Journal of Chromatography A, 1225 (2012) 91–98

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

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

Disaccharide analysis of glycosaminoglycan mixtures by ultra-high-performance liquid chromatography–mass spectrometry

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ARTICLE INFO

Article history:
Received 17 October 2011
Received in revised form
13 December 2011
Accepted 17 December 2011
Available online 26 December 2011

Keywords:
Heparin
Chondroitin sulfate
Hyaluronan
Disaccharide analysis
LC–MS
Fluorescently labeling

ABSTRACT

Glycosaminoglycans are a family of polysaccharides widely distributed in all eukaryotic cells. These polyanionic, linear chain polysaccharides are composed of repeating disaccharide units that are often differentially substituted with sulfo groups. The diversity of glycosaminoglycan structures in cells, tissues and among different organisms reflect their functional an evolutionary importance. Glycosaminoglycan composition and structure also changes in development, aging and in disease progression, making their accurate and reliable analysis a critical, albeit, challenging endeavor. Quantitative disaccharide compositional analysis is one of the primary ways to characterize glycosaminoglycan composition and structure and has a direct relationship with glycosaminoglycan biological functions. In this study, glycosaminoglycan disaccharides, prepared from heparan sulfate/heparin, chondroitin sulfate/dermatan sulfate and neutral hyaluronic acid using multiple polysaccharide lyases, were fluorescently labeled with 2-aminoacridone, fractionated into 17 well-resolved components by reverse-phase ultra-performance liquid chromatography, and analyzed by electrospray ionization mass spectrometry. This analysis was successfully applied to cell, tissue, and biological fluid samples for the picomole level detection of glycosaminoglycan composition and structure.

1. Introduction

Proteoglycans (PGs) are ubiquitously presented on the surface and in the extracellular matrix (ECM) of all eukaryotic cells where they participate in a variety of critical physiological and pathological processes, such as morphogenesis, embryonic development, pathogenic infection, immune response, inflammation mediation, tumor progression and invasion, angiogenesis and tissue regeneration [1–4]. PGs are comprised of one or more glycosaminoglycan (GAG) chains covalently linked to a core protein. GAG components of PGs are sulfated, linear polysaccharides consisting of repeating disaccharides units of hexuronic acid (D-glucuronic acid (GlcA) and/or its C5-epimer L-iduronic acid (IdoA)) and hexosamine (D-glucosamine (GlcN) or D-galactosamine (GalN)). Their structures can be extremely complex resulting from variations in the degree and pattern of sulfo group substitution and occurrence of two hexuronic acid epimers [2,5]. This structural heterogeneity and diversity endows GAGs with their crucial biological functions, such as the regulation and signaling of events through their interaction with various proteins, ligands, and receptors [5,6].

The major GAGs in animals are heparan sulfate (HS)/heparin (HP), chondroitin sulfate (CS)/dermatan sulfate (DS) and hyaluronic acid (HA). The classification of these GAGs depends on their disaccharide composition. HS/HP are constructed from a β-1,4-linked GlcA and α-1,4-linked GlcNAc (where Ac is acetyl) backbone. CS/DS and HA have alternating 1,3- and 1,4-linkages positions consisting of 1,3-linked β-GlcA that is 1,4-linked to either β-GalNAc in CS/DS, or β-D-GlcNAc in HA [5]. The simplest GAG, HA, contains neither sulfo groups nor is it attached to a core protein. HA is biosynthesized through the HA synthase-catalyzed copolymerization of uridine diphosphate (UDP)-GlcNAc and UDP-GlcA at the cell membrane and extruded into the ECM [7]. The biosynthesis of HS/HP and CS/DS begins with chain initiation of a tetrasaccharide linker on a core protein serine residue through a common pathway in the endoplasmic reticulum [8]. Then, chain elongation of HS/HP in the Golgi occurs through the stepwise addition of UDP-GlcNAc and UDP-GlcA catalyzed by EXT enzyme. In the Golgi-based biosynthesis of CS/DS, UDP-GalNAc and UDP-GlcA are added sequentially by alternate action of GalNAc transferase II and GlcA transferase II. The structural heterogeneity and diversity of

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doi:10.1016/j.chroma.2011.12.063

Table 1
Structure and mass of disaccharides studied and the assignment of found ion in the mass spectrum.

Disaccharides	Structure	Theoretical mol mass	Mw-AMAC[a]	Observed ions (charge)	Assignment
HS/HP disaccharides					
$0S_{HS}$	ΔUA-GlcNAc	379.1	573.1	571.5 (−1)	$[M-H]^-$
NS_{HS}	ΔUA-GlcNS	417.1	611.1	609.5 (−1)	$[M-H]^-$
$6S_{HS}$	ΔUA-GlcNAc6S	459.1	653.1	651.5 (−1)	$[M-H]^-$
$2S_{HS}$	ΔUA2S-GlcNAc	459.1	653.1	651.5 (−1)	$[M-H]^-$
$NS6S_{HS}$	ΔUA-GlcNS6S	497.0	691.0	689.4 (−1)	$[M-H]^-$
				344.0 (−2)	$[M-2H]^{2-}$
$NS2S_{HS}$	ΔUA2S-GlcNS	497.0	691.0	689.4 (−1)	$[M-H]^-$
				344.0 (−2)	$[M-2H]^{2-}$
$2S6S_{HS}$	ΔUA2S-GlcNAc6S	539.0	733.0	731.4 (−1)	$[M-H]^-$
				753.2 (−1)	$[M+Na-2H]^-$
				365.0 (−2)	$[M-2H]^{2-}$
Tri S_{HS}	ΔUA2S-GlcNS6S	576.9	770.9	769.3 (−1)	$[M-H]^-$
				384.0 (−2)	$[M-2H]^{2-}$
				344.0 (−2)	$[M-SO_3-2H]^{2-}$
CS/CD disaccharides					
$0S_{CS}$	ΔUA-GalNAc	379.1	573.1	571.5 (−1)	$[M-H]^-$
$2S_{CS}$	ΔUA2S-GalNAc	459.1	653.1	651.5 (−1)	$[M-H]^-$
$6S_{CS}$	ΔUA-GalNAc6S	459.1	653.1	651.5 (−1)	$[M-H]^-$
$4S_{CS}$	ΔUA-GalNAc4S	459.1	653.1	651.5 (−1)	$[M-H]^-$
SB_{CS}	ΔUA2S-GalNAc4S	539.0	733.0	731.4 (−1)	$[M-H]^-$
				753.2 (−1)	$[M+Na-2H]^-$
				365.0 (−2)	$[M-2H]^{2-}$
SD_{CS}	ΔUA2S-GalNAc6S	539.0	733.0	731.4 (−1)	$[M-H]^-$
				753.2 (−1)	$[M+Na-2H]^-$
				365.0 (−2)	$[M-2H]^{2-}$
SE_{CS}	ΔUA-GalNAc4S6S	539.0	733.0	731.4 (−1)	$[M-H]^-$
				753.2 (−1)	$[M+Na-2H]^-$
				365.0 (−2)	$[M-2H]^{2-}$
Tri S_{CS}	ΔUA2SGalNAc4S6S	619.0	812.0	811.2 (−1)	$[M-H]^-$
				404.9 (−2)	$[M-2H]^{2-}$
				365.0 (−2)	$[M-SO_3-2H]^{2-}$
$0S_{HA}$	ΔUA-GlcNAc	379.1	573.1	571.5 (−1)	$[M-H]^-$

[a] Mw-AMAC: the molecular weight of AMAC-tagged disaccharides.

HS/HP and CS/DS are the result of their subsequent and differential post-polymerization enzymatic modification. In HS/HP chain modification, *N*-deacetylase/*N*-sulfotransferases (NDST) catalyzes partial *N*-deacetylation/*N*-sulfonation. C-5 epimerase converts some of the GlcA into its C5-epimer, IdoA, which can then be modified by 2-*O*-sulfotransferase. Modification of the GlcNAc residue by 6-*O*-sulfotransferase and 3-*O*-sulfotransferase can also take place. In CS/DS, 4-*O*- and 6-*O*-sulfo groups can be introduced into certain GalNAc residues by 4-*O*- or 6-*O*-sulfotransferases, and in DS, C-5 epimerization of GlcA into IdoA can also be accompanied by the addition of 2-*O*-sulfo groups [9–11].

The structural characterization of the GAG polysaccharides pose significant challenges for analytical chemists due to their high negative charge, the lability of their sulfo groups, their polydispersity, and sequence heterogeneity [12]. A general method of GAG analysis relies on their enzymatic depolymerization to obtain disaccharide units that can then be related to the particular GAG(s) present and its (their) composition or structural heterogeneity. Typically, the enzymatic depolymerization process relies on the specificity of the heparin lyases and chondroitin lyases to distinguish between HS/HP and CS/DS and HA classes of GAGs [13,14]. These polysaccharide lyases leave a double bond, absorbing at 232 nm, in their product's non-reducing end uronic acid residue (ΔUA) [15,16]. Exhaustive heparin lyase treatment of HS/HP or chondroitin lyase treatment of CS/DS or HA afford twelve HP/HS-derived disaccharides or eight CS/DS disaccharides and one HA disaccharide. Of the twelve possible HP/HS-derived disaccharides, only eight disaccharides are commonly found in normal organisms (Table 1), the other four *N*-unsubstituted glucosamine disaccharides (ΔUA-$GlcNH_2$, ΔUA-$GlcNH_2$6S, ΔUA2S-$GlcNH_2$, and ΔUA2S-$GlcNH_2$6S) are rare products resulting from incomplete *N*-deacetylase/*N*-sulfotransferase modification during HS/HP biosynthesis or loss of labile *N*-sulfo groups during isolation. Following the sequential treatment with polysaccharide lyases, quantitative disaccharide compositional analysis is then used to characterize the structure of each GAG, as these structures have a direct relationship to the biological functions of each GAG. Several modern techniques have been used for disaccharide compositional analysis including high-performance liquid chromatography (HPLC) [17,18], ultra-performance liquid chromatography (UPLC) [19–21], and capillary electrophoresis (CE) [22,23].

Liquid chromatography–mass spectrometry (LC–MS) and LC–MS/MS, have recently become widely used to provide independent assessments of the retention time and mass of disaccharide without interference by impurities present in biological samples [24,25]. Reverse-phase ion-pair (RPIP)-HPLC generally relies on volatile lipophilic ion-pairing reagents in the mobile phase to aid in analyte retention on a hydrophobic C18 stationary phase and to evaporate in electrospraying, making these ion-pairing reagents compatible with ESI-MS [26,27]. RPIP-HPLC–MS analysis is applicable for a wide variety of analytes, ranging from unsulfated heparosan disaccharide to highly sulfated HS/HP and CS/DS disaccharides [19–21]. The application of UPLC, performed at high pressures (up to 10^8 Pa) with columns packed with 1.7 μm particles, provides even higher resolution, peak capacity, sensitivity, efficiency and speed of analysis [19,20]. Rapid, robust, and simple RPIP-UPLC–MS methods have been established for both HS/HP and CS/DS disaccharides and each set of analytes require less than a five minute analysis time [28]. The effect of several factors on RPIP-UPLC–MS separation, such as ion pairing regent concentration, counter-ion and pH, were systematically studied for disaccharide analysis [21]. Unfortunately, two distinct RPIP-UPLC–MS methods are required to analyze the CS/DS and HS/HP disaccharide components present in a single sample [29,30]. Thus, the routine analysis

of the disaccharide composition of HS/HP, CS/DS and HA GAGs in a cell, tissue or biological fluid sample currently requires multiple selective polysaccharide lyase treatments, multiple disaccharide isolation steps, multiple HPLC or UPLC columns using different mobile phases and different ESI-MS detection conditions.

Despite the outstanding performance of RPIP-UPLC–MS in GAG analysis it has a number of severe limitations. (1) Multiple independent analyses are required for determining the disaccharide composition of samples containing more than one type of GAG, particularly common in biological samples. (2) RPIP performance is particularly sensitive to the concentration mobile phase components and is often not considered sufficiently robust for analysis in clinical laboratories. (3) The ion-pairing reagents in mobile phase decrease the sensitivity of analytes on electrospray ionization mass. (4) The ion-pairing reagents seriously and permanently contaminate the ion source, making this analytical method difficult to use on shared LC–MS instruments. Here we report an alternative approach relying on RP-UPLC. Although hydrophilic, anionic disaccharides are not retained on RP-UPLC, by first labeling these disaccharides with a hydrophobic fluorophore, their chromatographic properties can be improved and their detection sensitivity enhanced. Several highly sensitive analytical methods have involved labeling GAG-derived disaccharides through their reductive amination with 2-aminoacridone (AMAC), 2-aminobenzamide (2-AB), and 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (BODIPY) fluorophores. These labeled disaccharides were then detected with high sensitivity by CE with laser-induced fluorescence detection (CE-LIF) [31,32] or using strong anion exchange (SAX)-HPLC coupled with in-line fluorescence detection [33]. Mixtures of HS/HP disaccharides and ones of CS/DS/HA disaccharides labeled with AMAC have been analyzed by RP-HPLC with in-line fluorescence detection [34,35]. Such methods have been applied to the compositional analysis HS/HP and CS/DS disaccharides derived from GAGs recovered from cultured cells and LMWH [33]. Fluorescent detection affords high sensitivity with a low picomole level detection limits. Furthermore, RP-HPLC mobile phases composed of volatile salts and organic solvents are compatible with MS detection. Indeed, AMAC-tagged CS/DS/HA disaccharides of GAGs, purified from endogenous normal human plasma, have recently been successfully analyzed using RP-HPLC with a fluorimetric detection before on-line detection using ESI-MS [36]. Recently, this method was used for the quantitative analysis heparin and LMWH [35]. All the current methods still require the separate recovery of HS/HP disaccharides and CS/DS disaccharides from multiple digestion steps, followed by separate and distinctly different analyses affording a very time-consuming and complicated analytical process. Moreover, the resulting data from multiple analyses are often difficult to compare and can provide inaccurate calculations of the ratio of HS/HP and CS/DS present in a sample. This study describes a simplified and highly sensitive method to analyze 17 AMAC-tagged disaccharides from HS/HP, CS/DS and HA by a single RP-UPLC–MS experiment and demonstrates its capability in performing the GAG-derived disaccharide profile of a cultured cell sample, an animal tissues sample, and a biological fluid sample.

2. Experimental

2.1. Materials

The 17 unsaturated disaccharide standards listed in Table 1 were obtained from Iduron Co (Manchester, UK). AMAC and $NaCNBH_3$ were purchased from Sigma–Aldrich (St. Louis, USA). The cloning, *Escherichia coli* expression and purification of the recombinant heparin lyase I (EC 4.2.2.7), heparin lyase II (no EC assigned), and heparin lyase III (EC 4.2.2.8) from *Flavobactrium heparinum* were performed in our laboratory as previously described [37–39]. Chinese hamster ovary (CHO)-S cells were grown in suspension culture on CD-CHO medium supplemented with 2% HT (Hypoxanthine/Thymidine mixture, Gibco-Invitrogen) and 8 mM glutamine [40]. Arabian camel liver tissue and camel urine were obtained from young (1–2 yr old animals) at a slaughterhouse in Egypt [29,41]. All other chemicals were of HPLC grade.

2.2. Sample preparation and GAG disaccharide recovery

Isolation and purification of GAGs from liver tissue, urine and CHO cells were previously described [29,42]. Briefly, the samples were defatted (when necessary), proteolyzed, the GAG purified using a strong anion exchange spin column, released with salt and alcohol precipitated. The recovered GAGs were next completely depolymerized using polysaccharides lyases. Chondroitin lyase ABC (5 m-units) and chondroitin lyase ACII (2 m-units) in 10 μL of 0.1% BSA were added to ~5 μg GAG sample in 25 μL of distilled water and incubated at 37 °C for 10 h. After boiling to inactivation the chondroitinase enzymes at 100 °C for 2 min and cooling to room temperature, a mixture of heparin lyase I, II, and III (10 mU each) in 5 μL of 25 mM Tris, 500 mM NaCl, 300 mM imidazole buffer (pH 7.4) were added and incubated at 37 °C for 10 h. The products were recovered by centrifugal filtration using an YM-10 spin column, and the disaccharides were collected in the flow-through and freeze-dried.

2.3. Derivatization of unsaturated disaccharides with AMAC

The freeze-dried biological sample containing GAG-derived disaccharides (~5 μg, determined by micro carbazole assay [43]) or a mixture of 17 disaccharide standards (5 μg/per each disaccharide or 0.5 nmoL/per each disaccharide) was added 10 μL a 0.1 M AMAC solution in acetic acid (AcOH)/dimethyl sulfoxide (DMSO) (3:17, v/v) and mixed by vortexing for 5 min. Next, 10 μL of 1 M $NaBH_3CN$ was added in the reaction mixture and incubated at 45 °C for 4 h [44]. Finally, the AMAC-tagged disaccharide mixtures were diluted to different concentrations (0.5–100 ng) using 50% (v/v) aqueous DMSO and LC–MS analysis was performed.

2.4. UPLC–MS

LC–MS analyses were performed on an Agilent 1200 LC/MSD Instrument (Agilent Technologies, Inc., Wilmington, DE) equipped with a 6300 ion-trap and a binary pump followed by a UV detector equipped with a high-pressure cell. The column used was an Acquity UPLC BEH C18 column (2.1 mm × 150 mm, 1.7 μm, Waters, Milford, MA, USA) at 45 °C.

For constant ammonium acetate concentration with methanol gradient, eluent A was water/methanol (88/12, v/v), and eluent B was water/methanol (12/88, v/v). Both eluents contained the same concentrations ammonium acetate (either 20 mM, 40 mM, 60 mM, 80 mM, or 100 mM). Solution A for was flowed (100 μL/min) through the column for 5 min followed by linear gradients 0–4% solution B from 5 to 15 min, 4–15% solution B from 15 to 30 min and 15%-100% solution B from 30 to 60 min.

For dual ammonium acetate and methanol gradient, eluent A was ammonium acetate solution (20 mM, 40 mM, 60 mM, 80 mM, 100 mM) and eluent B was methanol. Solution A and 12% solution B was flowed (100 μL/min) through the column for 5 min followed by linear gradients 12–15% solution B from 5 to 15 min, 15–30% solution B from 15 to 30 min and 30–100% solution B from 30 to 60 min.

The column effluent entered the ESI-MS source for continuous detection by MS. The electrospray interface was set in negative

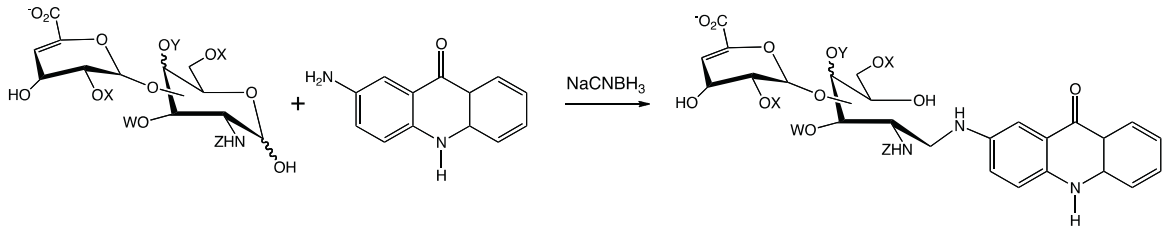

Fig. 1. Fluorophore derivatization reaction of unsaturated disaccharides with AMAC. A generalized GAG disaccharide is shown that can either be 1,3 or 1,4 linked and can contain a GalN or GlcN residue at the reducing end where W = H or glycosidic, X = H or SO_3^-, Y = H or SO_3^- or glycosidic, and Z = Ac or SO_3^-.

ionization mode with a skimmer potential of −40.0 V, a capillary exit of −40.0 V, and a source temperature of 350 °C, to obtain the maximum abundance of the ions in a full-scan spectrum (150–1200 Da). Nitrogen (8 L/min, 40 psi) was used as a drying and nebulizing gas.

2.5. Calibration

Quantification analysis of AMAC-labeled disaccharides was performed using calibration curves constructed by separation of increasing amounts of unsaturated disaccharide standards (0.1, 0.5, 1, 5, 10, 20, 50, 100 ng/each disaccharide or 0.02, 0.03, 0.05, 0.1, 0.2, 0.3 nM/each disaccharide). Linearity was assessed based on amount of disaccharide and peak intensity in $UV_{255\,nm}$, mass spectrometry total ion chromatography (TIC) and extract ion chromatography (EIC). All analyses were performed in triplicate.

3. Results and discussion

The quantitative analysis of disaccharide composition is extremely important to develop a deeper understanding of GAG structure–activity relationships. Some progress has been made in the disaccharide compositional analysis of either HS/HP or CS/DS. However, there is currently no rapid, sensitive and reliable method for the quantitative analysis of the GAG-derived disaccharides arising from mixtures of HS/HP, CS/DS and HA. The challenge is to design a single analytical method to determine 17 GAG-derived disaccharides, some containing structural isomers with identical charge and mass. Hyphenated techniques, such as LC–MS, provide multiple ways (retention time and mass, or MS/MS) to identify each disaccharide. Ideally, LC-MC relies on volatile mobile phase components that can be reliably formed, do not contaminate the ion-source, and provide high sensitivity detection.

The AMAC fluorophore can be conveniently introduced into the reducing end of GAG-derived disaccharides by reductive amination. Reductive amination relies on the efficient formation of Schiff base between the reducing end aldehyde of the disaccharide and the AMAC amino group followed by sodium cyanoborohydride reduction (Fig. 1). AMAC-labeling offers high sensitivity fluorescence (λ_{ex} 428 nm, λ_{em} 525 nm) detection and also a strong UV absorbance at 255 nm. More importantly, the hydrophobic AMAC-label facilitates the chromatographic separation of GAGs-derived disaccharides by RP chromatography eliminating the needs for ion-pairing reagents.

3.1. Optimization of mobile phase

It was necessary to optimize the UPLC fractionation to separate the 17 AMAC-disaccharides through a systematic study of mobile phase composition. An ammonium acetate–acetonitrile elution system has been previously reported for the analysis of AMAC-glycans [34,36]. However, we discovered that acetonitrile was too non-polar a mobile phase component to completely separate the 17 AMAC-disaccharides with all the gradients that were examined (data not shown). Instead, we selected to use methanol as the non-polar mobile phase component.

We next investigated the mobile phase having a constant ammonium acetate concentration with a methanol gradient (Fig. 2A, a–e). The results showed that several AMAC-disaccharides were difficult to separate at constant ammonium acetate concentrations (Fig. 2A, a-e, Fig. S1). As the ammonium acetate concentration increases, the retention times of each AMAC-disaccharide increase. A comparative study on this relationship was next undertaken that showed the retention time shift (ΔT) as a function of ammonium acetate concentrations (Table S1). The results shows that the Tri S_{CS}, SB_{CS}, SD_{CS}, $2S6S_{HS}$, $6S_{HS}$ show a significantly greater shift (ΔT = 2–3.5 min) with ammonium acetate concentrations than the other AMAC-disaccharides. Based on the results, we next decided to examine a gradient with a decreasing ammonium acetate concentration and an increasing methanol concentration (Fig. 2B). Using this approach, the separation of $0S_{HS}$ and $6S_{CS}$ (14 and 15) improved with 60 mM ammonium acetate in solution A (Fig. 2B-c) and $0S_{CS}$ was successfully separated from the AMAC peak with the 80 mM ammonium acetate in solution A (Fig. 2B-d). The 17 AMAC-disaccharides also separated completely in 100 mM ammonium acetate as elution solution A using a modified gradient by increasing the flow rate to 120 μL/min (data not shown) but the system pressure was considerably higher.

The pH of the mobile phase can also play a key role in the separation of AMAC-disaccharides. We found that a decrease in mobile phase pH lead to a gradual reduction in AMAC retention time (Fig. S2). However, the shift of AMAC-disaccharides vary based on their structures with the more highly sulfated AMAC-disaccharides showing elongated retention times and the non-sulfated and mono-sulfated AMAC-disaccharides showing shorter retention times with decreased mobile phase pH. The optimal value for the separation of the 17 AMAC-disaccharides was found to be pH 6.8.

3.2. Optimization of RP-UPLC–MS analysis of 17 AMAC-disaccharide standards

We qualitatively confirmed the identity of each disaccharide from their mass by injecting one AMAC-disaccharide at a time. The molecular weight (Mw) of AMAC-disaccharides (Fig. 1) were calculated from the sum of the disaccharide and AMAC molecular weights, minus water and plus 2H and are listed in Table 1. The mass spectra of the 17 AMAC-disaccharides (Fig. 3) show that only an $[M\text{-}H]^-$ molecular-ion peak for non-sulfated and mono-sulfated disaccharides and an $[M-2H]^{2-}$ molecular-ion peak the di-sulfated and tri-sulfated disaccharides. Because sulfo groups are relatively labile, minor doubly charged peaks, corresponding to loss of SO_3^-, were observed at m/z 344.0 and 365.0 for the tri-sulfated disaccharides $TriS_{HS}$ and $TriS_{CS}$, respectively. Detailed assignments of these mass spectra are provided in Table 1.

All 17 AMAC-tagged disaccharides were completely separated using 60 mM or 80 mM ammonium acetate as elution solution A. Two of the most commonly occurring disaccharides found in biological samples, the $0S_{HS}$ and $6S_{CS}$ (peaks 14 and 15), elute

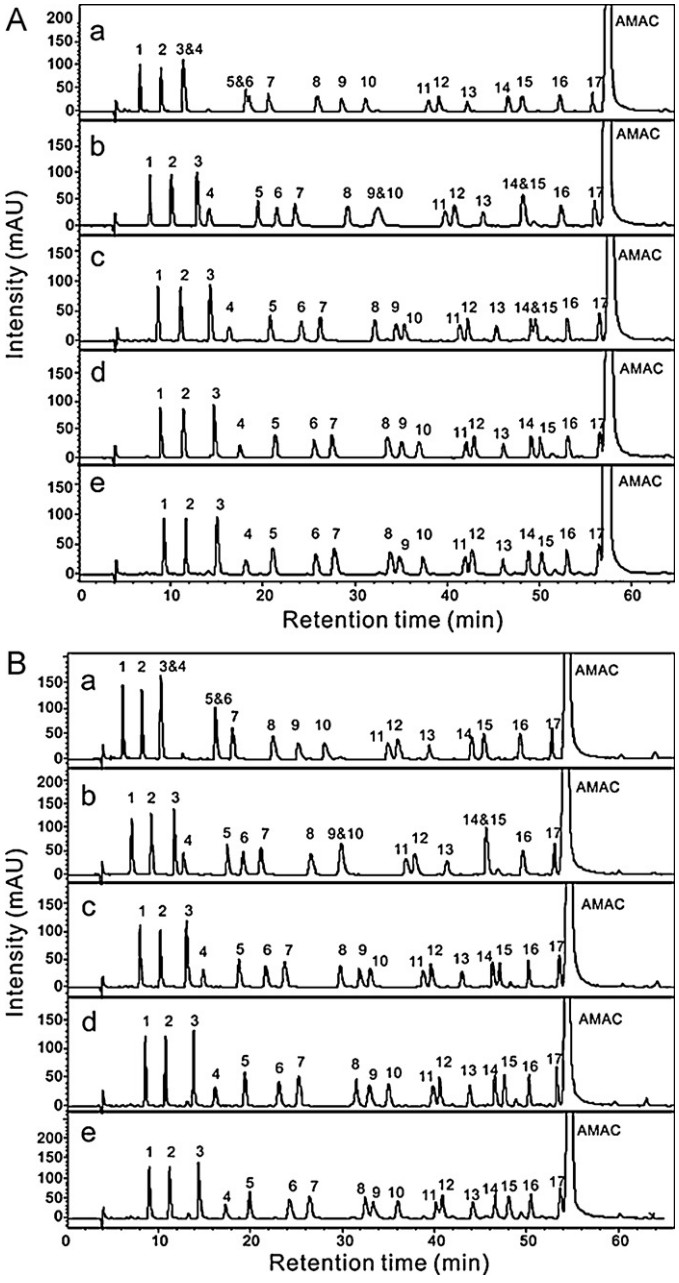

Fig. 2. Effect of the different ammonium acetate concentrations on the retention time by UV detection at 255 nm. (A) Constant ammonium acetate concentration with methanol gradient. Eluent A was water/methanol (88/12, v/v), and eluent B was water/methanol (12/88, v/v). Both eluents contained the same concentration ammonium acetate either 20 mM (a), 40 mM (b), 60 mM (c), 80 mM (d) or 100 mM (e). (B) Ammonium acetate and methanol gradient. Eluent A was ammonium acetate solution either 20 mM (a), 40 mM (b), 60 mM (c), 80 mM (d), or 100 mM (e). Eluent B was methanol. 1. Tri S_{HS}, 2. $NS6S_{HS}$, 3. NS2SHS, 4. Tri S_{CS}, 5. NS_{HS}, 6. SB_{CS}, 7. $2S6S_{HS}$, 8. SD_{CS}, 9. $6S_{HS}$, 10. SE_{CS}, 11. $2S_{HS}$, 12. $2S_{CS}$, 13. $4S_{CS}$, 14. $0S_{HS}$, 15. $6S_{CS}$, 16. $0S_{HA}$, and 17. $0S_{CS}$.

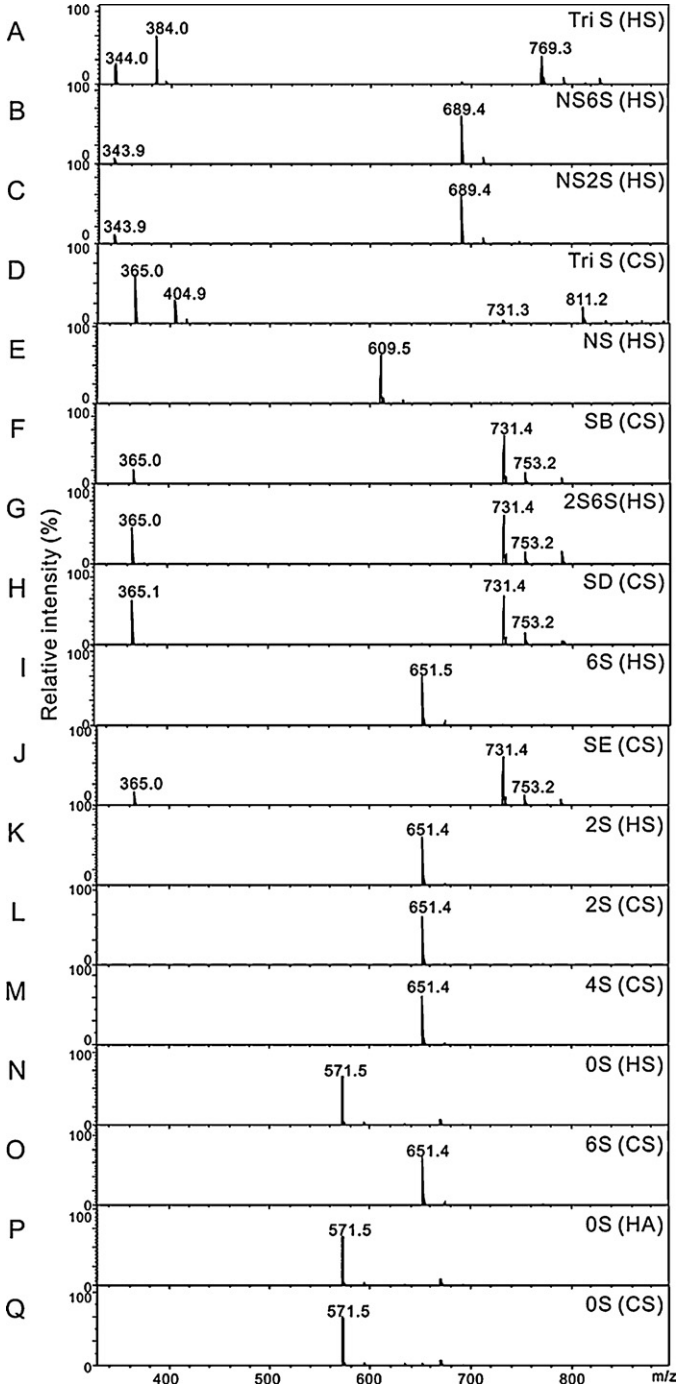

Fig. 3. Mass specta of heparin/HS-derived disaccharide: (A) Tri S_{HS}, (B) $NS6S_{HS}$, (C) $NS2S_{HS}$. (D) Tri S_{CS}, (E) NS_{HS}, (F) SB_{CS}, (G) $2S6S_{HS}$, (H) SD_{CS}, (I) $6S_{HS}$, (J) SE_{CS}, (K) $2S_{HS}$, (L) $2S_{CS}$, (M) $4S_{CS}$, (N) $0S_{HS}$, (M) $6S_{CS}$, (O) $0S_{HA}$, and (P) $0S_{CS}$.

very close to one another when using 60 mM ammonium acetate as elution solution A. Therefore, we selected 80 mM ammonium acetate as eluent A and methanol as eluent B at pH 6.8 for optimal RP-UPLC–MS elution conditions. In Fig. 4, 17 AMAC-tagged disaccharides (50 pmoL/disaccharide) were baseline-separated and detected by TIC (Fig. 4A), UV at 255 nm (Fig. 4B), and extracted ion chromatography (EIC) (Fig. 4C). Detection by UV exhibits the highest sensitivity, while EIC detection provides the flattest baseline so may be particular useful for the analysis of small amounts of biological samples containing protein, peptides, and other sample impurities [29]. When this mobile phase system was applied for analysis of either just the eight AMAC-HS/HP disaccharides or just the nine AMAC-CS/DS/HA disaccharides, a steeper gradient can be used in each case to afford baseline-separated within 25 min (Fig. S3).

3.3. Quantitative analysis of AMAC-disaccharides

Equal-mass (0.1–100 ng/each disaccharide) and equal-molar (0.02–0.3 nM/each disaccharide) mixtures of the 17 AMAC-disaccharides were analyzed by RPIP-UPLC–MS to evaluate the

Table 2
Quantification analysis of disaccharide mixtures containing known amounts of disaccharides by quality equations.

Disaccharides	M-1 (ng)				M-2 (ng)				M-3 (ng)			
	Known amount	Calculated amount by TIC	Calculated amount by UV	Calculated amount by EIC	Known amount	Calculated amount by TIC	Calculated amount by UV	Calculated amount by EIC	Known amount	Calculated amount by TIC	Calculated amount by UV	Calculated amount by EIC
Tri S_{HS}	100	98.8 ± 0.12	99.7 ± 0.06	99.1 ± 0.20	60	57.8 ± 0.18	58.3 ± 0.06	58.2 ± 0.09	10	9.92 ± 0.19	9.81 ± 0.07	9.96 ± 0.08
$NS6S_{HS}$	100	99.5 ± 0.08	99.8 ± 0.05	99.4 ± 0.08	60	58.9 ± 0.10	59.4 ± 0.08	59.1 ± 0.06	10	10.5 ± 0.09	9.96 ± 0.02	10.1 ± 0.13
$NS2S_{HS}$	80	81.4 ± 0.05	80.6 ± 0.31	81.2 ± 0.01	40	40.8 ± 0.04	40.1 ± 0.12	40.5 ± 0.14	20	21.8 ± 0.13	20.8 ± 0.09	22.3 ± 0.34
Tri S_{CS}	80	80.4 ± 0.12	79.8 ± 0.15	80.1 ± 0.16	40	42.4 ± 0.27	41.8 ± 0.18	41.2 ± 0.09	20	22.1 ± 0.19	21.2 ± 0.10	20.5 ± 0.21
NS_{HS}	80	81.3 ± 0.07	80.1 ± 0.10	80.8 ± 0.26	40	41.2 ± 0.08	40.2 ± 0.10	40.7 ± 0.03	20	21.3 ± 0.09	20.9 ± 0.17	21.5 ± 0.12
SB_{CS}	60	58.5 ± 0.03	59.2 ± 0.06	59.1 ± 0.15	10	10.2 ± 0.04	10.4 ± 0.07	9.99 ± 0.05	80	80.8 ± 0.34	81.0 ± 0.18	81.3 ± 0.11
$2S6S_{HS}$	60	59.1 ± 0.03	59.8 ± 0.06	59.3 ± 0.13	10	11.2 ± 0.09	10.4 ± 0.04	10.9 ± 0.14	80	82.1 ± 0.31	80.9 ± 0.07	81.5 ± 0.22
SD_{CS}	40	41.9 ± 0.11	40.4 ± 0.12	40.8 ± 0.11	5	4.96 ± 0.12	4.87 ± 0.03	4.98 ± 0.09	100	99.7 ± 0.18	99.2 ± 0.02	100.2 ± 0.17
$6S_{HS}$	40	41.7 ± 0.02	40.8 ± 0.10	41.7 ± 0.14	5	5.03 ± 0.11	4.92 ± 0.09	4.97 ± 0.12	100	98.6 ± 0.04	99.0 ± 0.02	99.4 ± 0.05
SE_{CS}	40	40.7 ± 0.13	40.2 ± 0.10	40.3 ± 0.10	5	4.88 ± 0.24	4.82 ± 0.11	4.87 ± 0.26	100	100.5 ± 0.14	99.3 ± 0.02	100.7 ± 0.15
$2S_{HS}$	20	18.9 ± 0.15	19.8 ± 0.05	19.3 ± 0.09	80	80.2 ± 0.15	79.5 ± 0.05	81.3 ± 0.19	5	5.37 ± 0.27	5.24 ± 0.16	5.40 ± 0.39
$2S_{CS}$	20	21.4 ± 0.13	20.2 ± 0.10	21.5 ± 0.03	80	81.5 ± 0.12	80.1 ± 0.10	80.9 ± 0.18	5	5.12 ± 0.11	5.07 ± 0.09	5.24 ± 0.12
$4S_{CS}$	20	21.0 ± 0.07	20.4 ± 0.15	20.8 ± 0.05	80	80.9 ± 0.03	79.5 ± 0.13	81.2 ± 0.12	5	5.26 ± 0.21	5.12 ± 0.13	5.31 ± 0.32
$0S_{HS}$	10	9.87 ± 0.02	10.1 ± 0.03	9.97 ± 0.05	100	101.3 ± 0.18	100.2 ± 0.02	100.9 ± 0.17	40	41.0 ± 0.14	40.6 ± 0.11	41.4 ± 0.07
$6S_{CS}$	10	10.6 ± 0.14	10.1 ± 0.06	10.4 ± 0.11	100	100.7 ± 0.11	99.7 ± 0.05	100.2 ± 0.08	40	40.2 ± 0.07	40.7 ± 0.04	40.4 ± 0.13
$0S_{HA}$	5	4.88 ± 0.02	4.85 ± 0.03	4.98 ± 0.05	20	21.2 ± 0.09	20.5 ± 0.10	21.7 ± 0.15	60	58.2 ± 0.19	59.4 ± 0.07	59.0 ± 0.21
$0S_{CS}$	5	4.94 ± 0.07	4.84 ± 0.07	4.92 ± 0.08	20	20.5 ± 0.02	21.4 ± 0.19	20.8 ± 0.11	60	59.9 ± 0.10	60.6 ± 0.24	59.7 ± 0.19

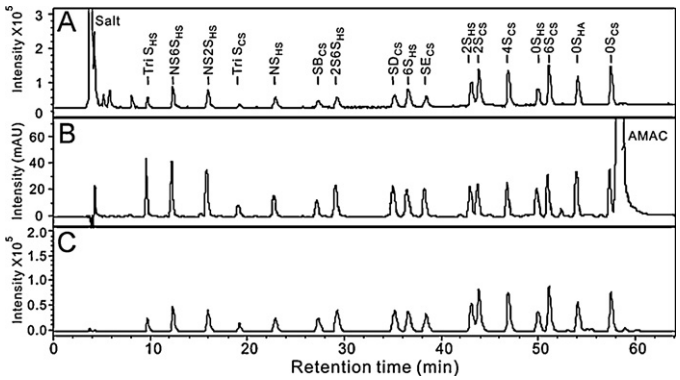

Fig. 4. RP-UPLC–MS chromatograms of 17 AMAC-tagged disaccharide standards with TIC (A), $UV_{255\,nm}$ (B), and EIC (C) detection.

sensitivity and linearity of disaccharide determination using UV, TIC and EIC detection (Fig. S4, Tables S2 and S3). The integrated disaccharide peak areas showed good linearity when plotted as a function of their amount. The linearity based on the mass-peak areas (R^2 = 0.9762–0.9999) was slightly better than the mole-peak areas (R^2 = 0.9476–0.9940). Monosulfated CS/DS disaccharides $2S_{CS}$, $4S_{CS}$, $6S_{CS}$ and unsulfated disaccharides $0S_{HS}$, $0S_{CS}$ showed a greater capacity to ionize, their limit of detection (LOD) was 0.1 ng, while trisulfated disaccharide Tri S_{CS}, Tri S_{HS} and monosulfated NS_{HS} showed lower ionization efficiencies and an LOD of >1 ng. Detection by $UV_{255\,nm}$ and by EIC exhibits a 10-fold higher sensitivity than detection by TIC. Three known disaccharide-mixtures designated M1, M2 and M3 were prepared, derivatized by AMAC, and analyzed using this optimized RP-UPLC–MS method. The results showed that the calculated amounts for the individual components in each mixture were consistent with their known amounts by TIC detection, UV detection at 255 nm and by EIC detection (Table 2). The combination of RP-UPLC separation and UV and MS detection represents a fast, highly sensitive, and reliable method for the qualitative and quantitative analysis of GAG disaccharide standards.

3.4. Analysis of GAG-containing biological samples

Quantitative disaccharide compositional analysis is one of the most important ways to characterize the structures of GAGs and is directly related to their important biological functions. However, many biological GAG samples are not very abundant making GAG detection and quantitative compositional analysis problematic. There is an increased interest in GAG structural characterization of genetically engineered cells and knockout organisms and for the comparison GAGs from different tissues and cell lines. This has resulted in the development of a number of analytical methods for the compositional profiling of GAGs from biological sources. Our laboratory had established methods for the quantitative recovery of GAGs from cells, tissue and biological fluids that rely on SAX spin columns [42]. While studies on GAG extraction from selected tissues have shown excellent recoveries [45], additional research will be necessary to optimize the rapid micro-scale recovery of GAGs from a wide variety of biological samples. Methods have also been developed for the analysis of GAG disaccharides composition by multiple RPIP-UPLC–MS systems [29]. However, several issues still require resolution: (1) the quantification of 0S is difficult due to its very short retention time and its elution with the salt peak; (2) there are difficulties in cross-comparison of analytical data obtained on different families of GAGs; and (3) time-consuming and complicated multiple sample recoveries and analyses are required.

Three biological samples, a tissue sample (camel liver), a biological fluid (camel urine) and a cell sample (cultured CHO cells)

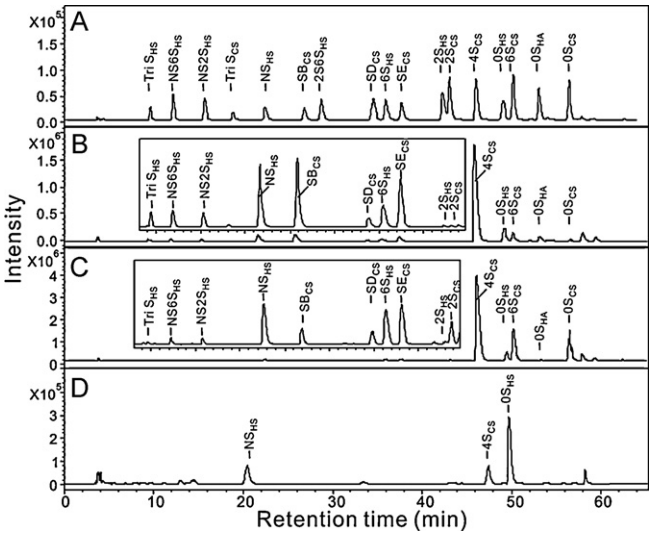

Fig. 5. EIC of AMAC-tagged disaccharide analysis of GAGs from different biological sources. (A) disaccharide standards; (B) disaccharides from camel liver; (C) disaccharides from camel urine and (D) disaccharides from CHO cells.

were selected for GAG analysis using our newly developed RP-UPLC–MS method. After recovery of GAGs from each sample and complete GAG digestion using multiple polysaccharide lyases, the resulting GAG-derived disaccharide mixtures were derivatized by reductive amination with AMAC. Liver and urine samples containing AMAC-disaccharides were directly injected onto LC–MS, without the removal of residual fluorescent AMAC reagent. Qualitative separation of GAG-derived disaccharides was accomplished (Fig. 5B and C) from which quantitative disaccharide composition could be calculated (Table 3). The results show that the $4S_{CS}$ was the major component (26.7%) in the liver GAG sample. The $0S_{HS}$, NS_{HS} and $6S_{HS}$ accounted for 20.5, 14.8 and 8.2% of the total disaccharides, respectively. The Tri SHS was 5.9% of the total disaccharides and 10.3% of HS/HP disaccharides. Previous comparative studies, undertaken in our laboratory, on the HS/HP compositions of camel and other liver samples by RPIP-HPLC and NMR showed a unique disaccharides composition for camel liver HS. The $TriS_{HS}$ level is considerably lower in camel liver compared to human liver and porcine liver. The reported compositions of camel liver HS using RPIP-HPLC, 19.7 mol% $0S_{HS}$, 25.9 mol% NS_{HS} and 27.2 mol% $6S_{HS}$ [46,47], are consistent with the current study relying on RP-UPLC. Furthermore, based on the total disaccharides analysis, we found a considerable amount of CS/DS/HA disaccharides also exist in camel liver samples with the HS/HP to CS/DS ratio of 1.5:1. In the urine sample, the $4S_{CS}$ was a dominant component (43.7%) and there were nearly equal amounts of $6S_{CS}$ (12.4%), $0S_{CS}$ (14.2%) and $0S_{HS}$ (12.3%), comparable to previous results [29]. The ratio of CS/DS to HS/HP is 1:2.9. With exception of $2S6S_{HS}$ and $TriS_{CS}$, the other 15 disaccharides were unambiguously detected in GAGs from liver and urine samples using high sensitivity RP-UPLC–MS.

Chinese hamster ovary (CHO) cells are widely used in the biotechnology/biopharmaceutical industry for the production of recombinant therapeutic proteins. They are also accepted by the FDA as a host for the production of human therapeutics, and protocols exist for removal of host cell proteins, nucleic acids, and viral contaminants. CHO cells are known to biosynthesize HS but not heparin. Current research in our laboratory is aimed at the metabolic engineering of the HS biosynthetic pathway to produce secreted CHO cell heparin. AMAC-disaccharides obtained from CHO cell GAGs were analyzed by RP-UPLC–MS (Fig. 5C). The data shows that only four different disaccharides were present, $0S_{HS}$ (72.2%), NS_{HS} (14.7%), $6S_{HS}$ (3.8%) and $4S_{CS}$ (8.3%), and the ratio of HS/HP to

Table 3
Disaccharide composition analysis of GAG samples from biological source.

Samples	HS/HP disaccharides								CS/DS disaccharides									Radio of HS/HP and CS/DS
	Tri S	NS6S	NS2S	NS	2S6S	6S	2S	0S	Tri S	SB	SD	SE	2S	4S	6S	0S	$0S_{HA}$	
Camel liver	5.9	3.5	4.5	14.8	nd	8.2	0.1	20.5	nd	5.8	1	2.6	0.2	26.7	3.1	0.6	2.5	1.5:1
Camel urine	0.8	0.7	0.7	5.4	nd	4.7	0.8	12.3	nd	1.1	0.6	1.2	0.3	43.7	12.4	14.2	1.1	1:2.9
CHO-S cells	nd	nd	nd	14.7	nd	3.8	1.0	72.2	nd	nd	nd	nd	nd	8.3	nd	nd	nd	11.2:1

CS/DS was 10. These results are consistent to our previous analysis using RPIP-HPLC methods [29].

Previous studies in our laboratory have demonstrated that EIC is a particularly useful means of detection means in the analysis of very small amounts of GAGs from the biological sources as this means of detection is not interfered with by proteins, peptides and other sample impurities. For AMAC-tagging biological samples, TIC and UV detection also display excellent chromatograms (Fig. S5). Qualitative analysis by MS detection also confirms all disaccharides, initially identified on the basis of retention time were correctly assigned. Several impurity peaks, such as Peak 3 in TIC and UV detection (Fig. S6) are still observed that might interfere with the integration of tiny amounts of certain disaccharides.

UPLC takes advantage of technological strides made in resolution, peak capacity, sensitivity, efficiency and speed of analysis. It has been widely used in many analytical fields as advanced technology. In our previous research, we found that both UPLC and traditional HPLC columns show sufficiently well separated peaks for the analysis of eight heparin/HS disaccharides standards and pharmaceutical heparin samples. But in the analysis of "real" biological samples from cells and tissues, UPLC significantly improves separation, sensitivity, and analysis speed without being subject to interference from proteins, peptides, and other sample impurities ultimately allowing us to separate 17 different GAG-derived disaccharides.

4. Conclusions

A new method has been developed that allows the analysis of the GAG content in tissue, biological fluid and cell samples. This method does not rely on differences in the specificity of polysaccharide lyases to distinguish between different types of GAGs and, thus, requires a single multi-enzyme digestion to convert the GAGs in these samples to a mixture of disaccharides. The AMAC labeling of these disaccharides makes their high-resolution separation possible by using RP-UPLC on a standard C18 column. The application of eluents with volatile components (water, methanol and ammonium acetate) provides a mobile phase that is compatible with ESI-MS analysis. Detection using UV, TIC and EIC provides picomole sensitivity. Future studies will be aimed and further simplification of the analytical method and improved sensitivity through the coupling of LIF detection.

Acknowledgements

The authors are grateful for funding from the National Institutes of Health in the forms of grants GM38060, GM090127, HL096972, HL101721 and the NY State Stem Cell Foundation N08G-264.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.12.063.

References

[1] R.V. Iozzo, Nat. Rev. Mol. Cell Biol. 6 (2005) 646.
[2] H.E. Bulow, O. Hobert, Annu. Rev. Cell Dev. Biol. 22 (2006) 375.
[3] J.R. Couchman, Annu. Rev. Cell Dev. Biol. 26 (2010) 89.
[4] R.V. Iozzo, R.D. Sanderson, J. Cell. Mol. Med. 15 (2011) 1013.
[5] I. Capila, R.J. Linhardt, Angew. Chem. Int. Ed. 41 (2002) 391.
[6] L. Kjellen, U. Lindahl, Annu. Rev. Biochem. 60 (1991) 443.
[7] R.L. Whistler, E.J. Olson, Adv. Carbohydr. Chem. 12 (1957) 299.
[8] K. Prydz, K.T. Dalen, J. Cell Sci. 113 Pt 2 (2000) 193.
[9] J.D. Esko, S.B. Selleck, Annu. Rev. Biochem. 71 (2002) 435.
[10] K. Sugahara, H. Kitagawa, Curr. Opin. Struct. Biol. 10 (2000) 518.
[11] D.O. Pinto, P.L. Ferreira, L.R. Andrade, H. Petrs-Silva, R. Linden, E. Abdelhay, H.M. Araujo, C.E. Alonso, M.S. Pavao, Glycobiology 14 (2004) 529.
[12] N. Volpi, R.J. Linhardt, Nat. Protoc. 5 (2010) 993.
[13] K.A. Jandik, K. Gu, R.J. Linhardt, Glycobiology 4 (1994) 289.
[14] R.J. Linhardt, Curr. Protoc. Mol. Biol. Chapter 17 (2001) Unit 17 13B.
[15] K.A. Jandik, D. Kruep, M. Cartier, R.J. Linhardt, J. Pharm. Sci. 85 (1996) 45.
[16] D.M. Cohen, R.J. Linhardt, Biopolymers 30 (1990) 733.
[17] K.G. Rice, Y.S. Kim, A.C. Grant, Z.M. Merchant, R.J. Linhardt, Anal. Biochem. 150 (1985) 325.
[18] S. Yamada, K. Sakamoto, H. Tsuda, K. Yoshida, M. Sugiura, K. Sugahara, Biochemistry 38 (1999) 838.
[19] M.E. Swartz, J. Liq. Chromatogr. Relat. Technol. 28 (2005) 1253.
[20] J.E. MacNair, K.C. Lewis, J.W. Jorgenson, Anal. Chem. 69 (1997) 983.
[21] C.J. Jones, S. Beni, C.K. Larive, Anal. Chem. (2011).
[22] N. Volpi, F. Maccari, R.J. Linhardt, Anal. Biochem. 388 (2009) 140.
[23] S.L. Eldridge, L.A. Higgins, B.J. Dickey, C.K. Larive, Anal. Chem. 81 (2009) 7406.
[24] Z. Liu, S. Masuko, K. Solakyildirim, D. Pu, R.J. Linhardt, F. Zhang, Biochemistry 49 (2010) 9839.
[25] B. Li, H. Liu, Z. Zhang, H.E. Stansfield, J.S. Dordick, R.J. Linhardt, Methods Mol. Biol. 690 (2011) 285.
[26] T. Cecchi, P. Passamonti, J. Chromatogr. A 1216 (2009) 1789.
[27] T. Cecchi, F. Pucciarelli, P. Passamonti, Anal. Chem. 73 (2001) 2632.
[28] A.M. Brustkern, L.F. Buhse, M. Nasr, A. Al-Hakim, D.A. Keire, Anal. Chem. 82 (2010) 9865.
[29] B. Yang, A. Weyers, J.Y. Baik, E. Sterner, S. Sharfstein, S.A. Mousa, F. Zhang, J.S. Dordick, R.J. Linhardt, Anal. Biochem. 415 (2011) 59.
[30] K. Solakyildirim, Z. Zhang, R.J. Linhardt, Anal. Biochem. 397 (2010) 24.
[31] M. Militsopoulou, F.N. Lamari, A. Hjerpe, N.K. Karamanos, Electrophoresis 23 (2002) 1104.
[32] A.M. Hitchcock, M.J. Bowman, G.O. Staples, J. Zaia, Electrophoresis 29 (2008) 4538.
[33] J.A. Deakin, M. Lyon, Glycobiology 18 (2008) 483.
[34] M. Ambrosius, K. Kleesiek, C. Gotting, J. Chromatogr. A 1201 (2008) 54.
[35] F. Galeotti, N. Volpi, Anal. Chem. 83 (2011) 6770.
[36] N. Volpi, Anal. Biochem. 397 (2010) 12.
[37] E. Yoshida, S. Arakawa, T. Matsunaga, S. Toriumi, S. Tokuyama, K. Morikawa, Y. Tahara, Biosci. Biotechnol. Biochem. 66 (2002) 1873.
[38] D. Shaya, A. Tocilj, Y. Li, J. Myette, G. Venkataraman, R. Sasisekharan, M. Cygler, J. Biol. Chem. 281 (2006) 15525.
[39] R. Godavarti, M. Davis, G. Venkataraman, C. Cooney, R. Langer, R. Sasisekharan, Biochem. Biophys. Res. Commun. 225 (1996) 751.
[40] B. Li, P.W. Ryan, B.H. Ray, K.J. Leister, N.M. Sirimuthu, A.G. Ryder, Biotechnol. Bioeng. 107 (2010) 290.
[41] M. Warda, E.M. Gouda, T. Toida, L. Chi, R.J. Linhardt, Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 136 (2003) 357.
[42] F. Zhang, P. Sun, E. Munoz, L. Chi, S. Sakai, T. Toida, H. Zhang, S. Mousa, R.J. Linhardt, Anal. Biochem. 353 (2006) 284.
[43] T. Bitter, H.M. Muir, Anal. Biochem. 4 (1962) 330.
[44] H. Kitagawa, A. Kinoshita, K. Sugahara, Anal. Biochem. 232 (1995) 114.
[45] S.E. Guimond, T.M. Puvirajesinghe, M.A. Skidmore, I. Kalus, T. Dierks, E.A. Yates, J.E. Turnbull, J. Biol. Chem. 284 (2009) 25714.
[46] M. Warda, R.J. Linhardt, Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 143 (2006) 37.
[47] P. Vongchan, M. Warda, H. Toyoda, T. Toida, R.M. Marks, R.J. Linhardt, Biochim. Biophys. Acta 1721 (2005) 1.