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Abstract

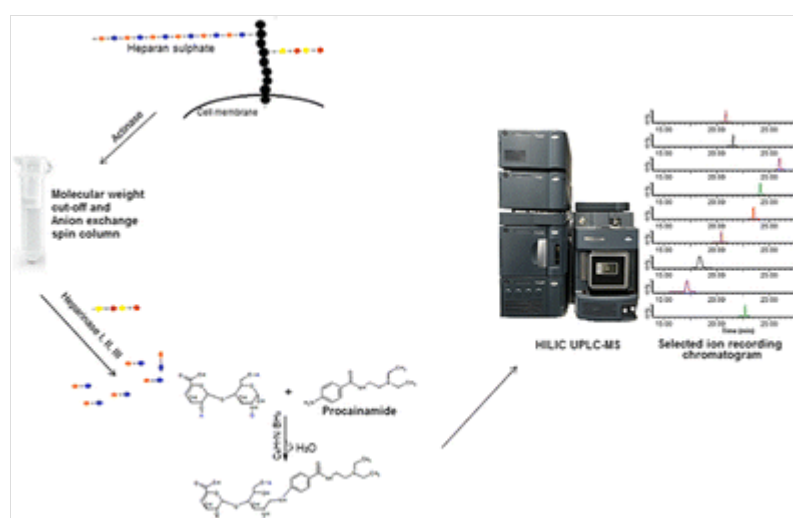
Glycosaminoglycans (GAGs) are a family of linear heteropolysaccharides made up of repeating disaccharide units that are found on the surface and extracellular matrix of animal cells. They are known to play a critical role in a wide range of cellular processes including proliferation, differentiation and invasion. To elucidate the mechanism of action of these molecules, it is essential to quantify their disaccharide composition. Analytical methods that have been reported involve either chemical or enzymatic

depolymerisation of GAGs followed by separation of non-derivatised (native) or derivatised disaccharide subunits and detection by either UV/fluorescence or MS. However, the measurement of these disaccharides is challenging due to their hydrophilic and labile nature. Here we report a pre-column LC-MS method for the quantification of GAG disaccharide subunits. Heparan sulphate (HS) was extracted from cell lines using a combination of molecular weight cutoff and anion exchange spin filters and digested using a mixture of heparinases I, II and III. The resulting subunits were derivatised with procainamide, separated using hydrophilic interaction liquid chromatography and detected using electrospray ionisation operated in positive ion mode. Eight HS disaccharides were separated and detected together with an internal standard. The limit of detection was found to be in the range 0.6–4.9 ng/mL. Analysis of HS extracted from all cell lines tested in this study revealed a significant variation in their composition with the most abundant disaccharide being the non-sulphated Δ UA–GlcNAc. Some structural functional relationships are discussed demonstrating the viability of the pre-column method for studying GAG biology.

Graphical abstract

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Extraction and HILIC UPLC-MS analysis of procainamide-labelled heparan sulphate disaccharides



Keywords

Procainamide derivatisation

Glycosaminoglycan disaccharides

HILIC UPLC-MS
Heparan sulphate
Cancer cell lines
Selected ion recording (SIR) chromatogram

Electronic supplementary material

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Introduction

Glycosaminoglycans (GAGs) are anionic linear heteropolysaccharides comprising repeating disaccharides units of uronic acid and an amino sugar and are classified into four families: hyaluronic acid, chondroitin/dermatan sulphate, heparin/heparan sulphate and keratan sulphate [1, 2, 3]. Of their repeating disaccharides, the amino sugar can either be D-galactosamine (GalN) or D-glucosamine (GlcN) while the uronic acid can be either L-iduronic acid (IdoA) or D-glucuronic acid (GlcA) except for keratan sulphate which has a D-galactose instead [4, 5]. GAGs are differentially sulphated with the exception of hyaluronic acid, and with heparan sulphate (HS), its disaccharide consists of IdoA or GlcA $\beta(1 \rightarrow 4)$ GlcN with or without a sulphate group on position C2 of GlcA/IdoA or N or C6 of the GlcN or occasionally C3 [3, 6]. The N position of GlcN can also be acetylated.

In addition to the well-known anticoagulant role of heparin, they also play a role in inflammation, cell growth, angiogenesis, differentiation and invasion [7, 8, 9]. However, there is increasing evidence of their involvement in a wide range of pathologies either as polysaccharides or disaccharides. In placental malaria, 4-sulphated chondroitin sulphate (CS) binds *Plasmodium*-infected erythrocytes expressing VAR2CSA [10] and sulphated GAGs are decreased in disorders of pregnancies such as pre-eclampsia [11]. Altered levels are also associated with a variety of carcinoma. For example, elevated levels of 6-sulphated and non-sulphated CS disaccharides are seen in pancreatic carcinoma [12]. Dysregulated GAG disaccharide composition is also seen in human chondrodysplasia [13]. Degraded forms of GAGs enhance tumour cell motility [14] and the initial attachment and entry of Merkel cell polyomavirus have been shown to be largely dependent on HS sulphation patterns [15].

Compositional analysis of these GAGs is therefore vital in understanding their

function and their biomarker potential. GAGs can be depolymerised into their composite disaccharides either enzymatically or chemically using specific polysaccharide lyases or mild hydrazinolysis followed by nitrous acid induced deaminative cleavage, respectively [16]. Chemical depolymerisation is used when the original uronic acid epimer is required, but with the resulting anhydrohexose, information about N-sulphation/acetylation is lost [16]. Enzymatic depolymerisation is often employed and involves the cleavage of the glycosidic bond between the hexosamine and hexuronic acid leaving a double bond between the C4 and C5 position of the non-reducing end uronic acid which absorbs at 232 nm allowing for spectrophotometric detection [17, 18]. For HS, enzymatic cleavage with heparin lyases yields eight disaccharides commonly seen in biological samples. Although four additional rare disaccharides have been reported, these are believed to result from the loss of labile sulphonic acid groups during isolation or incomplete modification during its biosynthesis [19].

Historically, the analysis of GAG disaccharides involved the spectrophotometric measurements at 232 nm of disaccharides separated using paper [20] and thin-layer chromatography [21]. The advent of mass spectrometry revolutionised the analysis of these disaccharides due to its increased specificity and sensitivity. However, non-derivatised (native) disaccharides are not well separated using reverse phase liquid chromatography (RPLC) on standard C18 columns as these disaccharides are hydrophilic and thus poorly retained. In a bid to improve separation and detection of these GAG disaccharides, pre-column derivatisation with 2-aminoacridone (AMAC) has been used [3, 22, 23]. AMAC is a hydrophobic fluorophore. Thus, retention of AMAC-GAG disaccharide derivative is significantly increased enabling RPLC separation. The fluorescent nature of AMAC also enables detection of lower concentrations of GAG disaccharides using fluorescent detection [24]. Other derivatisation reagents including 2-aminobenzamide [25] and aniline ($^{12}\text{C}_6$ and $^{13}\text{C}_6$) [26] have also been used to improve resolution and detection of disaccharides by RPLC coupled to a fluorescent detector and MS, respectively.

Ion-pairing reagents such as dibutylammonium acetate and tetrabutylammonium [27] have been employed in RPLC separation of native GAG disaccharides as they increase retention and improve resolution [28]. However, these ion-pair reagents often contaminate the MS interface [29] and may induce ion suppression [28]. Liquid chromatographic separation of native

disaccharides using graphitised carbon [30] and size exclusion chromatography (SEC) [31, 32] has also been reported. However, here the recovery of tri-sulphated disaccharides is problematic with graphitised carbon [16] and long run times of around 2 h are seen with SEC. Importantly, both approaches require tandem-MS to resolve co-eluting sulphation positional isomers [16]. These methods all require mass spectrometry to be used in negative ionisation mode which is often less sensitive than positive ionisation. Indeed, it has been reported that obtaining stable electrospray is more difficult in negative than in positive ion mode [33].

Hydrophilic interaction liquid chromatography (HILIC) is well suited for the separation of polar molecules such as GAGs. Furthermore, the relatively higher organic solvent composition employed is more compatible with mass spectrometry [34]. The advantage here is that the high organic solvent composition reduces the extent of salt deposition at the mass spectrometer cone. Amine or amide bonded HILIC columns are commonly used for the separation of charged and uncharged carbohydrates [28]. For the separation of native GAG disaccharides, amide-HILIC column with ESI in negative ionisation mode has previously been used [16] but a lack of complete baseline separation between sulphation positional isomers was observed [15].

Derivatisation using reagents with a greater affinity for positive ionisation can be used to improve MS sensitivity [35]. Derivatisation reagents including 2-(diethylamino) ethyl ester (ABDEAE) [36], procaine and procainamide [37] have been used for oligosaccharide derivatisation and analysis using MALDI-MS resulting in a better signal-noise ratio compared to native oligosaccharides. This is attributed to their higher proton affinity.

Derivatisation with these reagents involves the condensation of the primary amine group of the reagent with the aldehyde group of the reducing end disaccharide resulting in the formation of a Schiff base which is subsequently reduced to a secondary amine (reductive amination) [19, 38]. Consequently, the hydrophobicity of GAG disaccharides is increased, thus improving the affinity of the ions for the droplet surface entering the gas phase more readily and increasing ESI-MS response [39, 40]. Mutarotation seen as peak splitting is a significant complication of several HPLC-based analysis of native disaccharides [41]. A significant advantage of GAG disaccharide derivatisation is that mutarotation is prevented and thus anomeric species as the anomeric hydroxyl group is eliminated [41, 42].

Here, we present a highly sensitive and optimised amide-HILIC UPLC-MS

method for the analysis of HS disaccharides using pre-column derivatisation with procainamide. We have used this method for compositional analysis of HS disaccharides extracted from different cell lines. In addition, the reducing agent (2-methylpyridine borane) used here is less toxic compared to the commonly used cyanoborohydride. To the best of our knowledge, UPLC-MS analysis of procainamide-labelled glycosaminoglycan disaccharides has not been previously reported.

Materials and methods

Materials

Eight unsaturated HS disaccharides (Δ UA-GlcNAc; Δ UA-GlcNAc,6S; Δ UA,2S-GlcNAc; Δ UA-GlcNS; Δ UA-GlcNS,6S; Δ UA,2S-GlcNS; Δ UA,2S-GlcNAc,6S; Δ UA,2S-GlcNS,6S), Δ UA,2S-GlcNCOEt,6S (internal standard) and heparinases (from *Flavobacterium heparinum*) I, II and III were purchased from Iduron (Manchester, UK). Acetic acid, HPLC grade acetonitrile (ACN), ammonium acetate, UPLC water and LC-MS grade dimethyl sulphoxide (DMSO) were purchased from Fisher Scientific (Loughborough, UK). Procainamide hydrochloride, 2-methylpyridine borane complex, actinase (from *Streptomyces griseus*), anion exchange spin columns (Q Mini H, Sartorius), 0.22 μ m Millex-GP filters (Millipore) heparan sulphate (sodium salt from bovine kidney) and molecular weight cutoff filters (3 and 10 kDa, Millipore) were purchased from Sigma-Aldrich (Poole, UK). A Scan Vac CoolSafe 55-4 freeze dryer (Labogene, Lyngø, Denmark) was used for lyophilisation.

UPLC-MS conditions

A Waters Acquity H-Class UPLC system coupled to a diode array, fluorescence and SQ detector (Elstree, UK) was used. The fluorescent detector was set at excitation and emission wavelengths of 330 and 380 nm, respectively. An ACQUITY Glycan BEH Amide column (1.7 μ m, 2.1 \times 150 mm; Waters, Ireland, UK) maintained at 60 °C was employed for the separation of procainamide-derivatised HS disaccharides. Solvents A and B were ammonium formate (pH 4.4) and ACN respectively pumped at a flow rate of 0.3 mL/min. Different LC elution profiles were employed to optimise separation of analytes. Samples were maintained at 10 °C in an autosampler. The needle draw rate was set to 140 μ L/min and an injection volume of 5 μ L was used.

MS detection of procainamide-derivatised HS disaccharides was achieved in the positive ESI mode. The following MS parameters were used: capillary voltage, 3.1 kV; source gas flow, 800 L/h; cone gas flow 1 L/h; desolvation temperature, 250 °C; and probe position optimised to 7 mm vertical and 4.83 mm horizontal using procainamide-derivatised *n*-acetyl glucosamine. MassLynx® (v 4.1) was used to operate the system and acquire the data.

Mobile phase optimisation

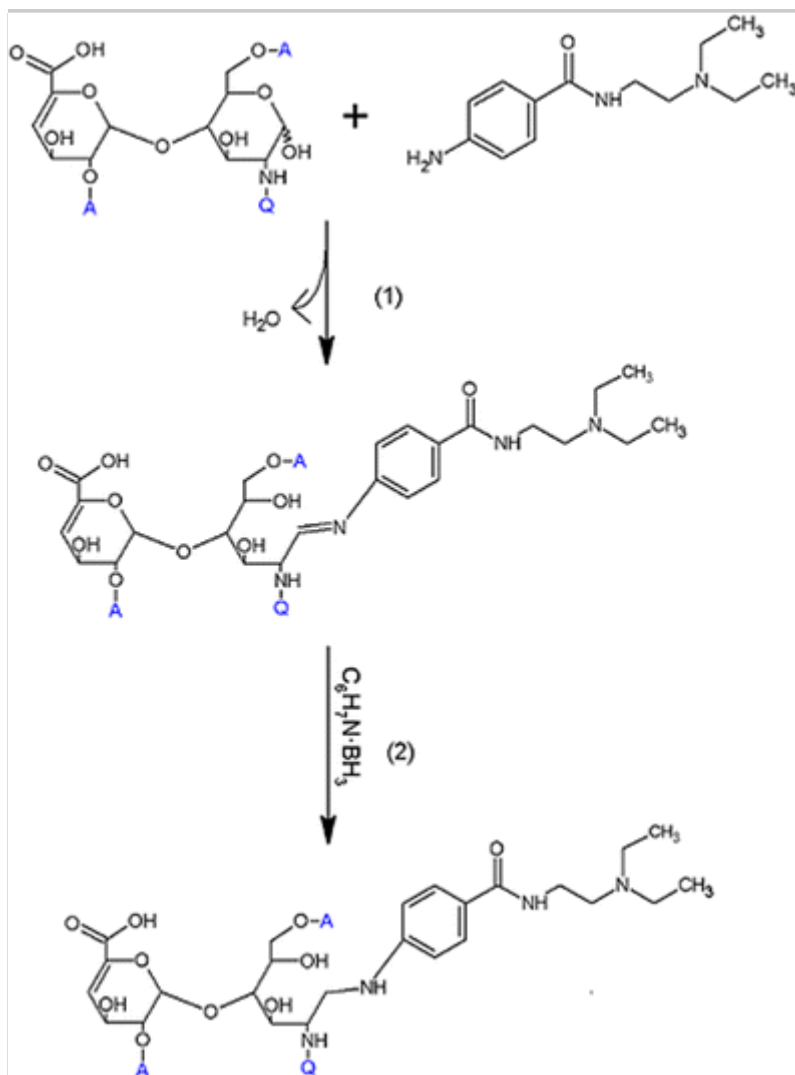
Solvents A and B were 50 mM ammonium formate (pH 4.4) and ACN, respectively. For the UPLC separation of procainamide-derivatised HS disaccharides, different elution profiles were studied; initially, linear gradient elution was used and then a combined isocratic/gradient elution steps were used to resolve the analytes.

Derivatisation

The derivatisation reaction used for HS disaccharides is shown in Fig. 1. This involves condensation and reduction steps.

Fig. 1

Procainamide-GAG disaccharide derivatisation reaction. A = SO_3^- or OH, Q = SO_3^- or $\text{CH}_3\text{CO}\cdot\text{C}_6\text{H}_7\text{N}\cdot\text{BH}_3$ (2-methylpyridine borne complex). (1) condensation, (2) reduction



Samples containing HS disaccharides were lyophilised and redissolved in 400 mM procainamide hydrochloride solution prepared in DMSO/acetic acid (7:3, v/v). This was left at RT for 15 min after which 1 M 2-methylpyrimidine borane complex dissolved in ACN was added. This mixture was then incubated at 45 °C for 2–8 h and reconstituted in 80% ACN. An aliquot was loaded onto the HPLC column. The effects of derivatisation reagent concentration, temperature and incubation time were investigated.

Additional MS optimisation

The effect of MS source temperature on MS signal intensity of procainamide-labelled HS disaccharides was studied. For the optimisation of cone voltage employed for detecting in selected ion recording (SIR) mode, the cone voltage was varied between 35 and 75 V.

Quantitative analysis

Eight HS disaccharides were prepared over a concentration range of

0.6–5000 ng/mL and derivatised using optimal derivatisation conditions. The samples were analysed in duplicate to determine linearity and LoD and LoQ, defined as signal/noise of 3:1 and 10:1, respectively. To determine assay precision, derivatised disaccharides (39–1250 ng/mL) were injected five times and the coefficient of variation (% CV) was determined. Variations in retention time of the disaccharides and internal standard were calculated from 38 injections of samples across the concentration range 0.6–5000 ng/mL.

Analysis of HS standard and cell-extracted HS

Human metastatic breast adenocarcinoma (MDA-468), ovarian carcinoma (HEY) and acute myeloid leukemic (MOLM-13) cell lines were cultured under standard conditions (RPMI medium with 10% foetal calf serum, 1% streptomycin and 1% penicillin in a humid atmosphere containing 5% CO₂). The method used for the extraction of HS from cell cultures was adapted from [3, 43]. Cells were grown to confluence in 3T-75 flasks and were gently scraped, washed in phosphate buffered saline at 4 °C, lyophilised and weighed. Cells were then resuspended in 750 µL of water, to which 250 µL of 20 mg/mL actinase was added. The mixture was incubated at 55 °C for 18 h. The sample was then filtered (0.22 µm) and the resulting peptides were removed using a 10-kDa MWCO filter. The retentate was lyophilised, resuspended in 300 µL of 8 M urea containing 2% CHAPS and the GAGs extracted using anion exchange spin columns. GAGs were then eluted using 16% NaCl and the solution desalted using a 3-kDa MWCO filter. The retentate was then lyophilised and resuspended in 20 µL of 50 mM phosphate buffer (pH 7.0). The resulting samples and HS standard were then incubated at 37 °C for 48 h with 3 mIU of heparinases I, II and III (30 µL). Enzymes were denatured by heating the sample in boiling water for 10 min. These enzymes were then pelleted upon centrifugation at 10,000×g for 10 min. The supernatant containing the disaccharides was collected and 0.5 µg of internal standard (Δ UA,2S-GlcNCOEt,6S) was added and the sample lyophilised. Samples were then derivatised and analysed.

Results and discussion

Optimisation of LC separation

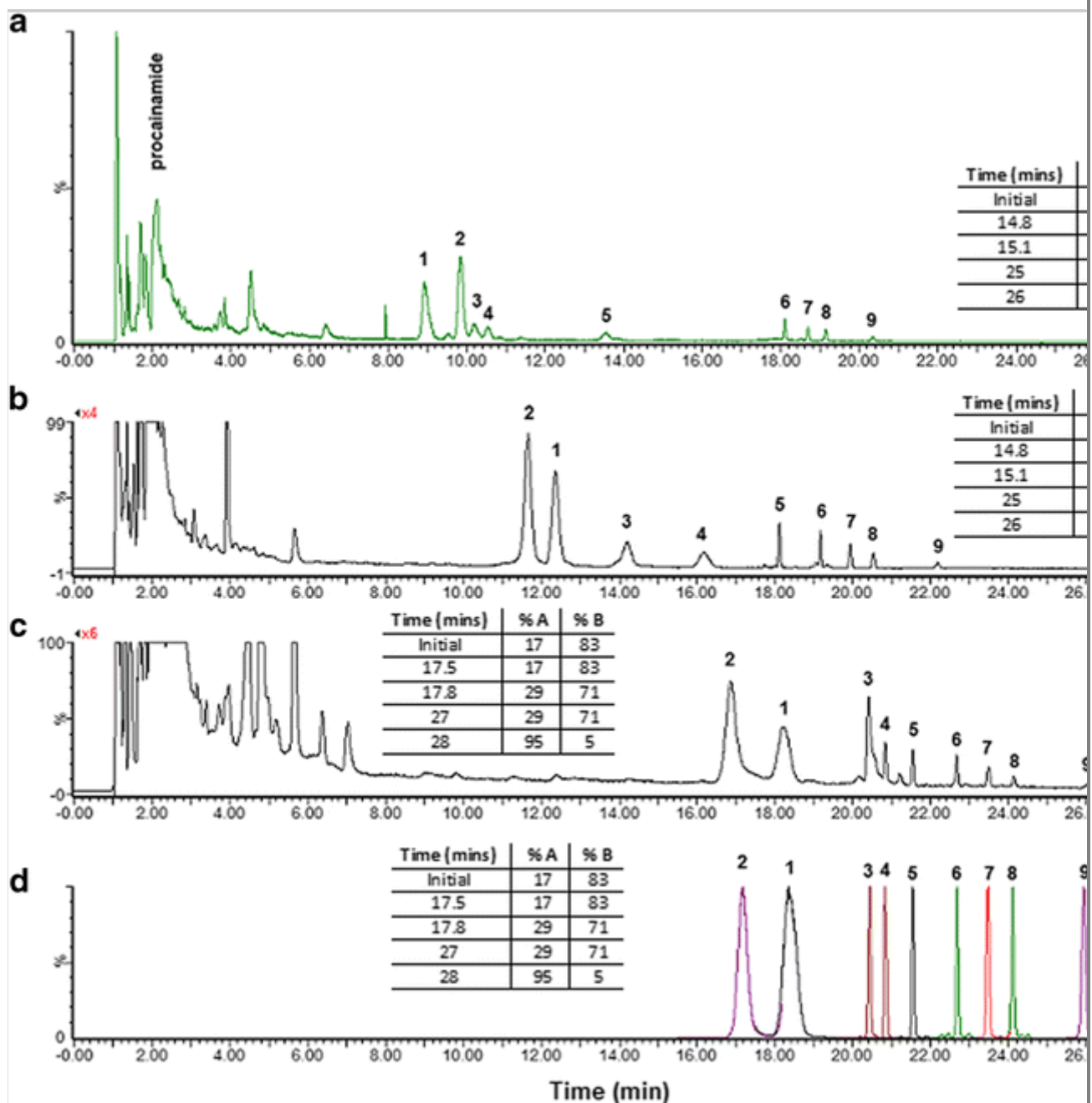
Initial chromatographic optimisation studies immediately identified procainamide-derivatised Δ UA–GlcNAc and Δ UA,2S–GlcNAc as the critical pair. The *m/z* values of Δ UA–GlcNAc and Δ UA,2S–GlcNAc were 599

$[M + H]^+$ and $679 [M + H]^+$, respectively. However, in-source fragmentation of $\Delta\text{UA}_{,2\text{S}}\text{-GlcNAc}$ also yielded ions with m/z values of $599 [M - \text{SO}_3 + H]^+$. In-source fragmentation and the labile nature of sulphonic groups in these molecules are well-known phenomena [19]. Thus, it was essential to separate these two disaccharides which were then separated using isocratic elution conditions (s. Electronic Supplementary Material, Fig. S1).

The effects of different buffer concentrations and the percentage used in the mobile phase on disaccharide separation were investigated. Using 50 mM ammonium formate (pH 4.4) as the aqueous mobile phase, all eight procainamide-derivatised HS disaccharides and the internal standard were resolved to baseline. In a bid to reduce the chances of salt deposition at the MS cone, we decided to reduce the buffer concentration to 20 mM. This produced a similar elution pattern to that seen using 50 mM buffer concentration (Fig. 2a, b). However, the more hydrophilic $\Delta\text{UA}_{,2\text{S}}\text{-GlcNAc}$ eluted before $\Delta\text{UA}\text{-GlcNAc}$ and the resolution between $\Delta\text{UA}\text{-GlcNAc}_{,6\text{S}}$ and $\Delta\text{UA}_{,2\text{S}}\text{-GlcNCOEt}_{,6\text{S}}$ were poor. This is in contrast to a previous study [16] where no change in elution pattern was observed using a decreased buffer concentration. However, the analysis reported by these authors was for the separation of native disaccharide. It is possible that using the amide-HILIC column for separation of procainamide-derivatised disaccharides, electrostatic interactions between analyte and stationary phase are masked at higher buffer concentrations. Also, in the current work, a reduction in retention time was observed for all the analytes when 20 mM buffer was used for separation. For these reasons, 50 mM ammonium formate (pH 4.4) was used for all further experiments.

Fig. 2

Separation of eight different procainamide-derivatised HS disaccharides and an internal standard using (a) TIC using 20 mM ammonium formate (pH 4.4, 18%), (b) TIC using 50 mM ammonium formate (pH 4.4, 18%), (c) TIC using 50 mM ammonium formate (pH 4.4, 17%) and (d) overlaid SIR using 50 mM ammonium formate (pH 4.4, 17%). The elution profile used to acquire the data is shown as an insert. 1, $\Delta\text{UA}_{,2\text{S}}\text{-GlcNAc}$; 2, $\Delta\text{UA}\text{-GlcNAc}$; 3, $\Delta\text{UA}\text{-GlcNAc}_{,6\text{S}}$; 4, $\Delta\text{UA}_{,2\text{S}}\text{-GlcNCOEt}_{,6\text{S}}$ (internal standard); 5, $\Delta\text{UA}_{,2\text{S}}\text{-GlcNAc}_{,6\text{S}}$; 6, $\Delta\text{UA}\text{-GlcNS}$; 7, $\Delta\text{UA}_{,2\text{S}}\text{-GlcNS}$; 8, $\Delta\text{UA}\text{-GlcNS}_{,6\text{S}}$; 9, $\Delta\text{UA}_{,2\text{S}}\text{-GlcNS}_{,6\text{S}}$



A reduction in the initial gradient composition from 18 to 17% buffer further improved resolution (Fig. 2c, d) of the eight procainamide-derivatised HS disaccharides and the internal standard. The final elution conditions (Fig. 2c, d) were as follows: solvent A was maintained at 17% from 0 to 17.5 min, 29% from 17.8 to 27 min and 95% from 28 to 29 min, returning to 17% from 30 to 55 min. The flow rate was set at 0.3 mL/min from 0 to 30 min, 0.5 mL/min from 33 to 47 min and returned to 0.3 mL/min from 48 to 55 min. These elution conditions showed resolution greater than 1.5 for all eight procainamide-derivatised disaccharides and the internal standard. Excess unreacted procainamide was well resolved from analyte peaks. As expected, procainamide-derivatised HS disaccharides generally eluted in order of

increasing polarity (hydrophilicity). Thus, the non-sulphated disaccharide ($\Delta\text{UA-GlcNAc}$) eluted first followed by the mono-sulphated, di-sulphated and finally the tri-sulphated disaccharide. The only exception was the mono-sulphated $\Delta\text{UA-GlcNS}$ which eluted amongst the di-sulphated disaccharides. All the N-acetylated disaccharides eluted before the N-sulphated disaccharides. A similar elution profile has been reported in the analysis of native disaccharides using HILIC chromatography [16]. These authors also used a column temperature of 60 °C which prevents peak broadening caused by anomeric separation. However, in comparison to their study, we achieved baseline separation of the N-acetylated mono-sulphated positional isomers ($\Delta\text{UA}_{,2\text{S}}\text{-GlcNAc}$ and $\Delta\text{UA-GlcNAc}_{,6\text{S}}$) and the HPLC peaks were sharper. It is likely that this is due to the slightly smaller particle size (1.7 μm) of the stationary phase used in our method.

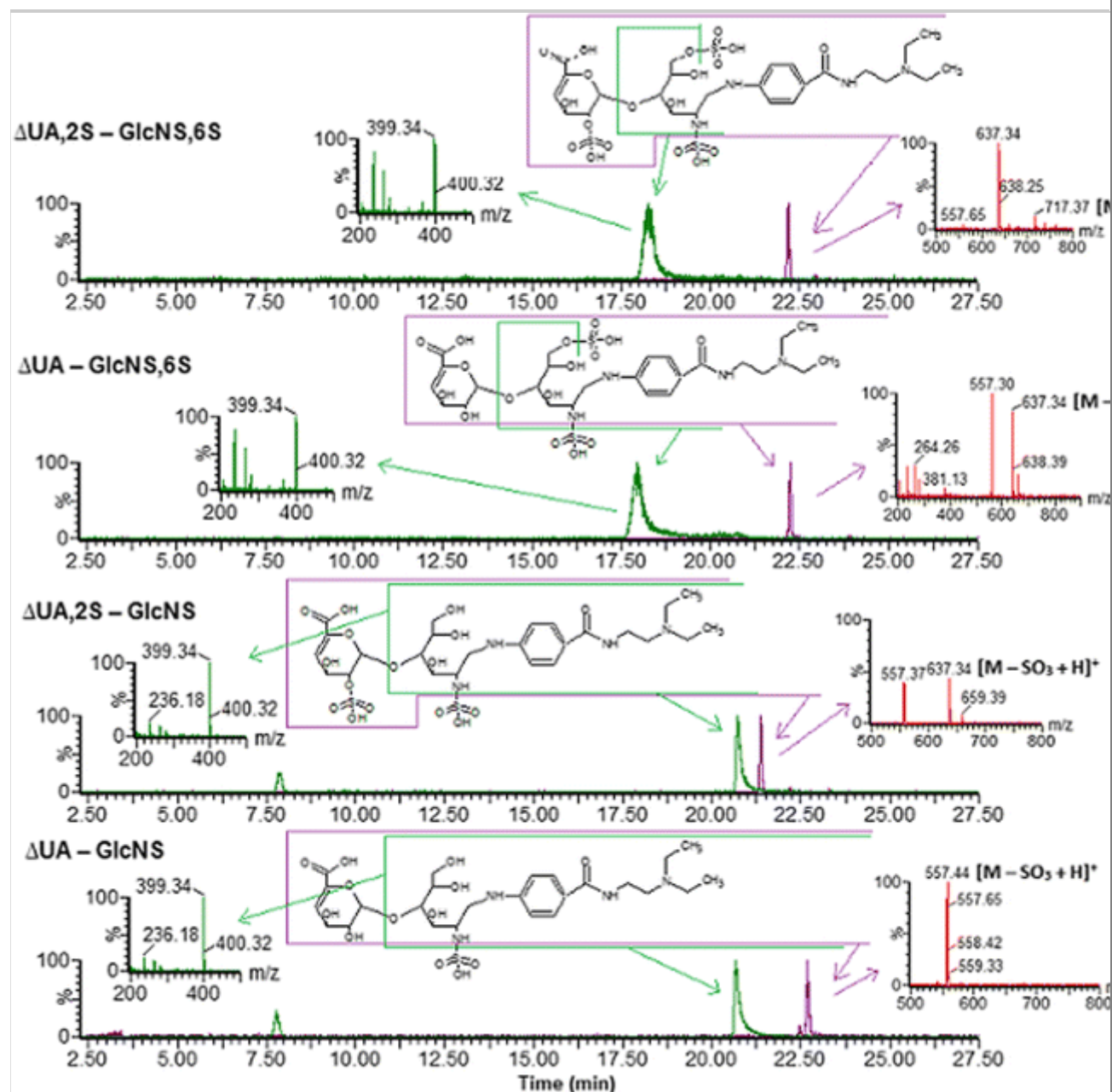
Optimisation of derivatisation conditions

Derivatisation with procainamide increases the disaccharide mass by $[\text{M} + 219 + \text{H}]^+$. In this study, we have investigated the effect of derivatisation temperature on the separation and integrity of the stable HS disaccharide derivatisation products. A derivatisation temperature of 65 °C has previously been used for the procainamide derivatisation of glycans [35, 44]. However, we found that at this temperature there was degradation of some disaccharides (ESM, Fig. S2b). Specifically, procainamide derivatisation products of $\Delta\text{UA}_{,2\text{S}}\text{-GlcNS}$ co-eluted with $\Delta\text{UA}_{,2\text{S}}\text{-GlcNAc}_{,6\text{S}}$, while $\Delta\text{UA}_{,2\text{S}}\text{-GlcNS}_{,6\text{S}}$ co-eluted with $\Delta\text{UA-GlcNS}_{,6\text{S}}$.

Analysis of the mass spectra acquired at this temperature revealed no intact procainamide-derivatised disaccharide $[\text{M} + \text{H}]^+$ species for the N-sulphated disaccharides. However, $[\text{M}-2\text{SO}_3^- + \text{H}]^+$ and $[\text{M}-\text{SO}_3^- + \text{H}]^+$ species could be detected (Fig. 3). Interestingly, derivatisation at 65 °C also revealed a peak attributable to GlcN-procainamide fragments (m/z 399.3) arising from all of these N-sulphated disaccharides (Fig. 3). The presence of this ion is likely to result from the loss of all the sulphonic groups on the glucosamine residue and the breakage of the glycosidic bond between uronic acid and glucosamine. This fragment was not observed for the N-acetylated disaccharides suggesting that the N-acetyl bond was not cleaved at this temperature. The initial loss of SO_3^- from the N position occurred during derivatisation. The difference in retention time of fragment arising from NS and the NS-6S disaccharides may possibly be due to the presence of the 6S SO_3^- group which is then lost within the ion source as evidenced by their identical mass spectra (Fig. 3).

Fig. 3

Overlaid extracted ion chromatograms of N-sulphated procainamide-derivatised (65 °C) HS disaccharides showing GlcN-procainamide fragments. Mass spectra are shown as inserts

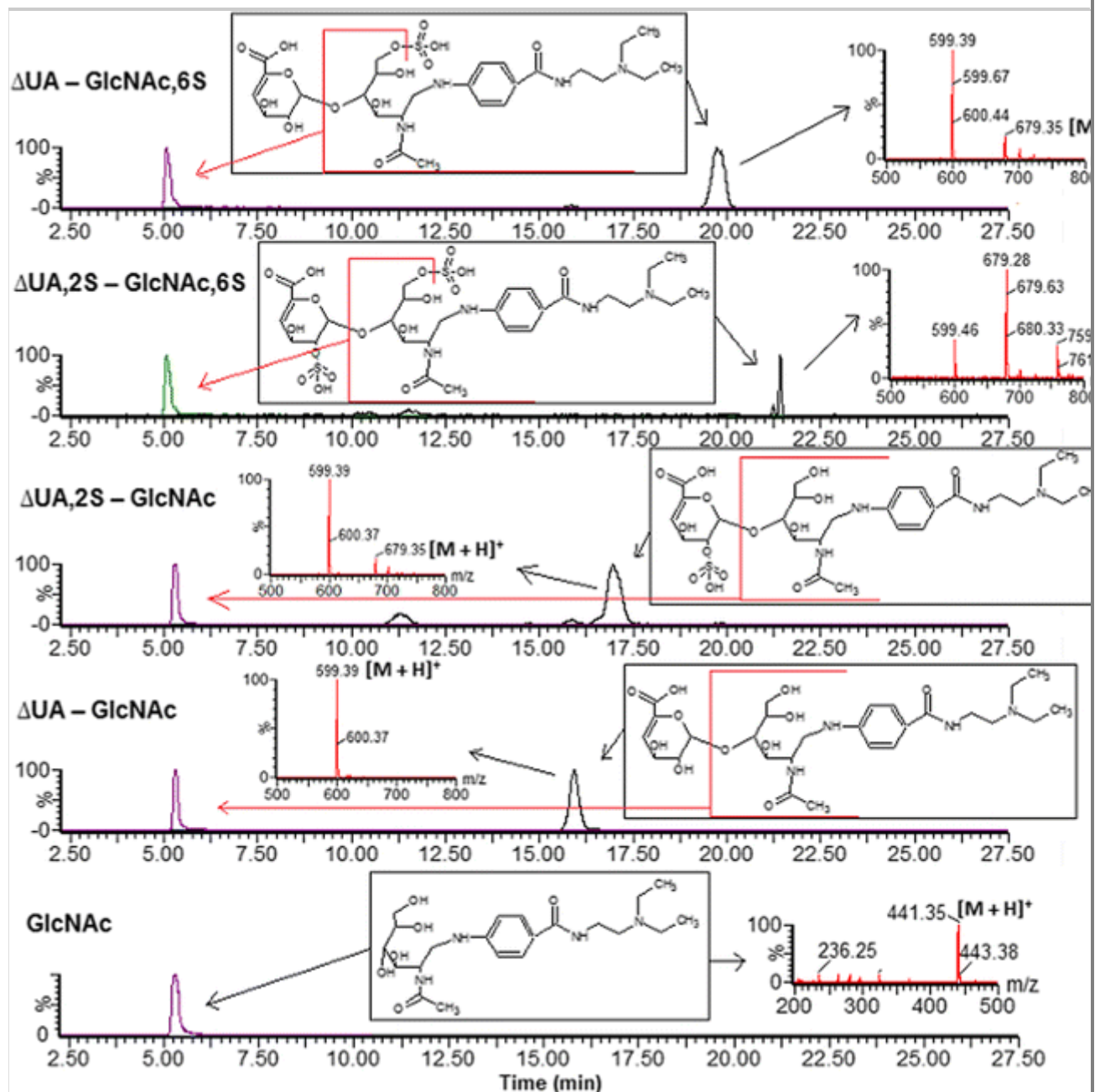


In contrast, the intact non-sulphated and *N*-acetylated disaccharide ions $[M + H]^+$ were still detected at 65 °C. However, in-source fragmentation resulted in the loss of one or two sulphonic groups (Fig. 4). Thus, it appears that the sulphated disaccharides with sulphonic groups attached to *N*-position of glucosamine are relatively heat-labile compared to disaccharides sulphated at other positions. Also, at 65 °C derivatisation temperature, a peak

attributable to GlcNAc-procainamide fragments observed for N-acetylated disaccharides was detected since injection of procainamide-derivatised GlcNAc standard showed the same m/z value and retention time (441.3 and 5.2 min, respectively, Fig. 4).

Fig. 4

Overlaid extracted ion chromatograms of N-acetylated procainamide-derivatised (65 °C) HS disaccharides showing GlcNAc-procainamide fragments with similar retention times to procainamide-derivatised GlcNAc standard. Mass spectra are shown as inserts



Thus, in order to minimise the loss of sulphonic groups, procainamide

derivatisation of HS disaccharides was carried out at 45 °C. At this temperature, good separation of all disaccharides was seen and MS signal for intact disaccharide was observed. In addition, the GlcN-procainamide and GlcNAc-procainamide fragments were not observed at this derivatisation temperature. The extracted ion chromatograms of procainamide-derivatised HS disaccharides and an internal standard are shown in ESM, Fig. S3. The mass spectra confirmed the presence of the parent ion. Table 1 shows the various ions produced following MS for each of the derivatised HS disaccharides analysed. For detection of all disaccharides, the most intense ion was used. For the non-sulphated disaccharide, mono- and di-sulphated disaccharides and the tri-sulphated disaccharide were $[M + H]^+$, $[M-SO_3^- + H]^+$ and $[M-2SO_3^- + H]^+$, respectively.

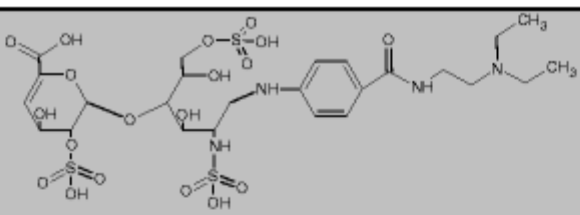
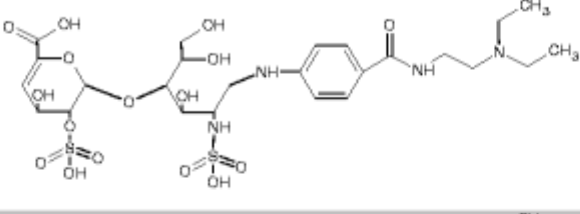
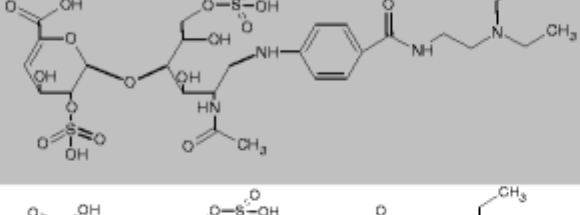
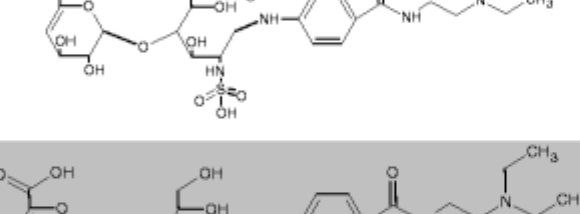
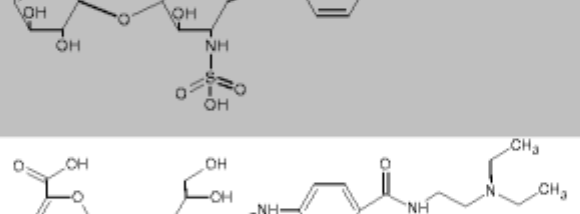
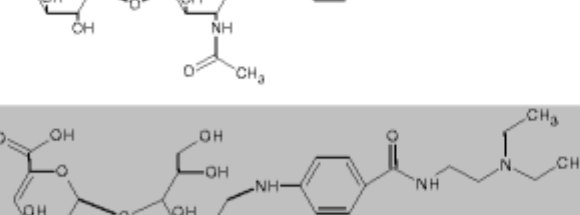
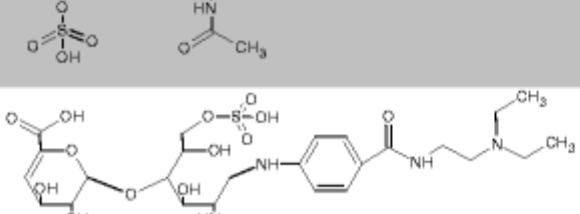
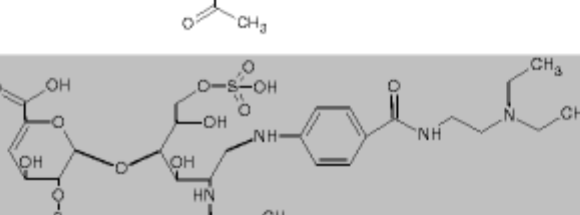

HS disaccharide code	HS disaccharide + procainamide (molecular structure)	Observed m/z [assignment]
Δ UA,2S – GlcNS,6S		797.2 717.3 [M] 637.3 [M]
Δ UA,2S – GlcNS		717.1 637.2 [M] 557.3 [M]
Δ UA,2S – GlcNAc,6S		759.3 679.2 [M] 599.4 [M]
Δ UA – GlcNS,6S		717.2 637.2 [M] 557.3 [M]
Δ UA – GlcNS		637.3 557.3 [M]
Δ UA – GlcNAc		599.3
Δ UA,2S – GlcNAc		679.3 599.3 [M]
Δ UA – GlcNAc,6S		679.4 599.3 [M]
Δ UA,2S – GlcNCOEt, 6S (internal standard)		773.3 693.4 [M] 613.3 [M]

Table 1

Procainamide-derivatised disaccharide structure and MS ions observed using optimum derivatisation conditions

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The *m/z* of the most intense ions are shown in bold and these were used for detection. Other ions formed, as a result of MS source-induced loss of sulphonic acid groups, are also shown

The effect of derivatisation reagent (procainamide hydrochloride) concentration on LC-peak area was studied (ESM, Fig. S4). The concentration of disaccharides used here (1.25 µg/mL) is higher than what was expected from cell-extracted HS. As expected, the LC-peak area of all eight disaccharides increased with procainamide hydrochloride concentration, plateauing at 10.9 mg/mL. The lack of a further increase above this concentration is most likely due to the exhaustion of free disaccharides. Differential ionisation efficiency of the monitored ions of the disaccharides was also observed. For equal mass of disaccharide, the LC-peak area was highest for the non-sulphated disaccharide (Δ UA–GlcNAc) with decreasing peak areas observed in order of increasing sulphation. The tri-sulphated disaccharide (Δ UA,₂S–GlcNS,₆S) yielded the lowest peak area.

Using the optimal derivatisation temperature (45 °C) and procainamide concentration (10.9 mg/mL), we studied the effect of derivatisation incubation time (ESM, Fig. S5). The LC-peak area for all the disaccharides increased with increasing incubation time from 2 to 4 h and plateaued thereafter for most of the analytes. However, a decrease was noticed at 8 h for some of the disaccharides. It is likely that the prolonged incubation for 8 h promotes heat-induced fragmentation of some of the disaccharides during derivatisation leading to the subsequent loss of signal. A 4-h incubation time was employed in all further experiments since no loss of signal was observed at this time point.

MS optimisation

For initial studies, an MS source temperature of 130 °C and cone voltage of 50 V were used. To improve method sensitivity, MS conditions were further optimised. The effect of MS source temperature on the intensity of the monitored ions was studied which revealed that 110 °C was the ideal MS source temperature (ESM, Fig. S6). A lower temperature (100 °C) produced

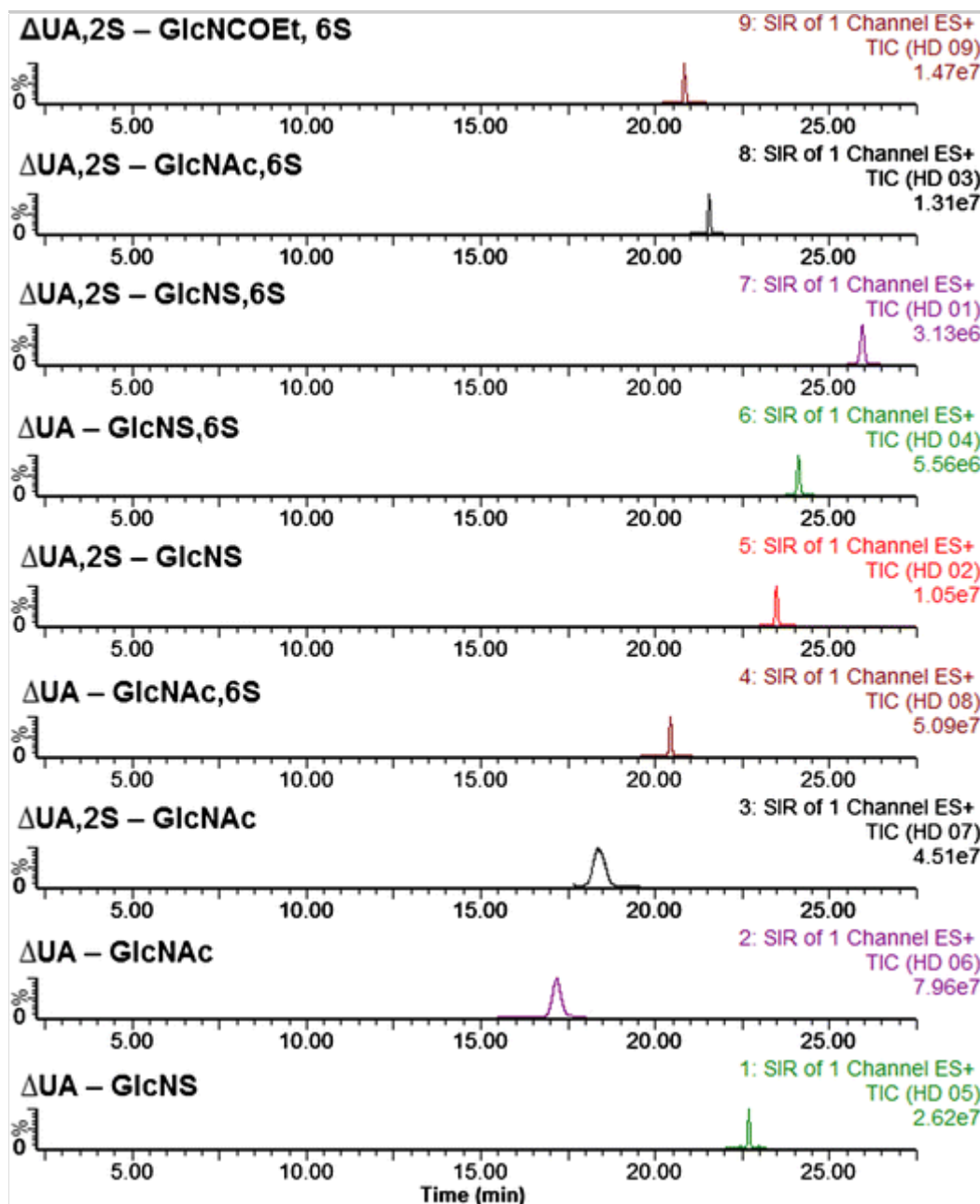
less intense ions and this may be explained by a significant reduction in in-source fragmentation thereby reducing the intensity of the monitored $[M-SO_3^- + H]^+$ and $[M-2SO_3^- + H]^+$ ions. However, this temperature was found to be too low for efficient desolvation. This resulted in a reduction in signal intensity for non-sulphated Δ UA–GlcNAc. This temperature may also promote the deposition of salts at the MS cone as a result of inefficient desolvation leading to erratic MS signals. Conversely, at 130 °C, an increased heat-induced in-source fragmentation was observed which lead to a corresponding drop in the intensity of the monitored ion.

The effect of cone voltage on signal intensity is shown in ESM, Fig. S7 and the optimum cone voltage used for each disaccharide is shown in ESM, Table S1. The LC-peak area for all procainamide-derivatised disaccharides increased with increasing cone voltage up to 55 V. However, the di-sulphated disaccharides peak area decreased beyond this point. We have previously shown the loss of sulphonic acid groups with increasing cone voltages [23]. A further increase in cone voltage leads to loss of more sulphonic acid groups resulting in the formation of the $[M-2SO_3^- + H]^+$ ion at the expense of the monitored $[M-SO_3^- + H]^+$ ions. This is also the reason for the continuous increase in LC-peak area observed for the tri-sulphated disaccharide ion $[M-2SO_3^- + H]^+$ above 55 V.

The SIR chromatogram of procainamide-derivatised HS disaccharides with optimised derivatisation, chromatography, and MS conditions is shown in Fig. 5. As it can be seen that using these conditions all eight derivatised disaccharides are well resolved. In comparison to TIC, using extracted ion chromatograms (SIR) improves sensitivity by up to 10 times [19]. Another advantage of our method is that attachment of procainamide tag allows both UV and fluorescent detection as an alternative to MS. The fluorescent and UV chromatograms of procainamide-derivatised HS disaccharides are shown in ESM, Fig. S8. Relative to UV, fluorescence detection was more selective. This is evident from the UV chromatogram that there is interference close to the elution of Δ UA–GlcNAc,6S and Δ UA,2S–GlcNCOEt, 6s.

Fig. 5

SIR chromatograms of procainamide-derivatised HS disaccharides using optimal derivatisation and UPLC-MS conditions. HS disaccharide concentration = 10 μ g/mL, injection volume = 5 μ L



Quantitative analysis

The LC-peak area exhibited an excellent linear relationship with the concentration of procainamide-derivatised disaccharide analysed over a range of 0.6–5000 ng/mL. The r^2 values ranged from 0.9985 to 1.0. The LoD, LoQ, retention time and r^2 of the respective procainamide-derivatised HS disaccharide subunits are shown in Table 2. The LoD and LoQ ranged from 0.6 to 4.9 and 0.6 to 9.8 ng/mL, respectively. Relative to the other disaccharides, Δ UA–GlcNAc, Δ UA,2S–GlcNAc and Δ UA–GlcNAc,6S showed the best ionisation capacity both with LoQs of 0.6 ng/mL (Table 2). We observed up to 30 times lower LoDs on our single quadrupole mass spectrometer in comparison to RP UPLC-MS analysis of AMAC-derivatised

HS disaccharides using ESI operated in negative ionisation mode [23]. A similar increase in sensitivity has been reported for glycans labelled with procainamide relative to 2-aminobenzamide-labelled glycans using HILIC-UPLC-MS [35]. Also, a 5000-fold improvement in sensitivity has been reported for procaine-labelled relative to native oligosaccharide using ESI-MS [45]. The intra-assay precision (ESM, Table S2) measured using 39.1, 312.5 and 1250 ng/mL procainamide-derivatised disaccharides ranged from 3.7 to 5.1, 2.5 to 3.7 and 2.7 to 6.0% CV, respectively, using a UPLC syringe draw rate of 140 $\mu\text{L}/\text{min}$. As expected, relatively poor reproducibility was seen at a lower UPLC syringe draw rate (25 $\mu\text{L}/\text{min}$, data not shown). This is most likely due to the significantly lower viscosity of the sample solvent that was used (80% ACN). The variation in retention time is shown as standard deviation in Table 2. Methods based on analysis of native HS disaccharides using ES in negative ionisation mode with MS and MS/MS detection have reported that a starting HS quantity of 10 μg is required [32]. We have previously reported that 5 μg of HS can be used with precolumn procedure based on AMAC derivatisation followed by RP-UPLC-MS. Here, our method, based on using ESI in positive ionisation mode shows an improvement as we are able to detect HS disaccharides from as little as 2.5 μg of starting material.

Table 2

LoD, LoQ, retention time and r^2 of procainamide-derivatised HS disaccharides

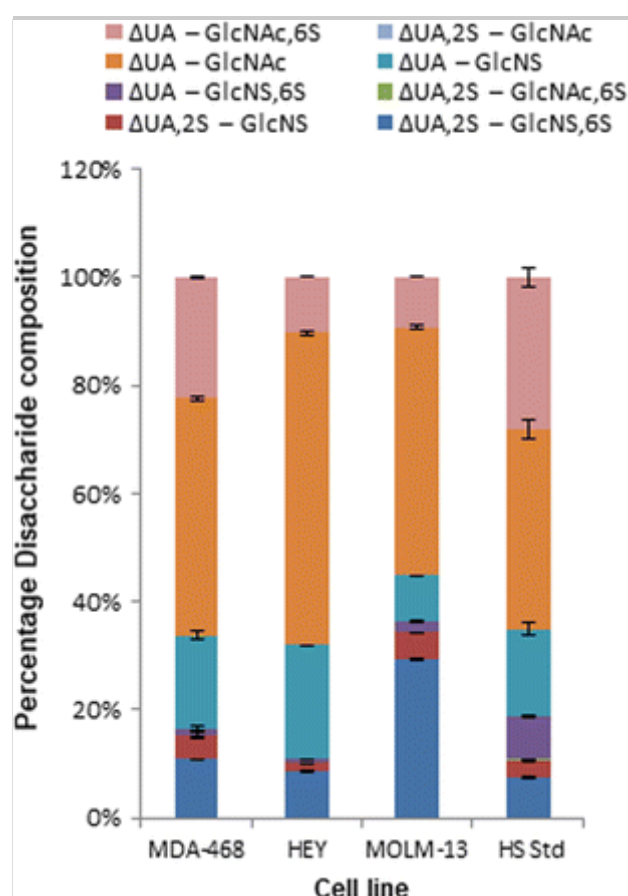
HS disaccharide subunits	r^2	LOD (ng/mL)	LOQ (ng/mL)	Retention time \pm SD
$\Delta\text{UA},2\text{S-GlcNS},6\text{S}$	0.9995	4.9	9.8	25.9 \pm 0.03
$\Delta\text{UA},2\text{S-GlcNS}$	0.9997	1.2	4.9	23.5 \pm 0.09
$\Delta\text{UA},2\text{S-GlcNAc},6\text{S}$	0.9999	1.2	2.4	21.5 \pm 0.01
$\Delta\text{UA-GlcNS},6\text{S}$	0.9993	2.4	9.8	24.1 \pm 0.02
$\Delta\text{UA-GlcNS}$	0.9984	0.6	1.2	22.7 \pm 0.02
$\Delta\text{UA-GlcNAc}$	0.9989	<0.6	0.6	17.1 \pm 0.09
$\Delta\text{UA},2\text{S-GlcNAc}$	0.9985	<0.6	0.6	18.4 \pm 0.09
$\Delta\text{UA-GlcNAc},6\text{S}$	0.9984	<0.6	0.6	20.4 \pm 0.02
$\Delta\text{UA},2\text{S-GlcNCOEt},6\text{S}$	1	1.2	2.4	20.8 \pm 0.01
<i>SD</i> standard deviation				

Analysis of HS standard and cell-extracted HS

The non-sulphated Δ UA–GlcNAc was found to be the most abundant disaccharide in the HS standard (37% of total, Fig. 6). This was also the most abundant disaccharide (53%) reported in a previous study by Deakin and Lyon [24]. In comparison to this work, we found higher levels of Δ UA–GlcNAc,6S (28.1%) but lower levels of Δ UA–GlcNS (16.2%). This may be associated with a difference in origin of HS used in their study (HS was derived from porcine intestinal mucosa) suggesting an organ-specific variation in HS. A HS composition of about 25 and 35% Δ UA–GlcNAc and Δ UA–GlcNAc,6S, respectively, has previously been reported for bovine kidney-derived HS [16].

Fig. 6

Percentage disaccharide composition of cell line-extracted HS



The non-sulphated disaccharide was also the most abundant in all the cell lines tested with the percentage composition highest in HEY cell lines (57.6%, Fig. 6 and ESM, Table S3). The disaccharide Δ UA,2S–GlcNS was more abundant than its positional isomer Δ UA–GlcNS,6S. The reverse was found in the HS standard. The di-sulphated Δ UA,2S–GlcNAc,6S was not detected in any of the cell lines and was the least abundant disaccharide in HS standard

(0.2%). The relative abundance of $\Delta\text{UA},2\text{S}\text{-GlcNAc},6\text{S}$ in HS is consistent with a previous study [16]. However, this disaccharide was not observed in another study of HS disaccharides using porcine mucosal HS [24]. The slight differences in the percentage disaccharide composition of HS reported in these studies may be attributed to batch to batch variation or to the origin of the HS.

With respect to HS standard and HS extracted from the other cell lines studied, MOLM-13 had a significantly higher relative abundance of the tri-sulphated disaccharide $\Delta\text{UA},2\text{S}\text{-GlcNS},6\text{S}$ (29.3%) (Fig. 6 and ESM; Table S3). MOLM-13 is an acute monocytic leukaemia cell line in origin exhibiting monoblastoid morphology established from the peripheral blood of a relapsed leukaemia patient [46]. This is consistent with a previous study that showed a high relative composition of $\Delta\text{UA},2\text{S}\text{-GlcNS},6\text{S}$ in leucocyte-extracted HS [47]. These authors suggest that HS may play a role in protease cell-surface binding, transport and storage of intracellular proteases by leukocytes. Previous studies have also shown that the relative composition of $\Delta\text{UA},2\text{S}\text{-GlcNS},6\text{S}$ in organ-derived HS other than from porcine intestinal mucosa is low [31, 47]. This is consistent with the results obtained in the current work where the relative abundance of this disaccharide was 8.5 and 10.8% in epithelium-derived HEY and MDA468, respectively.

There were other significant differences in HS disaccharide composition between the different cell lines studied. The disaccharides $\Delta\text{UA},2\text{S}\text{-GlcNAc},6\text{S}$ and $\Delta\text{UA},2\text{S}\text{-GlcNAc}$ were not detected in any of the cell lines studied. Low levels of $\Delta\text{UA}\text{-GlcNS},6\text{S}$ were detected but high levels of $\Delta\text{UA}\text{-GlcNAc}$ were seen in all cell lines. Concentrations of individual disaccharides and total disaccharide concentrations were considerably higher in MOLM-13 cells compared to HEY and MDA-468 (ESM, Table S3). These results are comparable to our previous work which also reported on the relative abundance of these disaccharides in cancer cell lines [23]. This variation in HS disaccharide expression could be due to tissue specificity. Both MDA-468 and HEY represent solid tissue tumours, whereas MOLM-13 is a relapsed acute myeloid leukaemia cell line composed of abnormal monocytes. The high levels of glycosaminoglycan expression could have a significant function in leukaemia relapse as they have been implicated in acute myeloid leukaemia progression where they directly induce cell angiogenesis and proliferation both in vivo and in vitro [48]. This increased disaccharide expression seen here in a monocyte-derived cell line is consistent

with the proposed important role for glycosaminoglycans in regulating leukocyte responses to inflammation, tumour cell angiogenesis and proliferation [48, 49]. Further research into the disaccharide composition of HS from different stages of cell and tumour growth will be useful in understanding their involvement in normal cell growth, proliferation and tumour development.

Conclusions

In this article, we have described a highly sensitive HILIC UPLC-MS method for the analysis of procainamide-derivatised HS disaccharides. Positive ionisation is made possible by procainamide derivatisation. Positional isomers were completely resolved and the solvent composition of the mobile phase used is compatible with mass spectrometry. Disaccharide compositional analysis of HS from different cell lines revealed significant differences in the relative abundance of the different disaccharides detected. Thus, this method can be applied to the quantification of HS subunits derived from biological material.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Electronic supplementary material

AQ4

ESM 1

(PDF 1651 kb)

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