Analysis of glycosaminoglycan-derived disaccharides by capillary electrophoresis using laser-induced fluorescence detection

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A B S T R A C T

A quantitative and highly sensitive method for the analysis of glycosaminoglycan (GAG)-derived disaccharides that relies on capillary electrophoresis (CE) with laser-induced fluorescence detection is presented. This method enables complete separation of 17 GAG-derived disaccharides in a single run. Unsaturated disaccharides were derivatized with 2-aminoaacidinone to improve sensitivity. The limit of detection was at the attomole level and approximately 100-fold more sensitive than traditional CE–ultraviolet detection. A CE separation timetable was developed to achieve complete resolution and shorten analysis time. The relative standard deviations of migration time and peak areas at both low and high concentrations of unsaturated disaccharides are all less than 2.7 and 3.2%, respectively, demonstrating that this is a reproducible method. This analysis was successfully applied to cultured Chinese hamster ovary cell samples for determination of GAG disaccharides. The current method simplifies GAG extraction steps and reduces inaccuracy in calculating ratios of heparin/heparan sulfate to chondroitin sulfate/dermatan sulfate resulting from the separate analyses of a single sample.

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Proteoglycans (PGs) are a group of glycoconjugates ubiquitously presented in the extracellular matrix and on the surface of all eukaryotic cells, as well as in basement membranes of various tissues, where they participate in many cellular, physiological, and pathological processes such as chemokine and cytokine activation, microbial recognition, tissue morphogenesis during embryonic development, immune response, and tumor progression and invasion [1–6]. PGs are composed of various core proteins posttranslationally modified with 1 to more than 100 long, unbranched, and anionic polysaccharides called glycosaminoglycans (GAGs) [7]. GAGs are composed of repeating disaccharide units of hexuronic acid, β-glucuronic acid (GlcA) and/or its C-5 epimer l-iduronic acid (IdoA), and hexosamine, β-glucosamine (GlcN) or β-galactosamine (GalN) [8–10]. With the exception of hyaluronic acid (HA), GAGs are sulfated polysaccharides with complex structures that are different based on degree of charge, pattern of sulfo group substitution, and hexuronic acid epimerization [4,5]. There are three major classes of GAGs in animals differing by their polysaccharide backbone structure: HA, heparan sulfate (HS)/heparin (HP), and chondroitin sulfate (CS)/dermatan sulfate (DS). HA, the simplest GAG that neither contains sulfo groups nor is attached to a core protein, is composed of a → 3) β-GlcNac (1 → 4) β-GlcA (1 → repeating unit (where Ac is acetyl). HS/HP are O-sulfo group substituted GAGs with → 4) α-GlcNac or α-GlcNS (1 → 4) β-GlcA or α-IdoA (1 → repeating units (where S is sulfite). CS/DS are O-sulfo group substituted GAGs with → 3) β-GalNAc (1 → 4) β-GlcA or α-IdoA (1 → repeating units [5]. Biosynthesis of GAGs takes place in Golgi and begins with the stepwise addition of four monosaccharides acting as a tetrasaccharide linker on a core protein serine residue in the endoplasmic reticulum [7]. Sugar chain elongation of GAGs then occurs as the sequential addition of uridine diphosphate (UDP)-GlcNAc and UDP–GlcA, determining that the GAG chain belongs to the HS/HP family, or as the addition of

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1 Abbreviations used: PG, proteoglycan; GAG, glycosaminoglycan; GlcA, β-glucuronic acid; IdoA, l-iduronic acid; GlcN, β-glucosamine; GalN, β-galactosamine; HA, hyaluronic acid; HS, heparan sulfate; HP, heparin; CS, chondroitin sulfate; DS, dermatan sulfate; Ac, acetyl; S, sulfite; UDP, uridine diphosphate; HPCL, high-performance liquid chromatography; UPLC, ultra-performance liquid chromatography; GPC, gel permeation chromatography; PAGE, polyacrylamide gel electrophoresis; CE, capillary electrophoresis; UV, ultraviolet; MS, mass spectrometry; NMR, nuclear magnetic resonance; LIF, laser-induced fluorescence; ΔUA, 4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid; 2-AP, 2-aminopyridine; APTS, 8-aminopyrene-1,3,6-trisulfonate; ANTS, 8-aminoanilhen-1,3,6-trisulfonic acid; AMAC, 2-amino-acridone; Et, ethyl; IS, internal standard; CHO, Chinese hamster ovary; HT, hyposan-thine/thymidine mixture; MWCO, molecular weight cutoff; RSD, relative standard deviation; IQQ, limit of quantification; RP, reversed-phase.
UDP–GalNAc and UDP–GlcA, determining that the GAG chain belongs to the CS/DS family. Then the growing chains of GAGs can be modified at various positions: N-deacetylation/N-sulfonation of GlcNAc units catalyzed by N-deacetylas/N-sulfotransferases in HS/HP chains, C-5 epimerization of GlcA to IdoA catalyzed by C-5 epimerase in HS/HP and also DS chains (and modification of IdoA catalyzed by 2-O-sulfotransferase can then take place), O-sulfonation in GlcNAc units catalyzed by 3-O-sulfotransferase and 6-O-sulfotransferase in HS/HP, and O-sulfonation in GalNAc units catalyzed by 4-O-sulfotransferase or 6-O-sulfotransferase [7,11,12]. These postpolymerization enzymatic modification steps result in the heterogeneity and diversity in the disaccharide residues of GAG polysaccharide chains as well as in the polydispersity of chain sizes, all of which are responsible for the crucial biological functions of GAGs; for example, HS/HP are implicated in critical biological processes, such as regulation of enzymatic catalysis in the coagulation cascade and cell–cell interactions [13,14], and CS/DS may be involved in participating and mediating cell–cell interactions and cellular communication [15].

GAGs are extremely difficult to analyze because of their negative charge, polydispersity, and structural heterogeneity [16]. A common strategy of detailed structural analysis of GAGs involves the enzymatic depolymerization of GAGs to obtain their disaccharide constituents. The GAG disaccharides produced by exhaustive lyase-catalyzed digestion contain a Δ-unsaturated hexuronic acid at their nonreducing end with a unique absorbance at 232 nm [17,18] and a molar extinction coefficient of approximately 6000 M⁻¹ cm⁻¹ [19]. Exhaustive heparin lyase treatment of HS/HP affords eight major HS/HP-derived disaccharides. There are also several rare HS/HP-derived disaccharides that can be formed from among the 23 possible known disaccharide sequences [11]. Chondroitin lyase treatment of CS/DS or HA produces eight CS/DS disaccharides and one HA disaccharide. The structures of 17 commercially available, lyase-prepared HS/HP, CS/DS, and HA disaccharides are shown in Table 1. Following the sequential enzymatic treatment, quantitative disaccharide analysis was applied to explore the structural information, which is directly related to biological functions of GAGs. Modern separation techniques, such as high-performance liquid chromatography (HPLC) [20–22], ultra-performance liquid chromatography (UPLC) [23,24], gel permeation chromatography (GPC) [25,26], polyacrylamide gel electrophoresis (PAGE) [27], and capillary electrophoresis (CE), have been applied to GAG analysis to help solve many complex structures [28–31].

CE is one of the most powerful techniques for GAG analysis because of its high sensitivity, resolving power, and separation efficiency combined with its short analysis time, straightforward operation [32], and compatibility with a variety of detection methods, including ultraviolet (UV) spectroscopy, mass spectrometry (MS) [33], nuclear magnetic resonance (NMR) spectroscopy, and laser-induced fluorescence (LIF) [28,34,35]. Analysis of GAGs, performed by CE with both normal and reversed polarity [36,37], has been reviewed previously [32,38]. In reversed polarity, low pH is used to reduce electroosmotic flow, and the analyte, applied at the cathode, migrates under electrophoresis toward the detector at the anode. Determination of disaccharides from complex biological samples containing only a few micrograms of GAGs often requires higher sensitivity than is typically associated with UV detection, and some GAG disaccharides do not have a 4-deoxy-α-D-threo-hex-4-enopyranosyluronic acid (ΔUA) residue for UV detection at 232 nm [39,40]. Moreover, limited repeatability of migration time and peak areas resulting from complex biological matrix and microheterogeneity in separation environment poses significant challenges to GAG analysis by CE. The conjugation of a fluorophore can greatly increase detection sensitivity using LIF [41]. One of the most frequently used derivatization methods is reductive amination of carbohydrates, which frequently relies on aromatic fluorescent amines, including 2-aminopyridine (2-AP) [42], 8-aminopyrene-1,3,6-trisulfonate (APTS) [43], 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) [44], and 2-aminoacridone

### Table 1

Structures of 17 ΔUA–disaccharide standards from HP/HS, CS/DS, and HA

<table>
<thead>
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<th>Disaccharide</th>
<th>Structure</th>
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<tr>
<td>1</td>
<td>Tris&lt;sub&gt;rs&lt;/sub&gt;</td>
<td>ΔUA(25)–GlcNS(6S)</td>
<td>R&lt;sub&gt;0&lt;/sub&gt;</td>
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<td>ΔUA(25)–GlcNAc(6S)</td>
<td>SO&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>CS/DS disaccharides, up middle</td>
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Materials and methods

Materials

Vivapure Q Mini H spin columns were purchased from Sartorius Stedim Biotech (Boehemia, NY, USA). Unadsorbed disaccharide standards of CS/DS (0S\textsubscript{SC}: ΔUA–GalNAc; 4S\textsubscript{SC}: ΔUA–GalNAc4S; 6S\textsubscript{SC}: ΔUA–GalNAc6S; 2S\textsubscript{SC}: ΔUA2S–GalNAc; SB\textsubscript{SC}: ΔUA2S–GalNAc4S; SD\textsubscript{SC}: ΔUA2S–GalNAc6S; SE\textsubscript{SC}: ΔUA–GalNAc4S6S; TriS\textsubscript{SC}: ΔUA2S–GalNAc4S6S), unsaturated disaccharide standards of HS/HP (0S\textsubscript{HC}: ΔUA–GlcNAc; N\textsubscript{SHC}: ΔUA–GlcNS; 6S\textsubscript{SHC}: ΔUA–GlcNAc6S; 2S\textsubscript{SHC}: ΔUA2S–GlcNAc; 2SNS\textsubscript{SHC}: ΔUA2S–GlcNS; 2SNS\textsubscript{SHC}: ΔUA–GlcNS6S; 2SNS\textsubscript{SHC}: ΔUA2S–GlcNC6S; TriS\textsubscript{SHC}: ΔUA2S–GlcNS6S), and unsaturated disaccharide standard of HA (0S\textsubscript{HA}: ΔUA–GlcNAc) were obtained from Seikagaku (Japan). Internal standard (IS: ΔUA2S–GlcNC\textsubscript{Et6S}) was obtained from Iduron (Manchester, UK). AMAC and sodium cyanoborohydride were obtained from Sigma–Aldrich (St. Louis, MO, USA). Actinase E was obtained from Flavobacterial heparin lyases I, II, and III were expressed in our laboratory using Flavobacterium and the lyase acetylation protocol was published [49].

CHO-S cell culture

Suspension adapted Chinese hamster ovary (CHO)-S cells were grown in CD–CHO medium supplemented with 2% hypoxanthine/thymidine mixture (HT) (Gibco–Invitrogen, Carlsbad, CA, USA) and 8 mM Glutamax (Gibco–Invitrogen). The cells were incubated in a 5% CO\textsubscript{2} and 37 °C incubator. For routine maintenance, cells were seeded at 2 × 10\textsuperscript{5} cells/ml fresh medium and cells were subcultured every 3 or 4 days. Cell viability was measured with the Trypan blue exclusion method.

Recovery of GAGs from CHO-S cells by strong anion exchange column

CHO-S cells (1 × 10\textsuperscript{7}) were freeze-dried overnight and incubated with actinase E (2 mg/ml) in 1 ml of solution at 55 °C for 20 h [46]. After removing the particulates from the resulting solutions by a 0.22-μm membrane, the peptides were removed by a Microcon centrifugal filter unit (YM-10, 10,000 MWCO [molecular weight cutoff]) by centrifuging at 10,000 g. Residual GAGs on membrane were collected and purified by a Vivapure Q Mini H spin column by elution with three 300-μl washes each of water and 0.2 M, 0.5 M, and 16% NaCl. The 0.5-M and 16% aqueous NaCl washes were each desalted by a Microcon centrifugal filter units (YM-10, 10,000 MWCO) by centrifuging at 10,000 g. Then GAGs were recovered from the top layer and lyophilized for disaccharide analysis.

Preparation of Δ-disaccharides from GAGs

CS/DS GAGs from CHO-S cells were converted to disaccharides by enzymatic treatment with chondroitin lyase ABC (10 m-units) and chondroitin lyase AClI (10 m-units) for 10 h at 37 °C. After boiling the lyase solution at 100 °C for 2 min to inactivate the lases and cooling to room temperature, the HS/HP in GAGs were converted to disaccharides by enzymatic treatment with a mixture of heparin lyses I, II, and III (10 μM each) for 10 h at 37 °C. All of the disaccharides were recovered using a 30-kDa MWCO spin column (Millipore, Billerica, MA, USA) and freeze-dried.

Derivatization of Δ-unsaturated disaccharides with AMAC

A mixture of 17 ΔUA–disaccharide standards (5 μg/disaccharide) or freeze-dried ΔUA–disaccharides from biological samples (~5 μg/disaccharide) and internal standard ΔUA2S–Glc\textsubscript{NC}Et\textsubscript{6S} was added to 10 μl of 0.1 M AMAC solution in acetic acid/dimethyl sulfoxide, 3:17 (v/v) and mixed by vortexing for 5 min. Next, 10 μl of 1 M sodium cyanoborohydride was added to the reaction mixture and incubated at 45 °C for 4 h [47]. Finally, the AMAC-derivatized ΔUA–disaccharide mixtures were diluted to different concentrations with 50% (v/v) aqueous dimethyl sulfoxide and CE–LIF analysis was performed.

Conditions for CE–LIF analysis

CE analyses were performed on an HPCE system (Agilent Technologies, Wilmington, DE, USA) equipped with a ZETALIF (Picometrix, France) detector (λ\textsubscript{ex} = 488 nm). Resolution and analysis were performed on an uncoated fused-silica capillary column (50 μm i.d., 85 cm total length, 70 cm effective length) at 25 °C using 50 mM phosphate buffer (pH 3.5) at different voltages (as shown in figures) and reversed polarity. New capillary was treated with methanol, 1 M HCl, 1 M NaOH, 0.1 M NaOH, water, and operating buffer until the baseline became constant. Between each run, the capillary was flushed with 0.1 M NaOH (3 min), HPLC-grade water (3 min), and operating buffer (4 min). The operating buffer was filtered through a 0.22-μm membrane filter. All solutions were degassed. Samples were introduced using the injection buffer (50 mbar × 5 s) at the cathode.

Conditions for UPLC–MS analysis

LC–MS analyses were performed on an Agilent 1200 LC/MS instrument (Agilent Technologies) equipped with a 6300 ion trap. The column used was an Acquity UPLC BEH C18 column (2.1 × 150 mm, 1.7 μm, Waters, Milford, MA, USA) at 45 °C. For dual ammonium acetate and methanol gradient, eluent A was 80 mM ammonium acetate solution and eluent B was methanol. Solution A and 12% solution B were flowed through the column (95 μl/min) for 15 min, followed by linear gradients: 12 to 15% solution B from 15 to 30 min, 15 to 30% solution B from 30 to 60 min, and 30 to 100% solution B from 60 to 62 min [40].
Results and discussion

Derivatization of unsaturated disaccharides

The AMAC derivatization procedure chosen in this study was simple and rapid [28]. The AMAC fluorophore can be efficiently introduced at the reducing end of GAG-derived disaccharides by reductive amination (Fig. 1). This process relies on the initial formation of a Schiff base between the AMAC amino group and the hemiacetal at the disaccharide’s reducing end and is followed by sodium cyanoborohydride reduction. A 50- to 100-fold excess of AMAC was applied for high efficiency derivatization. After AMAC labeling, the derivatized HS/HP, CS/DS, and HA disaccharides mixtures were monitored by comparing UV absorbance at 232 and 255 nm (AMAC-labeled ΔUA–disaccharides absorb at 255 nm). The absence of residual starting ΔUA disaccharides suggests high derivatization efficiency, satisfactory for CE–LIF analysis. Caution must be applied because it is possible to assess derivatization efficiency only at concentrations sufficient for UV detection, and biological samples often afford disaccharides at very low concentrations.

Under the separation conditions, excess AMAC receives a positive charge, making it impossible for AMAC to enter the capillary under reversed polarity mode. This was demonstrated by injecting a mixture of AMAC, reagents, and GAG-derived disaccharides into the capillary under the same CE conditions. No peaks were found in the electropherogram, so cleanup of excess AMAC was unnecessary.

**CE timetable for separation of 17 AMAC-labeled HP/HS, CS/DS, and HA ΔUA–disaccharides with internal standard**

It was necessary to develop a timetable for CE separation of 17 AMAC-labeled, GAG-derived disaccharides because they cannot be completely resolved under a single separation voltage within an acceptable analysis time. The separation efficiency relies significantly on the length and inner diameter of the capillary [48], so in our study a fused-silica column of 50 μm internal diameter was used to minimize thermal effects, and because the separation efficiency is relatively constant at long lengths [45], a capillary of an effective length of 70 cm was selected for use in this study. Buffer species can have a significant impact on separation efficiency. Phosphate buffer had been proven to be effective for separation of AMAC-labeled HS/HP, CS/DS, and HA disaccharides [28,31,41]. Moreover, the effect of ionic strength on separation efficiency is straightforward [48]; decreasing the ionic strength of the operating buffer decreases conductivity, lowering current flow, decreasing temperature gradients within the capillary column, and increasing separation efficiency. However, low conductivity of operating buffer can cause triangular-shaped peaks that can degrade the separation efficiency. In the current study, optimizing the concentration of buffer has only a minor effect on resolution. Another critical parameter governing CE separation of 17 AMAC-labeled disaccharides is the separation voltage. HS/HP and CS/DS disaccharides can be completely resolved under a certain voltage in separate experiments [31]. Disulfated HS/HP and CS/DS disaccharides with added internal standard disaccharide cannot be resolved until the separation voltage has been decreased to 20 kV, and mono-, tri-, and unsulfated HA disaccharides can be quickly and completely separated at 30 kV. A lower separation voltage increases disaccharide migration time, requiring longer analysis time. Under the buffer conditions in this study, the separation of 17 AMAC-labeled disaccharides with added internal standard disaccharide takes more than 90 min at 20 kV. In addition, electrolysis of buffer can occur during a long analysis, resulting in incomplete separation and poor repeatability. Electropherograms of analyses performed under different separation voltages of 20 and 30 kV are shown in Fig. 2A and B. Under separation voltage of 20 kV, only 14 components with internal standard were resolved in 70 min, and under separation voltage of 30 kV, 17 components with internal standard were detected in 60 min; however 2S6S N and internal standard were not completely resolved.

As a result, a timetable was applied to the CE separation, shown in Table 2. Although this timetable is more complex than the conditions previously reported for the individual separation of HS/HP- and CS/DS-derived disaccharide mixtures [31], such programmed elution is relatively straightforward on modern CE instruments. The electropherogram shows complete resolution of 17 AMAC- derivatized disaccharides (Fig. 2C). All disaccharides and internal standard were well resolved in a relatively short analysis time. An analysis time of approximately 1 h for a mixture of AMAC-derivatized HS/HP and CS/DS disaccharides compares favorably with two separate analyses of 20 to 30 min reported previously [31].

![Fluorophore derivatization reaction of ΔUA–disaccharides with AMAC. Shown is a generalized GAG disaccharide that can be either 1,3- or 1,4-linked and can contain a GalN or GlcN residue at the reducing end, where Y = O or glycosidic, Z = Ac or SO₃.](image)
4.4 fmol for different ΔUA–disaccharides. The limits of quantification (LOQs) by LIF detector (λex = 488 nm) were also estimated from the measurement as a signal-to-noise of 10:1 and ranged from 8 to 567 fmol for different disaccharides. A calibration curve and the LOQ for each ΔUA–disaccharide are presented in Table 3. These results show approximately 100-fold higher sensitivity when compared with UV detection of nonderivatized ΔUA–disaccharides at 232 nm. This clearly demonstrates that fluorescent labeling is very useful for the analysis of the GAGs present in trace amounts in complex biological samples. There might be several reasons for the variability in the LOQ for the various disaccharides (Table 3). The analyte is a 17-component mixture of disaccharides prepared from three classes of polysaccharides (HS/HP, CS/DS, and HA), and the derivatization reaction can be truly optimized only for a single disaccharide and at a single concentration. Although derivatization appeared to be quantitative for all components at a concentration sufficiently high to be analyzed by CE using UV detection, this may not be true when lower concentrations of disaccharides are derivatized with AMAC. Indeed, it appears that the more highly sulfated disaccharides (i.e., TriS6S and TriS6S) may be less efficiently derivatized. Moreover, 0S6S, eluting last from the capillary, shows a much broader peak, suggesting a reason for its relatively high LOQ. Thus, the use of a standard curve as well as an internal standard is strongly recommended in analyzing sample containing low concentrations of unknown GAGs.

Application of the method to biological samples

Quantitative disaccharide compositional analysis is one of the most common strategies for structural characterization of GAGs and is directly related to their biological function study. However, the fact that GAGs are not very abundant in many biological samples poses a great challenge to GAG-derived disaccharides analysis. In our laboratory, the macro-scale extraction method was developed for compositional profiling of GAGs from biological sources and excellent recoveries were achieved [46], but additional study is also necessary to optimize and simplify the extraction procedure necessary for the reversed-phase (RP) ion-pairing method; and (iii) a normal UPLC–MS injection is at the level of several microliters, whereas CE–LIF analysis consumes only 10 to 100 nl for each run.

CHO cells are widely used in the biopharmaceutical industry for the production of recombinant therapeutic proteins, and they are also known to be effective in the biosynthesis of HS but not HP.
Currently, our laboratory research is focused on the metabolic engineering of the HS biosynthetic pathway to produce secreted CHO cell heparin. AMAC-derivatized ΔUA−disaccharides obtained from endogenous GAGs from CHO-S and CHO-D29 cells were analyzed by CE–LIF (Fig. 3). The data show that in CHO-S cells only four different disaccharides were present (4S$_{CS}$ [36.5%], 6S$_{CS}$ [2.3%], NS$_{HS}$ [15.4%], and 0S$_{HS}$ [45.8%]) and the ratio of HS/HP to CS/DS was 1.6, whereas in CHO-D29 cells only NS2S$_{HS}$ (4.6%), 4S$_{CS}$ (2.3%), NS$_{HS}$ (88.0%), and 0S$_{HS}$ (5.0%) were present and the ratio of HS/HP to CS/DS was 42.5. These results are consistent with our analysis using RP–UPLC–MS (Fig. 4) and previously published results [49]. It is noteworthy that CHO-S and CHO-D29 cells show different disaccharide compositions than CHO-K cells, possibly because CHO-S and CHO-D29 cells are grown in suspension, whereas CHO-K is an adherent cell line. GAG compositions of cultured cell lines also vary based on species, cell type, cell line, and environmental and cell growth conditions and are the subject of ongoing studies in our laboratory [49].

Conclusions

The CE–LIF protocol described here provides a fast, simple, and reproducible method for analysis of HS/HP and CS/DS disaccharide samples. A complete separation of all 17 AMAC-labeled ΔUA−disaccharides was obtained in 70 min. This is the first time that CE separation has been applied to resolve HS/HP, CS/DS, and HA disaccharides in one run from a single sample, so the steps for enzymatic degradation of GAGs can be simplified. An internal standard ΔUA2S–GlCNCTet6S was also introduced to improve the quantitative ability of the method. The derivatization procedure was rapid, simple, and quantitative, and no cleanup step was required to remove excess labeling reagent. This method can be
applied for the analysis of GAGs in biological sample because of its high sensitivity and ease of quantification.

Acknowledgments

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References


Fig. 4. Extracted ion chromatograms (EIC) of AMAC-labeled AUA–disaccharides from endogenous GAGs present in CHO-S and CHO-D29 cells recovered in 16% NaCl wash. (A) The 17 standard disaccharides from HS/HP, CS/DS, and HA: (1) TriS9S, (2) NS6S9S, (3) NS2S9S, (4) TriS9S, (5) NS9S, (6) S9S, (7) 2S9S9S, (8) 4S9S, (9) S9S9S, (10) S9S9S, (11) 2S9S, (12) 2S9S, (13) 4S9S, (14) 0S9S, (15) 5S9S, (16) 0S9S, and (17) 0S9S. (B) Endogenous GAGs in CHO-S cells recovered in 16% NaCl wash. (C) Endogenous GAGs in CHO-D29 cells recovered in 16% NaCl wash.


