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LC-MS/MS method for simultaneous quantification of heparan sulfate and dermatan sulfate in urine by butanolysis derivatization

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ABSTRACT

Mucopolysaccharidoses are a group of lysosomal storage disorders (LSDs) characterized by the accumulation of glycosaminoglycans (GAGs). Recently, LC-MS/MS has been widely applied in GAGs analysis combined with different sample preparations for cleaving GAGs to disaccharide units.

The aim of the present is paper is to present a new method for the simultaneous quantification of urinary dermatan sulfate (DS) and heparan sulfate (HS) by LC-MS/MS, after butanolysis reaction. Chromatographic separation was achieved with a gradient of acetonitrile and water in 0.1% formic acid on a Kinetex Biphenyl analytical column in 21 min. Calibration curves ranging from 0.78 to 50 μg/mL for HS and from 1.56 to 100 μg/mL for DS were prepared and the coefficient of determination (r^2) was higher than 0.99 for both analytes. Intra-day and inter-day imprecisions and the bias for both compounds were < 10.0%.

Up to now, most analytical procedures for quantifying GAGs have not had a high level of reproducibility among laboratories, despite the availability of various techniques. The adoption of a new protocol incorporating the methods outlined in this paper could significantly improve the quality and reproducibility of MS results.

A procedure using simple steps for preparing samples and reagents that are easily available on the market could promote the standardization of analytical procedures and increase the use of these measurements in clinical practice.

1. Introduction

Glycosaminoglycans (GAGs) are long unbranched polysaccharides with repeating disaccharide units containing an amino sugar and a uronic acid. They are usually covalently linked to a protein forming proteoglycans. These negatively charged molecules are essential components of extracellular matrices and connective tissue and play an important role in physiological processes such as angiogenesis and cell proliferation [1].

The lysosomal catabolism of proteoglycans is part of the natural turnover of extracellular matrices. The degradation process of these macromolecules occurs in the lysosomal compartment through four different pathways catalyzed by eleven enzymes depending on GAGs type: dermatan sulfate (DS), heparan sulfate (HS), keratan sulfate (KS) and chondroitin sulfate (CS). The same enzyme can be involved in one or more degradation pathway. A loss of function of one of these enzymes causes a progressive accumulation of specific GAGs in various tissues and organs [2–4]. These pathological conditions are known as mucopolysaccharidoses (MPSs), a group of inherited metabolic disorders with an overall incidence of 1 in 22.000 live births [5].

To date, seven different types of MPSs have been reported in literature. Most patients do not show any symptoms at birth. When clinical manifestations appear they may include organomegaly, dysostosis multiplex and abnormal facies. MPSs have overlapping clinical symptoms that can lead to their misdiagnosis [2].

Diagnosis of an MPS is confirmed by demonstrating lysosomal

Abbreviations: CS, Chondroitin sulfate; CV, Coefficient of variation; DS, Dermatan sulfate; ESI, Electrospray ionization; GAGs, Glycosaminoglycans; HPLC, High Performance Liquid Chromatography; HS, Heparan sulfate; KS, Keratan sulfate; LC-MS/MS, Tandem mass spectrometry coupled with liquid chromatography; LLOD, Low limits of detection; LLOQ, Low limits of quantification; LSD, Lysosomal Storage Disorders; MRM, Multiple reaction monitoring; MPSs, Mucopolysaccharidoses; Q1, First Quadrupole; Q3, Third Quadrupole; QC, Quality Control

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enzyme deficiency in leukocytes or fibroblasts [6]. GAGs analysis is important for making differential diagnoses as it can determine which MPS a patient is affected by. Biochemically, patients are classified on the basis of increased urinary excretion patterns of GAGs.

Several methods have been proposed for qualitative and quantitative analysis of complex polysaccharides in biological samples, including, separation by paper or thin layer chromatography, gas chromatography, capillary electrophoresis, enzyme-linked immunosorbent assay and high-performance liquid chromatography [7–9]. Unlike alcian blue and dimethylmethylen blue dye-binding assays, used to quantify total urinary GAGs, these techniques are able to separate specific GAGs. However, they have a number of limitations. They cannot be applied to all matrices, the sample preparation is complicated and time consuming and they are often resource intensive. [10–12].

In recent years, there have been significant improvements in LC-MS/MS technology making it possible to measure different GAGs at the same time in smaller volumes of different biological matrices [13]. However, to make GAGs suitable targets for mass spectrometry they must first be subject to enzymatic digestion or chemical cleavage.

Here we present an improved LC-MS/MS method for the simultaneous quantification of dermatan and heparan sulfates that is cheaper and less complicated than existing methods. We have introduced steps to simplify sample preparation and chromatographic modifications which result in greater accuracy and reproducibility.

2. Materials and method

2.1. Chemicals and reagents

DS and HS sodium salts were purchased from Sigma-Aldrich (Steinheim, Germany). 3 N HCL in N-Butanol was purchased from Regis Thecnologies (Morton Grove, IL, USA). A certified synthetic urine matrix (Surine®, Cerilliant, TX, USA) was used for preparation of calibrators. Acetonitrile and water (HPLC grade) were supplied by Panreac (Barcelona, Spain). Formic acid (> 96%, reagent grade) was obtained from Sigma-Aldrich (Steinheim, Germany).

2.2. Calibration curve and quality control (QC)

HS and DS stock solution, containing 1 mg/mL and 3 mg/mL respectively, were separately prepared in water and stored at −20 °C until analysis. Calibration curves were prepared, covering a concentration range from 0.39 to 50 μg/mL for HS and from 1.56 to 100 μg/mL for DS using seven different levels. Quality controls (QC) were also prepared by spiking synthetic urine with HS and DS standard solution at three different concentrations: low (1 μg/mL HS and 3 μg/mL DS), medium (10 μg/mL HS and 30 μg/mL DS) and high (20 μg/mL HS and 90 μg/mL DS).

2.3. Sample preparation

Calibration curves, QCs and urine specimens were treated with the same procedure. Urine samples were filtered through 0.22-μm syringe filters (Merck KGaA, Darmstadt, Germany) and diluted in deionized water to give a final urinary creatinine (uCr) concentration of 100 μg/mL. HS and DS digestion were performed using two distinct butanolysis reactions characterized by different temperatures and incubation times. For each diluted sample, 5 μL were placed in two glass tubes (for HS and DS analysis) and dried under nitrogen at 45 °C. After drying, 75 μL of 3 N HCL in N-Butanol were added to each vial. HS samples were heated to 90 °C for 60 min whereas DS samples were heated to 65 °C for 25 min. After incubation, all vials were completely dried and each pair was reconstituted in the same vial with 1 mL of a water/acetonitrile solution (30:70, v/v) containing 0.1% of formic acid. A fully detailed operating procedure is reported in [14].

2.4. Patient samples

A total of 14 urine samples from patients with MPS (I $n = 2$, II n = 2, III $n = 3$, VI $n = 4$, VII $n = 1$) were evaluated in this study after informed consent was obtained. Urine specimens were stored and frozen at −20 °C until analysis. In addition, urine samples were collected anonymously from healthy subjects ($n = 101$, age: 0 months – 1 year; $n = 83$, age: 1–3 years; $n = 102$, age: 3–12 years; $n = 107$, age: > 12 years) and used to obtain age related reference ranges. Moreover, urine samples from 4 newborns, identified by an LSD newborn screening pilot project and later confirmed as pseudo-deficient for MPS I were tested.

2.5. Liquid chromatographic and mass spectrometric conditions

HS and DS analysis was performed using a QTRAP 5500 (AB SCIEX, Toronto, Canada) equipped with the Turbo Ion Spray source operating in positive ion mode. The Ion spray voltage was set to 5500 V and the temperature was 300 °C.

To identify HS and DS two transitions were monitored in Multiple Reaction Monitoring (MRM) mode. The MRM transitions for each compound were optimized by direct infusion of standard solution. MS/MS parameters used for the detection are shown in Table 1.

Chromatographic separation was achieved using an Agilent 1260 Infinity HPLC capillary system (Agilent Technologies, Waldbronn, Germany), operating in gradient mode, coupled with a thermostated autosampler (Agilent Micro ASL) and controlled by Analyst Software (Version 1.5.2).

Chromatographic separation was carried out using a Kinetex Biphenyl column 2.6 μm, 100 × 2.1 mm, (Phenomenex, Torrance, CA) maintained at 40 °C with a flow rate of 0.2 mL/min. Acquisition time was 11 min; the eluate was introduced into the ESI interface without splitting. The mobile phase A consisted of 0.1% formic acid in water while solvent B was 0.1% formic acid in acetonitrile. Total run time was 21 min with gradient elution: 0.0–2.0 min (15% B); 2.1–14.1 min (35% B); 14.2–17.1 min (100%); 17.2–21.0 min (15% B). The injection volume was 1 μL.

3. Validation procedure

Validation procedures were conducted according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guideline [15].

3.1. Linearity, accuracy and precision

Calibration curves from 0.78 to 50 μg/mL for HS and from 1.56 to 100 μg/mL for DS were prepared in synthetic urine and analyzed in triplicate. Linearity was evaluated by applying least-square regression analysis.

Table 1
MS/MS detection parameters for HS and DS.

Compound	Q1 Mass (amu)	Q3 Mass (amu)	Entrance Potential EP (V)	Collision Energy CE (V)	Declustering Potential DP (V)	Cell Exit Potential CXP (V)
HS	468.4	162.2	9	21	85	13
DS	510.4	278.2	4	13	85	18

For each analyte, the low limits of detection (LLOD) and low limits of quantification (LLOQ) were determined using a six-point calibration curve (0.02–0.78 μg/mL for HS and 0.09–3.12 μg/mL for DS) prepared in synthetic urine. The LLOD value was defined by signal-to-noise ratio > 3:1. For evaluation of LLOQ, six sets of calibration curves were analyzed. The residual standard deviation (σ) of y-intercepts of regression lines and the slope (S) were used to calculate LLOQ according to the formula: LLOQ = 10σ/S.

Intra-day and inter-day imprecision studies were carried out by measuring the three QC concentrations in five replicates on the same batch and in triplicate on five different days. From the results obtained, the imprecision was expressed as CV (%) and the bias as the percentage difference between the measured value and the nominal concentration.

3.2. Matrix effect and stability studies

The matrix effect was evaluated using the standard addition method applied to synthetic urine samples. Analysis was conducted in triplicate for low and high QC concentrations. Corresponding spiked samples deionized in water were used as controls. The matrix effect was calculated comparing the mean peak area of the analyte in synthetic urine (B) to the mean area of the analyte in water (C) at the same concentration level, using the following equation: (C/B)-1 × 100.

In order to investigate the impact of three freeze-thaw cycles on the stability of both analytes, low and high QCs were analyzed in triplicate, subsequently frozen at −20 °C for 24 h and then thawed. After three freeze-thaw cycles, the samples were again analyzed and the results compared with those obtained from fresh samples.

In addition, we conducted stability studies on the low and high QCs stored under different conditions: -20 °C, +4 °C, +37 °C and room temperature. Samples were tested in triplicate (one replicate for each of three urine samples stored at the different temperatures) every week up to one month. The stability was evaluated comparing the mean concentrations obtained at different times with the initial concentrations.

4. Results

4.1. Optimization of butanolysis reactions

To optimize the reaction conditions for HS and DS butanolysis, several experiments were conducted to study the effect of different solvent volumes, times and temperatures of incubation on product yields. Based on results obtained by Trim and colleagues on HS butanolysis [16], different temperatures around 100 °C were tested. Our results showed a comparable sensitivity with Trim's method after reducing the reaction temperature from 100 to 90 °C; both repeatability and reproducibility of the assay improved remarkably at 90 °C (see Fig. 1 in [14]). Derivatization reaction time was monitored every 30 min for 6 h. HS reached a maximum yield at approximately 60 min, remained constant up to 90 min and then decreased gradually (see Fig. 2 in [14]).

Another series of experiments was performed to estimate the best temperature setting and time of incubation for DS butanolysis. Because disaccharides deriving from DS degradation were not detectable at temperatures above 65 °C, as reported in literature [16,17], we initially tested the reaction yield every 30 min until 360 min. A dramatic decrease in product yields was observed when the time was extended beyond 30 min at 65 °C (Supplementary Fig. 2a). Different reaction times were investigated to establish a suitable incubation period for DS derivatization. Further experiments to determine optimal reaction times were carried out within a window ranging from 5 to 30 min. A temperature lower than 50 °C was also tested but the signal compared to the response at 65 °C decreased considerably. The optimal conditions for butanolysis of DS were found to be 65 °C for 25 min (see Fig. 3 in [14]).

To verify the effect of the derivatization reagent quantity on reaction efficiency, different volumes were added to the HS and DS QC samples. For each analyte, the same QC was incubated at specific temperatures with 75, 100, 150, 200 and 250 μL of 3 N HCl in n-Butanol. No significant differences associated with volume variation were observed, so a volume of 75 μL was chosen to cover the bottom of the vial and to avoid unnecessary reagent overload (see Fig. 4 in [14]).

4.2. LC-MS/MS analysis

For DS and HS quantification, dimers derived from butanolysis reactions were chromatographed on a biphenyl based column using a variety of eluting solvents. The most suitable mobile phase was found to be acetonitrile and water containing 0.1% formic acid for ionization. In order to reduce the column back pressure, the column oven was set at 40 °C. The increase in the analytical signal produced by standard addition identified the peaks corresponding to the specific dimers with a retention time of 8.1 and 8.5 min for DS and HS respectively, as shown in Fig. 1.

4.3. Analytical performance

Method validation studies were conducted to determine method linearity, specificity, sensitivity, accuracy and precision.

The linearity range was established by a calibration curve constructed with seven level concentrations. Regression analysis of calibration lines was used to calculate the slope, intercept and correlation coefficient. A good linearity was obtained in the range of investigated concentrations for both HS (r^2 = 0.9999) and DS (r^2 = 0.9998).

The sensitivity of the method was determined by assessing the LLOD and LLOQ. The LLOD was set at 0.05 μg/mL for HS and 0.19 μg/mL for DS. The LLOQ was found to be 0.16 μg/mL for HS and 0.71 μg/mL for DS.

Bias and imprecision were evaluated by analysing QC samples at low, medium and high concentrations. Intra-day and inter-day variations are reported in Table 2.

4.4. Matrix effect and stability

The matrix effect was evaluated by comparing the signal response of QC samples (low and high) prepared in synthetic urine and in pure solvent. The difference percentage showed no significant variations except for the low QC of DS (−15.8%).

Stability studies of HS and DS showed no significant change under tested conditions with a CV% lower than 15% for all temperatures (see Fig. 5 in [14]).

4.5. Analysis of urine samples

Reference intervals for each biomarker were determined in healthy individuals divided into four age groups from 0 to 80 years with over 80 persons in each group. In Table 3, age-related mean and upper limit reference values for both biomarkers are reported. The cut off values are based on the 97.5% percentile

Urinary HS and DS concentrations were also quantified in 14 confirmed MPS patients and the results are presented in Fig. 2.

5. Discussion

Triple quadrupole mass spectrometers are typically limited in mass range to approximately 2000–4000 m/z, so large molecules such as entire GAGs can only be measured by LC-MS/MS after they have been broken down into smaller molecules.

The use of specific enzymes which digest GAGs followed by mass spectrometry analysis allows sensitive quantification of GAGs [18–22]. However, this methodology is characterized by long incubation periods and the use of expensive reagents and there is the additional problem

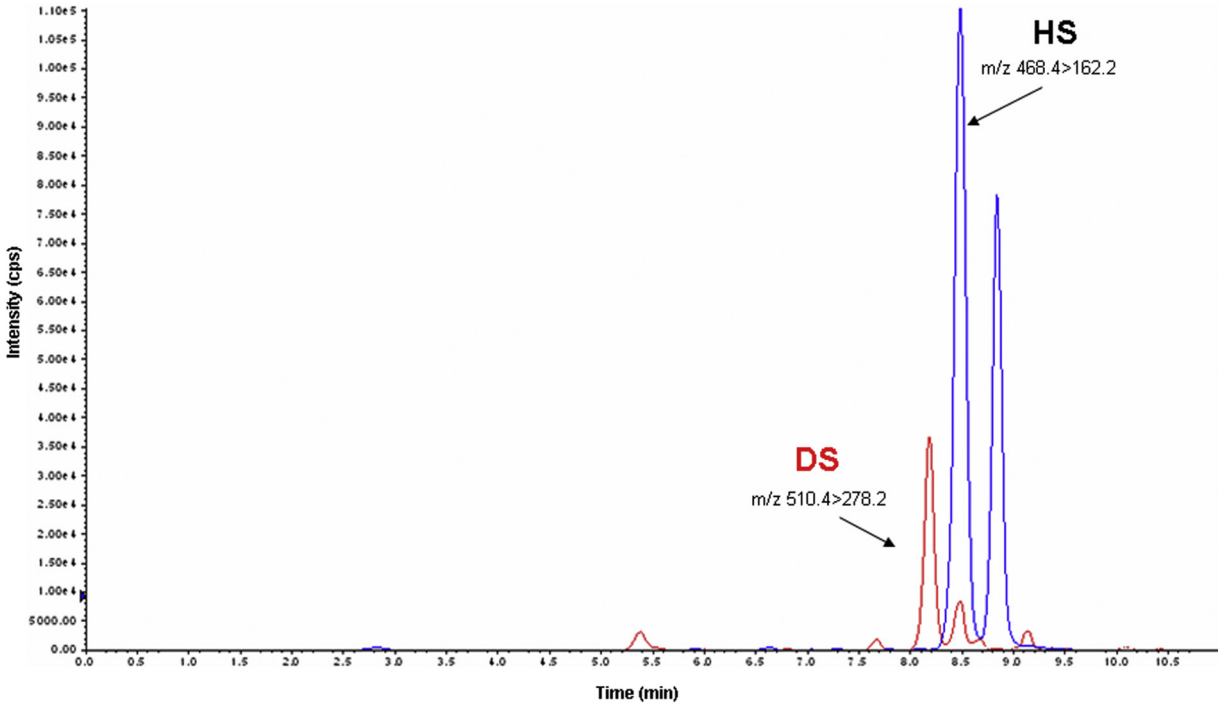

Fig. 1. Mass chromatogram of a standard solution of HS and DS (6.25 and 25 μg/mL, respectively) contained typical dimers released from GAGs by butanolysis.

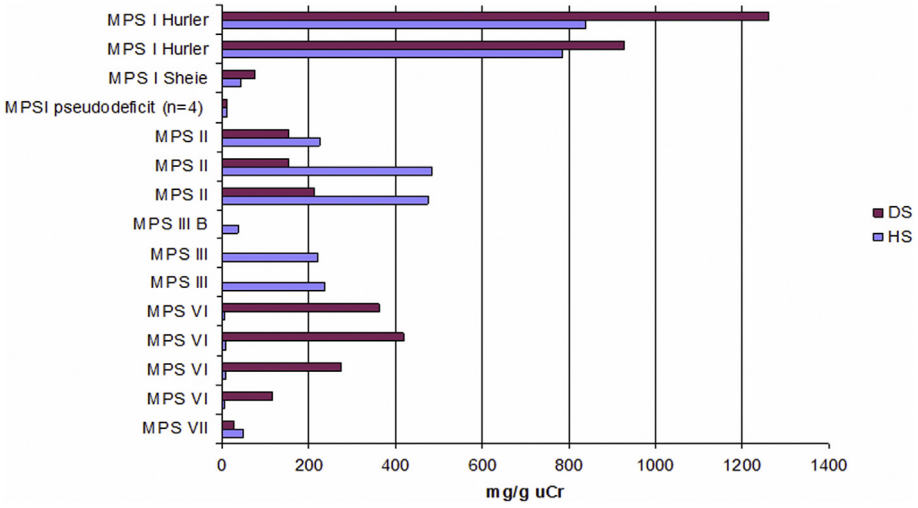

Fig. 2. Urinary concentration of HS and DS measured in confirmed MPSs patients.

Table 2
Validation data for the proposed method.

					Intraday (n = 5)			Interday (n = 3, day = 5)		
Analyte	LLOD (μg/mL)	LLOQ (μg/mL)	QC level	Added concentration (μg/mL)	Imprecision (CV%)	Bias (%)	SD	Imprecision (CV%)	Bias (%)	SD
HS	0.05	0.16	Low	1.0	7.4	104.4	0.1	7.8	102.5	0.1
			Medium	10.0	4.2	102.0	0.3	8.0	100.2	0.8
			High	20.0	5.0	92.3	1.1	5.4	101.7	1.1
DS	0.19	0.71	Low	3.0	3.1	95.9	0.1	5.4	96.8	0.2
			Medium	30.0	1.4	93.6	0.4	5.0	100.5	1.5
			High	90.0	1.0	108.3	0.1	3.5	105.4	3.3

that the enzymes may not completely degrade the total amount of GAGs molecules [16,17].

Chemical cleavage is an alternative to enzymatic digestion. It provides clear advantages including shorter processing times, lower costs and higher reaction rates. Methods for quantification of HS and DS based on chemical cleavage coupled with LC-MS/MS have been widely reported [23–29].

Recently Trim and colleagues developed a new method using

Table 3
Age related reference values for HS and DS measured in urine by LC-MS/MS.

	HS (mg/g uCr)				DS (mg/g uCr)			
Age	0–1 y	1–3 y	3–12 y	> 12 y	0–1 y	1–3 y	3–12 y	> 12 y
n°	101	83	102	107	101	83	102	107
Maximum	26.7	24.3	22.9	12.6	21.3	10.1	ND	ND
Median	10.3	5.4	4.1	2.3	6.0	3.5	2.0	1.1
97.5th percentile	23.1	15.5	9.6	6.7	18.7	8.0	ND	ND

ND-not determined.

butanolysis to quantify HS showing signal responses higher than those generated with other chemical reagents [16].

The present work describes a new sensitive, specific and fast LC–MS/MS assay for simultaneous quantification of HS and DS using small amounts of urine, which adopts butanolysis derivatization to make sample preparation less complicated. It is also less time consuming than the reported enzyme-digestion process.

To optimize the butanolysis process we investigated the influence of solvent addition, incubation temperature and time. According to our results, DS and HS yields markedly depend on reaction temperature and time. The effect of temperature on derivatization reaction is crucial. Trim and colleagues previously obtained a higher assay sensitivity using HS butanolysis at 100 °C compared to HS derivatization with other reagents [16]. However, this procedure is not easy to repeat or reproduce because the reagent evaporates during the incubation stage.

Our study on the effects of temperature on HS derivatization showed a comparable sensitivity to Trim's reaction after reducing the incubation temperature from 100 to 90 °C. Both repeatability and reproducibility of the assay improved remarkably and overall method validation parameters were satisfactory.

The development of reliable analytical methods for quantifying different GAGs would be useful in the diagnosis and clinical management of MPS patients [30–32]. Our data show differences in HS and DS concentrations between normal and pathological samples and also highlight the wide variations in biomarker excretion based on MPS type; no overlap with the group of normal controls was observed. In addition, during a newborn screening pilot project for LSD, 4 newborns showed low enzyme activity of alpha-iduronidase indicative of MPS I. Molecular analysis of these newborns revealed they were pseudo-deficiencies. Fig. 2 shows the mean urinary excretion of HS and DS for these four newborns. The values were within the normal range, as expected. Our results suggest that age effects the concentrations of both biomarkers: concentrations decreased with increasing age in normal controls.

In conclusion, this specific and sensitive analytical method for GAGs quantification could prove useful for the differential diagnosis of MPS patients and their follow up. We are assessing the feasibility of using dried blood spots as specimens for this method to improve newborn screening procedures.

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Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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