (n = 10) and 10.4% (n = 40). For TSA intra- and inter-assay variation was 9.7% (n = 10) and 12.8% (n = 40). Accuracy of FSA was 98.8% and 97.3% for TSA. LOD was 0.6 μmol/L and LOQ was 2.0 μmol/L for FSA as well as for TSA. The assay was found to be linear till 6402 μmol/L with a correlation coefficient (r^2) of 0.999. Lower range calibration curve for quantification of FSA and TSA showed a similar correlation (r^2 = 0.999, n = 10). Recovery of FSA was 94–101% and 91–105% for TSA (n = 6). Signal reduction of peak area IS was 94% for FSA and 86% for TSA compared with peak area IS in prepared standards. Short-term stability test of SA in prepared samples showed a variation of 6.0% for FSA and 5.2% for TSA. No change in area counts for SA as well as for IS was observed.

Table 1
Linear gradient profile for chromatographic separation of SA in CSF with HPLC/MS/MS.

HPLC/MS/MS		
Time (min)	Solvent A (%)	Solvent B (%)
0	100	0
2.0	0	100
2.5	0	100
2.8	100	0
6.0	100	0

3.2. Analysis of CSF

Analyzing CSF fractions 2 and 6 from three individuals showed similar concentrations (p = 0.12) in SA metabolites. Furthermore, no effect of freeze–thaw cycles (n = 10) was found.

Reference ranges for FSA, TSA and CSA were established (Table 2). No age and gender dependency was observed and reference values showed normal distribution for FSA, TSA and CSA. Table 3 gives the SA content of CSF in patients suffering from meningitis (n = 6), patients with leukaemia (n = 5), patients with brain tumour (n = 2), and one patient with Salla disease. From six analyzed CSF samples of meningitis patients, two (patients 1 and 4) showed increased amount of FSA, while other four had a FSA concentration within reference range (samples 2, 3, 5 and 6). All six meningitis patients showed increased TSA as well as increased CSA. Four (sample 7, 8, 9 and 11) out of five patients with leukaemia showed increased FSA in CSF while all patients showed an increased level of TSA. In sample 8, CSA was within reference range, in the remaining four leukaemia patients CSF was increased.

Table 2
Reference values of free sialic acid (FSA), total sialic acid (TSA) and conjugated sialic acid (CSA) in CSF samples from control subject, age 0.1–85.9 years (n = 217, 134 males and 83 females).

Compound	Concentration in μmol/L		
	Mean	SD	Reference range
FSA	11.8	4.2	4–20
TSA	28.4	9.2	10–46
CSA	16.6	6.0	6–29

Table 3
Results of free sialic acid (FSA), total sialic acid (TSA) and conjugated sialic acid (CSA) in CSF samples of patients with meningitis, leukaemia, brain tumour and one patient with Salla disease compared with reference values (Table 2) ↑, above established reference value; *N*, normal regarding to reference values.

Sample	Disorder	FSA, TSA and CSA in CSF (μmol/L)					
		FSA		TSA		CSA	
1	Meningitis	22	↑	259	↑	237	↑
2	Meningitis	12	*N*	1008	↑	996	↑
3	Meningitis	3	*N*	1800	↑	1797	↑
4	Meningitis	83	↑	366	↑	283	↑
5	Meningitis	10	*N*	399	↑	389	↑
6	Meningitis	9	*N*	69	↑	60	↑
7	Leukaemia	33	↑	68	↑	35	↑
8	Leukaemia	32	↑	60	↑	27	*N*
9	Leukaemia	23	↑	53	↑	30	↑
10	Leukaemia	13	*N*	50	↑	37	↑
11	Leukaemia	21	↑	51	↑	30	↑
12	Brain tumour	4	*N*	1056	↑	1052	↑
13	Brain tumour	22	↑	299	↑	277	↑
14	Salla	134	↑	272	↑	137	↑

Both patients with a brain tumour (samples 12 and 13) showed increased concentrations of TSA and CSA and only one patient had normal FSA content in CSF (sample 12).

The patient suffering from Salla disease (sample 14) showed increased levels of FSA, TSA and CSA levels in CSF compared with reference values.

4. Discussion

Changes in CSF SA metabolites have been described in several diseases [3,6–8,15]. Moreover, in a yet unidentified neurometabolic disease increased FSA has been reported in CSF while FSA content was normal in the urine and fibroblasts of these patients [9]. This urged the need for accurate detection of SA acid concentrations in CSF for diagnostic purposes.

Our validation experiments showed linearity till 6402 μmol/L to determine SA concentrations. The absolute recovery was reproducible. Matrix effects and losses during sample preparation were compensated by use of isotope labelled internal standard.

A critical issue in the evaluation of CSF metabolites is the influence of sampling and storing methods on the stability of CSF metabolites. FSA and TSA concentrations were not affected by the ventriculospinal gradient meaning that SA can be quantified in a random CSF sample. Moreover, freeze–thaw experiments showed acceptable stability over 10 cycles, with no significant change in concentration of FSA and TSA.

Established reference range for FSA in CSF is compatible with previous studies. Hayakawa et al. [11] reported a mean FSA in 7 CSF samples (age range 0.3–20 years) of 15.7 ± 9.4 μmol/L and Mochel et al. [9] reported a mean FSA in 227 CSF samples of 9.9 ± 5.6 μmol/L.

Different metabolites (for example homovanillic acid) in CSF have been described as showing wide variations related to age [16]. In contrast of what we observed in urine [14], the present study shows that the reference values of FSA and TSA in CSF in childhood are not age-dependent which is in agreement with the study by Mochel et al. [9] who reported no age dependency in FSA between 1 and 80 years. Additionally, an age independency of SA of apolipoprotein E in CSF was observed by Kawasaki et al. [10] who reported no significant difference in the amounts of CSF apolipoprotein E SA between the young ($n = 7$, 5.4 ± 2.3 years) and aged groups ($n = 7$, 72.0 ± 3.2 years).

It has been reported that SA in serum from healthy subjects is largely bound (TSA 1.5–2.5 mmol/L) with small amounts of FSA (1–3 μmol/L) [17]. In contrast to serum, FSA in CSF accounts for almost 50% of TSA and the physiological role of these large amounts of FSA remains to be elucidated. FSA levels can be influenced by a number of factors, such as the rate of synthesis of glycoconjugates or the level of neuraminidase activity. The reason for the observed increased FSA in CSF remains unknown in patients with cerebellar ataxia [9]. In addition, we could not support this observation in a subpopulation of patients with ataxia (data not shown) in which extensive metabolic workup in a tertiary diagnostic center [18] has been performed. FSA and TSA in CSF have been studied in various neurological disorders. FSA and TSA have been found to be increased in bacterial meningitis. All patients tested with meningitis showed increased levels of TSA as well as CSA (Table 3). We suggest that SA concentrations in CSF may be a good indicator for meningitis. Measuring these metabolites in CSF may be an advantage since culture results are often much delayed.

In a number of other potential applications our method of quantification of SA in CSF may be of use, for instance in patients with brain tumours. Although the number of patient tested was limited our observations are in line with data from the literature [8] and SA in CSF may be a source of biological indicators. Some caution is required to use SA metabolites as a marker for leukaemia, since concentrations found in the CSF of these patients were only marginally increased. Another potential application is the analysis of SA metabolites in CSF in patients with psychiatric illness.

Since our developed HPLC/MS/MS method has a good sensitivity (LOQ = 2.0 μM) it is possible to detect decreased SA concentrations as well. This is special interest for patients with schizophrenia in which the lower SA content in the glycoproteins is correlated with the seriousness of the psychosis [19].

In conclusion, in this paper we describe a method for the quantification of FSA, TSA and CSA in human CSF. The fast, convenient methodology presented here could increase our knowledge about SA concentrations in CSF in various disease states and aid to its diagnosis.

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