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Journal of Chromatography B, 848 (2007) 251–257
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Quantification of free and total sialic acid excretion by LC–MS/MS

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Received 23 June 2006; accepted 20 October 2006
Available online 22 November 2006

Abstract

Background: The main purpose for measuring urinary free sialic acid (FSA) is to diagnose sialic acid (SA) storage diseases. Elevated amounts of conjugated sialic acid (CSA) are observed in several diseases indicating the need to quantify CSA as well. A LC–MS/MS method for quantification of FSA and total sialic acid (TSA) in urine is developed and validated.

Methods: FSA is analyzed directly after filtration of urine samples. For determination of TSA an enzymatic (neuraminidase) and a chemical (acid) hydrolysis were compared. $^{13}C_3$-sialic acid was used as internal standard. LC–MS/MS was performed in negative electrospray ionisation mode with multiple reaction monitoring of transitions m/z 308.2 → 87.0 (SA) and m/z 311.2 → 90.0 ($^{13}C_3$-SA). CSA was calculated by subtracting FSA from TSA.

Results: Limit of detection for FSA and TSA was 0.3 and 1.7 μmol/L, respectively. Limit of quantification for FSA and TSA was 1.0 and 5.0 μmol/L. Intra- and inter-assay variations of FSA were 4.6% and 6.6% ($n = 10$) for FSA and 6.5% and 3.6% ($n = 10$) for TSA. Linearity was tested till 7800 μmol/L ($r^2 = 0.9998$). Values of SA analyzed after neuraminidase- or acid hydrolysis treatment were comparable. Urine samples from patients with inborn errors of SA (related) metabolism were analyzed and compared with age-related reference values.

Conclusion: A method has been developed for routine determination of urinary FSA and TSA. The method is rapid, specific, robust and sensitive. Age-related reference values for FSA, TSA and CSA were determined and improved diagnostic efficacy.

Keywords: Sialic acid; Salla disease; Sialuria; Sialidosis; Galactosialidosis; Hemolytic uremic syndrome

1. Introduction

The term sialic acid (SA) denotes a member of a family of more than 20 compounds derived from neuraminic acid. In humans, the predominant SA is *N*-acetyl-neuraminic acid and circulates in tissues and body fluids in two forms: as free sialic acid (FSA) and as conjugated sialic acid (CSA). SA is found to be a component of oligosaccharides, glycoproteins and sphingolipids called gangliosides. While CSA is involved in cell-functioning and protein–protein interactions, the biological role of FSA is unknown [1–4].

Synthesis of SA begins with glucose, which undergoes several modifications to become activated uridine-diphosphate-*N*-acetylglucosamine. This central metabolite is converted to *N*-acetylmannosamine by an enzyme that is feedback inhibited by cytidine monophosphate-SA. Failure of this feedback mechanism results in overproduction and elevation of cytoplasmatic SA, as seen in the autosomal dominant disorder sialuria [5]. After synthesis, SA is incorporated into glycoconjugates in the Golgi-apparatus. The degradation of sialoglycoconjugates occurs within lysosomes by the enzyme sialidase (α-neuraminidase). A genetic defect of this enzyme leads to accumulation of undegraded sialoglycoconjugates

Abbreviations: FSA, free sialic acid; SA, sialic acid; CSA, conjugated sialic acid; TSA, total sialic acid; ISSD, infantile sialic storage disease; HUS, hemolytic uremic syndrome; TLC, thin layer chromatography; IS, internal standard; α-NAGA, α-*N*-acetylgalactosaminidase; QC's, quality control samples; MS, mass spectrometer; LOD, limit of detection; LOQ, limit of quantification

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doi:10.1016/j.jchromb.2006.10.066

and subsequently to an increase in CSA as found in sialidosis (mucolipidosis type I) [6]. Galactosialidosis results from neuraminidase and β-galactosidase deficiencies due to a primary deficiency in a lysosomal protein: the bifunctional Protective Protein/Cathepsin A [7,8]. SA is transported out of the lysosome via a transporter called sialin [9]. In healthy subjects, this SA transporter ensures the efflux of FSA outside the lysosome. Salla disease and infantile sialic storage disease (ISSD) are lysosomal storage diseases caused by this defective SA transporter in lysosomal membrane. In summary, while several inborn errors of SA metabolism are associated with an increased FSA (sialuria, Salla disease and ISSD) others are associated with an increased CSA (sialidosis and galactosialidosis).

Since SA is rapidly eliminated via the kidney, urine can be used to screen for inborn errors of SA metabolism. Subjects with a disorder in SA metabolism present a great variability in clinical phenotype (even within one specific defect) [10–15]. As a consequence, there is need for a reliable, routine based and rapid (high throughput) method to quantify FSA and TSA. During the past years, several analytical methods based on colorimetry [16,17], ^{1}H-NMR spectroscopy [18,19], thin layer chromatography (TLC) [20] and ultraviolet-enzymatic assay [21] have been developed for quantification of urinary FSA and/or TSA. Although, intra-assay, inter-assay and recovery improved, these assays were not suitable for high throughput screening. Recently, a rapid method based on tandem mass spectrometry was described for quantification of FSA [22]. However, disorders characterized by increased CSA will be missed by measuring only FSA.

Quantification of urinary SA is not only restricted to the field of inborn errors of metabolism. TSA excretion is also increased in diabetes mellitus type II [23], and in subjects with renal failure [24]. In addition, TSA excretion can be used for diagnosis and follow-up of patients with bladder tumors [25,26]. Another abnormality in SA metabolism has been observed in patients with hemolytic uremic syndrome (HUS). This disease of nonimmune hemolytic anemia, thrombocytopenia, and renal failure is caused by platelet thrombi in the microcirculation of the kidney and other organs [27,28]. The presence of bacterial neuraminidase activity in plasma of patients with *S.pneumoniae*-associated HUS explains the increased FSA excretion.

In the present study, a new method was developed enabling quantification of FSA, TSA and CSA with minimal sample preparation and a total runtime of 6 min.

2. Material and methods

2.1. Reagents

Formic acid, sulphuric acid and potassium chloride were purchased from Merck (Darmstadt, Germany). *N*-acetylneuraminic acid (sialic acid, SA), 1,2,3-$^{13}C_3$ *N*-acetyl-D-neuraminic acid (internal standard, IS), ammonium formate, potassium phosphate, sialyllactose from bovine colostrum and neuraminidase type V from Clostridium perfringers were obtained from Sigma–Aldrich Chemie (Steinheim, Germany). Acetonitrile was obtained from Rathburn Chemicals (Walkerburn, Scotland).

2.2. Urine samples

Urine samples were obtained from 589 individuals (ages 0–70 years), who were examined in our university hospital for reasons other than inborn errors of SA metabolism. Urine was collected without any restriction and stored at −20 °C or analyzed immediately. Urine samples of eleven patients with a defect affecting SA metabolism were obtained. These samples include Salla disease ($n=2$), sialuria ($n=2$), sialidosis ($n=2$), galactosialidosis ($n=1$), aspartylglucosaminuria ($n=1$), α-*N*-acetylgalactosaminidase (α-NAGA) deficiency ($n=1$) and (HUS, $n=2$). In addition, two patients were included, who had an abnormal sialyloligosaccharide pattern on TLC, in which the primary defect is still unknown.

Creatinine concentrations in urine samples were measured with standard laboratory methods on a Vitros 950 (Johnson & Johnson, Clinical Diagnostics, NY, USA).

2.3. Sample preparation

2.3.1. Free sialic acid (FSA)

For analysis of FSA, 25 μL IS (0.85 mmol/L) was added to 125 μL urine in a 96-wells collection plate (Waters, Massachusetts, USA). After mixing, urine was subsequently filtered using a 96-wells plate (0.2 μm protein precipitation plate, Sirocco, Waters, Massachusetts, USA). An eight points calibration curve with concentrations of 8, 34, 68, 136, 272, 340, 508 and 676 μmol/L SA were prepared in milli-Q water.

2.3.2. Total sialic acid (TSA)

For analysis of TSA, 25 μL IS (0.85 mmol/L) was added to 25 μL urine followed by addition of 100 μL sulphuric acid (63 mmol/L) and placed at 80 °C for 1 h to obtain all CSA in free form. Samples were prepared in glass HPLC vials (Waters, Massachusetts, USA).

2.3.3. Quality control samples (QC's)

Urine from healthy subjects was pooled and used for preparation of QC's. For quantification of FSA, urine was spiked with SA (0.85 mmol/L) to obtain a concentration of 85 and 255 μmol/L (QC 1 and QC 2), respectively. QC's were stored at −20 °C until analysis.

For quantification of TSA, urine was spiked with sialyllactose. Urinary diluted sialyllactose (100 mg/L) (QC 3) and sialyllactose (1 g/L in milli-Q water) (QC 4) were used as QC's and stored at −20 °C. Acid hydrolysis with sulphuric acid obtains a concentration of approximately 165 μmol/L SA (QC 3) and 935 μmol/L SA (QC 4), respectively.

2.4. Instruments

A Quattro Ultima triple quadrupole mass spectrometer (tandem MS) (tandem MS) (Waters, Manchester, UK) interfaced with an electron spray ionisation source and equipped with an Alliance 2795 HPLC (Waters, Etten-Leur, The Netherlands) was used. Masslynx software (Version 4.0, SP 4, Waters, Manchester,

Table 1
Linear gradient profile for the chromatographic separation of SA (FSA and TSA) in urine

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

Solvent A (0.05 M ammonium formate, pH 3.0), solvent B (100% acetonitrile).

UK) was used for instrument control, data acquisition and data processing.

2.5. Chromatographic and mass spectrometric conditions

Chromatographic separation was performed using an Atlantis dC18 guard column (3 μm, 3.0 mm × 20 mm) and an Atlantis dC18 analytical column (3 μm, 3.0 mm × 100 mm)(Waters, Massachusetts, USA). The analytical column temperature was kept at 30 °C and the injection volume was 10 μL. A two min linear gradient at a flow rate of 0.3 mL/min between solvent A (0.05 M ammonium formate, pH = 3.0) and solvent B (100% acetonitrile) was used. After 0.5 min at 100% B, the system returns in 0.3 min to 100% A (Table 1). For column equilibration a total cycle time of 6 min was needed. Retention time of SA and IS was 2.6 min, including a delay time of approximately, 1.5 min. To reduce contamination of cone and ion source, a solvent delay-diverting valve was interpolated between the HPLC and MS system and was switched to the MS system between 2 and 3.7 min.

The MS was operated in negative electron spray ionisation mode. A capillary voltage of 3.2 kV and a cone voltage of 35 V were used. Source temperature was 100 °C and desolvation temperature was 300 °C. Ultra high purity nitrogen was used for cone gas (150 L/h), desolvation gas (500 L/h) and nebulising gas (100 L/h). For collision induced dissociation (CID) a collision energy of 12 eV and ultra high purity argon at 0.003 mbar was used. Negatively single charged ions $[M-H]^-$ of SA (*m/z* 308.2, Fig. 1A) and IS (*m/z* 311.2, Fig. 2A) were selected as parent ions for CID. The daughter ion *m/z* 87.0 was the most abundant for SA, while the IS resulted in a dominant daughter ion of *m/z* 90.0 (Fig. 1B and Fig. 2B). For multiple reaction monitoring, the transitions *m/z* 308.2 → 87.0 and *m/z* 311.2 → 90.0 for SA and IS were measured.

2.6. Method validation

QC 1–4 were used for method validation (intra- and inter-assay variation, limit of detection (LOD) and limit of quantification (LOQ). LOD is the result of three times signal to noise ratio (S/N), while LOQ is calculated as ten times the S/N ratio.

2.6.1. Quantification of FSA

Low range linearity of FSA was tested using an eight points calibration curve with concentrations of 8, 34, 68, 136, 272, 340, 508 and 676 μmol/L SA, respectively. High range linearity

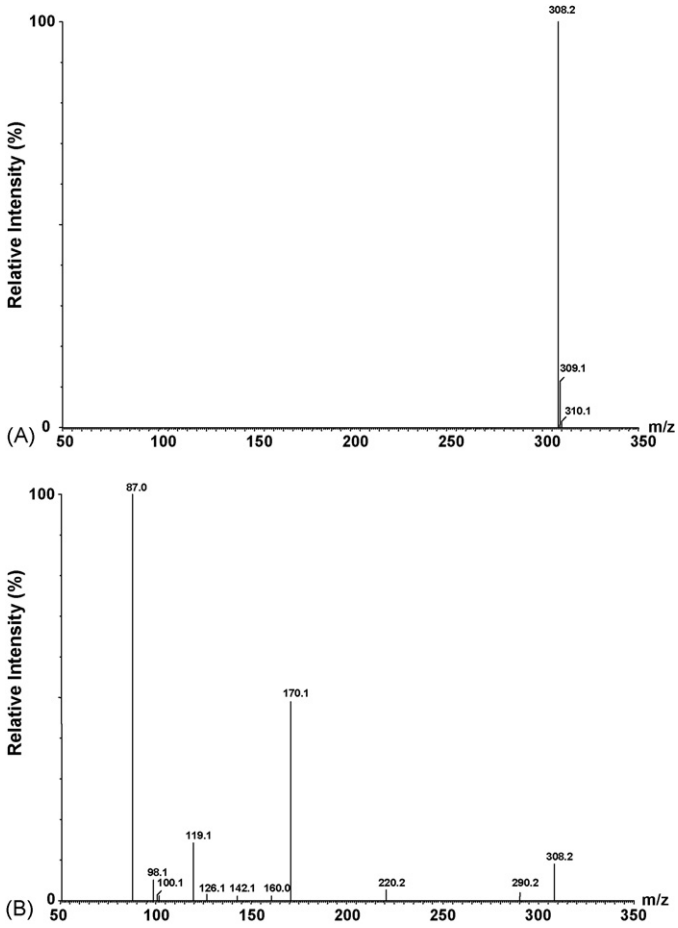

Fig. 1. (A) Negative single charged ions $[M-H]^-$ spectra of sialic acid (SA) (*m/z* 308.2); (B) daughter scan of sialic acid (SA) with *m/z* 308.2 as parent ion.

of FSA was tested using an eight points calibration curve with concentrations of 935, 1870, 2805, 3740, 4676, 5612, 6546 and 7793 SA μmol/L, respectively. All calibrators were prepared in milli-Q water.

2.6.2. Conversion of CSA into FSA for quantification of TSA

QC 3 and QC 4 were used to test the effect of variations in temperature (60, 70, 80, 90 °C) and incubation time (30, 60 and 90 min) on the acid hydrolysis with sulphuric acid. In addition, QC 3 and QC 4 were treated with different ratio's of neuraminidase (0.1 mg/mL in 5 mmol/L phosphate buffer pH 6 containing 50 mM potassium chloride) varying from 1:1 to 100:1 v/v urine:neuraminidase and incubated for 16 h at 37 °C. The conversion of CSA into FSA was tested using TLC as described in [20].

2.7. Calculation and statistical analysis

CSA is the result of subtracting FSA from TSA. Control samples were categorized by age and for each group mean and 95% reference interval (±2SD) were determined. Normal concentrations were defined as mean ± 2SD. Correlations were performed by linear regression analysis (SigmaStat 3.0).

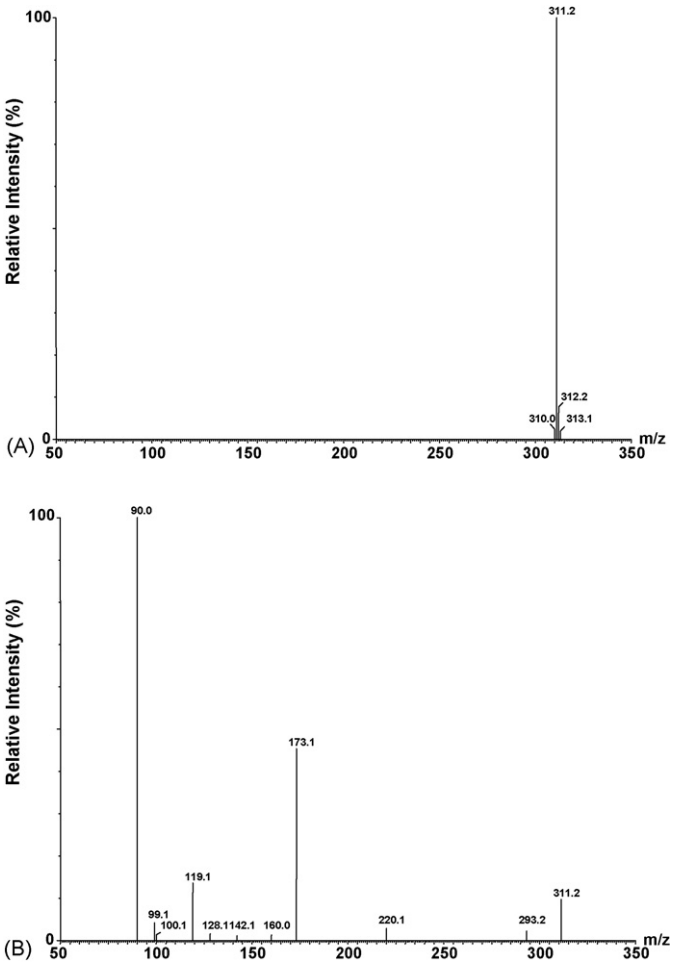

Fig. 2. (A) Negative single charged ions $[M-H]^-$ spectra of 1,2,3-$^{13}C_3$ *N*-acetyl-D-neuraminic acid (internal standard, IS) (*m/z* 311.2); (B) daughter scan of 1,2,3-$^{13}C_3$ *N*-acetyl-D-neuraminic acid (internal standard, IS) with *m/z* 311.2 as parent ion.

3. Results

3.1. Quantification of FSA

Intra- and inter-assay variation in urine was determined by analyzing QC 1 and QC 2. Intra- and inter-assay variation was 4.6% and 6.6%, respectively ($n = 10$). LOD was 0.3 μmol/L and LOQ was 1.1 μmol/L. The calibration curve of FSA was linear over a wide range (tested till 7800 μmol/L, $r^2 = 0.9998$). All measured concentrations (in μmol/L) were within the linear range of the calibration curve. Reference values for FSA are shown in Table 2a. Mean urinary FSA excretion decreases from 45.0 ± 37.8 mmol/mol creatinine (0–0.5 years) to 7.4 ± 5.8 mmol/mol creatinine (>20 years).

3.2. Quantification of TSA

Intra- and inter-assay variation in urine was determined by analyzing QC 3 and QC 4 after acid hydrolysis with sulphuric acid. Intra- and inter-assay variation was 6.5 and 3.6%, respectively ($n = 10$). LOD was 1.7 μmol/L and LOQ was 5.6 μmol/L. All measured concentrations (in μmol/L) were within the linear range of the calibration curve. Reference values for TSA are shown in Table 2b. Mean urinary TSA excretion decreases from 156.4 ± 124.0 mmol/mol creatinine (0–0.5 years) to 31.0 ± 25.4 mmol/mol creatinine (>20 years). Reference values for CSA are calculated by subtracting FSA from TSA and are shown in Table 2c. Mean calculated urinary CSA excretion decreases from 111.5 ± 97.2 mmol/mol creatinine (0–0.5 years) to 23.6 ± 25.6 mmol/mol creatinine (>20 years).

Table 2a
Free sialic acid (FSA) excretion (expressed as mmol/mol creatinine) in urine samples from controls categorized by age

Age (year)	FSA excretion (mmol/mol creatinine)		
	Mean	2SD	*n*
0–0.5	45.0	37.8	125
0.5–1	32.2	28.2	49
1–2	28.7	21.0	61
2–3	27.1	23.2	52
3–5	21.2	13.6	94
5–10	15.2	11.8	108
10–20	9.7	8.0	67
>20	7.4	5.8	33

Data are presented as mean ± 2SD.

Table 2b
Total sialic acid (TSA) excretion (expressed as mmol/mol creatinine) in urine samples from controls categorized by age

Age (year)	TSA excretion (mmol/mol creatinine)		
	Mean	2SD	*n*
0–0.5	156.4	124.0	125
0.5–1	100.5	68.4	49
1–2	90.2	56.8	61
2–3	83.3	72.0	52
3–5	63.7	36.8	94
5–10	51.1	36.8	108
10–20	38.8	34.8	67
>20	31.0	25.4	33

Data are presented as mean ± 2SD.

Table 2c
Conjugated sialic acid (CSA) excretion (expressed as mmol/mol creatinine) in urine samples from controls categorized by age

Age (year)	Calculated CSA excretion (mmol/mol creatinine)		
	Mean	2SD	*n*
0–0.5	111.5	97.2	125
0.5–1	68.3	45.0	49
1–2	61.5	42.4	61
2–3	56.1	50.4	52
3–5	42.5	26.2	94
5–10	35.9	25.8	108
10–20	29.1	29.6	67
>20	23.6	25.6	33

CSA is calculated by subtracting FSA from TSA. Data are presented as mean ± 2SD.

Table 3
Urinary free sialic acid (FSA), total sialic acid (TSA) and calculated conjugated sialic acid (CSA) concentration (expressed as mmol/mol creatinine) in samples from patients with a defect affecting sialic acid (SA) metabolism ($n = 10$)

Sample	Disorder	Age (year)	FSA, TSA and CSA excretion (mmol/mol creatinine)					
			FSA		TSA		CSA	
1	Salla disease	12	49	↑	74	↑	25	N
2[a]	Salla disease	7	285	↑	386	↑	101	↑
2[b]	Salla disease	7	176	↑	236	↑	60	N
3[a]	Sialuria	0.4	508	↑	574	↑	66	N
3[b]	Sialuria	5	362	↑	406	↑	44	N
4	Sialuria	?	8653	↑	9478	↑	825	↑
5	Sialidosis	5	9	N	255	↑	246	↑
6	Sialidosis	48	7	N	47	N	40	N
7	Galactosialidosis	41	7	N	91	↑	84	↑
8[a]	Aspartylglucosaminuria	5	11	N	115	↑	105	↑
8[b]	Aspartylglucosaminuria	5	12	N	108	↑	97	↑
9	α-NAGA deficiency	4	12	N	127	↑	115	↑
10[a]	HUS	0.6	703	↑	832	↑	130	↑
10[b]	HUS after 7 days	0.6	49	N	191	↑	143	↑
11[a]	HUS	0.4	408	↑	708	↑	300	↑
11[b]	HUS after 18 days	0.4	2	N	65	N	63	N
12[a]	Abnormal sialyloligosaccharidosis	8	11	N	179	↑	168	↑
12[b]	Abnormal sialyloligosaccharidosis	9	<0.3	–	106	↑	106	↑
13	Abnormal sialyloligosaccharidosis	9	39	↑	478	↑	439	↑

In addition, data from two subjects with an abnormal sialyloligosaccharide (primary defect unknown) are shown. [a/b]samples from one patient at different time points; ↑, increased regarding to age-related reference values; N, normal regarding to age-related reference values; α-NAGA deficiency, α-*N*-acetylgalactosaminidase deficiency; HUS, hemolytic uremic syndrome.

The effect of temperature and incubation time upon treatment with sulphuric acid of QC 4 is shown in Fig. 3. From this figure it is clear that the conversion of CSA into FSA at 60 °C (30, 60 and 90 min) and 70 °C (30 min) is incomplete. Although 60 min incubation at 70 °C and 30 min at 80 °C was sufficient to obtain all CSA in a free form, a 60 min incubation period at 80 °C was used for additional experiments. No significant differences in TSA concentration were observed during a 60 min incubation period at 70, 80 or 90 °C. Although no change in peak ratio for FSA/internal standard is observed, a 25% reduction in peak area was found for both FSA and IS at 90 °C compared to 70 °C. Treatment with neuraminidase and sulphuric acid of QC 4 and a urine specimen of a patient with sialidosis (sample 5, Table 3) were checked by TLC-plates (Fig. 4). Both samples showed complete conversion of CSA into FSA, implying that both the enzymatic- and chemical treatment can be used for complete conversion of CSA into FSA. No significant variation was observed between both methods ($n = 6$, variation was below the inter day variation of 6.5%).

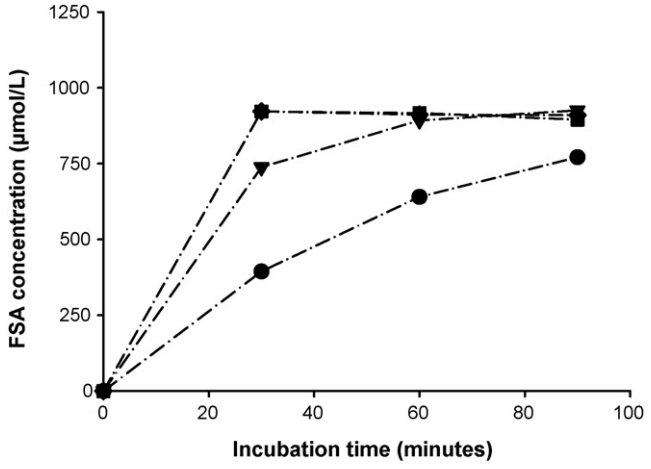

Fig. 3. The effect of temperature variation and incubation time on the acid hydrolysis with sulphuric acid of QC 4 (1 g/L siallyllactose in milli-Q water). The symbols represent the incubation temperature (●), 60 °C; (▼), 70 °C; (■:), 80 °C and (♦), 90 °C.

3.3. Patient samples

Urinary FSA, TSA and calculated CSA concentrations for patients with a defect affecting SA metabolism are shown in Table 3. In samples 1, 2[a], 2[b], 3[a], 3[b] and 4 both urinary excretion of FSA and TSA were above the 95 reference limit (compared with controls in the corresponding age group). Strikingly, in urine sample 2[a] (Salla disease), CSA excretion was elevated, while in a second urine specimen (sample 2[b]), a normal CSA excretion was observed. In urine samples from three patients with a disorder in CSA degradation (samples 5, 7 and 9) urinary excretion of CSA and TSA was above the 95 reference limit (compared with controls in the corresponding age group). Two urine samples from a patient with aspartylglucosaminuria (samples 8[a] and 8[b]) also revealed increased CSA and TSA excretions. Surprisingly, in one patient with sialidosis, urinary excretions of both CSA and TSA were normal (sample 6).

In two patients with HUS (10[a] and 11[a]), urinary excretions of FSA, TSA and CSA were increased. After a 7 days' recovery period (sample 10[b]), urinary excretion of FSA returned to normal, while TSA and CSA excretions were still elevated. The other patient (sample 11[b]) showed complete recovery from

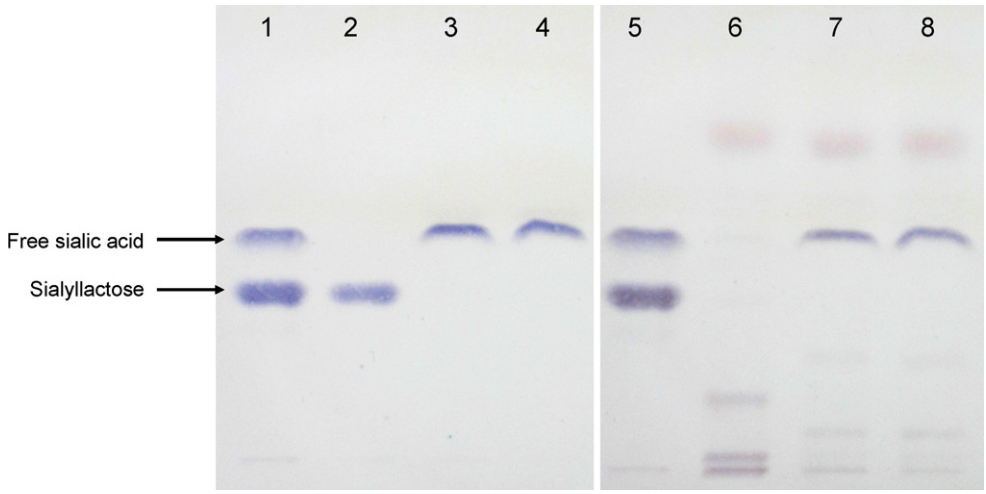

Fig. 4. Thin layer chromatography (TLC) of QC 4 and sample 5. TLC plates are coloured as described in [34]. Lane 1: standard containing both free sialic acid (1 g/L) and sialyllactose (5 g/L); lane 2: QC 4 (1 g/L siallyllactose in milli-Q water); lane 3: QC 4 treated with neuraminidase (0.1 mg/mL, 25:1 v/v urine:neuraminidase) incubated 16 h at 37 °C; lane 4: QC 4 treated with sulphuric acid (63 mM) for 60 min at 80 °C; lane 5: standard containing both free sialic acid (1 g/L) and sialyllactose (5 g/L); lane 6: urine specimen of patient with sialidosis (sample 5, Table 3); lane 7: neuraminidase treatment (0.1 mg/mL, 25:1 v/v urine:neuraminidase) of urine specimen incubated 16 h at 37 °C of patient with sialidosis; lane 8: sulphuric acid treatment (63 mM) for 60 min at 80 °C of urine specimen of patient with sialidosis.

HUS after 18 days, which is clearly expressed in normal excretions of FSA, TSA and CSA. Two brothers with an abnormal sialyloligosaccharide pattern on TLC revealed increased CSA excretions (samples 12^a, 12^b and 13). FSA in sample 12^a was normal, while in sample 12^b FSA was below LOQ. In sample 13 FSA excretion was elevated.

4. Discussion

Affected subjects with inborn errors in SA metabolism present a clinical phenotype varying from very mild to severe [10–15,29,30]. This implies that screening of SA excretion should be routinely performed. In this study, we describe for the first time a high throughput method for quantifying FSA, TSA and CSA.

Until now, the key to identify inborn errors in SA metabolism is determination of FSA and sialyloligosaccharides in urine. In most laboratories, TLC related techniques are still the method of choice, despite disadvantages such as low sample throughput and quantification problems. However, as an alternative, the ultraviolet-enzymatic assay described by Crook et al. [21] can be used for quantification of FSA and CSA, but this assay is not appropriate for high sample throughput. The introduction of LC–MS/MS involved new possibilities for rapid and reliable quantification. Recently, a method for quantification of FSA, based on LC–MS/MS, was described [22]. Using this approach, patients with increased concentrations of CSA (e.g., (galacto)sialidosis, aspartylglucosaminuria and α-NAGA deficiency) will be missed. To overcome this problem, a method was developed that quantifies both TSA and CSA.

In the present study, 50 mM ammonium formate buffer was used instead of aqueous ammonia (0.114 g/L ≈ 5 mM) [22] improving the ionization yield, resulting in a 10-fold lower LOD. The sensitivity gain makes this method, in potential, suitable for measurement of FSA concentrations in, e.g. cell culture systems. In addition, dilution of urine to a certain level of creatinine as described in [22] to reduce ion-suppression was no longer necessary. Linearity of the method was tested till 7800 μmol/L ($r^2 = 0.9998$). However, sample 4 (Table 3) was analyzed at three different concentrations (undiluted, 10- and 50-fold diluted, respectively) and after correction, a FSA concentration of 40000 μmol/L was found (8653 mmol/mol creatinine). This implies that linearity of the current method can be expanded to 40000 μmol/L.

Urinary FSA quantification was compared with two methods, the Warren method [16,17] and the method recently described by Valianpour [22]. As expected, our method correlated well with the method described by Valianpour ($r^2 = 0.981$, $n = 22$), but correlated poorly with the colorimetric Warren method ($r^2 = 0.616$, $n = 6$). This discrepancy can be explained by the fact that the Warren method is more sensitive for interferences [3] in comparison with the specific tandem MS based methods. For quantification of TSA, CSA must be converted into FSA and CSA is calculated by subtracting FSA from TSA. From the present study it is clear, that both the enzymatic- and chemical treatment (acid hydrolysis with sulphuric acid) can be used for complete conversion of CSA into FSA. The chemical approach is preferred (time-profit and low costs).

Urinary FSA, TSA and calculated CSA concentrations are age-related and normal reference values were determined in 589 control samples and categorized by age. The applicability of reference values was evaluated by analyzing urinary FSA and TSA in eleven patients with a defect affecting SA metabolism. In patients with Salla disease (sample 1, 2^a, and 2^b, Table 3) and sialuria (sample 3^a, 3^b and 4, Table 3), urinary FSA excretion was increased 5- to 27-fold compared with age-related reference values, indicating that patients with Salla disease or sialuria can easily be detected with the present method. In patients with sialidosis (sample 5, Table 3), galactosialidosis (sample 7, Table 3), aspartylglucosaminuria (sample 8^a and 8^b, Table 3)

and α-NAGA deficiency (sample 9, Table 3) increased CSA and TSA excretions were found. In a urine specimen from a 48 year-old individual (sample 6, Table 3) with a proven diagnosis of sialidosis (α-neuraminidase deficiency), normal SA excretions were observed. Such observation is in agreement with other reports [31,32] showing that patients with sialidosis can excrete normal amounts of SA in urine. Interestingly, analysis of urinary specimens of two brothers (sample 12^a, 12^b and 13) resulted in increased CSA excretions concomitantly with an increased excretion of FSA only in sample 13. In sample 12^a, FSA excretion was normal, while in sample 12^b FSA excretion was below the LOD. Unfortunately, until now the metabolic defect in these two siblings is still not clear.

Neuraminidase produced and released by *S.pneumoniae* in patients with HUS is capable of removing SA residues from glycoconjugates. This is reflected by an increased concentration of FSA (sample 10^a and 11^a, Table 3). The reason for the observed increased CSA excretion in HUS patients is unknown. In its typical presentation, HUS manifests as an acute disease and 80% to 90% of cases recover without sequelae, either spontaneously (as in most cases of childhood HUS) or after plasma infusion or exchange (as in adult or severe forms of HUS) [28,33]. In sample 10^b lower FSA and CSA concentrations are found in comparison with urine specimen 10^a, indicating that the patients condition is improved. Urinary excretion of FSA and TSA in sample 11^b normalized indicating complete recovery.

In summary, we have developed a method for routine determination of FSA and TSA excretion. The method is rapid, specific, robust and sensitive. A semi-automated sample preparation procedure in combination with a runtime of 6 min makes this method suitable for high throughput analysis. Age-related reference values for FSA, TSA and CSA were determined and improved the diagnostic efficacy.

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