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Heparin Isomeric Oligosaccharide Separation Using Volatile Salt Strong Anion Exchange Chromatography

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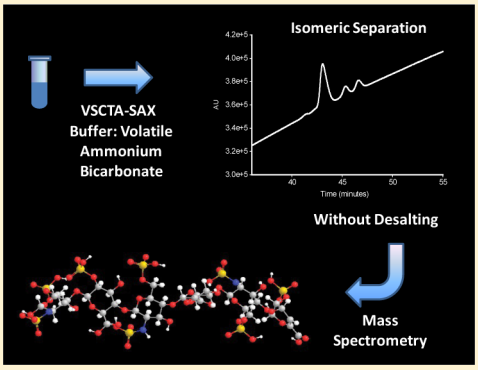
Supporting Information

ABSTRACT: The complexity of heparin and heparan sulfate saccharides makes their purification, including many isomeric structures, very challenging and is a bottleneck for structure−activity studies. High-resolution separations have been achieved by strong anion exchange (SAX) chromatography on Propac PA1 and cetyltrimethylammonium (CTA)-C_{18} silica columns; however, these entail subsequent desalting methodologies and consequent sample losses and are incompatible with orthogonal chromatography methodologies and, in particular, mass spectrometry. Here, we present the CTA-SAX purification of heparin oligosaccharides using volatile salt (VS) buffer. In VSCTA-SAX, the use of ammonium bicarbonate buffer for elution improves resolution through both weaker dissociation and conformational coordination of the ammonium across the sulfate groups. Using ion mobility mass spectrometry, we demonstrate that isomeric structures have different structural conformations, which makes chromatographic separation achievable. Resolution of such structures is improved compared to standard SAX methods, and in addition, VSCTA-SAX provides an orthogonal method to isolate saccharides with higher purity. Because ammonium bicarbonate is used, the samples can be evaporated rather than desalted, preventing substantial sample loss and allowing more effective subsequent analysis by electrospray mass spectrometry. We conclude that VSCTA-SAX is a powerful new tool that helps address the difficult challenge of heparin/heparan sulfate saccharide separation and will enhance structure−activity studies.



Heparin and heparan sulfate (HS) are part of the glycosaminoglycan (GAG) family of sugars. Heparin was first discovered in 1916 and then later developed into an anticoagulant-based drug that was first used in 1935. Heparin and HS play an important role in the regulation of biological systems with chemical synthesis, enzymatic synthesis, and oligosaccharide purification being used to obtain pure structures to understand structure−function relationships.[1−5] Heparin and HS are highly anionic structures with their synthesis being nontemplate driven with enzymatic specificity creating variably sulfated regions and acetylated regions identified in HS. Sulfate groups can reside on the 2-O position of the uronic acid group, and the 6-O, 3-O, and N-position of the glucosamine with the N-positions also able to adopt an acetate group and occasionally an amine. It is this micro-heterogeneity in sulfation that changes between organs,[6] stage of development,[7,8] communication, and environment,[9] providing heparin and HS with a dynamic conformational versatility and thus a vast range of biological functions.[10]

Size exclusion chromatography (SEC) is often the first method used in heparin/HS oligosaccharide purification.[11] SEC separation depends not just on the number of saccharide units but also on the number of sulfate groups, thus increasing the likelihood of oligosaccharides with different sequences and structures having equivalent hydrodynamic radii.[12] Nevertheless, in isolation of structures ranging from dp4 to dp12, SEC will separate roughly in dp2 increments due to the disaccharide cleavage specificity of various depolymerization methods.[13] Further purification of structures isolated by SEC is most commonly achieved using strong anion exchange (SAX).

SAX is a powerful heparin/HS separation method, as it offers higher oligosaccharide resolution over other methods currently available, including hydrophilic interaction chromatography (HILIC),[14] reverse phase ion pairing (RP-IP),[15,16] and capillary electrophoresis (CE).[17,18] With small saccharides (for example, disaccharides), there is a significant degree of separation dependent on the presence of 2OS, 6OS, and NS groups, but as the oligosaccharide becomes longer, this resolution decreases,[19] necessitating the use of additional SAX or orthogonal chromatography methods.

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Cetyltrimethylammonium SAX (CTA-SAX) is an alternative SAX method that uses a C8 or C18 column matrix derivatized with CTA.[20,21] The coating of CTA is dynamic, and fine-tuning of this coat results in the resolution of oligosaccharides containing up to 20 sulfate groups. The extent of CTA derivatization is determined by the water−methanol (v/v) concentration in which the column is derivatized. However, a drawback of this method arises from the use of the ammonium methanesulfonic acid mobile phase at pH 2.5 to create the eluting gradient. This method has not been widely used due to the difficulty in producing the derivatized column, weaker functional end groups, and acidic elution.

RP-IP chromatography is also used to separate oligosaccharides using di- and tributyl amines.[22] All eight disaccharides can be separated using a tributyl quaternary amine,[23] and for larger oligosaccharides, a triethyl amine is generally used as the ion pairing agent.[24] This method has been employed in the analysis of sugars from model organisms, such as *Caenorhabditis elegans* and *Drosophila*.[25] It has also been used in the compositional analysis of HS from human tissues, such as liver,[26] and in pharmacokinetics in the analysis of contaminated oral heparin.[27] RP-IP has high resolving capabilities, but removal of the ion pairing reagent is problematic, resulting in oligosaccharide losses through desalting and causing in-source contamination when coupled with a mass spectrometer.

Heparin and HS have polar carboxylates, sulfates, and hydroxyl groups and are good candidates for the HILIC technique,[28] where HILIC has been used to separate a wide range of GAG saccharides as well as O- and N-glycans.[29−31] Recently, HILIC LC−MS has been used in the analysis of GAGs from tissues with studies focusing on chondroitin sulfate (CS)/dermatan sulfate (DS) in connective joint tissues[14,32] and profiling heparin and HS[33−37] using the parent ion and collision-induced dissociation (CID) differences. HILIC uses volatile buffers and is compatible with mass spectrometry but has low oligosaccharide resolution.

In this paper, we describe an ammonium-based (volatile salt) CTA-SAX method in conjunction with SEC and conventional SAX that provides improved separation of isomeric heparin oligosaccharides. The purification of heparin oligosaccharides using the VSCTA-SAX method avoids the need for desalting and improves column resolution, and the products are directly compatible with mass spectrometry analysis.

EXPERIMENTAL METHODS

Materials and Reagents. All chemicals of analytical or HPLC-grade purity were purchased from Sigma (Gillingham, UK) and VWR (Lutterworth, UK) unless otherwise stated. Disaccharide standards 1−8 were purchased from Iduron (Manchester, UK). Heparin was purchased from Alfa Aesar (Massachusetts, USA). Heparinase I−III enzymes were purchased from IBEX (Canada).

Disaccharide CTA-SAX Chromatography. CTA-SAX was performed based on the method of Mourier and Viskov (2004)[20] utilizing a Discovery C_{18} silica column (250 × 4.6 mm, 5 μm; Supelco), which was derivatized with 1 mM cetyltrimethylammonium in a water:methanol ratio of 50:50 (v/v). Separations were performed using a Shimadzu SPD 10A instrument using a UV−visible spectrophotometric detector. Eluent A was HPLC grade water, pH 3, using methanesulfonic acid, and eluent B was ammonium methanesulfonic acid (2 M), pH 2.5. The elution profiles were monitored by absorbance at 232 nm. Eight commonly occurring authentic HS disaccharides (Dextra Laboratories) were injected and eluted with a 0−50% linear gradient of eluent B at a flow rate of 1 mL/min over 60 min. The disaccharide separation was repeated using HPLC grade water as eluent A and 2 M ammonium bicarbonate as eluent B.

Digestion of Heparin. Heparin (1 g) was dissolved in 500 μL of lyase buffer (100 mM sodium acetate, 10 mM calcium acetate) and digested into oligosaccharide products using 1 mU of heparinase I (1 mU per 1 μL) to 10 mg of heparin at 37 °C. At 2, 3, 4, 6, and 8 h, a 120 μL aliquot was taken from the reaction and quenched by denaturing the heparinase enzyme at 98 °C for 3 min. The resulting products were pooled and further separated as described below.

Size Exclusion Chromatography (SEC). Heparin was separated using a Biorad Econo column packed in-house with prep grade Sephadex 30 beads (15 mm x 170 cm, bead size 34 μm; GE Healthcare) on a Delta 600 HPLC system (Waters). A 500 mg sample of the pooled digested heparin was made up to 1 mL with 0.5 M ammonium bicarbonate and injected into the system. The digested heparin sample was eluted using 0.5 M ammonium bicarbonate with a flow rate of 0.1 mL/min. The elution profile was monitored with absorbance at 232 nm. Fractions were pooled and repeatedly freeze-dried using water until all the ammonium bicarbonate was removed.

Strong Anion Exchange Chromatography (SAX). SEC fractions were separated using a Propac PA1 column (4.6 mm × 250 mm, 5 μm bead size; Thermo Scientific) on a Delta 600 HPLC system (Waters). Eluent A was HPLC grade water, and eluent B was 2 M NaCl. The concentration of each oligosaccharide fraction loaded was dependent on its previous purification method, as stated in the text. The initial hexasaccharide separation was performed on a 0−1.4 M NaCl gradient over 90 min using the Propac PA1 column. Subsequent bespoke elution gradients for each oligosaccharide were calculated based on the first round of SAX separation. Isolated peaks were collected and subjected to further orthogonal techniques.

Oligosaccharide VSCTA-SAX Chromatography. The C18 column (4.6 mm × 250 mm, 5 μm bead size; Sigma) was derivatized with 1 mM cetyltrimethylammonium in a water:methanol ratio of 40:60 (v/v). Oligosaccharides collected from the Propac PA1 SAX column separation were diluted 1 in 10, and multiple injections were performed to load the entire sample onto the VSCTA-SAX column. VSCTA-SAX separations were performed using a Waters Delta 600 HPLC with a UV−visible spectrophotometric detector. Eluent A was HPLC grade water, and eluent B was 2 M ammonium bicarbonate using a flow rate of 1 mL/min at a temperature of 40 °C. The elution profiles were monitored with an absorbance at 232 nm. Gradient details for each sample are in the text and figure legends. Each fraction was dried on a SPD121B speed vac (Thermo Scientific) prior to mass spectrometry and compositional analysis.

Disaccharide Compositional Analysis. Purified oligosaccharide structures were digested using 10 μL of 1 mU/1 μL, heparinase I, heparinase II, and heparinase III. Each reaction was incubated at 30 °C for 24 h to achieve complete digestion. The resulting disaccharide products were then separated on a SAX Propac PA1 (4.6 mm × 250 mm, 5 μm bead; Dionex) column using a Waters Delta 600 HPLC with a UV−visible spectrophotometric detector. Each sample was separated on a 0−1 M NaCl gradient over 60 min. Elution profiles were monitored with an absorbance of 232 nm. Disaccharide

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standards (1 μg of each standard) were loaded onto the same SAX Propac PA1 column and separated using the same gradient, so that the samples could be compared.

Ion Mobility-Mass Spectrometry (IM-MS) of Isomeric Hexasaccharides. IM-MS was performed on a Synapt G1 mass spectrometer equipped with a T-wave mobility cell (Waters Corp.). Sample concentration was calculated based on its 232 nm absorbance and then adjusted to a concentration of 0.5 μM in water/acetonitrile (50:50 v/v) with 500 mM ammonium hydroxide. The borosilicate tips were made in-house as stated in previous publications.[38,39] Oligosaccharides were sprayed in a borosilicate gold-coated tip, and mass spectra were acquired in negative ion mode with a capillary voltage of 0.55 kV, a sample cone voltage of 7 V, and an extraction cone voltage of 0.6 V. The ion mobility parameters for each tetra- or hexasaccharide can be found in the Supporting Information. MS/MS was performed on selected ions and collisionally activated at 15 and 20 V in the transfer cell with the mobility cell turned off to produce comparable CID data for each isomer.

RESULTS AND DISCUSSION

Disaccharide Analysis by CTA-SAX Employing Volatile Buffers. The natural diversity of heparin and HS provides a rich source for structure–function studies, but many oligosaccharides are isomeric structures, making purification difficult. This arises from the structures having identical negative charge and is compounded as the chain length increases.[12,40] The separation problems deepen due to the molecule being linear and the distance between the groups being close.[41,42] As the number of adjacent negative charges increases, the charge–charge repulsion makes this task even more difficult. Low pH buffers (e.g., pH 1) result in protonation of 50% of the sulfate groups and 99% of the carboxylic acid groups, potentially altering conformation and interaction with columns. At neutral pH, most sulfate groups will be negatively charged, causing charge–charge repulsion as well as adduct retention. It is known that coordination of salts causes different structural conformations,[43] so here we used ammonia-based salts to enhance the level of diversity for isomeric separation. The other major obstacle for purification is the use of nonvolatile salt buffers, which necessitates subsequent desalting methods that for hydrophilic HS oligosaccharides result in interactions and material loss (with desalting column recoveries of only 60–70% observed compared to >90% recovery with VSCTA-SAX; data not shown). Nonvolatile salts also affect mass spectrometry analysis by increasing the number of adducts attached to the sulfate groups and via signal suppression. We reasoned that the use of a volatile salt SAX method would overcome these problems. A conventional SAX method using a Propac PA1 column was tested with volatile ammonium bicarbonate salts; however, neither disaccharides nor oligosaccharides eluted from the column, indicating the interaction was too strong for ammonium bicarbonate to achieve effective dissociation (data not shown). To overcome this, we investigated CTA-SAX[19] in which a C-18 reverse phase column is first derivatized using CTA, imparting a positive charge on the matrix and allowing the column to be used for SAX. First, eight commonly occurring HS disaccharide standards were separated on CTA-SAX using a 0–1 M ammonium methanesulfonic acid gradient over 60 min (Figure 1a). All disaccharides eluted with baseline resolution; furthermore, the column separated α and β anomers

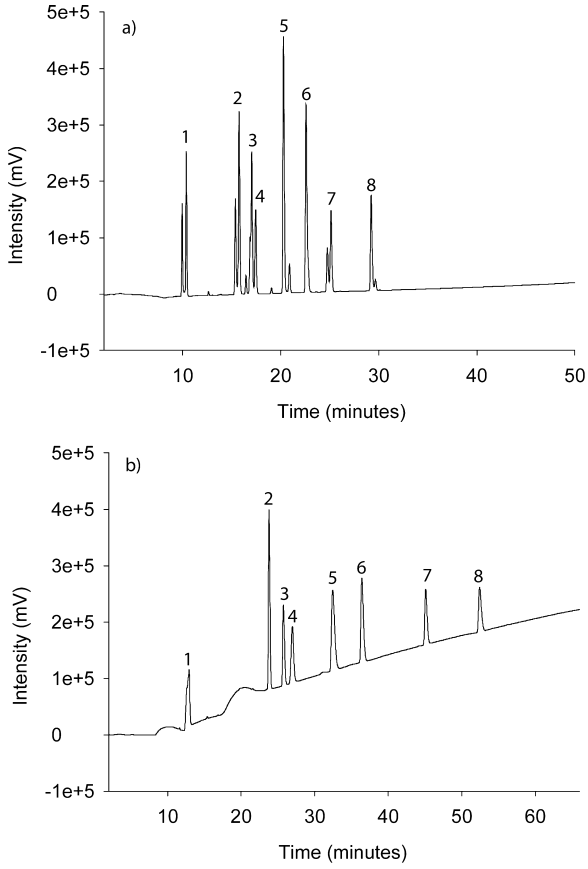

Figure 1. CTA-SAX separation of HS standards using nonvolatile and volatile buffers. Disaccharide standards were injected and eluted from CTA-derived Discovery C18 column using a 0–1 M gradient over 60 min of (a) ammonium methanesulfonic acid, pH 2.5 and (b) ammonium bicarbonate. Standards: 1, ΔUA-GlcNAc; 2, ΔUA-GlcNS; 3, ΔUA-GlcNAc6S; 4, ΔUA2S-GlcNAc; 5, ΔUA-GlcNS6S; 6, ΔUA2S-GlcNS; 7, ΔUA2S-GlcNAc6S; 8, ΔUA2S-GlcNS6S.

for the disaccharides, resulting in double peaks for many of the standards. The standards were then separated on the CTA-SAX column using the volatile ammonium bicarbonate salt (Figure 1b). As with the ammonium methanesulfonic acid, all eight disaccharides were separated but without separation of the anomeric structures with the minor drawback of increased baseline absorbance. The ability of the ammonium bicarbonate to dissociate disaccharides from the CTA-SAX column but not the Propac PA-1 column suggested that the VSCTA-SAX system might be ideal as an additional purification step for oligosaccharides.

Major and Minor Oligosaccharide Peak Separation. To prove the effectiveness of VSCTA-SAX to prevent losses, improve separation, and increase compatibility with other techniques such as mass spectrometry, we isolated a heparin hexasaccharide using traditional SEC and SAX followed by VSCTA-SAX and determined its sequence using mass spectrometry. Full length heparin was digested with heparinase I, and the oligosaccharide mixture (ranging from dp2 to dp30) was separated using SEC (data not shown). The dp6 oligosaccharide fraction isolated from SEC separation was subjected to further chromatographic separation using Propac PA1 SAX (Figure 2). Separation of the dp6 SEC fraction is shown in Figure 2; peaks A–C were selected for further

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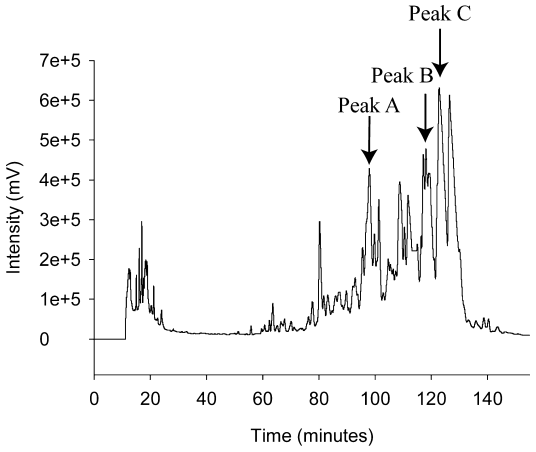

Figure 2. SAX separation of a heparin hexasaccharide mixture. The dp6 oligosaccharide fraction isolated from SEC (data not shown) was collected and separated using a Propac PA1 SAX column on a 0−1.4 M NaCl gradient over 90 min as described in the Experimental Methods. Arrowed peaks were collected for further separation using subsequent methodologies.

purification. Previous work has focused on using such partially resolved SAX fractions.[44]

Peak A was separated further using a Propac PA1 column employing a shallower salt elution gradient to improve oligosaccharide resolution (Figure 3a). However, using this gradient, full oligosaccharide separation could not be obtained. Peak D from this run was then further separated by VSCTA-SAX chromatography using ammonium bicarbonate as the elution buffer, demonstrating resolution of two separate oligosaccharide structures (Figure 3b). The reasons for these two peaks being separated could be related to the spacing of the CTA linker on the column or because an ammonium salt is weaker in its displacement than a sodium salt. It could also be because ammonium ions are known to coordinate and separate oligosaccharides differently than sodium adducts as shown through graphite LC−MS (Miller, Karlsson, and Turnbull; unpublished data). The larger peak, peak E (Figure 3b), was selected for mass spectrometry analysis and compositional analysis to determine whether the structure was pure and establish whether it could be sequenced (Figure 3c−e). MS showed that this peak was pure with an m/z 509.9 $[M - 3H]^{3-}$, which corresponds to a hexasaccharide with six sulfates and one acetyl group (dp6 + $6SO_3$ + 1Ac; Figure 3c). Data from tandem mass spectrometry and compositional analysis were both used to inform sequencing (Figure 3d and e).[45−48] Compositional analysis of peak E (Figure 3e) showed that this structure contains three different disaccharides: ΔUA-GlcNAc6S, ΔUA2S-GlcNS, and ΔUA2S-GlcNS6S. The order of the disaccharides can be determined based on the acetate group, as this does not dissociate in the mass spectrometer and displays a distinct mass signature compared to sulfate groups. The ions m/z 486 (Y_3, UA-GlcNAc6S-UA2S-GlcNS), 429.7 (C_5, ΔUA2S-GlcNS6S-UA-GlcNAc6S-UA2S), and 636 (B_5, ΔUA2S-GlcNS6S-UA-GlcNAc6S-UA2S) show that the GlcNAc is positioned in the middle of the structure and that the GlcNS is at the reducing terminal. If the GlcNAc residue was the reducing terminal disaccharide, it would be expected that ions corresponding to m/z 536 (C_4), 615 (B_5), and 624 (C_5) would be observed; none of these ions were observed in the product ion spectra. Therefore, the sequence can be defined as ΔUA2S-GlcNS6S-UA-GlcNAc6S-UA2S-GlcNS (Figure S-1).

Separation of Isomeric Structures. The majority of structures within heparin and HS have multiple isomers due to variation in positioning of sulfate and acetate groups in different saccharides of the same mass, making isolation of single structures challenging. For example, peak B (Figure 2) clearly contains multiple species and has peak shoulders and therefore likely represents multiple oligosaccharide structures. For tackling this problem, the middle peak was collected with the aim of isolating the major product with only minor overlapping products. Peak B (Figure 2) was separated a second time using the SAX column with a shallower gradient (0.6−1.1 M NaCl over 60 min) in an effort to improve its separation (Figure 4a). In this case, a greater level of separation was observed. It is likely that the resolving power of these peaks could be further enhanced by using a shallower salt gradient and reduced sample loading. Because the resulting major peak (peak F) was not baseline resolved, as commonly observed for heparin/HS oligosaccharides,[11] there is a need to have additional orthogonal separation methodologies. To avoid desalting and consequent losses, peak F (containing a high concentration of NaCl) was loaded onto the VSCTA-SAX column through dilution and multiple injections. Peak F oligosaccharide structures were then eluted from the VSCTA-SAX column with a 0.7−1.4 M ammonium bicarbonate gradient (Figure 4b). This method of separation resulted in close to baseline resolution of four apparent oligosaccharide compounds, demonstrating the enhanced resolution of VSCTA-SAX chromatography. The ammonium bicarbonate salt was readily removed via centrifugal evaporation (data not shown). Peaks G and H were analyzed by offline ESI-MS. They both displayed an m/z of 549.3, confirming that they were mass isomers as anticipated and correspond to a dp6 with $8SO_3$ (Figure 4c). Furthermore, IM-MS of peaks G and H revealed that they displayed different arrival time distributions (ATDs) of 3.39 and 3.67 ms, respectively (Figure 4d). This suggests different structural conformations with peaks G and H displaying more compact and extended conformations, respectively. Because both compounds could be separated though a charge-dependent interaction method, this result indicates that this separation is at least partly dependent on their different conformational properties.

For the structure of the isolated peaks to be determined further, tandem mass spectrometry and compositional analysis were performed on both peaks G and H. MS revealed very different product ion spectra (Figure 4e). Compositional analysis showed that peak G contained a single ΔUA-GlcNS6S and two ΔUA2S-GlcNS6S disaccharides, whereas peak H contained one ΔUA2S-GlcNS and two ΔUA2S-GlcNS6S (Figure 4f). Because there are two ΔUA2S-GlcNS6S, the first piece of information to identify in the product ion spectra is whether these disaccharides are adjacent. In peak G, no doubly charged 576 product ion was observed (Figure 4e), whereas in peak H, a doubly charged 576 product ion was observed, indicating that the two trisulfated disaccharides must be adjacent. Therefore, the sequence of peak H is ΔUA2S-GlcNS-UA2S-GlcNS6S-UA2S-GlcNS6S or ΔUA2S-GlcNS6S-UA2S-GlcNS6S-UA2S-GlcNS (Figures S-1 and S-2). In contrast, peak G has a major triply charged product ion of 464 corresponding to Z_4 (GlcNS6S-UA-GlcNS6S-UA2S-GlcNS6S). The Y_3 ion (UA-GlcNS6S-UA2S-GlcNS6S) and Y_2 ion (GlcNS6S-UA2S-GlcNS6S) confirmed that the UA-

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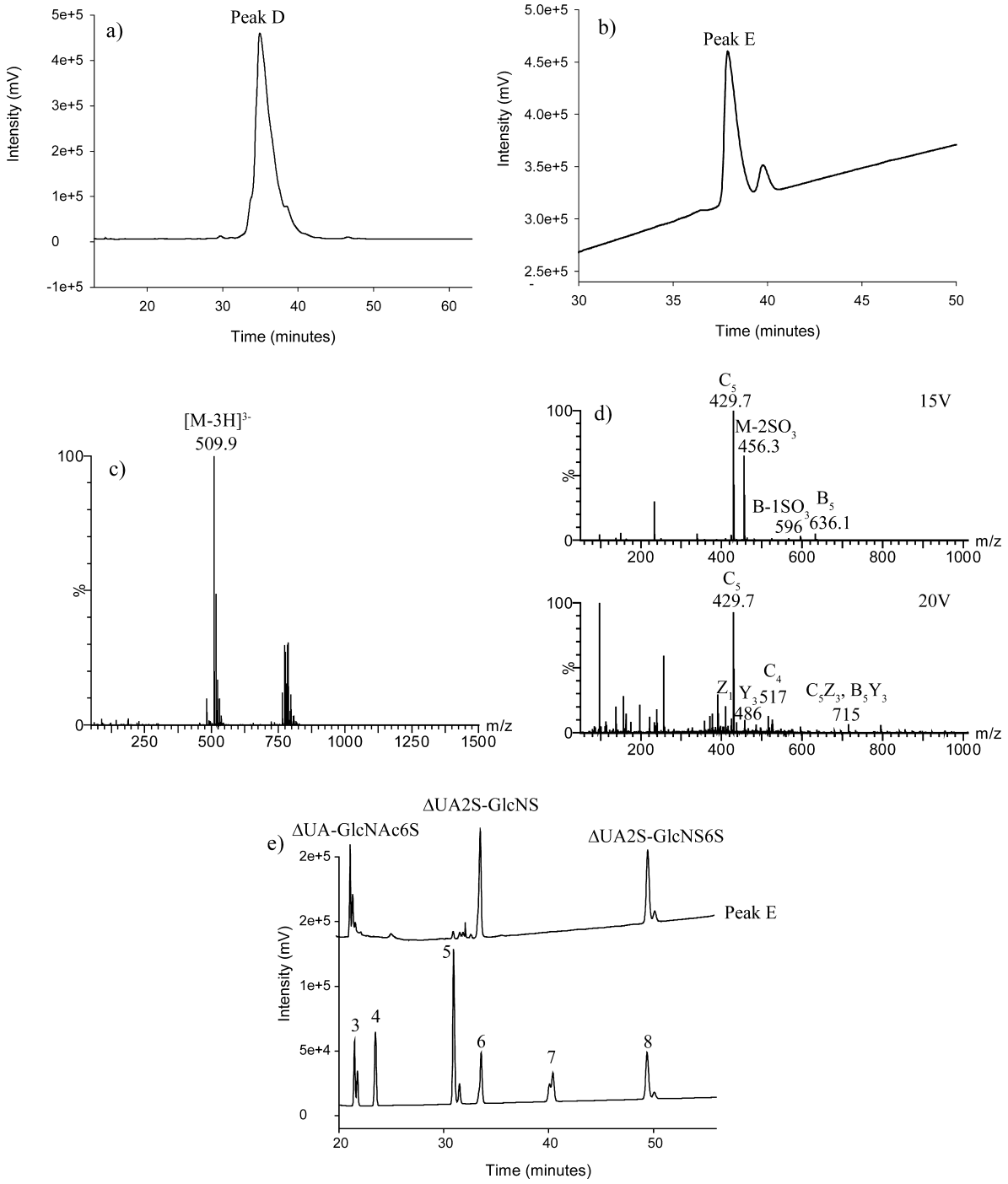

Figure 3. Purification of minor and major oligosaccharide products using SAX and VSCTA-SAX. (a) SAX separation of peak A on a Propac PA1 SAX column using a 0.6−1.1 M NaCl gradient over 60 min. (b) Peak D was separated further using a VSCTA-SAX column on a 0.7−1.4 M ammonium bicarbonate gradient B in A over 60 min. (c) MS of peak E showing an m/z of 509.9, which corresponds to dp6 + $6SO_3$ + 1Ac. (d) MS/MS of peak E at 15 and 20 V. (e) Disaccharide analysis of peak E. The lower chromatograms correlate to disaccharide standards. The upper trace is disaccharide analysis of peak E. This information defined the structure with an overall sequence of ΔUA2S-GlcNS6S-UA-GlcNAc6S-UA2S-GlcNS. Standards: 3, ΔUA-GlcNAc6S; 4, ΔUA2S-GlcNAc; 5, ΔUA-GlcNS6S; 6, ΔUA2S-GlcNS; 7, ΔUA2S-GlcNAc6S; 8, ΔUA2S-GlcNS6S.

GlcNS6S is in the middle of the oligosaccharide structure (Figure 4e). Therefore, the sequence of the oligosaccharide isolated from peak G is ΔUA2S-GlcNS6S-UA-GlcNS6S-UA2S-GlcNS6S (Figures S-1 and S-2).

In a further demonstration of the utility of VSCTA-SAX chromatography, we were also able to separate another three hexasaccharides (dp6 + $8SO_3$) from the same dp6 SEC fraction (peak C; Figure 2). This peak was further separated on a shallow gradient (0.84−1.2 M NaCl over 60 min) on a SAX Propac PA1 column (Figure 5a). The structures were separated into two peaks, I and J (although not to baseline resolution), and were collected separately. It should be noted that collecting partial peaks and reseparating on a shallower gradient can also lead to improved resolving power. Peaks I and J were both subjected to separation on the VSCTA-SAX column with a 0.8−1.5 M ammonium bicarbonate gradient. This resulted in

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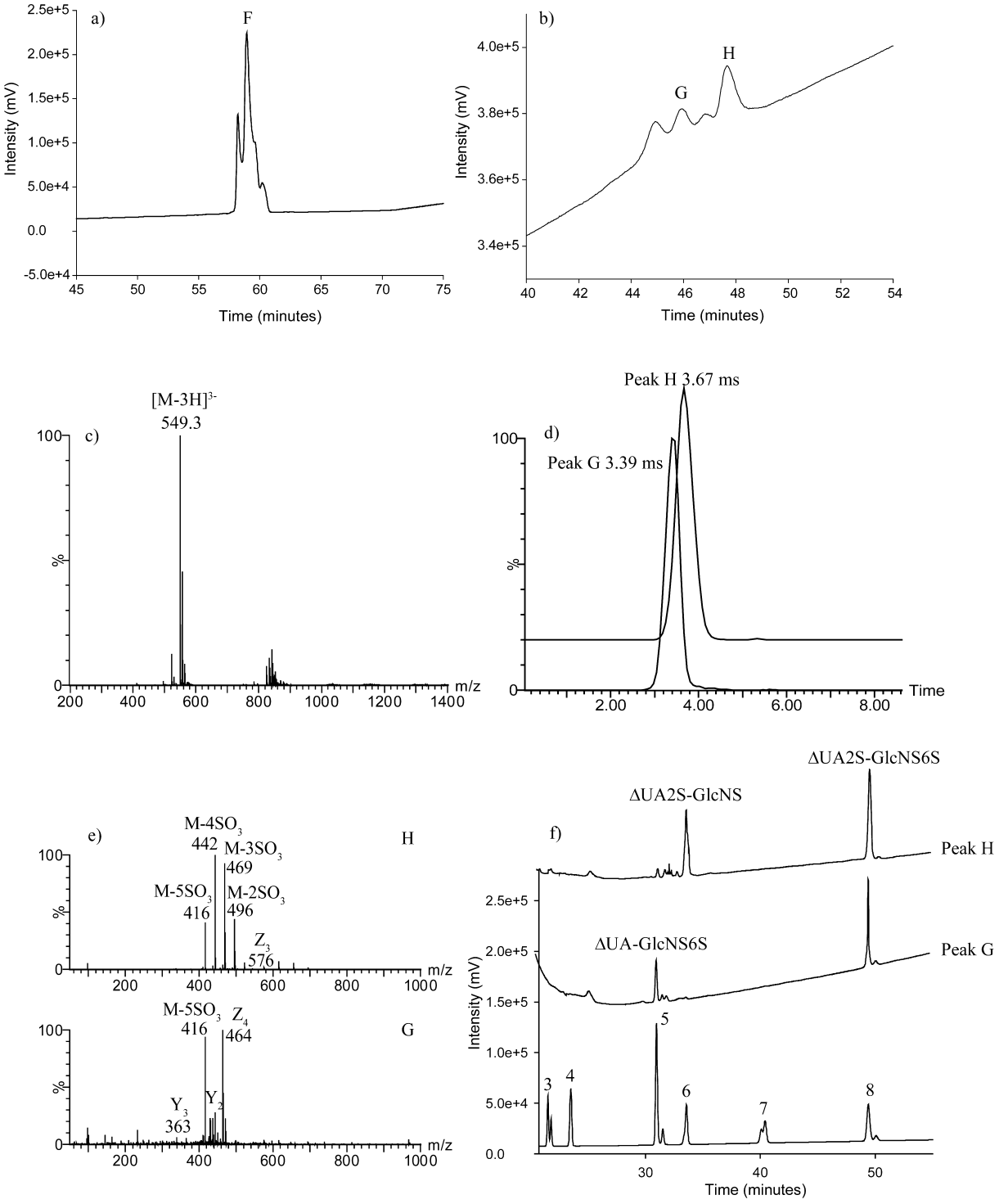

Figure 4. Purification and analysis of isomeric heparin hexasaccharide products from peak B (Figure 2) using SAX and VSCTA-SAX. (a) SAX separation of peak B on a Propac PA1 SAX column using a 0.6−1.1 M NaCl gradient over 60 min. (b) Peak F was separated further using a VSCTA-SAX column on a 0.7−1.4 M ammonium bicarbonate gradient over 60 min. (c) MS of peaks G and H displayed an m/z of 549.3, which corresponds to dp6 + 8SO_3. (d) IM-MS of peaks G and H. (e) MS/MS of peaks G and H at 15 V. (f) The lower chromatogram correlates to disaccharide standards, and the upper two chromatograms correlate to disaccharide analysis of peaks G and H. This information gave peak G an overall sequence of ΔUA2S-GlcNS6S-UA-GlcNS6S-UA2S-GlcNS6S and peak H a sequence of ΔUA2S-GlcNS-UA2S-GlcNS6S-UA2S-GlcNS6S. Standards: 3, ΔUA-GlcNAc6S; 4, ΔUA2S-GlcNAc; 5, ΔUA-GlcNS6S; 6, ΔUA2S-GlcNS; 7, ΔUA2S-GlcNAc6S; 8, ΔUA2S-GlcNS6S.

improvements in peak resolution (Figure 5b and c). MS showed that all three structures had the same m/z ratio of 549.3 $[M - 3H]^{3-}$ (data not shown). It was particularly noteworthy that IM-MS on these three isomeric structures revealed that each structure had different ATDs. Peak K displayed the most extended conformation with an ATD of 3.74 ms, peak M a slightly more compact conformation (3.60 ms), and peak L a much more compact structural conformation (3.46 ms) (Figure 5d). MS/MS and compositional analysis were completed on all three of these structures (Figure 5e and f). Compositional

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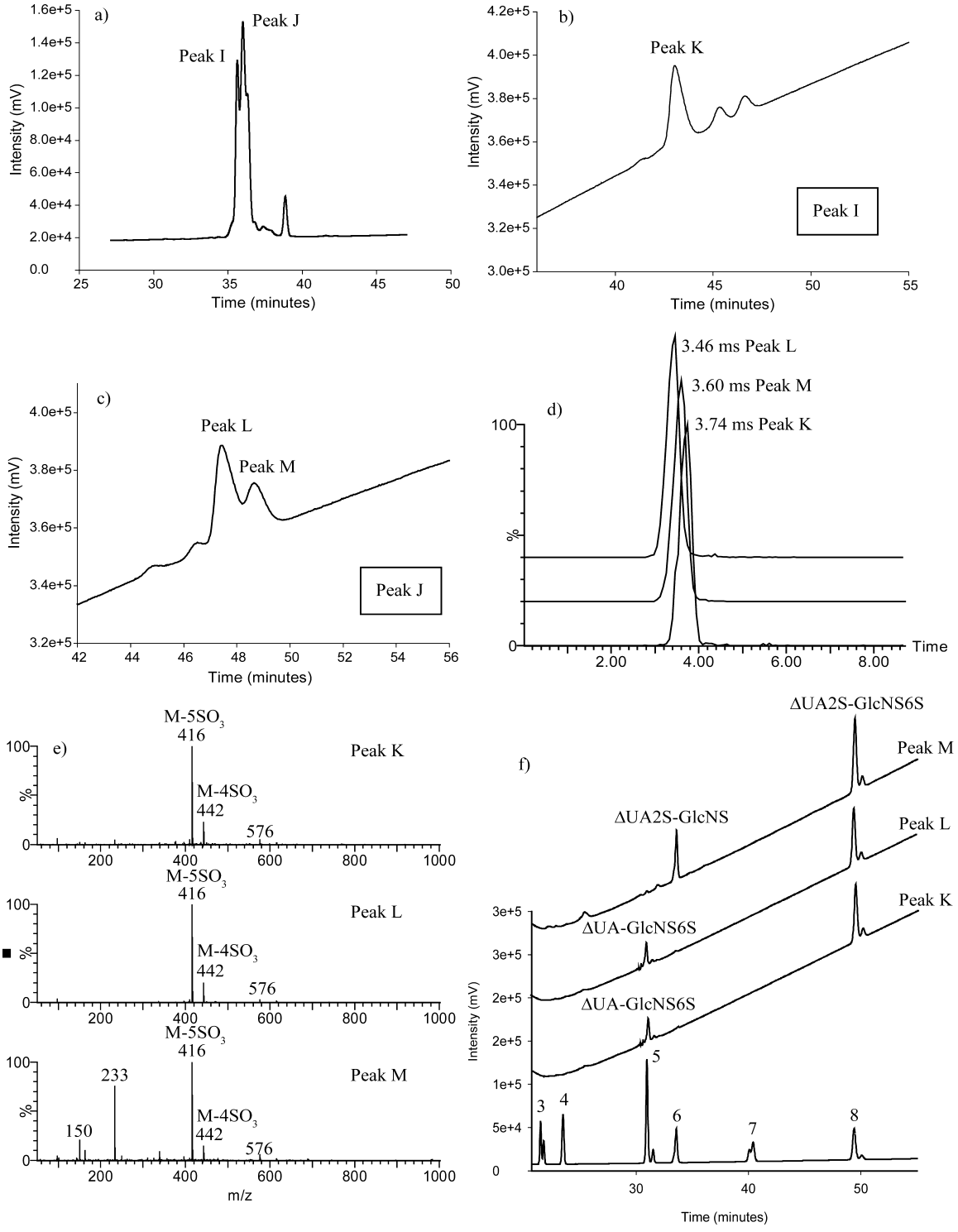

Figure 5. Separation of three isomeric structures. (a) Propac PA1 SAX separation of peak C on a 0.84−1.2 M NaCl gradient over 60 min. (b) VSCTA-SAX separation of peak I on a 0.8−1.5 M ammonium bicarbonate gradient over 60 min, isolating peak K. (c) VSCTA-SAX separation of peak J on a 0.8−1.5 M ammonium bicarbonate gradient over 60 min, isolating peaks L and M. (d) IM-MS separation of peaks K−M. (e) MS/MS of peaks K−M at 15 V. (f) The lower chromatogram correlates to disaccharide standards, and the upper three chromatograms correlate to disaccharide analysis of peaks K−M. The sequence of peak K is ΔUA-GlcNS6S-UA2S-GlcNS6S-UA2S-GlcNS6S, peak L is ΔUA2S-GlcNS6S-UA2S-GlcNS6S-UA-GlcNS6S, and peak M is ΔUA2S-GlcNS6S-UA2S-GlcNS6S-UA2S-GlcNS. Standards: 3, ΔUA-GlcNAc6S; 4, ΔUA2S-GlcNAc; 5, ΔUA-GlcNS6S; 6, ΔUA2S-GlcNS; 7, ΔUA2S-GlcNAc6S; 8, ΔUA2S-GlcNS6S.

analysis showed that peaks K and L each contained one ΔUA-GlcNS6S and two ΔUA2S-GlcNS6S disaccharides, whereas peak M contained one ΔUA2S-GlcNS and two ΔUA2S-GlcNS6S disaccharides. MS/MS of these structures showed that all three contained a doubly charged product ion of 576 corresponding to an intact dp4 + 6SO_3 (UA2S-GlcNS6S-UA2S-GlcNS6S). Thus, the sequences of peaks K and L are defined as ΔUA-GlcNS6S-UA2S-GlcNS6S-UA2S-GlcNS6S and

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ΔUA2S-GlcNS6S-UA2S-GlcNS6S-UA-GlcNS6S, respectively, and the sequences of peaks M and H are ΔUA2S-GlcNS6S-UA2S-GlcNS6S-UA2S-GlcNS or ΔUA2S-GlcNS-UA2S-GlcNS6S-UA2S-GlcNS6S, respectively (Figure S-1). These two structures with identical compositional analysis but disaccharide UA-GlcNS6S at either the reducing end or the nonreducing end displayed the largest ATD difference (3.74 and 3.46 ms). This suggests that oligosaccharide conformation can have an effect on separations using SAX columns, possibly via the different distribution of free electrons affecting the strength of the interaction.

CONCLUSIONS

Here, we demonstrate the isolation, separation, and analysis of multiple hexasaccharide structures from heparin oligosaccharide starting material. The VSCTA-SAX methodology we describe resulted in enhanced resolution compared to previous methods, likely resulting from a combination of the weaker displacement salt, the coordination of ammonium with the sulfate groups, and differences in structural conformation and electron distribution. We have demonstrated that VSCTA-SAX provides improved separation of structural isomers compared to that of HILIC and RP-IP and also removes the need for traditional desalting methods. Volatile salt methods for heparin/HS chromatography techniques are increasingly in demand to improve yields, which is crucial because only very small amounts of individual structures are often purified from complex starting mixtures. Thus, the practicality of subsequent analysis and screening is enhanced using VSCTA-SAX. We demonstrate here that the combination of established SEC and SAX separation techniques with VSCTA-SAX allowed the separation of five isomeric structures (dp6 + $8SO_3$) and their sequence determination in a manner not possible with traditional methods alone. We have also applied the method to purification of isomeric heparin saccharides for studying selectivity of interactions with the chemokine MCP-1/CCL2.[49] We conclude that VSCTA-SAX is a powerful additional tool to enhance structure−activity studies on heparin/HS saccharides.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b02801.

Figure S-1, structural representation of each sequenced heparin saccharide; Figure S-2, workflow procedure involving the purification and sequencing of oligosaccharides; and Supporting Methods (PDF)

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Notes

The authors declare no competing financial interest.

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