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Analysis of Glycosaminoglycans in Cerebrospinal Fluid from Patients with Mucopolysaccharidoses by Isotope-Dilution Ultra-Performance Liquid Chromatography–Tandem Mass Spectrometry

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BACKGROUND: New therapies for the treatment of mucopolysaccharidoses that target the brain, including intrathecal enzyme replacement, are being explored. Quantitative analysis of the glycosaminoglycans (GAGs) that accumulate in these disorders is required to assess the disease burden and monitor the effect of therapy in affected patients. Because current methods lack the required limit of quantification and specificity to analyze GAGs in small volumes of cerebrospinal fluid (CSF), we developed a method based on ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS).

METHODS: Samples of CSF (25 μL) were evaporated to dryness and subjected to methanolysis. The GAGs were degraded to uronic acid-*N*-acetylhexosamine dimers and mixed with internal standards derived from deuteriomethanolysis of GAG standards. Specific dimers derived from heparan, dermatan and chondroitin sulfates (HS, DS and CS) were separated by UPLC and analyzed by electrospray ionization MS/MS using selected reaction monitoring for each targeted GAG product and its corresponding internal standard.

RESULTS: CSF from control pediatric subjects (n = 22) contained <0.38 mg/L HS, 0.26 mg/L DS, and 2.8 mg/L CS, whereas CSF from patients with Hurler syndrome (n = 7) contained concentrations of DS and HS that were at least 6-fold greater than the upper control limits. These concentrations were reduced by 17.5% to 82.5% after allogeneic transplantation and treatment with intrathecal and intravenous enzyme replacement therapy.

CONCLUSIONS: The method described here has potential value in monitoring patients with mucopolysaccharidoses receiving treatment targeted to the brain.

Hurler syndrome (OMIM 607014) *(1)* and Hunter disease (OMIM 309900) *(2, 3)*, also referred to as mucopolysaccharidoses types I and II (MPSIH and MPS II),[4] are caused by deficiency of the enzymes α-L-iduronidase (EC 3.2.1.76) and iduronate-2-sulfatase (EC 3.1.6.13), respectively. Hurler syndrome is an autosomal recessive disorder; Hunter disease is X-linked. These conditions are characterized by the progressive accumulation of specific glycosaminoglycans (GAGs) in all cells, leading to severe physical and neurological impairment in most affected patients *(4)*. Treatment has proven difficult and has met with limited success. Allogeneic hematopoietic stem cell transplantation (HCT) has improved life expectancy, stabilized the neurologic deterioration observed in untreated patients, and led to improvements in many of the complications with Hurler syndrome, but has not alleviated the orthopedic manifestations of the disease, nor has it led to improvement in the valvular changes observed in the heart *(5)*. Enzyme replacement therapy (ERT) has also been able to stabilize the visceral manifestations of both MPSIH and MPSII, but its lack of ability to cross the blood–brain barrier (BBB) has prevented successful treatment of the neurologic aspects of these diseases through intravenous infusions of enzyme *(4, 6)*. Recently, the delivery of these enzymes through an intra-

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[4] Nonstandard abbreviations: MPSIH, mucopolysaccharidosis type I (Hurler syndrome); MPS II, mucopolysaccharidosis type II (Hunter disease); GAG, glycosaminoglycan; HCT, hematopoietic stem cell transplantation; ERT, enzyme replacement therapy; BBB, blood–brain barrier; LSD, lysosomal storage disease; ESI, electrospray ionization; CSF, cerebrospinal fluid; DS, dermatan sulfate; HS, heparan sulfate; CS, chondroitin sulfate; UPLC, ultra-performance liquid chromatography; IT, intrathecal; IV, intravenous; IRB, institutional review board; SRM, selected reaction monitoring; *m/z*, mass-to-charge ratio; CID, collision-induced dissociation.

thecal route has been explored as a means of improving neurological outcomes *(7–13)*.

The availability of effective therapy coupled with improved diagnostic technology is driving interest in screening newborns for MPSIH, MPSII, and other lysosomal storage diseases (LSDs) *(14–17)*. The impact of early diagnosis on these and other conditions is as yet uncertain, although the positive identification of affected patients at birth will enable the consideration of treatment options at an earlier stage than has been possible previously.

One of the considerations in determining the effectiveness of interventions is the assessment of the burden of disease at baseline and during therapy. At the time of diagnosis, the accumulation of GAGs may already be clinically significant. The GAGs are complex proteoglycans that are difficult to quantify in the body. In both MPSIH and MPSII, there is primarily an accumulation of dermatan and heparan sulfates, resulting in their enhanced excretion in the urine. Various methods have been developed to identify and quantify GAGs in urine, many of them targeted to uronic acid or degradation products containing this moiety *(18–23)*. Recently, a new method applicable to urine has been reported that is based on liquid chromatography–tandem mass spectrometry with electrospray ionization (LC-ESI-MS/MS) *(24)*. To date, there have been no reports of GAG quantification analysis in the cerebrospinal fluid (CSF) of patients with LSDs by stable isotope-dilution LC-MS/MS. We report here a procedure to determine dermatan sulfate (DS), heparan sulfate (HS), and chondroitin sulfate (CS) concentrations in small volumes (25 μL) of CSF using ultra-performance liquid chromatography (UPLC)-ESI-MS/MS. The method targets uronic acid–hexosamine dimers produced by methanolysis of the GAGs and uses isotope-labeled dimers derived from DS, HS, and CS as pseudo–internal standards. Our objectives were to establish pediatric control values in CSF for CS, DS, and HS and to determine the concentrations of these biomarkers in patients with MPSIH, both before and after receiving HCT in combination with intrathecal (IT) and intravenous (IV) ERT.

Materials and Methods

REAGENTS

Dermatan, chondroitin, and heparan sulfates were obtained from Sigma Aldrich. The reagents 3 mol/L HCl in methanol, deuterium (^{2}H)-labeled methanol and acetyl chloride were also from Sigma Aldrich. We prepared 2 mol/L ^{2}HCl in $C^2H_3O^2H$ by dropwise addition of CH_3COCl (80 μL) to $C^2H_3O^2H$ (500 μL) in an ice bath under dry conditions. Acetonitrile (MeCN) was from EMD Chemicals, ammonium acetate was from Sigma Aldrich, and deionized water was prepared in-house.

PATIENTS AND CONTROLS

We obtained CSF by spinal tap from 22 pediatric patients suspected of having inherited metabolic conditions that cause seizures, particularly nonketotic hyperglycinemia, but in whom no diagnosis was made and CSF amino acid concentrations were within normal limits. These discarded and deidentified samples were used as controls to establish pediatric concentrations of GAGs in CSF, under an institutional review board (IRB)-approved protocol. In addition, we obtained CSF from 7 patients with a confirmed diagnosis of Hurler syndrome, as part of their evaluations for pretransplantation on an IRB-approved protocol that provides consent for use of CSF in biomarker research (Table 2). We also obtained samples from 4 of these patients 100 days after HCT transplantation and IT-ERT plus IV-ERT (clinicaltrials.gov identifiers NCT00638547 and NCT00176891).

INTERNAL STANDARDS

Internal standards were prepared by deuteriomethanolysis of DS and HS. A quantity of 0.3 mg of each standard was incubated with 0.3 mL of the deuteriomethanolysis reagent, freshly prepared as described above, for 75 min at 65 °C. The solvent was removed by evaporation under nitrogen. After solvent evaporation, the residue was reconstituted in deionized water and stored at −20 °C. Working internal standard was a mixture of [$^2H_6$]DS and [$^2H_6$]HS dimers in aqueous solution, sufficient for analysis of a sample batch (40 samples/batch), stored at 4 °C for up to 1 week. The [$^2H_6$]DS dimer was used to quantify DS, and [$^2H_6$]HS dimer was used to quantify HS. There was also a signal corresponding to [$^2H_6$]CS dimer in the internal standard mixture, which was available to quantify CS.

CALIBRATION AND QC SAMPLES

Calibrators and QC materials were prepared from pooled control CSF (approximately 20 mL) that was subdivided into aliquots of 25 μL then spiked with 0.2, 0.4, 1.0, 2.0, 4.0, 8.0, or 12 mg/L CS, DS, or HS standard. The "low" and "high" QCs were 25 μL pooled control CSF that contained 0.8 and 10 mg/L of added DS or HS, respectively. We added a fixed amount of the internal standard solution (100 μL) to the methanolysate derived from each calibrator before analysis by UPLC-MS/MS. After analysis, we plotted the ratio of signals corresponding to each of the dimers derived from DS, CS, and HS to their respective internal standard signals against the added concentration of the GAG standards. We used the slope of each curve, de-

termined by linear regression, to quantify each biomarker in unknown CSF samples.

SAMPLE PREPARATION AND ANALYSIS BY UPLC-MS/MS

Aliquots of CSF (25 μL) were pipetted into 1.5-mL borosilicate vials and evaporated to dryness under nitrogen. The residues were incubated with anhydrous 3 mol/L HCl-methanol (200 μL) at 65 °C for 75 min, and again evaporated to dryness. Each residue was vortex-mixed with 100 μL internal standard solution and 100 μL MeCN. The solutions were filtered under centrifugation though a 0.2-μm membrane, transferred into an injection vial, dried under nitrogen, and reconstituted in the mobile phase [10 mmol/L NH_4OAc in MeCN:H_2O (90:10 (vol/vol), buffer A]. The samples (5 μL) were injected sequentially into a Xevo-TQ™ mass spectrometer equipped with an Acquity UPLC® system with autosampler (Waters Corp.). The Acquity UPLC® BEH Amide column (1.7 μm, 2.1 by 50 mm; Waters Corp.) was heated to 30 °C under a flow rate of 400 μL/min with a programmed linear gradient from 100% buffer A to 75:25 (vol/vol) buffer A:buffer B [10 mmol/L NH_4OAc in MeCN:H_2O 10:90 (vol/vol)] over 4 min, then returned to 100% A over 1.5 min. The column was equilibrated for a further 0.5 min. The column eluate was directly infused into the mass spectrometer. The capillary voltage was 3.5 kV; cone voltage was 20 V for CS and DS and 50 V for HS. The source block and desolvation temperatures were 150 °C and 500 °C, respectively. The collision energy was 9 eV for CS and DS and 29 eV for HS. Data were acquired by selected reaction monitoring (SRM) using the protonated molecular ion transition mass-to-charge ratio (*m*/*z*) 426 → 236 for dimers derived from CS and DS, plus the sodiated molecular ion transition *m*/*z* 406 → 245 for the HS dimer. Also monitored were the transitions *m*/*z* 432 → 239, corresponding to [$^2H_6$]CS and [$^2H_6$]DS dimers, plus *m*/*z* 412 → 251 for the [$^2H_6$]HS dimer.

Results

METHANOLYSIS OF CS, DS, AND HS

The primary products from the methanolysis of CS and DS were presumed to be uronic or iduronic acid-*N*-acetylhexosamine dimers, methylated at both the carboxylic acid and terminal hemiacetal function *(24)*. These dimers are derived from repeating units within the GAG polymer chain that become desulfated and cleaved during methanolysis, and are structurally related to those produced by enzymatic degradation reported previously *(21)*. Evidence for these structures was provided by their mass spectra (Fig. 1A), showing predominantly the expected protonated and sodiated molecular species at *m*/*z* 426 and 448, respectively. The

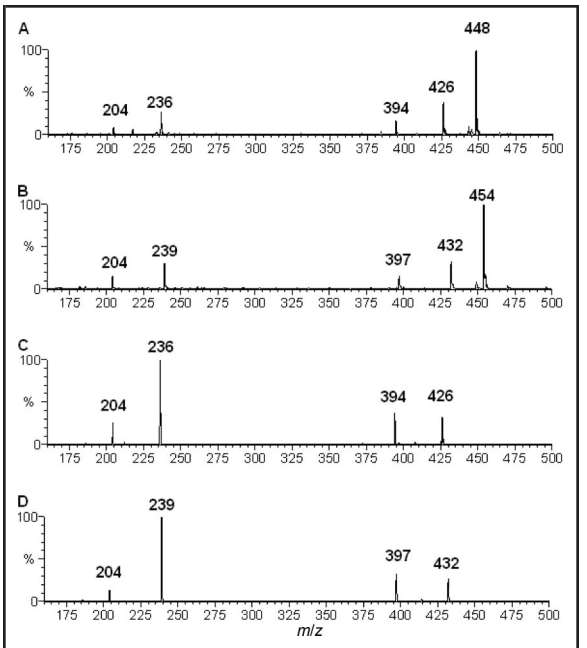

Fig. 1. (A), Full mass spectrum from methanolysate of DS showing predominantly the protonated (*m*/*z* 426) and sodiated (*m*/*z* 448) dimer.

(B), Mass spectrum of deuteriomethanolysis product of DS showing incorporation of +6 atoms of deuterium. (C), MS/MS spectrum of *m*/*z* 426 with major fragment at *m*/*z* 236. (D), MS/MS spectrum of *m*/*z* 432 with fragment at *m*/*z* 239.

presence of ions at *m*/*z* 204, 236, and 394 in these spectra was consistent with the production of *N*-acetylhexosamine monomer combined with fragmentation of the dimer in the ion source. The primary fragmentation of the protonated dimers under collision-induced dissociation (CID) in the tandem mass spectrometer yielded *m*/*z* 236 (Fig. 1C) and supported cleavage with charge retention on the hexosamine unit. The relative abundance of the dimers and monomers depended on the methanolysis conditions. The DS dimer concentration in the methanolysate was maximized after 75 min at 65 °C and diminished thereafter in favor of the monomer. The unique signal for the HS dimer continued to increase, but was adequate for detection at 75 min (see Supplemental Fig. 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol57/issue7).

Further evidence for the dimeric structures arose from the mass spectrum of the product of deuteriomethanolysis of chondroitin sulfate. The spectrum showed a shift of +6 Da for both the protonated and sodiated species, and a shift of +3 Da in the mass of the *N*-acetylhexosamine monomer (Fig. 1B). The incorpo-

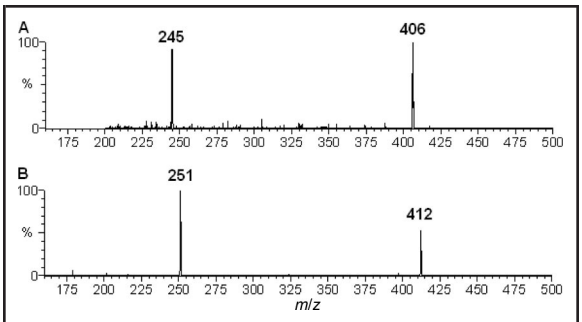

Fig. 2. (A), MS/MS spectrum of protonated methanolysis product derived specifically from HS (*m/z* 406) showing a major fragment at *m/z* 245.

(B), Corresponding MS/MS spectrum of deuteriomethanolysis product from HS (*m/z* 412) with a major fragment at *m/z* 251.

ration of labeled methyl groups in the dimer was in accord with the presumed structure. It is noteworthy that although the sodiated species was more dominant than the protonated form of the CS and DS dimers, the sensitivity to selected reaction monitoring was much higher for the protonated dimers because the sodiated species showed minimal fragmentation even at high collision energies. CID of the unlabeled and labeled protonated dimers derived from CS are shown in Fig. 1, C and D. The dominant transitions, *m/z* 426 → 236 and 432 → 239, were used for selected ion monitoring. The use of ammonium acetate in the elution buffer ensured a sufficient abundance of the protonated dimers for quantitative analysis.

HS has structural domains consisting of segregated blocks of repeating GlcA-(β1–4)-GlcNAc disaccharides (NA domains), similar to the GlcA-(β1–3)-GalNAcβ4 repeating unit in CS, and blocks of highly sulfated, heparin-like IdoA-(β1–4)-GlcNS disaccharides (NS domains).[5] The methylated disaccharide derived from the NA domains of HS behaved similarly to that derived from CS. The presumed dimeric product derived from the NS domain yielded predominantly a sodiated molecular ion at *m/z* 406 in the mass spectrum of the methanolysate HS (data not shown). Fragmentation of this ion by CID yielded a major fragment at *m/z* 245 (Fig. 2A). Deuteriomethanolysis of HS produced a corresponding signal at *m/z* 412 that fragmented to *m/z* 251 upon CID (Fig. 2B).

[5] GlcA, β-D-glucuronic acid; GalNAc, *N*-acetylated β-D-galactosamine; GlcN, β-D-glucosamine; GlcNAc, *N*-acetylated β-D-glucosamine; GlcNS, *N*-sulfated β-D-glucosamine; NS, *N*-sulfated; IdoA, α-L-iduronic acid.

CALIBRATION AND VALIDATION OF THE ASSAY

We assessed the suitability of the isotope-labeled dimers derived from CS, DS, and HS as pseudo–internal standards for each individual compound by analysis of residual partially and unlabeled homologs in the product, both before and after sample preparation and analysis by UPLC-MS/MS. The materials were also checked by repeated analysis up to 3 months after preparation. In all cases, the residual partially labeled or unlabeled species constituted <1% of the total, and their proportions were unaltered by the conditions of the analysis. We avoided possible exchange of methoxyl groups during the analysis by using acetonitrile instead of methanol as the organic modifier in the mobile phase. Representative chromatograms from the UPLC-MS/MS analysis of these materials are shown in Fig. 3. The chromatogram of the internal standards (Fig. 3A) shows strong signals for the transition *m/z* 432 → 239 for [$^2H_6$]DS and [$^2H_6$]CS dimers but no signal in the *m/z* 426 → 236 channel for native CS and DS, and a signal for the transition *m/z* 412 → 251 for the [$^2H_6$]HS dimer but no signal in the *m/z* 406 → 245 channel for native HS. Fig. 3B shows SRM traces from the calibrator with 1.0 mg/L added DS, showing clear and well-separated signals for the isomeric dimers derived from DS and CS and their respective internal standards. Note that there was no signal from the channel *m/z* 406 → 245 corresponding to the NS domain of HS. Fig. 4 compares the chromatogram from a normal control CSF sample with, on the same scale, a CSF sample from 1 of the patients with Hurler syndrome before treatment, showing the much higher concentrations of DS and HS in the patient. We calculated the areas of the peaks corresponding to the dimers derived from DS, CS, HS, and their corresponding IS peaks and their area ratios using TargetLynx® software (Waters Corp.), with manual correction of integration when needed. Calibration curves derived from these peak area ratios were linear over the calibration range 0.2–12 mg/L ($r^2 > 0.99$). The slopes of these curves for CS, DS, and HS were 1.77 (0.05), 1.74 (0.05), and 0.44 (0.05) [mean (SD), n = 7], respectively. We determined inaccuracy of the method by comparing measured concentrations back-calculated from the calibration curves with those of the known added concentrations in the calibrators. Relative SD (CV) varied from 2% to 6% (n = 7) for CS and DS, and from 6% to 17% for HS (n = 7). Mean inaccuracy was from 0.4% to 12% (n = 7) for CS, DS, and HS over the concentration range 0.2–12 mg/L.

We evaluated imprecision of the assay by replicate analysis of low and high QCs (Table 1). Intraassay imprecision (CV) of the low and high QCs was <5.9% for CS and DS and <21% for HS (n = 5). Interassay imprecision of low and high QCs was <13% for CS, DS, and HS (n = 7, over a 4-week period).

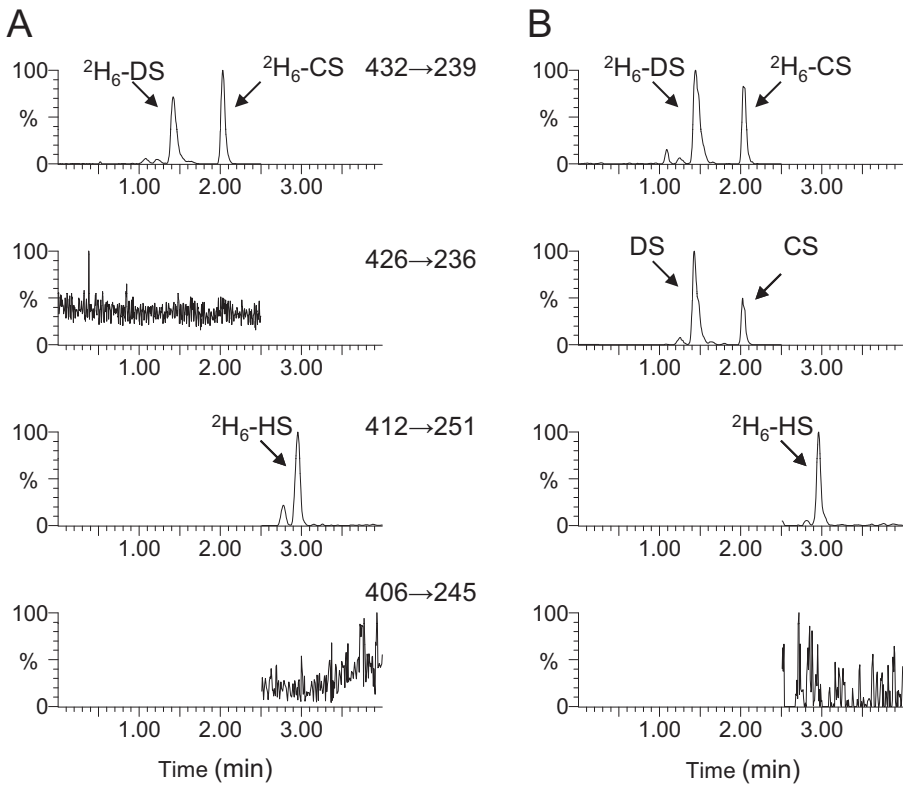

Fig. 3. UPLC-MS/MS chromatograms.

(A), Internal standard mixture showing strong signals corresponding to isotope-labeled CS, DS (*m/z* 432 → 239), and HS (*m/z* 412 → 251) and the lack of signals corresponding to their unlabeled counterparts (*m/z* 426 → 236 and 406 → 245). (B), A calibrator (CSF pool containing 1.0 mg/L added DS) showing clear signals for unlabeled CS and DS.

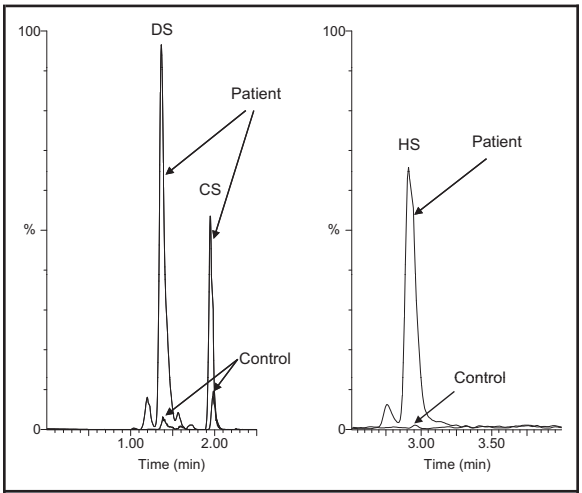

Fig. 4. UPLC-MS/MS chromatograms comparing signals on the same scale from unlabeled CS, DS, and HS in an untreated patient with Hurler syndrome with those from a control.

The limit of quantification was 0.2 mg/L, where target concentrations and imprecision were within acceptable limits (<20%) for CS, DS, and HS. The absolute limits of detection, defined by a signal-to-noise ratio of 3:1, were 2 pg on-column for CS and DS and 0.3 ng for HS. The CSF samples were stable after 3 freeze–thaw cycles, in storage at −80 °C for at least 6 months and at room temperature for at least 24 h, as judged by the analysis of CS, DS, and HS in selected patient and quality control samples.

ANALYSIS OF CSF SAMPLES

We analyzed CSF specimens (25 μL aliquots) from pediatric patients previously referred for amino acid analysis, but in whom no metabolic disease was apparent, for CS, DS, and HS dimers using the method described. As represented in Fig. 4, which shows a chromatogram from 1 of these controls, there are quantifiable signals from CS and DS but not from HS in this particular example. The data from 22 such samples show a mean concentration (SD) of 0.67 (0.57) mg/L for CS, 0.08

Table 1. Imprecision of the assays for CS, DS, and HS.[a]

Imprecision, relative SD %	n	Low QC			High QC		
		CS	DS	HS	CS	DS	HS
Intraassay	5	5.9	3.6	21	2.3	2.3	9.5
Interassay	7	4.5	3.9	13	5.3	2.6	11

[a] QCs derived from 25-μL aliquots of pooled control CSF spiked with either 0.8 mg/L (low QC) or 10 mg/L (high QC) of DS and HS standards, respectively.

(0.07) mg/L for DS, and 0.14 (0.12) for HS in those samples (10/22) where HS was detectable as the NS dimer. The only publication we are aware of that mentions GAG concentrations in human CSF used a colorimetric assay and reported a normal control value for total GAG of <12 mg/L and a single Hurler patient value of 13 mg/L, which is only marginally higher than the control limit *(9)*. In contrast, in our study, samples from patients with Hurler syndrome (n = 7) show markedly increased concentrations of both DS and HS compared with controls (Fig. 4 and Table 2). All of the patients in this group received IT-ERT as well as IV-ERT followed by allogeneic transplantation. When samples were available, posttreatment values 100 days after the HCT and IT/IV-ERT were also determined. The results are summarized in Table 2 and show that the DS concentration was reduced by more than 56.2%, and the HS concentration, by between 17.5% and 58.6% following IT-ERT and transplantation. Further clinical details are not available at this time.

ION SUPPRESSION

We investigated ion suppression or enhancement for CS and DS *(25)* by UPLC-MS/MS analysis of a CSF control sample during constant infusion of isotope-labeled internal standards from a separate inlet system using a syringe pump. There was no suppression of the signals for the isotope-labeled CS and DS at the elution times of their unlabeled counterparts. We tested for ion suppression of HS by infusing unlabeled HS, because there is only a minor signal from HS in control CSF. We observed a minor peak at the retention time of HS, probably due to the endogenous concentration; otherwise, suppression of the signal for HS during UPLC-MS/MS was observed as a general phenomenon of the buffer change (see online Supplemental Figs. 2 and 3). Furthermore, calibration and analysis were carried out in the same manner, using CSF matrix to prepare the calibrators.

Discussion

A recent report of IT-ERT in a single patient with Hurler disease mentioned the analysis of total GAGs in CSF using a colorimetric method that required several milliliters of CSF *(9)* and did not clearly discriminate the patient from normal controls. The method of analysis for GAGs described here has a limit of quantification at least 30 times lower than the recently published

Table 2. Concentrations of individual GAGs in CSF from 7 patients with Hurler syndrome before any treatment (Pre) and, for 4 of these same individuals, 100 days after hematopoietic stem cell transplant (Post).[a]

Patient	CS, mg/L			DS, mg/L			HS, mg/L		
	Pre	Post	Reduction, %	Pre	Post	Reduction, %	Pre	Post	Reduction, %
1	0.93	0.84	9.7	1.33	0.53	60.2	7.08	5.84	17.5
2	1.48	0.64	56.6	2.35	0.41	82.5	11.1	4.6	58.6
3	1.24			2.39			10.8		
4	1.0	0.83	16.5	1.1	0.5	56.2	7.53	6.09	19.1
5	0.82			1.61			10.1		
6	0.96			1.32			9.12		
7	1.1	0.8	31.1	2.56	0.81	68.1	9.02	5.49	39.2
Control mean	0.67			0.08			0.14		
Control SD	0.57			0.07			0.12		
Control min–max	0.16–2.80			0.00–0.26			0.00–0.38		

[a] These 4 patients received IV-ERT and IT-ERT in addition to transplantation.

method for urine analysis *(24)*. It has the required limit of quantification and specificity to detect and quantify signals specifically derived from DS and HS, the principal accumulating storage materials in the lysosomes of patients with both Hunter and Hurler syndrome, in 25-μL aliquots of CSF. In these patients treated with combination therapy including IV and IT enzyme replacement with allogeneic transplantation, there is complete separation of the signals derived from DS, HS, and CS on the UPLC column under the conditions described, and all 3 are quantified in a single analysis. The CS signal is not specific for CS, because it is also partially derived from DS and HS as shown by the analysis of standards. The method of quantification is based on stable isotope dilution. However, because the isotope-labeled material is added to the methanolysate of the CSF, it is not a true internal standard. This would require addition to the CSF before workup, and the lack of stable isotope–labeled precursor molecules (GAGs) precludes this possibility. The assay relies for its performance on the reproducibility of the methanolysis step, which must be performed with fresh reagent and with careful temperature and time control of the reaction. A further advantage of the method is that is suitable for batched analysis of multiple samples. For example, the preparation time for up to 96 samples of CSF in 96-well format is estimated to be approximately 3 h, and analysis time for each specimen is approximately 6.5 min.

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