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Sensitive method for glycosaminoglycan analysis of tissue sections

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Abstract

A simple, isocratic HPLC method based on HILIC-WAX separation, has been developed for analyzing sulfated disaccharides of glycosaminoglycans (GAGs). To our best knowledge, this is the first successful attempt using this special phase in nano-HPLC-MS analysis. Mass spectrometry was based on negative ionization, improving both sensitivity and specificity. Detection limit for most sulfated disaccharides were approximately 1 fmol; quantitation limits 10 fmol. The method was applied for glycosaminoglycan profiling of tissue samples, using surface digestion protocols. This novel combination provides sufficient sensitivity for GAG disaccharide analysis, which was first performed using prostate cancer tissue microarrays. Preliminary results show that GAG analysis may be useful for identifying cancer related changes in small amounts of tissue samples (ca. 10 μg).

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1. Introduction

Nano-scale liquid chromatography (nano-LC) coupled to mass spectrometry (MS) is an excellent technique used in routine characterization of different types of molecules (e.g. peptides[1,2], glycans [3], etc.), with still growing application areas. The field of proteomics greatly benefited from its constant improvements; however, a similar breakthrough is still waiting to occur in case of glycomics and especially glycosaminoglycan (GAG) analysis. GAGs are an often overlooked class of compounds, characterized by highly sulfated, long, linear polysaccharide chains. Their characterization poses several challenges [4]. The saccharide units can be sulfated at various positions and epimerization may also occur along the chain. Most GAGs are covalently attached to core proteins and form proteoglycans (PGs). They are localized in the extracellular matrix, cell surfaces and intercellular granules and mediate various physiological and pathophysiological processes including coagulation, cancer metastasis, and inflammation [5-8]. Involvement of both GAGs and PGs in cancer progression has been reported [7,9,10]. GAG chains may interact with different effector proteins (e.g. chemokines, cytokines and growth factors) and thus regulate processes such as tumor cell growth, metastasis, and angiogenesis [10]. Interaction between the GAG chains and the effector proteins strongly depends on sulfation motifs within the chain.

The building blocks of GAG chains are repeating disaccharide units of a uronic acid or galactose unit and an amino sugar (often abbreviated as HexA/Gal-HexNAc). GAGs are divided into four different classes: hyaluronan (HA), heparan sulfate (HS)/heparin, chondroitin sulfate/dermatan sulfate (CS/DS) and keratan sulfate (KS). Perhaps the most widely known group is HS, consisting of a hexuronic acid (HexA) and *N*-acetyl glucosamine (GlcNAc) disaccharide units.

Analytical characterization of GAGs is usually performed after hydrolysis of the polymeric chain into the constituent disaccharide units. Bacterial polysaccharide lyase enzymes can cleave the glycosidic bond between the HexA and HexNAc sugars via an eliminative mechanism and produce $\Delta^{4,5}$-unsaturated disaccharides with varying degrees of sulfation; HS disaccharides may be sulfated by up to three sulfate groups. The structures, masses and nomenclature [11] of HS disaccharides are summarized in Table 1. Determining the ratio of these different structures is important in understanding mechanisms underlying diseases such as cancer.

71   Several different chromatographic techniques can be used to analyze $\Delta^{4,5}$-unsaturated, and

72   variously sulfated disaccharide units of HS. These include derivatization followed by reversed-

73   phase chromatography (RP) [12-17]; reversed-phase ion-pairing chromatography (RPIP) [18-20];

74   size exclusion chromatography (SEC) [21-23]; graphitized carbon [24,25]; amide-HILIC [26,27];

75   or HILIC-WAX [28] chromatography. These separation methods can be on-line coupled to mass

76   spectrometry (MS) and yield useful information about the involvement of GAGs in various

77   biochemical processes [29][30]. These analytical approaches have been reviewed recently [31].

78   The main disadvantage of the above mentioned methods is their relatively low sensitivity, which

79   is generally in the low pmol range [23,28]. Perhaps the currently most sensitive method for GAG

80   disaccharide profiling is fluorescent labelling and HPLC separation [15]. Limit of detection of this

81   method for most HS disaccharides was ca. 0.3 pmol (~0.1 ng); for the trisulfated D2S6 it was ca.

82   2 pmol (~1 ng). This sensitivity is sufficient to study bulk tissues, but is insufficient to study tissue

83   slices or tissue microarrays (TMAs). In fact, recent examples of GAG analysis as much as 1 g

84   tissue amount were required [16].

85   The main reason for developing our method was the need to have a technology capable of GAG

86   analysis of small size tissue slices. Our plan is utilizing tissue microarrays (set of 1.5 mm diameter,

87   5 μm thick tissue slices arranged in array format, ca. 10 μg tissue), which provide an opportunity

88   to study relatively large number of well characterized, well matched human cancer tissue samples.

89   Target sensitivity is the low fmol range (for individual disaccharides), which is the amount

90   foreseen to be present in tissue microarray digests. To achieve this goal, special, self-packed,

91   commercially unavailable capillary columns were designed. A mixed mode resin combining

92   hydrophilic interaction (HILIC) and weak anion exchange (WAX) retention mechanisms was

93   chosen as packing material, as it enables separation of glycans based on charge, size and polarity

94   [28,32]. Use of capillary columns allows the use of on-line nano-UHPLC-MS coupling; needed to

95   achieve the necessary sensitivity. The packing material was obtained by opening a commercially

96   available analytical (2.1 mm diameter) column, and repacked into 100 μm i.d. capillaries.

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2. Materials & Methods

2.1 Chemicals and reagents

The following standards and enzymes were purchased from Iduron (Cheshire, UK): unsaturated heparan sulfate disaccharides, heparinase I, II and III enzymes. LC-MS grade solvents, ammonium formate solution were purchased from Sigma-Aldrich (Sigma-Aldrich Kft., Budapest, Hungary).

2.2 Capillary column packing

A GlycanPac™ AXH-1 1.9 μm analytical column (2.1x100mm, Thermo Fisher Scientific, Waltham, MA USA) was unpacked and repacked into 100 μm internal diameter capillaries using a pressure injection cell (Next Advance Inc., NY, USA). For this purpose, 20-30 cm 100 μm internal diameter capillaries were cut and fritted as previously reported [33]. Briefly, capillaries were dipped in a solution containing Kasil® 1624, Kasil 1 and formamide in a ratio of 3:1:1. The capillaries were then placed in an oven at 80 °C for 4 hours. Capillaries were examined under a microscope and in case of fully porous frit the excess was trimmed to 0.5 cm in order to reduce dead volume. Capillary was then placed in the pressure injection cell and was washed with methanol. A 1 mg/mL suspension was prepared from the GlycanPac™ AXH-1 resin in 75% Acetonitrile - 25% Methanol. The slurry was continuously vortexed using a magnetic stir bar and the column was packed using nitrogen at 2000 psi. After reaching the desired length – generally between 10 and 11 cm – the pressure was carefully released overnight. Due to the pressure drop inhomogeneity may appear, therefore 30 minute long compression step (at 5000 psi) was necessary following mounting the column on the nano-HPLC.

2.3 Liquid chromatography-mass spectrometry

The in-house packed capillary column was mounted on a Waters® nanoAcquity UPLC system (Waters, Milford, MA, USA) coupled to a high resolution Waters® Q-Tof Premier™ Mass Spectrometer (Waters, Milford, MA, USA) via nanoelectrospray ionization source. Precut silica

127   tips (360 μm OD, 20 μm ID, 10 μm tip ID, DNU - MS GbR, Berlin, Germany) were used as

128   emitters.

129

130   *2.3.1   LC parameters*

131   A flow rate of 0.6 μL/min was found to give stable signal and gave reasonable back pressure (2000

132   psi). Solvent A was 50 mM ammonium formate pH 4.4 (pH adjusted with formic acid) and solvent

133   B was 95% acetonitrile and 5% water, without any buffer. Method optimization was performed on

134   the self-packed columns using commercially available $\Delta^{4,5}$-unsaturated HS disaccharide standards.

135   The following isocratic methods were tested: 90%B, 85%B, 80%B, 75%B and 70% B.

136

137   *2.3.2   MS parameters*

138   Negative ionization mode was used. Correct tuning of MS parameters is critical when analyzing

139   labile molecules such as sulfated sugars. The instrument was tuned by direct infusing the most

140   labile triply sulfated heparan sulfate disaccharide (D2S6). Decreasing the cone voltage to 15 eV

141   from the generally used 35 eV resulted in minimal (<5%) sulfate loss. Extraction cone voltage was

142   also lowered to 1 V. MS data was acquired in the *m/z* 180-800 mass range.

143

144   *2.4 Enzymatic digestion of TMA cores*

145   Prostate cancer tissue microarrays T191a and T196 were obtained from US Biomax, Inc.

146   (Derwood, MD, USA). Tissue sections were processed (dewaxing and antigen retrieval) as

147   previously described [34]. TMA cores corresponding to normal (healthy prostate tissue, male, 33

148   years), grade 1 (adenocarcinoma, male, 72 years) and grade 2-3 (adenocarcinoma, male, 60 years)

149   cancer were chosen for the current analysis. In case of each patient the TMA contained 3 cores/case

150   allowing to assess the reproducibility of the developed method. Heparinase I, II and III digestion

151   was performed on the surface of the TMA cores as previously described [34]. Following 5 cycles

152   of enzyme addition (40 min 37 °C incubation/cycle) two additional cycles were carried out by

153   pipetting the enzyme solution without the enzymes to ensure completion of the enzymatic

154 digestion process. The HS disaccharides were extracted from the individual cores using 0.3%
155 ammonium-hydroxide solution, dried down and re-suspended in the LC starting conditions.
156 During the experiments a quality control sample followed by two blank injections was ran before
157 the tissue microarray samples.

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159 *2.5 Data analysis*

160 Data acquisition was controlled by MassLynx, quantitative results were evaluated by QuanLynx
161 software (Waters, Milford, MA). Non-supervised PCA analysis was performed by XLStat
162 software (Addinsoft, New York, USA).

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3. Results & discussion

Detection of both neutral and highly acidic disaccharides by mass spectrometry presents a challenge; initial trials showed that negative electrospray ionization provides the best sensitivity. High mass resolution was needed to improve selectivity of disaccharide analysis as extracts of tissue surface digestion contain a large amount of impurities, the target disaccharides being minor components only. Sulfated disaccharides fragment easily in the mass spectrometer ion source under conventional conditions, compromising analysis. In order to avoid this problem ion source conditions have to be specially tuned. The most critical parameter is the cone voltage, which, in our instrument, had to be reduced from the conventionally used 30-35 eV to as low as 15 eV.

Based on the literature [28] we have selected a mixed mode HILIC-WAX stationary phase, which was shown to be efficient for analysis of acidic oligosaccharides [32]. Capillary columns were packed in-house with this packing material. We have used medium ion strength ammonium formate buffer and acetonitrile solvents. Method development was carried out using commercially available standards with the goal of analyzing small amounts of tissue digests (tissue microarrays) containing disaccharides in the low fmol range.

Initial trials using gradient elution showed that mass spectrometry sensitivity significantly changes with solvent composition; as a result, the late-eluting doubly and triply sulfated disaccharides had the lowest sensitivity among those tested. For example, in equimolar mixtures the signal intensity for the trisulfated HS disaccharide was 30 times less than those of the neutral or the monosulfated disaccharides, due to changes in solvent composition. For this reason, we have decided to develop an isocratic elution method.

3.1 Method optimization

First, the required ion strength of the strong eluent was established. It was found that the method is very sensitive to the salt concentration: sulfated (especially highly sulfated) disaccharides do not elute from the column neither at lower (10-40 mM) nor at higher (80 mM) ammonium formate concentration. Medium ion strength (50 mM ammonium formate) resulted in a reasonable

192 chromatogram; and this was used in the following. The next step was the adjustment of the pH of
193 the buffer, using formic acid. We tested pH values of 4.0, 4.4 and 4.6, respectively. At pH 4.0
194 resolution of monosulfated, at pH 4.6 resolution of disulfated disaccharides were unsatisfactory.
195 The best results were achieved at pH 4.4 for which it was possible to detect and resolve the HS
196 disaccharides according to composition.

197 Subsequently we evaluated five different isocratic conditions (90% B, 85% B, 80%B, 75%B and
198 70% B, Fig.1). Note, Fig. 1 shows the sum of the peak intensities (sum of selected ion
199 chromatograms at high resolution) of the HS disaccharides. When the eluent contained a high
200 percentage of acetonitrile (90 and 85% B) the corresponding peaks were broad and shallow (Fig.
201 1E and Fig. 1D) for the highly sulfated structures and the triply sulfated disaccharide did not elute
202 within 15 minutes. In case of 75% and 70% B partial co-elution of various peaks was observed
203 (Fig. 1A and Fig. 1B). The isocratic method at 80% B was optimal (Fig. 1C) because it resolved
204 the HS disaccharides based on composition; peak shapes were symmetric, and all peaks eluted
205 within 15 minutes. Individual selected ion chromatograms of the HS disaccharides are shown in
206 Fig. 2. This isocratic method ameliorated the problem of low signal intensity for the triply sulfated
207 HS. The main reason was that using gradient separation water and buffer concentration is increased
208 at the elution of the triply sulfated disaccharide. Under such conditions the MS sensitivity
209 decreases, resulting in low sensitivity for the highly sulfated derivatives. Note that D2S6 (the triply
210 sulfated derivative) elutes in a fairly wide peak. This is due to the highly polar nature of this
211 compound, which binds strongly to the HILIC-WAX resin.

212 We have determined the repeatability of analysis as well (Table 2). For the 500 fmol sample
213 mixture intra-day repeatability of relative peak areas was 5% on average; inter-day repeatability
214 was 10% (relative standard deviation; 5 measurements in a day, measured on 3 different days).
215 Repeatability (relative standard deviation) of retention times was, on average 0.3% within a day,
216 and 1.2% between days.

217

218 *3.2 Linearity studies*

219 We also demonstrated the linearity (Fig. 3 and Supplementary material Fig. S1) of signal intensity
220 with increasing sample amount, and the limits of quantitation. The following concentrations of HS

221   disaccharide mixtures were tested with 80% B isocratic method: 10 fmol, 20 fmol, 50 fmol, 100

222   fmol, 200 fmol, 500 fmol and 1000 fmol. As 1 μL sample volume was injected always, these

223   sample amounts correspond to 10, 20, 50, 100 200, 500 and 1000 pmol/mL concentrations. Each

224   concentration was measured in triplicate. For the mono and disulfated components at 10 fmol

225   injection the signal-to-noise ratio was greater than 20:1 (indicating that the limit of quantitation

226   (LOQ, defined as S/N>10) is slightly better than 10 fmol). We estimate the limit of detection for

227   these components (S/N>3) to be approximately 1 fmol. For the non-sulfated D0A0 disaccharide,

228   the detection limit was ca. 10 fmol (due to interference from an abundant background ion at a

229   similar mass and retention time). The quantitation limit was ca. 20 fmol in this case. The trisulfated

230   compound gave very low signal intensity at low concentration, detection limit was ca. 20 fmol,

231   and quantitation limit was 50 fmol. We evaluated linearity of peak areas over a 10-1000 fmol range

232   that is appropriate for biological samples. Excellent linearity was observed for four disaccharides

233   in the 10-1000 fmol range (Fig. 3B, Fig. 3C, Fig. 3D and Fig. 3E), with an R^2 value > 0.99 for

234   each. The non-sulfated D0A0 disaccharide was measured in the 20-1000 fmol range (Fig. 3A), the

235   trisulfated HS in the 50-500 fmol range (Fig. 3F), both with R^2>0.99 linearity. Average standard

236   deviation of the HS disaccharides, as expected, decreased with increasing concentration of the

237   standard mixture (Table 3). Average standard deviations were found to be 4.2% for the 1000 fmol

238   mixture and 14% for the 100 fmol mixture. The present experiments do not distinguish the

239   positional isomers (D2A0/D0A6 and D2S0/D0S6). We have checked, that the ionization

240   efficiencies of the two isomer pairs are within 15%; well within the intended accuracy of the

241   present study. For this reason, quantitation will determine the sum of the two positional isomers.

242   Relative sensitivity of the isomers is shown in Supplementary material Table S1.

243

244   *3.3 Analysis of tissue microarrays*

245   The next phase of method development was establishing, whether the method developed on

246   standards would be suitable for analyzing small amounts of biological material. As test examples

247   we have chosen tissue microarrays (TMA), which are gaining importance in biomedical

248   workflows; the main challenge being the limited sample amount (1.5 mm diameter, 5 μm thin

249   cores). Sample amount in such cores is sufficient for proteomics workflows based on nanoLC-MS

250   methodologies. TMA cores were digested using heparinase enzymes on their surface (see details

251   in the Materials & Methods section), yielding $\Delta^{4,5}$-unsaturated HS disaccharides. The digestion

252   products were pipetted off the cores, and analyzed by the LC-MS method described above.

253   Selected example chromatograms are shown in Fig. 4 for a grade 2-3 prostate cancer TMA cores.

254   Based on the calibration curve obtained using the standards it was possible to estimate the amount

255   of the extracted disaccharides (Fig. 4).

256   In this study TMA sections were analyzed corresponding to prostate tissue biopsy from one healthy

257   individual, one grade 1 and one grade 2-3 patient, respectively. Three serial biopsies were analyzed

258   for each TMA sample. We observed the 4 most abundant disaccharides in these samples. Among

259   these, one doubly sulfated and the triply sulfated disaccharide were not detected. Approximate

260   quantitation based on Fig. 3 indicated that the most abundant component is the non-sulfated D0A0

261   disaccharide being in the 20-80 fmol range in the various samples. The big peak in the D0A0 ion

262   chromatogram, before the sample at Rt=3.5-6.5 min is coming from the background. This does not

263   interfere with detecting and quantifying D0A0. The two mono-sulfated components are present in

264   comparable amounts; 10-40 fmol in the case of D0S0 and 5-20 fmol in the case of D0A6/D2A0.

265   Among the higher sulfated components only the disulfated D2S0/D0S6 HS disaccharide was

266   observed in each case, but only in low amounts (less than 10 fmol).

267   Three biological samples were analyzed in triplicates. The results showed an increased abundance

268   in cancer of the mono-sulfated D0S0 and the D0A6/D2A0 components (Fig. 5).

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4.   Conclusion

We demonstrated that sulfated HS disaccharides can be advantageously studied using LC-MS with isocratic nano-chromatography conditions. Isocratic elution is advantageous for mass spectrometry analysis, as ion spray conditions and sensitivity issues do not complicate analysis. It is of particular importance in those cases, when the analyte has unfavorable MS characteristics. Using a combination of nano-HPLC and negative ionization, we detected HS disaccharides in the low fmol range.

We used the technique to analyze HS in tissue slices, using digestion directly on the tissue surface. Both sensitivity and specificity were sufficient to analyze small, ca. 2 mm^2 size areas on the tissue surface, opening up the possibility of studying tissue microarrays. We expect that this will allow systematic studies of human disease biospecimens. In the present paper, as an illustrative example, we studied TMA of healthy, grade 1 and grade 2-3 prostate tissue biopsies. These pilot studies demonstrated the ability to differentiate the biospecimens based on HS disaccharide abundances. This is consistent with known roles of HS proteoglycans in dysregulation of receptor tyrosine kinase signaling in cancer phenotypes [35-37]. In the future studies, we will extend to analyze GAGs from a large number of TMA cores, in order to build statistically relevant models correlating with disease progression.

Appendix A. Supplementary data

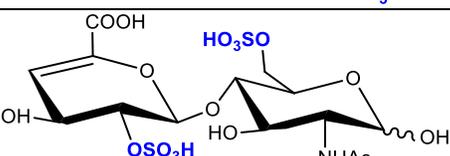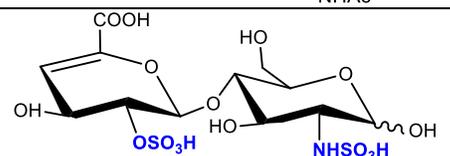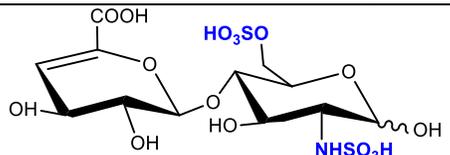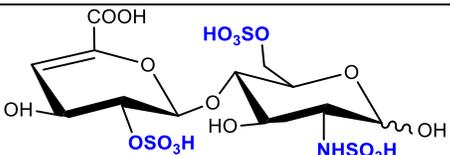Figure S1.   Linearity of the method (log-log plot), calibration curves for the individual HS disaccharide standards: D0A0 (A), D0S0 (B), D0A6/D2A0 (C), D2S0/D0S6 (D),

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297   D2A6 (E), and D2S6 (F). Excellent linearity was observed for four disaccharides

298   (B, C, D and E) in the 10-1000 fmol range with an R^2 value > 0.99 for each. The

299   non-sulfated disaccharide D0A0 (A) was measured in the 20-1000 fmol range, the

300   trisulfated HS (F) in the 50-500 fmol range, both with R^2>0.99 linearity.

301

302   Table S1.   Signal intensities of D0A6, D2A0, D2S0 and D0S6, and relative sensitivity of the

303   D0A6/D2A0 and D0S6/D2S0 isomer pairs. Data were measured using direct

304   infusion of the individual isomers in 20%A and 80%B eluent mixture, under the

305   same mass spectrometric tuning, as in the LC-MS studies described. Values are

306   given as the sum of singly and doubly deprotonated ion counts. Concentration of

307   the standards were 5 μM.

308

HS disaccharide structure	Traditional name	Lawrence code [11]	m/z in negative mode
	ΔHexA-GlcNAc	**D0A0**	378.1
	ΔHexA2S-GlcNAc	**D2A0**	458.1
	ΔHexA-GlcNAc6S	**D0A6**	458.1
	ΔHex-GlcNS	**D0S0**	416.1
	ΔHexA2S-GlcNAc6S	**D2A6**	538.1
	ΔHexA2S-GlcNS	**D2S0**	496.1
	ΔHex-GlcNS6S	**D0S6**	496.1
	ΔHexA2S-GlcNS6S	**D2S6**	576.1

310

Table 1.   Structure and nomenclature of the most common unsaturated HS disaccharides obtained following heparin lyases digestion. Note, that D2A0/D0A6 and D2S0/D0S6 are positional isomers and are not distinguished in the present study.

314

	RSD Peak Area		RSD Retention time	
HS disaccharide	Intra-Day	Inter-Day	Intra-Day	Inter-Day
D0A0	6.68%	7.91%	0.22%	0.45%
D0S0	5.15%	15.58%	0.27%	0.97%
D0A6/D2A0	3.11%	3.44%	0.26%	0.75%
D2S0/D0S6	3.67%	7.61%	0.48%	1.69%
D2A6	4.03%	7.37%	0.19%	1.35%
D2S6	10.18%	20.19%	0.35%	1.75%
Average	5.47%	10.35%	0.30%	1.16%

315

316 Table 2.   Repeatability of analysis. Intra-Day and Inter-Day relative standard deviation of
317   peak areas and retention times for the unsaturated HS disaccharides.

318

	10 fmol	20 fmol	50 fmol	100 fmol	200 fmol	500 fmol	1000 fmol
D0A0	ND	18.04%	10.98%	5.82%	4.57%	4.92%	6.31%
D0S0	18.79%	13.07%	11.43%	15.27%	10.28%	4.84%	1.45%
D0A6/D2A0	13.95%	18.21%	20.89%	14.86%	14.97%	10.81%	10.98%
D2S0/D0S6	15.84%	15.88%	17.11%	17.28%	9.94%	5.53%	2.86%
D2A6	8.53%	11.56%	12.40%	13.75%	7.50%	1.20%	1.96%
D2S6	ND	ND	ND	19.85%	7.58%	3.52%	1.73%

319

320   Table 3.   Relative standard deviation of peak areas for the unsaturated HS disaccharides at
321   different concentrations measured in triplicates

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