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Analysis of proteoglycans derived sulphated disaccharides by liquid chromatography/mass spectrometry

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Abstract

A method has been developed for the identification and quantitative determination of sulphated disaccharides derived from chondroitin sulphate (CS) and dermatan sulphate (DS) chains attached to proteoglycans (PGs). After digestion with Chondroitinase ABC, the pool of disaccharides can be directly separated by liquid chromatography on a porous graphitized carbon (PGC) column and identified by on-line electrospray mass spectrometry under negative ionization conditions. The relative intensities of the fragment ions obtained by MS/MS allow to distinguish the sulphate position. Calibration with standard disaccharides allows the quantification of the different isomers. The method showed good repeatability in terms of relative standard deviation (RSD < 2%) and linearity between 0.5 and 50 ng (total injected amount) for both 4- and 6-sulphated disaccharides. The limit of detection achieved in full scan mode was 0.1 ng. The methodology was applied to different types of biological samples obtained from patients suffering from chronic lung inflammation such as: lung tissue, bronchoalveolar lavage fluid (BALF), induced sputum and urine.

Keywords: Sulphated disaccharides; Glycosaminoglycans; Liquid chromatography; Mass spectrometry

1. Introduction

Proteoglycans (PGs) are key components of the extracellular matrix consisting of a protein core with one or more long polysaccharide chains (glycosaminoglycans, GAGs) covalently attached. These glycosaminoglycans are linear polymers of repeating disaccharide units (Fig. 1) of uronic acid or galactose and hexosamine with varying length and composition. They play an important role in protein binding, cell signaling and modulation of cell growth and differentiation [1–4].

PGs have a polyanionic nature due mainly to the sulphate substituents on carbohydrate residues and to the carboxyl group of the hexuronic acid moieties.

According to the type of monosaccharides and the glycosidic bonds between them, GAGs can be divided into four main categories: hyaluronan (HA); chondroitin sulphate (CS) and dermatan sulphate (DS); heparan sulphate (HS) and heparine; and keratan sulphate (KS). CS and DS contain galactosamine as the only hexosamine and are known as galactosaminoglycans. The rest contain glucosamine as the only hexosamine and are known as glucosaminoglycans [5].

There are indications that the content and composition of some proteoglycans (such as decorin) may be altered in patients with smoking-related emphysema [6].

In our research we are interested in studying the sulphated glycosaminoglycans attached to the leucine-rich repeat proteoglycan family present in lung tissue. The aim of this work was to develop an analytical method able to separate and identify the different types of sulphated disaccharides present in GAG chains derived from lung proteoglycans.

Total glycosaminoglycan concentration can be assayed spectrophotometrically with dimethyl methylene blue [7,8]. However, this methodology cannot provide any further structural information about the glycans.

A general strategy consists in the treatment of the GAG chains with specific lyases, such as Chondroitinase ABC (from *Proteus vulgaris*), which acts by eliminative cleavage

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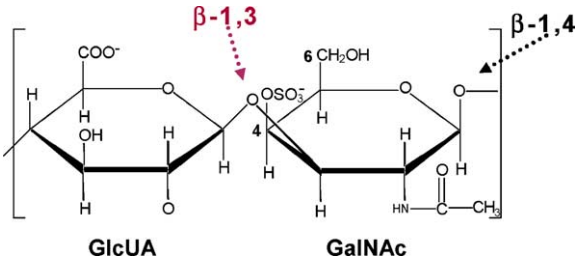

Fig. 1. Schematic representation of a disaccharide typical for chondroitin sulphate. The sulphate group can be at position 4 or 6 of GalNAc.

to produce disaccharides with an unsaturated C4–C5 bond on the uronic acid residue.

Chondroitinase ABC degrades CS and DS into Δ-disaccharides, which can be sulphated in position 4 or 6. The degradation products formed are further separated by chromatographic or electrophoretic methods [9] and detected/quantified using different types of detectors [10].

The negative charge of the sulphate group facilitates the use of capillary electrophoresis (CE) providing high efficiency separations, mass sensitivity and rapid analysis time [11]. However, the limited loading capacity restrict the use of this technique to concentrated samples.

Liquid chromatography has been also widely applied using size exclusion [12], ion pair [13] or amino-bonded stationary phases [14] due to the polar nature of the disaccharides.

The most common detection method coupled to these techniques has been UV absorption at λ 232 nm. However, due to its low sensitivity several derivatization procedures have been developed to apply fluorescence detection. The most common are the use of 2-aminoacridone [15] and dansylhydrazine [16].

In recent years mass spectrometry has become a useful tool for the structural characterization of saccharides released from proteoglycans. Fast atom bombardment (FAB) MS has been utilized to identify unusual sulphated disaccharide units in digests of chondroitin sulphate E from various species [17] and MALDI-TOF with a DBH matrix has also been applied to digests of chrondroitin sulphate with different enzymes [18]. Also electrospray ionization (ESI) MS combined with flow injection analysis has been quite widely used for the study of glycosaminoglycan composition [19–21]. In this case extensive optimisation of the MS parameters is necessary to distinguish *N*-acetylgalactosamine (GalNAc) residues sulphated at the 4- versus the 6-position.

Here, we report the development of a new method for the quantitative direct analysis of disaccharides released from proteoglycans by Chondroitinase ABC. The method combines a chromatographic separation using porous graphitized carbon (PGC) with mass spectrometric detection.

PGC has exceptional chemical and physical stability and can be used throughout the entire pH range. PGC exhibits unexpected and largely unexplained retention properties for molecules containing polar groups. It has been successfully applied to the analysis of N and O-linked glycans released from glycoproteins [22]. Several reports have been published showing an increase in retention when the polarity of the analyte increases [23]. The dissacharides released from proteoglycans can be eluted with a gradient of acetonitrile containing 0.1% formic acid, and detected as negative ions without any derivatization.

2. Materials and methods

Human lung tissue, bronchoalveolar lavage fluid, induced sputum and urine from patients suffering chronic lung inflammation were obtained using protocols approved by the medical ethics committee and provided by the Pulmonary Research Unit of the Groningen Academic Hospital (Groningen, The Netherlands).

Formic acid, ammonium acetate and acetonitrile were from Merck (Darmstadt, Germany).

Chondroitinase ABC from *P. vulgaris* affinity purified, chondroitin sulphate A sodium salt from bovine trachea, chondroitin sulphated disaccharide ΔDi-4S and ΔDi-6S sodium salt, 1,9-dimethyl-methylene blue (DMB), phenylmethylsulphonyl fluoride (PMSF), and propylene glycol were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). Ethylenediaminetetraacetic acid disodium salt (EDTA), tris (hydroxylmethyl) aminomethane (Tris) ultrapure, and glycine were from Duchefa (The Netherlands).

2.1. Sample preparation

2.1.1. Tissue extraction

Lung tissue (20 g), obtained from patients who underwent lobectomy or lung transplantation, was homogenised with a blender and incubated with extraction buffer (100 w/v) for 20 h at 4 °C, with continuous stirring. The extraction buffer was 20% propylene glycol, 50 mM sodium acetate, 300 mM NaCl, containing 10 mM EDTA and 1mM PMSF as proteases inhibitor, pH 6.0. The resulting extract was centrifuged at 40,000 × *g* for 30 min at 4 °C using a Beckman Coulter centrifuge equipped with a JA-3050 rotor. The supernatants were filtered through OE 66 membrane filters (Schleicher & Schuell, Dassel, Germany), 0.2 μm pore size, using a vacuum filtration system. Lung extract (250 ml) was subjected to FPLC separation (Amersham Pharmacia Biotech) in two tandemly connected HiTrap-Q Sepharose (5 ml) columns equilibrated with the extraction buffer. The column was washed with the same buffer until the A_{280} decreased to baseline and further with five column volumes of 50 mM sodium acetate and 300 mM NaCl, pH 6.0. The bound fraction was eluted with 1.5 M NaCl, pH 6.0, subjected to buffer exchange to 10 mM Tris, pH 7.8, using an Amicon 8050 stirred ultrafiltration cell with a Biomax PBGC (10,000 NMWL) ultrafiltration membrane (Millipore Corporation, Bedford, USA), and further digested with Chondroitinase ABC.

2.2. Biological fluids

Bronchoalveolar lavage fluid (BALF) supernatant samples, were obtained from chronic obstructive pulmonary disease (COPD) patients who underwent bronchoscopy. BAL was performed by instillation of saline solution. The lavage fluid was centrifuged and the supernatant stored at −80 °C until use. BALF samples were preconcentrated 25 times using a vacuum centrifuge. 100 μl preconcentrated samples were subjected to buffer exchange to 10 mM Tris, pH 7.8, using Protein Desalting spin columns from Pierce, according to the manufacturer instructions.

Sputum samples were diluted 1:1 in 10 mM Tris, pH 7.8.

For the urine samples the pH was adjusted to 7.8 using 50 μl of 10 mM Tris buffer, pH 7.8 per ml urine.

All samples (lung extract, BALF, sputum, and urine), after buffer exchange to 10 mM Tris or pH adjustment, were subjected to the DMB assay for GAG quantitation and further digested with Chondroitinase ABC, as described in the next two sections.

2.3. DMB measurements

Total chondroitin/dermatan sulphated GAGs were quantitated using the DMB assay as described by Farndale et al. [24]. Briefly, 100 μl samples were mixed with 2.5 ml DMB colour reagent into a disposable spectrophotometric cuvette and absorbance (λ 525 nm) was read immediately, using an UV-160 UV–Vis recording spectrophotometer (Shimatzu, Benelux). Concentrations were determined from standard curves of chondroitin sulphate A.

2.4. Chondroitinase ABC digestion

GAG containing samples were incubated overnight with 2.8 mU Chondroitinase ABC per μg GAG, at 37 °C, 450 rpm using an Eppendorf thermomixer. The enzyme was used in excess to ensure that all disaccharides have been removed from the chondroitin/dermatan chains.

2.5. Instrumental

The HPLC part of the analytical system consisted of an Agilent Series 1100 LC system (Waldbronn, Germany) comprising a degasser, a binary pump, an autosampler and a thermostatted column compartment. Chromatographic separation of the disaccharides took place in a porous graphitized carbon column Hypercarb (Thermo Electron Corporation, Cheshire, UK), 0.32 mm I.D. × 100 mm length operated at 5 ul/min flow rate.

Mobile phase A consisted of 0.1% formic acid in ultrapure water. Mobile phase B was 0.1% formic acid in acetonitrile. Separation was performed with an increasing gradient of B (5–90% in 60 min). The column temperature was kept constant at 28 °C.

The analytes were detected by an Agilent SL ion-trap mass spectrometer equipped with an ESI source operated in negative mode. MS data were acquired over a scan range of 50–1200 amu and 5500 *m*/*z* per second scan rate.

3. Results

Initial experiments were focused on the optimization of the chromatographic separation. After injection of the standard ΔDi-4S two chromatographic peaks were observed with the same spectrum, indicating that both peaks are isomers (Fig. 2A). The same was observed after injection of the ΔDi-6S standard (Fig. 2B).

Both 4- and 6-sulphated disaccharides were characterized by a single ion with *m*/*z* 458.2 corresponding to $[M-H]^-$. No sodiated or other adducts were observed.

When this parent ion (*m*/*z* 458.2) was submitted to negative ion CID, similar mass spectra were observed for both

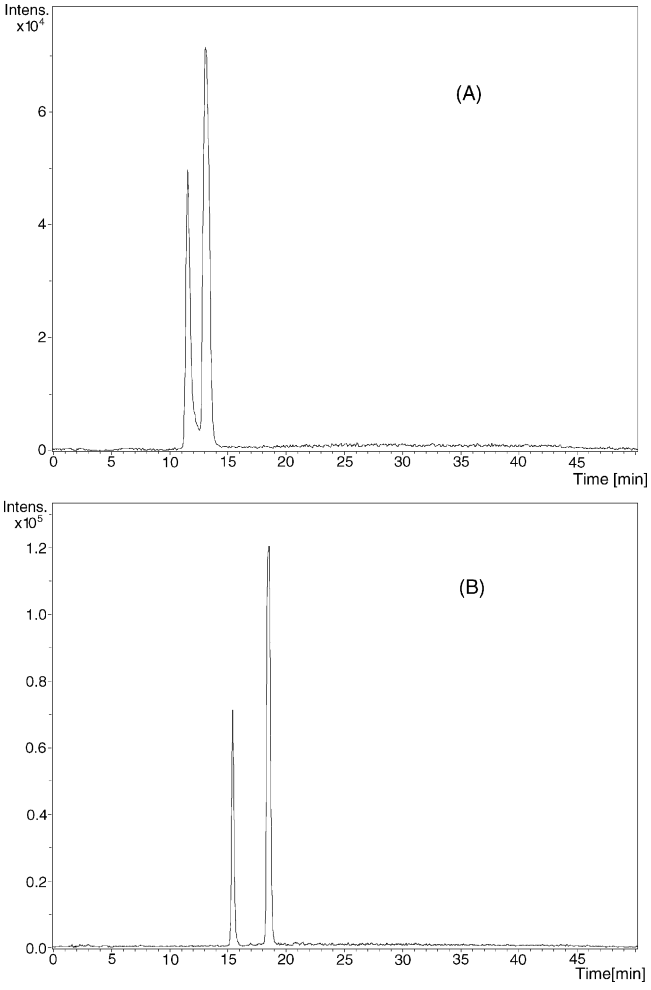

Fig. 2. EIC (*m*/*z* 458.2) corresponding to injection of 5 ng of each of the standard disaccharides. 2A = ΔDi-4S; 2B = ΔDi-6S. As observed two isomeric chromatographic peaks are obtained for each disaccharide. Gradient: 20–90% of B in 60 min. (B: acetonitrile containing 0.1% formic acid).

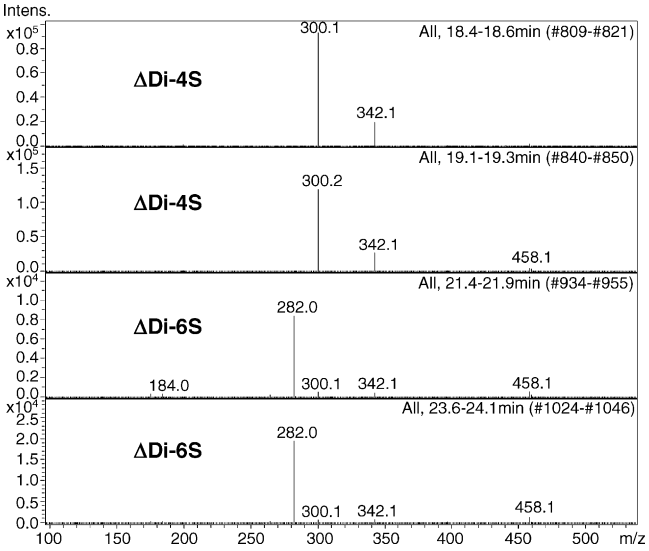

Fig. 3. MS/MS spectra corresponding to each isomer of both disaccharides (sulphated in position 4 or 6). As observed, the ratios of the different fragment ions depend on the position of the sulphate group.

disaccharides, but with quite different ratios between the daughter ions (Fig. 3) allowing to distinguish the position of the sulphate group.

For ΔDi-4S the most abundant fragment ions are Y_1 (*m*/*z* 300.1) and $^{0,2}X_1$ (*m*/*z* 342.1), Z_1 being absent, while for the ΔDi-6S the most abundant fragment ion is Z_1 at *m*/*z* 282. Also a small ion corresponding to C_1 at *m*/*z* 175 is seen (nomenclature according to Domon and Costello [25]). MS^3 of Z_1 (*m*/*z* 282) produced mainly one ion (*m*/*z* 97) corresponding to $[SO_4H]^-$. This is in agreement with the results obtained by Zaia et al. [12].

The influence of the pH on the chromatographic separation was also studied in the range 2–8, using mobile phases containing ammonium acetate at different pH values. Increase of the pH of the mobile phase resulted in decreased retention times for the 6-sulphated disaccharides and increased ones for the 4-sulphated isomers. However, peaks became broader and it was not possible to obtain a good separation of the isomers due to peak overlap.

3.1. Calibration graphs

In order to validate the method for quantitative analysis, calibration graphs were prepared by injecting 1 uL of different concentrations (0.5, 2, 8, 15, 20, 25, 35, 45, 50 ng/uL) of the standard 4- and 6-sulphated disaccharide solutions in Tris. The total area for each disaccharide was considered as the sum of the contribution of both isomeric peaks.

The calibrations were linear up to 50 ng (in terms of total injected amount).

The following equations were obtained: $y = 2.1 \times 10^5 x + 1 \times 10^3$ ($r^2 = 0.997$) for ΔDi-4S and $y = 1.7 \times 10^5 x + 8 \times 10^3$ ($r^2 = 0.997$) for ΔDi-6S.

The limit of detection (calculated as the minimum injected amount which gave a response in the detector higher than three times the signal to noise ratio using the EIC (*m*/*z* 458.2)) was 0.1 ng for both disaccharides in full scan mode.

Using MRM mode (458.2 > 300.1, 458.2 > 282.0), it was possible to detect 0.01 ng.

The repeatability (calculated by repeating the calibration graph four times) gave a relative standard deviation (RSD) of less than 2%.

Spiking of the standard disaccharides directly into the biological fluids showed that there was no interference from the matrix (e.g. ion suppression) with a RSD less than 3%.

To check the reproducibility of the whole procedure different concentrations of chondroitin sulphate A (0.5, 2 and 4 ug) were spiked into 300 uL of the different fluids (25 times preconcentrated BALF, induced sputum and urine) and the mixture digested with 2 mU of Chondroitinase ABC. After digestion, 5 uL were injected in the LC/MS system. The results showed that the amount of disaccharides obtained kept a good correlation with a digest of the corresponding amount of Chondroitin sulphate A (RSD less than 5%), indicating that the enzyme is not affected by the matrix, and therefore, the method is applicable for quantitative measurements.

3.2. Applications

The methodology developed was applied to the quantitation of 4- and 6-sulphated disaccharides present in biological samples.

3.3. Lung tissue extracts

The first application was the monitorization of the digestion of proteoglycans isolated from lung tissue by anion exchange chromatography.

In our research we are interested in the purification of small leucine-rich proteoglycans (e.g. decorin, byglycan) from lung tissue. One crucial step in this procedure is the digestion of the anion exchange fraction containing a mixture of proteoglycans with Chondroitinase ABC to release the core proteins which are recovered in the flow through after a new anion exchange run for further purification.

For the tissue extract fraction, eluted with 1.5 M NaCl and subjected to buffer exchange to 10 mM Tris, pH 7.8, DMB measurements showed the presence of 46 μg GAG/ml.

Due to the lack of information about the amount of CS and DS present in the tissue extract, it is difficult to estimate the amount of enzyme required for the digestion. Digests of lung extract were prepared with increasing concentrations of Chondroitinase ABC and the enzymatic reaction was followed by monitoring the amount of disaccharides released. Also different time points were selected during the digestion to find the optimal digestion time. In Fig. 4, one of these digest is presented showing clearly in the total ion current (TIC) chromatogram both disaccharides.

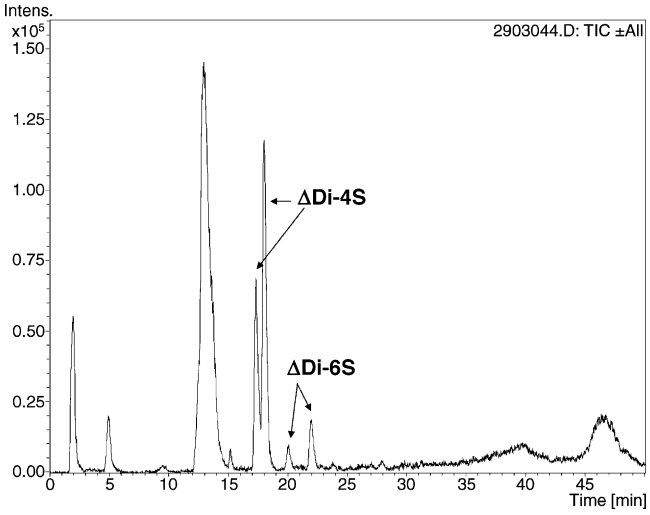

Fig. 4. TIC chromatogram obtained after injection of a digest of lung tissue extract containing proteoglycans with chondroitin/dermatan sulphate chains.

3.4. Biological fluids

The method was also applied to the quantitation of 4- and 6-sulphated disaccharides in biological fluids obtained from patients suffering chronic lung inflammatory diseases, such as chronic obstructive pulmonary disease. Two different BALF supernatant samples were preconcentrated (as described in the sample preparation section) and concentrations of GAGs measured by the DMB assay were 52 and 90 μg GAG/ml, respectively.

After digestion with Chondroitinase ABC, the resulting digest was diluted five times with Tris and 5 uL injected in the LC/MS system. In Fig. 5A the total ion current chromatogram obtained for one of the samples is presented. When the extracted ion chromatogram (EIC) corresponding to *m/z* 458.2 is selected (Fig. 5B), both disaccharides can be clearly detected. As observed the 4-sulphated form is more abundant than the 6-sulphated.

A similar procedure was followed with induced sputum. For the sputum sample, GAGs concentration was 75 μg/ml according to the DMB assay. In this case, after digestion, the sample was diluted 20 times with Tris before injection in the LC/MS system due to the high concentration of the disaccharides. Again the 4-sulphated form is present in higher abundance than the 6-sulphated (Fig. 6A and B). In Table 1 the calculated concentration of both disaccharides in some biological samples and the ratios ΔDi-4S/ΔDi-6S are shown.

Table 1
concentration of ΔDi-4S and ΔDi-6S in some biological samples

Sample	Concentration ADi-4S (ng/ml)	Concentration ADi-6S (ng/ml)	Ratio ADi-4S/ ADi-6S
BALF l	33.01	8.07	4.09
BALF 2	24.13	8.62	2.80
Sputum	105787.50	5185.66	20.4

When looking at the chromatogram for these biofluids, many other peaks can be distinguished. Tandem MS of these peaks revealed the presence of non-sulphated disaccharides. In Fig. 7, the EIC corresponding to the non-sulphated forms ΔDi-0S (*m/z* 378.3) in induced sputum shows also several

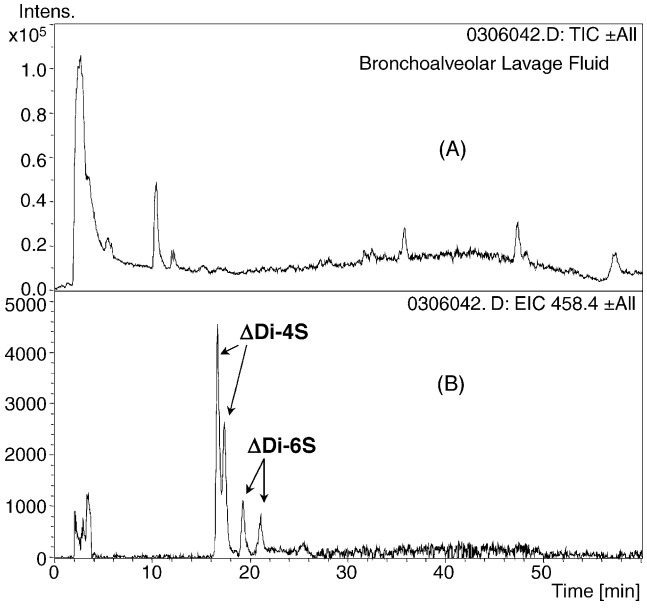

Fig. 5. LC/MS profiles corresponding to BALF from a patient with chronic obstructive pulmonary disease, digested with Chondroitinase ABC. The upper trace shows the total ion current chromatogram and the lower trace corresponds to the extracted ion chromatogram (*m/z* 458.4) showing the presence of 4- and 6-sulphated disaccharides.

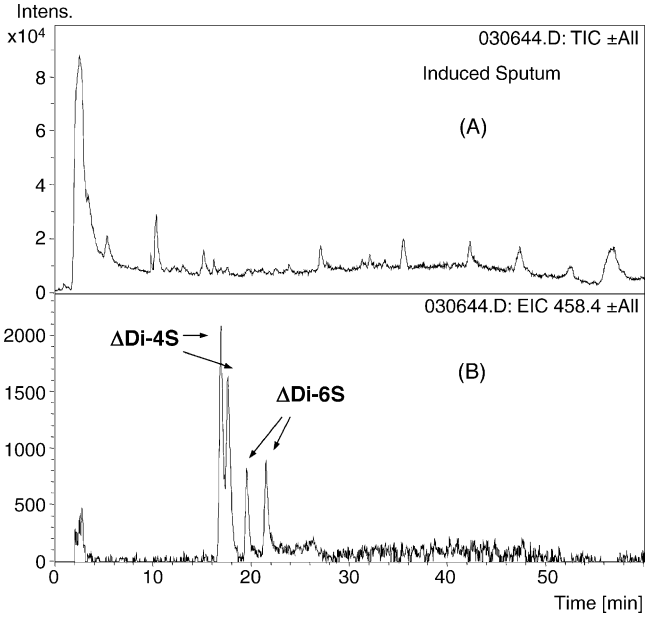

Fig. 6. LC/MS profile obtained after injection of a sample of induced sputum from a patient with chronic obstructive pulmonary disease (COPD) digested with Chondroitinase ABC. The upper trace shows the total ion current chromatogram and the lower trace corresponds to the extracted ion chromatogram (*m/z* 458.4) showing the presence of 4- and 6-sulphated disaccharides.

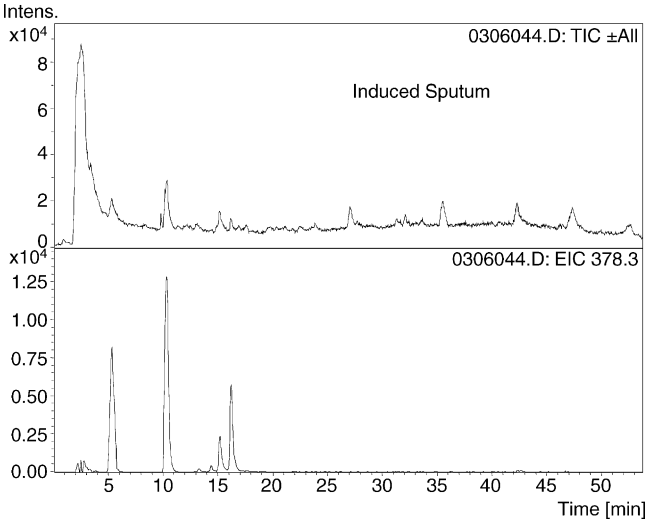

Fig. 7. TIC (upper trace) and EIC *m/z* 378.3 (lower trace) showing the presence of non-sulphated disaccharides in induced sputum.

peaks (isomers). This fact indicates that our approach is also valid for the analysis of other saccharides after calibration with the appropriate standards.

Application of the same methodology to two different urine samples did not show any disaccharide peaks, although the DMB assay indicated the presence of 1.2 and 5.2 μg/ml GAGs, respectively. If present, these disaccharides are in too low concentration to be detected.

This method developed opens the possibility to compare the relative amounts of 4- and 6-sulphated disaccharides in different biological samples as well as concentration variations which can be associated with disease progression.

4. Conclusions

The methodology developed allows the separation and quantitation of disaccharides after Chondroitinase ABC digestion of natural proteoglycans as well as the elucidation of their fine structure (e.g. determination of the sulphate position) using tandem mass spectrometry.

When compared with other methods, our is simple and fast (after digestion the samples can be directly injected in the column without further clean up) and can be applied to different types of biological samples like tissue extracts, BALF, induced sputum and urine. Even with these complex matrices the elution window of the disaccharides is free of coeluting peaks.

No derivatization strategies or isolation of the digestion products are required before LC/MS analysis.

If necessary, the limit of detection (0.1 ng) can be improved using MRM to 0.01 ng.

Glycosaminoglycans take part in several biological functions in the lung extracellular matrix through their specific structures. It has been reported that in certain diseases they undergo specific compositional and structural modifications [26]. In the future the method developed will be used to detect variations in the concentration and in the sulphation pattern of different galactosaminoglycans (chondroitin sulphate and dermatan sulphate) during the progression of COPD and other diseases involving chronic lung inflammation such as bronchiolitis obliterans.

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