Carbohydrate Polymers 106 (2014) 343–350

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Quantitative analysis of anions in glycosaminoglycans and application in heparin stability studies

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ARTICLE INFO

Article history:
Received 11 November 2013
Received in revised form 22 February 2014
Accepted 22 February 2014
Available online 12 March 2014

Keywords:
Glycosaminoglycans
Anions
High-performance anion exchange chromatography (HPAEC)
Structure
Quality
Heparin stability

ABSTRACT

The sulfo groups of glycosaminoglycans contribute to their high charge densities, and are critical for the role they play in various physiological and pathophysiological processes. Unfortunately, the sulfo groups can be hydrolyzed to inorganic sulfate. Thus, it is important to monitor the presence of these sulfo groups. In addition, free anions, including chloride, sulfate and acetate, are often present in glycosaminoglycans as a result of multiple purification steps, and their presence also needs to be monitored. In this report, ion chromatography with conductivity detection is used to analyze the anions present in glycosaminoglycans, including heparin, heparan sulfate, chondroitin sulfate and dermatan sulfate. This method allows quantitation over a wide range of concentrations, affording a limit of quantitation of 0.1 ppm and a limit of detection of 0.05 ppm for most anions of interest. The stability of heparin was also studied, providing data on the formation of both sulfate and acetate anions.

1. Introduction

Glycosaminoglycans (GAGs) are linear, acidic polysaccharides found within cells, on cell surfaces, and in the surrounding extracellular matrix. Through their interaction with proteins, GAGs participate in and regulate many cellular events as well as physiological and pathophysiological processes, such as cell proliferation and differentiation, cell–cell and cell–matrix interactions, and viral infection (Wu, Zhang, Beeler, Kuberan & Rosenberg, 2002; Capila & Linhardt, 2002; Bernfield et al., 1999). GAGs are composed of repeating disaccharide units (Fig. 1), and they are divided into four main categories, hyaluronic acid (HA), chondroitin sulfate/dermatan sulfate (CS/DS), heparan sulfate/heparin (HS/Hp) and keratan sulfate (KS), based on their monosaccharide composition and the configuration and position of their glycosidic linkages. Differences in the specificity of interaction between GAGs and their binding proteins result from the structural diversity of GAGs, including type, size, saccharide composition, charge density, sequence and molecular weight (Taylor & Gallo, 2006; Sasisekharan, Raman & Prabhakar, 2006). Charge density, a critical factor for GAG–protein interaction specificity, often depends on the degree of sulfation of a GAG (Zsila & Gedeon, 2006). The impact of charge density on the proper functioning of a GAG is usually greater than the other structural properties of GAG (Yard, Lorentz, Herr & van der Woude, 1998; Naimy, Buczek-Thomas, Nugent, Leymarie & Zaia, 2011; Mummery, Mulloy & Rider, 2007). The amount of sulfation present in a GAG sample is not only related to its biological function but also provides information on the quality of that sample and on the presence of impurities or contaminants (Bo, Muschin, Kanamoto, Nakashima, & Yoshida, 2013; Nilasaroya, Poole-Warren, Whitelock, Martens, 2008). Thus, an assessment of the sulfo group content of a GAG is necessary for appropriate quality control.

Heparin, one of a few carbohydrate drugs, is widely used as an anticoagulant and has the highest sulfo group content of all GAGs (Liu, Zhang & Linhardt, 2009). Because of its high charge

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http://dx.doi.org/10.1016/j.carbpol.2014.02.076
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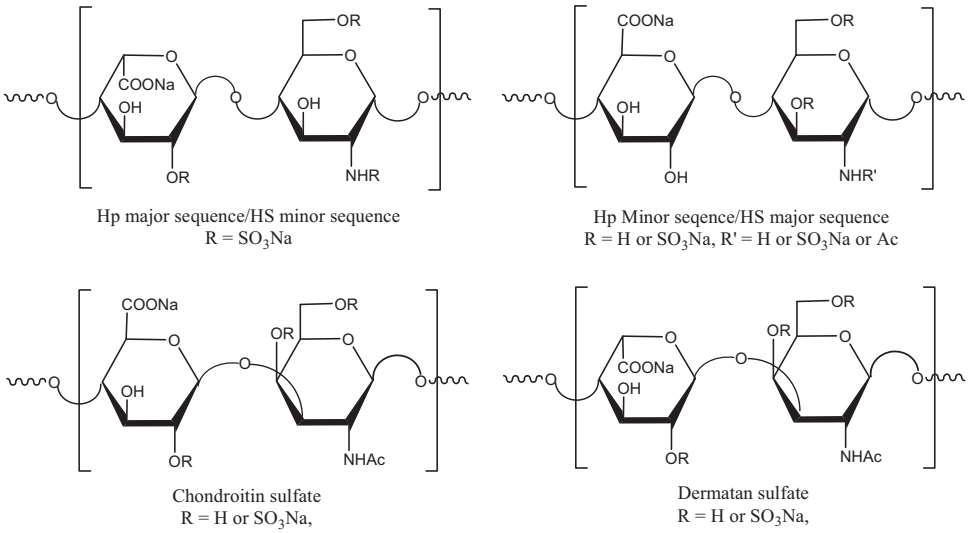

Fig. 1. Structures of heparin (Hp), heparan sulfate (HS), chondroitin sulfate (CS), and dermatan sulfate (DS).

density, heparin also shows many other biological activities through its binding to various proteins. Heparin was contaminated with oversulfated chondroitin sulfate (OSCS) in 2007–2008 (Guerrini et al., 2008; Zhang et al., 2008, 2009). This contamination crisis was associated with the deaths of nearly 100 Americans (Liu, Zhang & Linhardt, 2009). The structure and activity of OSCS were so similar to heparin that this contaminant was difficult to detect by the standard pharmacopeial methods in place at the time of the heparin crisis. Other GAGs, such as DS, are often found in both crude heparin and heparin active pharmaceutical ingredient (API) as impurities, as a result of inefficient purification (Guerrini et al., 2008; Zhang et al., 2009). Some methods have been recently developed to detect and analyze these GAGs in heparin products (Trehy, Reepmeyer, Kolinski, Westengerger, & Buhse, 2009; Somsen, Tak, Torano, Jongen, & deJong, 2009; Limtiaco, Jones, & Larive, 2009; Guerrini et al., 2009; Fu et al., 2013) but most of these assays are not straightforward. Because the sulfo group contents are different for different GAGs, methods that quantitate sulfation levels can be used to evaluate the purity of heparin API.

Heparan sulfate (HS) is related to heparin and is the most structurally complex GAG (Sugahara & Kitagawa, 2002). Because of its structural heterogeneity, HS is an important regulator of signaling molecules in many physiological and pathophysiological processes (Princivalle & de Agostini, 2002; Edwards & Edwards, 2012; Kennedy, 2012). Characterization of its structural properties, such as degree of sulfation, can help explain its various activities (Poole, 1986).

Chondroitin sulfate (CS) has also been used for many years as a nutraceutical and in medicine (Bartus, James, Bosch, & Bradbury, 2012; Sharma, Wood, Richardson, Roberts, & Kuiper, 2007). CS is divided into CS-A (GlcA-GalNAc4S), CS-B (DS, IdoA-GalNAc4S), CS-C (GlcA-GalNAc6S), CS-D (GlcA2S-GalNAc6S) and CS-E (GlcA2S-GalNAc4S) based on differences in sulfation pattern and sugar composition. These CS GAGs are primarily extracted from animal tissues. The different applications for each CS often depends on their degree of sulfation (Barroca & Jacquinet, 2002).

The sulfo groups covalently linked to GAGs are labile and can be released as inorganic sulfate anions on prolonged storage or on storage under improper conditions (Zaia, 2013; Zaia & Costello, 2003). Different salts and buffers are often used in the commercial production of GAGs (Liu, Zhang & Linhardt, 2009). Thus, it is critical to monitor the presence of anions, introduced in the production of GAGs or in GAG decomposition, to ensure GAG purity and stability.

High performance anion exchange chromatography (HPAEC) has been developed to quantify free sulfate and other anions with high resolution and high sensitivity (Morales, de Graterol & Mesa, 2000; Cole & Evrovski, 1997; McPhee, Atkinson & Cole, 1990; Singh & Nancollas, 1988; Morris & Levy, 1988). Compared to combustion analysis, titration and colorimetric methods (Greweling, Bache & Lisk, 1972; Lambert & Ramasamy, 1975; Harenberg et al., 2009), quantitative analysis of sulfate by HPAEC is faster, requires less labor and sample consumption, can differentiate between free or covalently bound sulfate before and after hydrolysis, can provide information on other ions present, and can improve analytical sensitivity, precision, and accuracy. The recent US Pharmacopeia monograph on enoxaparin for injection requires sulfate group content testing by HPAEC (United State Pharmacopeial Convention, 2012).

In this paper, we compare free anions, including chloride, acetate, phosphate and sulfate in GAG products; analyze the degree of sulfation of heparin, HS, CS-A, and DS; monitor the sulfate and acetate group levels in heparin stability studies; and inspect the release of sulfate and acetate under various conditions.

2. Experimental

2.1. Materials

Two heparin standards were purchased from United State Pharmacopeia (USP, Rockville, MD) and Chinese National Institutes for Food and Drug Control (NIFDC, Beijing, PR China), respectively. Heparin, HS and DS were purchased from Celsus (Cincinnati, OH). Heparin, CS-A, HA and Certified Multi-anion Standard Solution PRIMUS (10 mg/kg ± 0.2% F^-, Cl^-, Br^-, NO_3^-, SO_4^{2-}, PO_4^{3-} of each anion) were purchased from Sigma–Aldrich (St. Louis, MO). Two lots of heparin were provided by a plant in China. High-purity water (resistivity ≥ 18.2 MΩ cm, 25 °C) was used throughout this study. All chemicals and reagents were of HPLC grade.

2.2. Standard and sample preparation

Standards—Five multi-anion standard solutions were prepared at a series of concentrations (0.1, 1, 2, 4, 5 ppm of each anion) in

ultrapure water. Five sodium acetate solutions were also prepared at same concentrations.

Intact GAG samples—GAGs were dissolved in water and lyophilized to remove the water of hydration. These lyophilized GAG products were then prepared at 0.25 mg/mL to analyze their content of free anions.

Hydrolyzed GAG samples—Specific amounts of lyophilized GAGs were dissolved in a 2 M aqueous TFA solution to obtain a final solution of 5 mg/mL. Hydrolysis was carried out on 1 mL of solution at 100 °C over 24 h. The hydrolysates were monitored by TLC to confirm the completeness of hydrolysis (Zhang, Xie, Zhang, & Linhardt, 2007). Excess TFA was removed and the hydrolysates were dried using a SpeedVac concentrator (Labconco, Kansas City, MO), as previously described (Zhang, Khan, Nunez, Chess, & Szabo, 2012). The hydrolysates were re-dissolved in water and neutralized with dilute NaOH solution before they were diluted with water to 500 mL in volumetric flasks. The solutions at a final concentration of 10 ppm were ready for analysis.

Samples for heparin stability experiments—Heparin powder (Celsus) with a particle size of ≤5 mm was incubated at 30, 60 and 80 °C. Aliquots were removed at 0, 5, 12, 24, 48, and 96 h. Each sample was dissolved in ultrapure water to afford a final concentration of 0.5 mg/mL and stored at −40 °C before analysis. Heparin (Celsus) was dissolved in 0.1 M hydrochloric acid and 0.1 M sodium hydroxide solutions at final concentrations of 5 mg/mL. Aliquots of solutions stored at 30, 60 and 80 °C were taken out at 0, 1, 2, 5, 12, 24 h. Each aliquot was neutralized with dilute NaOH or dilute HCl, diluted with water to final concentration of 0.5 mg/mL, and stored at −40 °C before analysis.

The intact and hydrolyzed samples were analyzed in triplicate, while the standard curves and stability experiment were performed in duplicate. The standard deviations of results from hydrolyzed samples and stability experiments were calculated based on complete replicate analysis using the standard curves.

2.3. HPAEC analysis

The analysis was performed on a Metrohm 850 Professional system with a 919 IC auto-sampler plus, dual pumps and a conductivity detector (Herisau, Switzerland). Data was acquired and analyzed with software MagIC Net 2.3 (Herisau, Switzerland). Chromatography was performed on a Metrosep A Supp 5 analytical column (4 × 150 mm, Metrohm, Herisau, Switzerland) with a Metrosep A Supp Guard column (4 × 5 mm, Metrohm, Herisau, Switzerland) at 30 °C. The isocratic mobile phase consisted of 3.2 mM sodium carbonate and 1.0 mM sodium bicarbonate delivered at 0.7 mL/min. The injection volume was set to 20 μL and the data acquisition time was set to 20 min.

3. Results

3.1. Method development

The method developed for quantitation of fluoride, acetate, chloride, bromide, nitrate, phosphate and sulfate gave a limit of quantitation (LOQ) of 0.1 ppm (20 μL). These anions are observed in a chromatogram at about 4.1, 4.6, 5.9, 8.7, 9.8, 14.0 and 16.0 min, respectively. (Fig. 1a and b) The negative peak, observed at about 3.5 min, is believed to be solvent peak (water). The limit of detection (LOD) of the anions was 0.05 ppm with the exception of chloride, which was 0.01 ppm (see Supplemental Information Fig. S1a–c). The peak areas of each ion, plotted as a function of its concentrations, gave good linearity over a wide range of concentrations (0.1–5 ppm) and the representative standard curves of acetate, chloride and sulfate are shown in Fig. 2c–e. The standard curves of other anions were shown in Supplemental Information Fig. S1d–g. The standards were analyzed in duplicate. Some of their standard deviations were too small to be observed in Fig. 2c–e and Fig. S1d–g, suggesting good repeatability for the HPAEC method.

3.2. Free anion detection in GAG products

The chromatograms of free anions in six heparins from different companies, and one source each for HS, CS and DS are shown in Fig. 3. Free sulfate was observed at ~16.0 min. The sulfate contents of the most samples ranged from 0.1 to 1.3% (w/w). However, no free sulfate groups, above the LOQ, were observed in the heparin API obtained from a Chinese heparin manufacturer. The free sulfate could result from the purification, drying, or storage processes. Sodium chloride is applied abundantly in GAG production processes, so chloride is another major free anion observed in all of these GAGs at ~5.9 min. The chloride content of these samples reflects the inefficiency of desalting. The highest chloride content (~3.9%) was observed in one heparin from a Chinese manufacturer. The chloride contents of other samples were about 0.5% or lower. Phosphate was observed, at about ~14.0 min, only in the chromatogram of CS-A. Another peak, observed at ~4.8 min in all the samples, with the exception of the two standard heparins, was not included in standard ion solution and was confirmed to be acetate (Fig. 2b). The acetate anion could result from the instability of the N-acetylated polysaccharides and/or could be introduced in the production process. The contents of all observed anions are presented in Table 1. The standard deviations are low.

3.3. Determination of covalently bound sulfate in GAG products

TLC results showed that the carbohydrate hydrolysates were unsulfated (data not shown) and confirmed that TFA treatment released all of the sulfo groups from each GAG. In addition to the sulfate anion, residual TFA was also observed as its anion at ~8.05 min in all the chromatograms of the hydrolyzed samples (Fig. 4). A chromatogram of multi-anion standards (Fig. 4a), a representative chromatogram of hydrolyzed USP heparin standard (Fig. 4b), and a chromatogram of TFA (Fig. 4c) are presented. The sulfate content (%SO_3Na) of each GAG was calculated and is listed in Table 1. The sulfate contents of the heparin samples differed slightly, the heparin standard from the USP had the lowest sulfate content (~34.2%, w/w), while the heparin standard from NIFDC had the highest sulfate content (~38.2%, w/w). The standard deviations in this experiment are higher than that of free anion analysis. The order of sulfo group content was Hp > DS > HS > CS-A. An acetate anion signal was not observed in any of the hydrolyzed samples.

3.4. Stability study on heparin

Three conditions were applied to test heparin stability at temperatures of 30 °C, 60 °C, and 80 °C. Representative chromatograms of samples from this stability study are presented in Fig. 5. The chromatograms of heparin in dry powder treated at 80 °C for 96 h (Fig. 5a), of heparin in 0.1 M HCl at 80 °C for 24 h (Fig. 5b), and of heparin in 0.1 M NaOH at 80 °C for 24 h (Fig. 5c) are presented. Acetate and sulfate anions were observed at ~4.7 and ~16.0 min, respectively. In addition, a large peak for chloride anion was observed in chromatograms of acidic and alkaline treated samples. Chloride anion was introduced when each aliquot was treated or neutralized. Because this chloride anion was overloaded in the analysis, its retention time was observed later than that of standard at ~6.1 min, as confirmed by the analysis of a diluted sample. This chromatogram is shown in Supplemental Information Fig. S2. All experiments released an increasing amount of sulfate and acetate

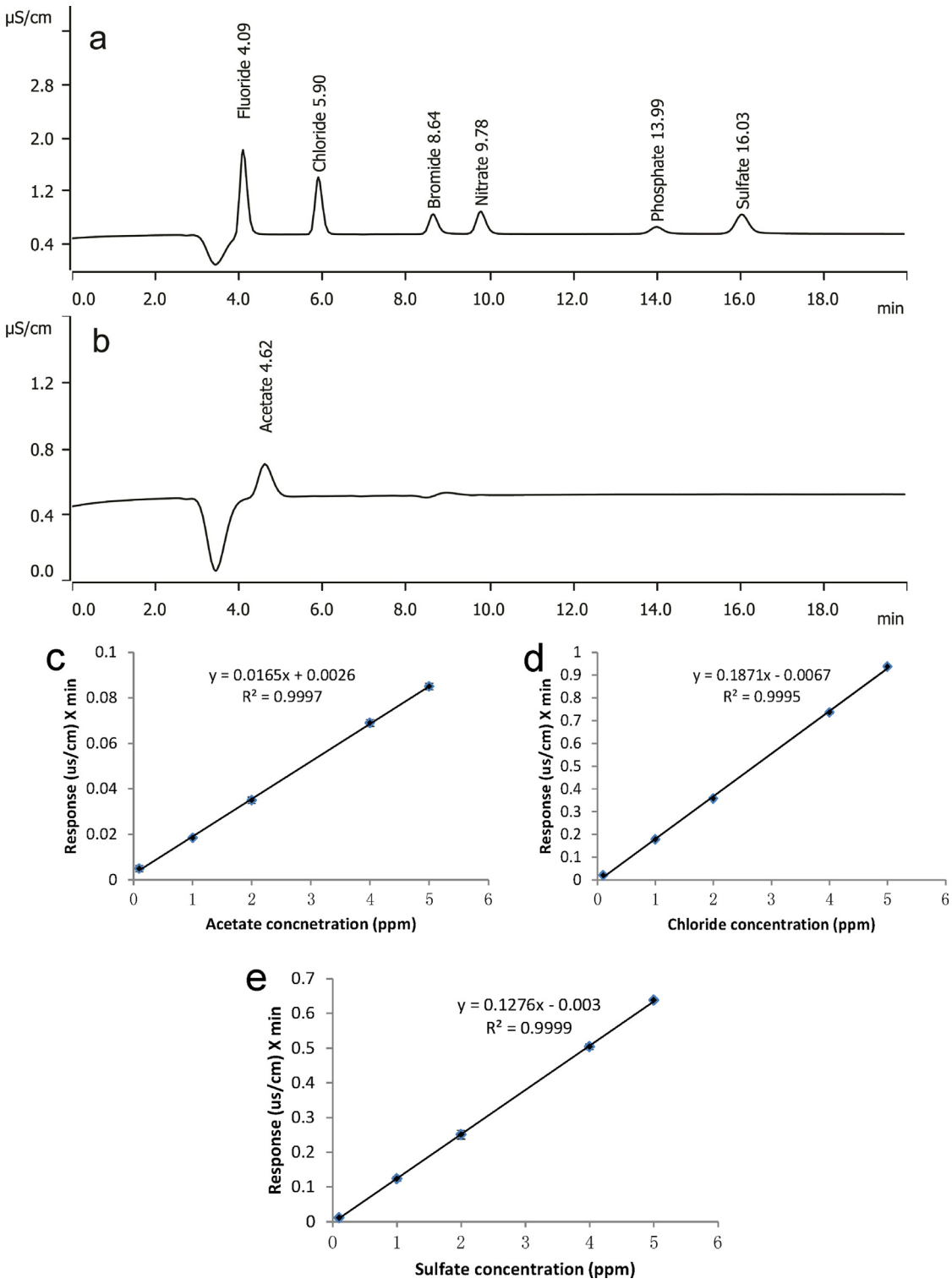

Fig. 2. Chromatograms and standard curves of anion standards. (a) Chromatogram of multi-anion standard; (b) chromatogram of acetate standard; (c) standard curve of acetate; (d) standard curve of chloride; and (e) standard curve of sulfate.

anions (SO_4^{2-} and OAc^-) over time and their concentrations are plotted as a function of time in Fig. 6.

In the experiment with dry powder sample, a small but an increasing amount of sulfate anion and acetate anion was observed as a function of time. At 30 °C and 60 °C, sulfate anion appeared slowly at a similar rate. The rate of sulfate anion appearance was much higher at 80 °C (Fig. 6a). The appearance of acetate anion was not significantly different at different temperatures. The rate of acetate anion appearance was high for the first 24 h, and then slowed afterwards (Fig. 6b).

In the experiment using 0.1 M HCl, an increasing amount of sulfate anion and acetate anion was observed as a function of time.

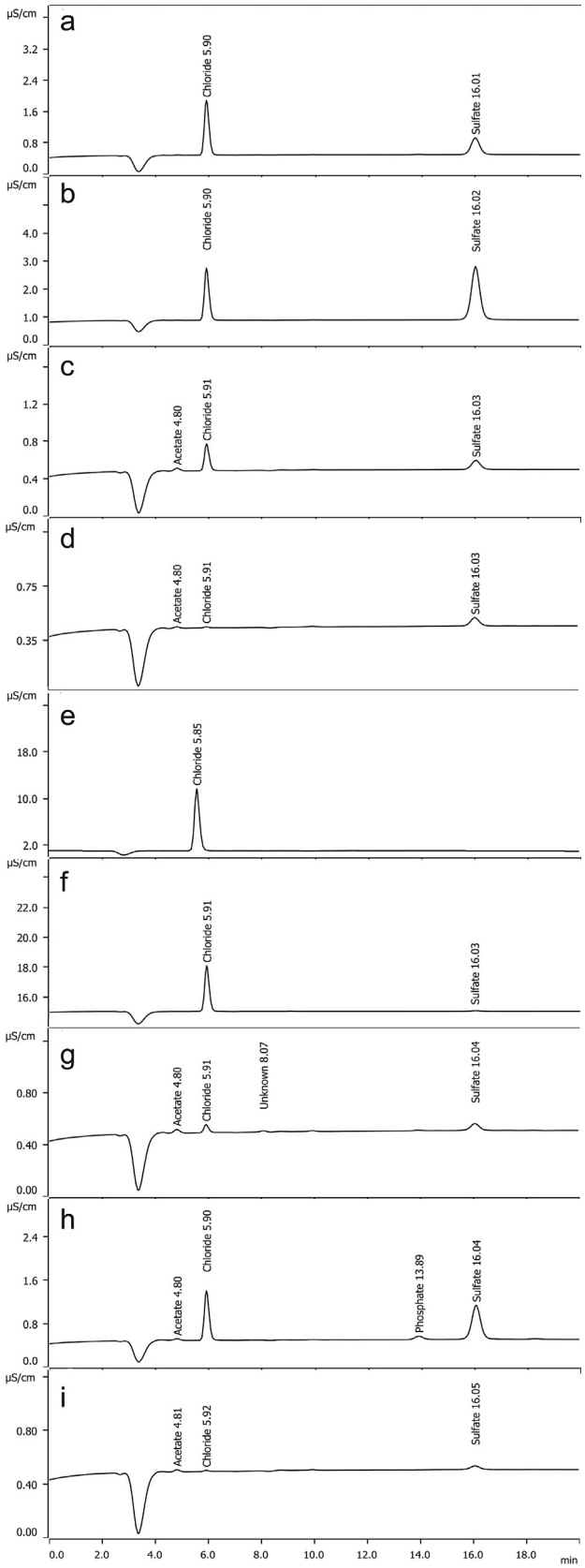

Fig. 3. Chromatograms of free anions in GAG products. (a) Chromatogram of free anions in heparin from the USP; (b) chromatogram of free anions in heparin from the NIFDC; (c) chromatogram of free anions in heparin from Sigma; (d) chromatogram of free anions in heparin from Celsus; (e) chromatogram of free anions in heparin-1 from a plant in China; (f) chromatogram of free anions in heparin-2 from a plant in China; (g) chromatogram of free anions in HS from Celsus; (h) chromatogram of free anions in CS-A from Sigma; and (i) chromatogram of free anions in DS from Celsus.

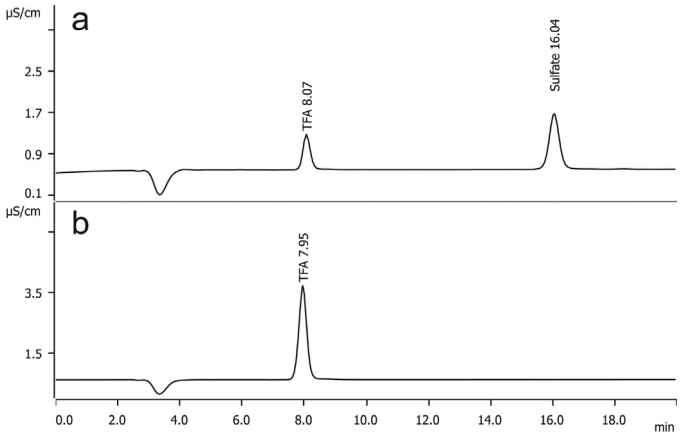

Fig. 4. Chromatograms of sulfate released from GAGs. (a) Chromatogram of released anions from heparin (USP) and (b) chromatogram of TFA.

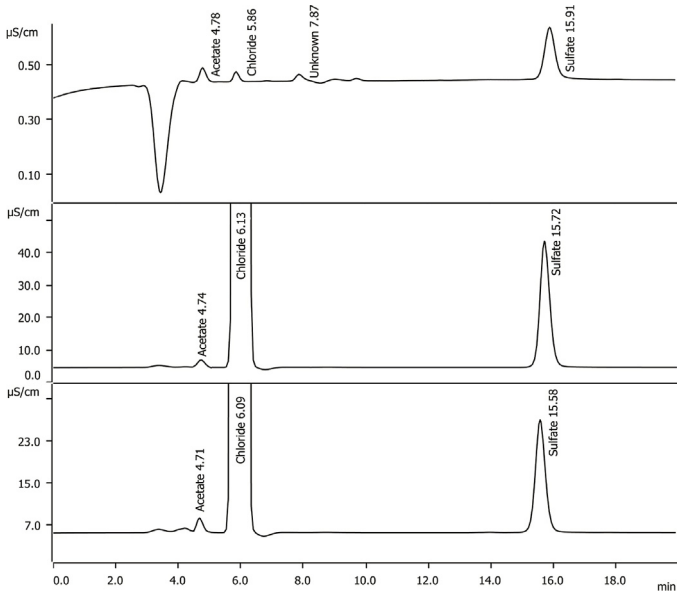

Fig. 5. Chromatograms of anions in heparin stability experiments. (a) Chromatogram of anions tested in solid heparin treated at 80 °C for 96 h; (b) chromatogram of anions formed from heparin in acidic solution at 80 °C for 24 h; and (c) chromatogram of anions formed from heparin in alkaline solution at 80 °C for 24 h.

Sulfate anion appearance at the three temperatures tested showed similar trends but resulted in significantly different levels of sulfate anion (Fig. 6c). At higher temperatures (60 °C and 80 °C), a large amount of sulfate anion was released from heparin at a high rate in the first 10 h, but slowed down gradually over time. At the lower temperature (30 °C), the release of sulfate anion was much lower. A significant amount of acetate anion was also released from heparin, and the rate of its appearance was different at different temperatures (Fig. 6d).

The results using 0.1 M NaOH were similar to those using 0.1 M HCl. A large amount of sulfate anion was also observed in 60 °C and 80 °C, which was released from heparin quickly in the first 10 h and then slowly afterwards. However, the level of release curve in 0.1 M NaOH was lower than that observed using 0.1 M HCl (Fig. 6e). A large amount of acetate anion was released from heparin under alkaline conditions at a high rate and the final concentration of acetate anion was about 4–5-times higher than observed in 0.1 M HCl (Fig. 6f).

Table 1
Anion content and sulfation level of various GAGs (TFA hydrolysis, $n = 3$).

% (w/w)	Hp USP	Hp NIFDC	Hp Sigma	Hp Celsus	Hp-1	Hp-2	HS Celsus	CS-A Sigma	DS Celsus
SO_4^{2-} [a]	0.4 ± 0.0	1.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	n.d.	n.d.	0.1 ± 0.0	0.4 ± 0.0	0.1 ± 0.0
Cl^- [a]	0.5 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	3.9 ± 0.1	0.4 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
PO_4^{3-} [a]	n.d.[d]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.1 ± 0.0	n.d.
Hydrolysate-SO_3Na [b]	34 ± 2	38 ± 3	35 ± 3	34 ± 2	37 ± 4	37 ± 3	18 ± 2	16 ± 3	19 ± 1
Degree of sulfate [c]	2.0	2.4	2.1	2.0	2.3	2.3	0.85	0.76	0.94

[a] The content of free anion was calculated from weight of free anion divided by total weight.
[b] The content of covalent sulfo group with sodium was calculated from weight of SO_3Na divided by the weight of the pure GAG.
[c] Degree of sulfate was calculated by moles of sulfate released divided by moles of disaccharide unit in GAG backbone.
[d] n.d. = non detectable.

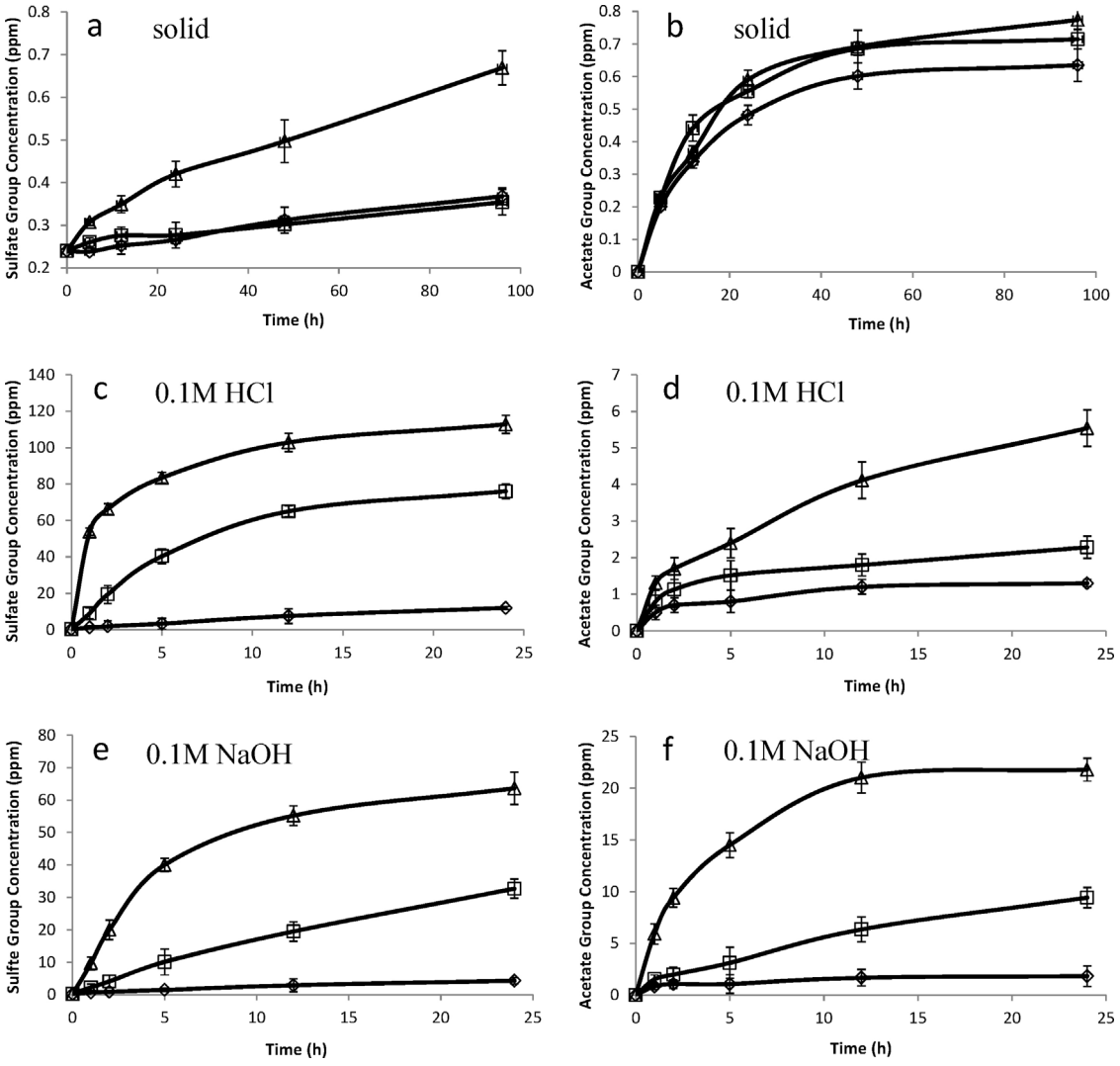

Fig. 6. Anion concentrations tested measured as a function of time in heparin stability experiments. (a) Sulfate anion concentrations from solid heparin treated at 30 °C, 60 °C and 80 °C were plotted as a function of time; (b) acetate anion concentrations in solid heparin treated at 30 °C, 60 °C and 80 °C were plotted as a function of time; (c) sulfate anion concentrations from heparin in acidic solution (0.1 M HCl) at 30 °C, 60 °C and 80 °C were plotted as a function of time; (d) acetate anion concentrations from heparin in acidic solution (0.1 M HCl) treated at 30 °C, 60 °C and 80 °C were plotted as a function of time; (e) sulfate anion concentrations from heparin in alkaline solution (0.1 M NaOH) at 30 °C, 60 °C and 80 °C were plotted as a function of time; and (f) acetate anion concentrations from heparin in alkaline solution (0.1 M NaOH) at 30 °C, 60 °C and 80 °C were plotted as a function of time. The symbols of -◊-, -□-, and -△- correspond to the results tested at 30 °C, 60 °C and 80 °C, respectively.

4. Discussion and conclusions

In this paper, a sensitive and precise method was developed to analyze free sulfate anion and sulfo group content of GAG samples. This method has a LOD of 0.05 ppm for the most anions, a LOQ of 0.1 ppm (20 μL) and a wide range of delectable concentrations, from 0.1 ppm to 5 ppm for the anions examined in this study. The current method detects sulfate as well as other anions commonly encountered in GAG samples and is not interfered with by cations or polymers present in the assay solution. The small standard deviations in determining anion standards and free anions in GAG samples suggest that this method has good precision. Hydrolysis and sample treatment steps appear to be the major factor contributing to higher standard deviations observed when determining sulfo group content of GAG samples, and in the GAG stability study.

Free sulfate, chloride, phosphate, and acetate anions were observed in the various GAG samples examined. Presumably, chloride remained from extraction and purification processes used to make these products. Sulfate anion detected could have been released from GAG samples during processing. Acetate anion detected could have been released from N-acetyl groups of GAG sample and/or during bleaching of GAG sample with peracetic acid and/or in another processing step. Acetate anion has been previously reported in heparin products (Zhang et al., 2009). The current method provides a comprehensive approach for monitoring the major anions present in GAG products and may be useful for analyzing other sulfated polysaccharides.

In addition, this work provides compositional information on GAG samples. In heparin, trisulfated disaccharides correspond to the major structural units (60–80%), containing 46.5% (w/w) sulfo groups (SO_3Na). The HS and undersulfated domain in heparin have much lower sulfo group content. The current study shows an average sulfate content of porcine intestinal heparin of ~36%. This corresponds to an average degree of sulfation of ~2.2 sulfo groups/disaccharide repeating unit, based on an average molecular weight of a disaccharide unit of ~600 Da. DS having a relatively homogeneous sequence, consisting of one 4-O-sulfo group/disaccharide unit, should theoretically correspond to a sulfo group content of 21.4%. The result in the current study was 19.4%, corresponding to 0.94 sulfo groups per disaccharide repeating unit, just below the theoretical value, suggesting the presence of a small number of unsulfated disaccharide units. The sulfo group content of CS-A was 16.3%, significantly lower than DS, suggesting that CS-A contains a larger content of unsulfated disaccharide units.

The determination of the acetyl group content of GAG samples was another important analytical target of this study. However, acetate anion was not observed in hydrolyzed GAG samples. Clearly TFA, was either insufficiently acidic for the hydrolysis of GAG N-acetyl groups, or, more likely, the acetate anion was converted to acetic acid and lost during the evaporative removal of TFA.

In the stability study on the dry powder heparin sample, little if any sulfate or acetate anion was observed after four days at 30 °C and 60 °C. Even at 80 °C, only ~0.6% sulfate anion formed, clearly demonstrating the stability of heparin in the solid state under these conditions.

However, a large amount of sulfate anion was observed in heparin stored in acidic solution (0.1 M HCl). The sulfo groups were released very quickly (in the first 10 h) in a temperature-dependent manner (Fig. 6c). At 80 °C, sulfate anion was formed very slow, and did not reach the plateau over the time examined. Thus, it was necessary to use a higher concentration of acid (2 M TFA) and a higher temperature (100 °C) to completely release covalently bound sulfate from GAG samples. Only about half of the total bound sulfate was released under base-catalyzed hydrolysis, confirming that the sulfo groups in heparin are more sensitive to acid than to base. A likely mechanism involves the protonation of the oxygen of the sulfo group by acid followed by hydrolysis. In contrast, 4–5-times higher amounts of acetate anion were observed in alkaline solution than in acidic solution. This result confirms the greater sensitivity of N-acetyl groups to base than to acid. A likely mechanism involves the nucleophilic attack of OH^- on the carbonyl of the N-acetyl groups, resulting in base hydrolysis. Moreover, the concentrations of released acetate anion reached a plateau (~22 ppm) after ~15 h under harshest conditions (Fig. 6c), implying that most acetate groups are released from heparin under these conditions. Based on a typical molecular weight of ~600 Da for a heparin disaccharide unit, the number of acetyl groups in heparin was calculated to be ~0.44 per disaccharide repeating unit. In conclusion, pH is an important factor impacting heparin stability in solution.

In this report, we used HPAEC for the efficient and precise analysis of anions in GAGs. This method allows an insight into the presence of impurities and the structural properties and stability of GAGs. Different types of GAGs were analyzed for the presence of free anions and their content of covalently bound sulfate. Free sulfate, chloride, acetate and/or phosphate anions were observed in some GAG products. The covalently bound anionic substituents could also be analyzed using this method. Sulfo groups, a particularly important structural element of GAGs could be determined. The current method can be used in the quality control of heparin and other GAGs. Heparin stability was also examined and we report the first data on the release of sulfate and acetate anions (Jandik, Kruep, Cartier & Linhardt, 1996). An increasing amount of sulfate and acetate ions were released as a function of time, giving important data on heparin stability at different temperatures and under high and low pH conditions. These results provide important information in establishing processes for heparin purification.

Acknowledgements

The authors are grateful to Soochow University staff start funding (Q413200412), Jiangsu Specially-Appointed Professor Research Funding (SR13200113), Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD, YX13200111), and the funding for Jiangsu Key Laboratory of Translational Research and Therapy for Neuro-Psycho-Diseases (BM2013003).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.02.076.

References

Bartus, K., James, N. D., Bosch, K. D., & Bradbury, E. J. (2012). Chondroitin sulphate proteoglycans: Key modulators of spinal cord and brain plasticity. *Experimental Neurology*, *235*, 5–17.

Barroca, N., & Jacquinet, J. C. (2002). An access to various sulfation patterns in dermatan sulfate: Chemical syntheses of sulfo forms of trisaccharide methyl glycosides. *Carbohydrate Research*, *337*, 673–689.

Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., et al. (1999). Functions of cell surface heparan sulfate proteoglycans. *Annual Reviews in Biochemistry*, *68*, 729–777.

Bo, S., Muschin, T., Kanamoto, T., Nakashima, H., & Yoshida, T. (2013). Sulfation and biological activities of konjac glucomannan. *Carbohydrate Polymers*, *94*, 899–903.

Capila, I., & Linhardt, R. J. (2002). Heparin–protein interactions. *Angewandte Chemie International Edition*, *41*, 391–412.

Cole, D. E., & Evrovski, J. (1997). Quantitation of sulfate and thiosulfate in clinical samples by ion chromatography. *Journal of Chromatography A*, *789*, 221–232.

Edwards, I., & Edwards, J. (2012). Proteoglycans in prostate cancer. *Nature Reviews. Urology*, *9*, 196–206.

Fu, L., Li, G., Yang, B., Onishi, A., Li, L., Sun, P., et al. (2013). Structural characterization of pharmaceutical heparins prepared from different animal tissues. *Journal of Pharmaceutical Sciences*, *102*, 1447–1457.

Greweling, T., Bache, C. A., & Lisk, D. J. (1972). Determination of sulfur in plant material by oxygen flask combustion. *Journal of Agricultural and Food Chemistry*, *20*, 438–439.

Guerrini, M., Beccati, D., Shriver, Z., Naggi, A., Viswanathan, K., Bisio, A., et al. (2008). Oversulfated chondroitin sulfate is a contaminant in heparin associated with adverse clinical events. *Nature Biotechnology*, *26*, 669–675.

Guerrini, M., Zhang, Z., Shriver, Z., Naggi, A., Masuko, S., Langer, R., et al. (2009). Orthogonal analytical approaches to detect potential contaminants in heparin. *Proceedings of the National Academy of Sciences of the United States of America*, *106*, 16956–16961.

Harenberg, J., Kakkar, D., Berrowcliffe, T., Casu, B., Fareed, J., Missmetti, P., et al. (2009). Recommendations on biosimilar low-molecular-weight heparins. *Journal of Thrombosis and Haemostasis*, *7*, 1222–1225.

Jandik, K. A., Kruep, D., Cartier, M., & Linhardt, R. J. (1996). Accelerated stability studies of heparin. *Journal of Pharmaceutical Sciences*, *85*, 45–51.

Lambert, J. L., & Ramasamy, J. (1975). Colorimetric determination of sulfate in water with barium violurate. *Analytica Chimica Acta*, *75*, 460–463.

Limtiaco, J. F., Jones, C. J., & Larive, C. K. (2009). Characterization of heparin impurities with HPLC-NMR using weak anion exchange chromatography. *Analytical Chemistry*, *81*(24), 10116–10123.

Liu, H., Zhang, Z., & Linhardt, R. J. (2009). Lessons learned from the contamination of heparin. *Natural Product Reports*, *26*, 313–321.

McPhee, M. D., Atkinson, S. A., & Cole, D. E. (1990). Quantitation of free sulfate and total sulfoesters in human breast milk by ion chromatography. *Journal of Chromatography*, *527*, 41–50.

Morales, J. A., de Graterol, L. S., & Mesa, J. (2000). Determination of chloride, sulfate and nitrate in ground water samples by ion chromatography. *Journal of Chromatography A*, *884*, 185–190.

Morris, M. E., & Levy, G. (1988). Assay of inorganic sulfate in biologic fluids by nonsuppressed (single-column) ion chromatography. *Analytical Biochemistry*, *172*, 16–21.

Mummery, R. S., Mulloy, B., & Rider, C. C. (2007). The binding of human betacellulin to heparin, heparan sulfate and related polysaccharides. *Glycobiology*, *17*, 1094–1103.

Naimy, H. J., Buczek-Thomas, A., Nugent, M. A., Leymarie, N., & Zaia, J. (2011). Highly sulfated nonreducing end-derived heparan sulfate domains bind fibroblast growth factor-with high affinity and are enriched in biologically active fractions. *Journal of Biological Chemistry*, *286*, 19311–19319.

Nilasaroya, A., Poole-Warren, L. A., Whitelock, J. M., & Martens, P. J. (2008). Structural and functional characterization of poly (vinyl alcohol) and heparin hydrogels. *Biomaterials*, *29*, 4658–4664.

Poole, A. R. (1986). Proteoglycans in health and disease: Structures and functions. *Biochemical Journal*, *236*, 1–14.

Princivalle, M., & de Agostini, A. (2002). Developmental roles of heparan sulfate proteoglycans: A comparative review in Drosophila, mouse and human. *International Journal of Developmental Biology*, *46*, 267–278.

Sasisekharan, R., Raman, R., & Prabhakar, V. (2006). Glycomics approach to structure-function relationships of glycosaminoglycans. *Annual Reviews in Biomedical Engineering*, *8*, 181–231.

Sharma, A., Wood, L. D., Richardson, J. B., Roberts, S., & Kuiper, N. J. (2007). Glycosaminoglycan profiles of repair tissue formed following autologous chondrocyte implantation differ from control cartilage. *Arthritis Research & Therapy*, *9*, R79.

Singh, R. P., & Nancollas, G. H. (1988). Determination of phosphate, sulfate and oxalate in urine by ion chromatography. *Journal of Chromatography*, *433*, 373–376.

Somsen, G. W., Tak, Y. H., Torano, J. S., Jongen, P. M., & deJong, G. J. (2009). Determination of oversulfated chondroitin sulfate and dermatan sulfate impurities in heparin by capillary electrophoresis. *Journal of Chromatography A*, *1216*(18), 4107–4112.

Sugahara, K., & Kitagawa, H. (2002). Heparin and heparan sulfate biosynthesis. *IUBMB Life*, *54*, 163–175.

Taylor, K. R., & Gallo, R. L. (2006). Glycosaminoglycans and their proteoglycans: Host-associated molecular patterns for initiation and modulation of inflammation. *FASEB Journal*, *20*, 9–22.

Trehy, M. L., Reepmeyer, J. C., Kolinski, R. E., Westengerger, B. J., & Buhse, L. F. (2009). Analysis of heparin sodium by SAX/HPLC for contaminants and impurities. *Journal of Pharmaceutical and Biomedical Analysis*, *49*(3), 670–673.

United State Pharmacopeial Convention Enoxaparin Sodium Injection (2012), 3402–3404.

Wu, Z. L., Zhang, L., Beeler, D. L., Kuberan, B., & Rosenberg, R. D. (2002). A new for defining critical functional groups on heparan sulfate. *FASEB Journal*, *16*, 539–545.

Yard, B. A., Lorentz, C., Herr, P. D., & van der Woude, F. J. (1998). Sulfation-dependent down-regulation of interferon-gamma-induced major histocompatibility complex class I and II and intercellular adhesion molecule-1 expression on tubular and endothelial cells by glycosaminoglycans. *Transplantation*, *66*, 1244–1250.

Zaia, J. (2013). Glycosaminoglycan glycomics using mass spectrometry. *Molecular & Cellular Proteomics: MCP*, *12*, 885–892.

Zaia, J., & Costello, C. E. (2003). Tandem mass spectrometry of sulfated heparin-like glycosaminoglycan oligosaccharides. *Analytical Chemistry*, *75*, 2445–2455.

Zhang, Z., Li, B., Suwan, J., Zhang, F., Wang, Z., Liu, H., et al. (2009). Analysis of pharmaceutical heparins and potential contaminants using (1)H-NMR and PAGE. *Journal of Pharmaceutical Sciences*, *98*, 4017–4026.

Zhang, Z., Weiwer, M., Li, B., Kemp, M. M., Daman, T. H., & Linhardt, R. J. (2008). Oversulfated chondroitin sulfate: Impact of a heparin impurity, associated with adverse clinical events, on low-molecular-weight heparin preparation. *Journal of Medicinal Chemistry*, *51*, 5498–5501.

Zhang, Z., Xie, J., Zhang, F., & Linhardt, R. J. (2007). Thin-layer chromatography for the analysis of glycosaminoglycan oligosaccharides. *Analytical Biochemistry*, *371*, 118–120.

Zhang, Z., Khan, N. M., Nunez, K. M., Chess, E. K., & Szabo, C. M. (2012). Complete monosaccharide analysis by high-performance anion-exchange chromatography with pulsed amperometric detection. *Analytical Chemistry*, *84*, 4104–4110.

Zsila, F., & Gedeon, G. (2006). Binding of anti-prion agents to glycosaminoglycans: Evidence from electronic absorption and circular dichroism spectroscopy. *Biochemical and Biophysical Research Communications*, *346*, 1267–1274.