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Assay of possible economically motivated additives or native impurities levels in heparin by ^{1}H NMR, SAX-HPLC, and anticoagulation time approaches

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ABSTRACT

New assays have been developed to detect the presence of economically motivated additives (EMAs) and poor manufacturing processes in heparin. Here, selected oversulfated glycosaminoglycans that are possible EMAs to heparin were synthesized and the utility of current ^{1}H NMR, SAX-HPLC or anticoagulation time protocols were evaluated for the detection of native impurities (chondroitin sulfate A or B (CSA or CSB), or heparan sulfate (HS)), or synthetic contaminants (oversulfated-(OS)-CSA, OS-CSB, OS-HS or OS-Heparin) spiked into heparin sodium active pharmaceutical ingredients (APIs). The range of w/w percent LOD values from the SAX-HPLC analysis for heparin spiked with CSA, CSB, HS, OS-CSA, OS-CSB, OS-HS, OS-Heparin and two partially oversulfated CSA analogs was 0.05–0.12%. The 500 MHz 1D-^{1}H NMR spectra of heparin spiked with 1.0–10% CSA, CSB, OS-CSA, or OS-CSB showed unique signal pattern changes while the samples spiked with HS, OS-HS, OS-Heparin or partially sulfated CSA were more difficult to identify using NMR data. The ratio of anticoagulation time values obtained with factor Xa and factor IIa were found to remain within USP specifications in the presence of 10% amounts of these impurities or contaminants. In a separate test, using OS-CSA spiked API heparin samples, the factor Xa or factor IIa to USP standard ratio were found to fall below the USP 0.9 specification value in samples spiked at *ca.* weight percent of 15% or greater of OSCS. We conclude that the SAX-HPLC assay is the most sensitive and robust assay to identify and quantitate possible GAG-based EMAs in heparin.

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1. Introduction

Heparin is a complex mixture of sulfated glycosaminoglycans (GAGs) primarily isolated from the intestinal mucosa of pigs [1,2] and is widely used as an anticoagulant or anti-thrombotic agent [3,4]. Heparin active pharmaceutical ingredients (APIs) are purified from crude heparin by proprietary processes and differences in these processes leads to variation in the level of native impurities in the heparin APIs produced. Therefore, heparin API and formulations of the processed heparin can contain low levels (normally less than 1%) of several natural GAG impurities (*e.g.* heparan sulfate or chondroitin sulfate A or B) that are not associated with adverse health effects [5].

In 2008 heparin raw materials and finished drug products were found to contain non-native contaminants that were linked with increased incidences of heparin product associated adverse events and over 100 deaths [6]. This contamination crisis led to action by a team of FDA, industry and academic scientist that identified oversulfated chondroitin sulfate A (aka OSCS) as a heparin contaminant associated with adverse reactions [7,8]. In addition, greater than 1% (w/w) levels of CSB (aka dermatan sulfate or DS) were also detected in many of the same samples contaminated with OSCS indicating that in addition to the presence of an EMA these samples also had poor process controls in manufacturing the drug. Because of this contamination, new proposed U.S. Pharmacopeia (www.usp.org/hottopics/heparin.html) assays for OSCS were developed and include: expansion of the current NMR technique, a new strong-anion-exchange high performance liquid chromatography (SAX-HPLC) protocol, and an assay that measures the delay in the coagulation time associated with purified IIa and Xa coagulation factors caused by heparin.

Analytical chromatography of heparin is difficult because the drug varies in the chain length and in the amount of *N*-acetyl, *N*-sulfation, *O*-sulfation and iduronic acid versus glucuronic acid content in the repeating 1-4 linked uronic acid-glucosamine disaccharide sequence [1]. Chromatographic and capillary electrophoresis methods have been developed for the analysis of heparin APIs [9,10–16]. In many of these approaches chemical- or

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enzyme-induced heparin depolymerization is employed to simplify the assay (*e.g.* [10,13]). Trehy et al. developed a robust SAX-HPLC protocol that separates intact OS-CSA and CSB from heparin with weight percent LODs of 0.03% and 0.1% for OSCS and CSB, respectively [17].

A common orthogonal approach to analyze heparin samples is NMR spectroscopy. For example, Beyer et al. analyzed more than 100 unfractionated (API) heparin samples by 400 MHz ^{1}H NMR at 315 K using a standard addition method with OSCS, heparin, and dermatan sulfate standards [18]. These authors used spectral subtraction and deconvolution of the standard addition data and reported an OSCS LOD of 0.1% for their NMR method. In another study, different NMR methods to determine OSCS in heparin were compared in six different labs and found that the best quantification (LOD 0.1%) was obtained by using the relative peak heights of heparin and OSCS methyl proton resonances [19]. Similarly, our laboratory observed a weight percent LOD of 0.1% for OSCS detection in crude heparins by 500 MHz ^{1}H NMR [20]. For comparison, in the same study we found that our SAX-HPLC protocol was more sensitive for detection of OSCS in crude heparins (w/w LOD of 0.02%).

Because OS-CSA was the contaminant found to be associated with adverse events in the spring of 2008 much of the assay development that has occurred since that time has focused on OS-CSA measurement. Looking to the future where EMAs based on oversulfation of GAGs other than CSA are possible, the assays in place need to be robust in their ability to identify a range of possible GAG-like contaminants. Two publications in 2009 establish the importance of orthogonal techniques to identify OSCS and other possible contaminants in heparin [5,21]. Guerrini et al. [5] applied 1-dimensional (1D) and 2D-NMR or capillary electrophoresis (CE) to the detection of persulfonated-(fully sulfated)-CSA, -CSB, -HS, -hyaluronic acid (HA) and -heparin contaminants in heparin. Zhang et al. [21] utilized 1D-^{1}H NMR analysis of heparinoid/heparin mixtures and polyacrylamide gel electrophoresis (PAGE) on nitrous acid digested mixtures to detect 20 different heparinoid impurities spiked into heparin sodium APIs that had been collected over the past 67 years. Zhang et al. reported heparinoid weight percent LODs with PAGE of 0.1–5% and 0.5–10% ^{1}H NMR with their approach [21].

Because we have observed that our SAX-HPLC protocol is the most sensitive assay to quantify OSCS contamination in crude heparin, we applied this approach to assay other possible synthetic oversulfated contaminants that could be made from impurities commonly found in heparin (*e.g.* CSA, CSB or HS). Two heparin APIs meeting USP specifications were selected and spiked at the 1.0%, 5.0% and 10.0% weight percent levels with CSA, CSB or HS or with oversulfated variants of these starting materials. In addition, we assayed these samples by 1D-^{1}H NMR as an orthogonal technique to determine if signals unique to each contaminant could be identified and used to flag possible adulteration of heparin by synthetic oversulfated GAGs.

Finally, we examine the sensitivity of anticoagulation time assays to the presence of native impurities and contaminants. The activity of coagulation factors IIa and Xa are potently inhibited by heparin but not by less sulfated GAGs such as CSA, CSB or HS [22]. The addition of sulfates by oversulfation reactions has different affects on the ability of these compounds to inhibit coagulation. For example, full *O*-sulfonation of heparin significantly reduces the anti-IIa and -Xa activity of this molecule [23]. By contrast, for chondroitin sulfates, the anti-IIa potency and anti-Xa activity increases with the addition of each sulfate group with anti-factor IIa potency disproportionately increased by the addition of a fourth sulfate group [22,23]. The disproportionate effect on factor IIa in part forms the basis for the current USP monograph test which specifies that the ratio of anti-Xa activity to anti-IIa potency should fall between 0.9 and 1.1.

In this work, we examine the utility of ^{1}H NMR, SAX-HPLC, and anticoagulation time assays as orthogonal techniques to help insure that heparin products in the market are free from contaminants that potentially can put consumers at risk. The assay of 9 native or oversulfated GAGs by the 1D-^{1}H NMR or the anticoagulation time protocols showed that these assays had limitations in sensitivity or specificity compared to the SAX-HPLC method. Importantly, we found that the SAX-HPLC method was found to be a sensitive (LODs of $\leq$0.1%) and robust (detects all nine compounds in the presence of heparin) assay for a range of native impurities or oversulfated GAGs in heparin.

2. Methods

2.1. Chemicals

Heparin API was collected from the marketplace by the FDA investigators. Chondroitin sulfate A sodium salt from bovine trachea and Trizma™ base (TRIS) were purchased from Sigma (St. Louis, MO, USA). Chondroitin sulfate B from porcine intestinal mucosa was purchased from Calbiochem (Darmstadt, Germany). Heparan sulfate fraction I from porcine intestinal mucosa was purchased from Celsus (Cincinnati, OH, USA). The percent sulfur content analysis was performed by pyrolysis on samples pressure dialyzed with a 1000 molecular weight cut off filter versus over 1 L of water followed by drying in a SpeedVac system (ThermoSavant SPD SpeedVac®). The percent sulfur assay has a quoted assay %RSD of 2.6% and a 98% recovery (ASTM D4239-08, Galbraith Laboratories, Memphis, TN). OmniSolv acetonitrile was purchased from EMD Chemicals. Phosphoric acid, 85%, was purchased from Mallinckrodt. Sodium chloride, >99.5% purity, was purchased from Sigma. Water purified to a conductivity <18 mΩ was obtained using a MilliQ system (Millipore, Billerica, MA, USA). Standard heparin sodium for factor IIa and factor Xa assays was purchased from USP (Heparin Sodium for Assay, USP, Rockville, MD Cat #1304016). Factor Xa, human α-thrombin, and antithrombin III (AT) were purchased from Sigma (St. Louis, MO). The chromogenic substrates S-2765 (for factor Xa assay) and S-2238 (for factor IIa assay) were from Chromogenix (Lexington, MA).

2.2. Synthesis

2.2.1. Preparation of oversulfated CSA, CSB, or heparan sulfate

Fifty milligrams of CSA, CSB, or heparan sulfate were converted to their tetrabutylammonium salts by being suspended in MilliQ water (6 mL) and vortexed for 1 min to afford a clear yellow solution. A glass column (1.5 cm diameter by 23 cm tall) was plugged with glass wool and loaded with Dowex 50W-X8 SCX beads up to the 8 cm mark. 1N HCl (40 mL) was eluted through the beads, followed by MilliQ water (200 mL) until the eluate was neutral (pH strips). The heparan sulfate (or other GAG) solution prepared above was loaded onto the beads and eluted with MilliQ water (50 mL). This 50 mL eluate was treated with tetrabutylammonium hydroxide (0.4 M, Fisher) until the pH was ~8.5 (pH strips). The mixture was stirred at room temperature for 5 min, and then dried via vacuum centrifugation in a Speedvac system (ThermoSavant SPD SpeedVac®) overnight at 40 °C. The crude tetrabutylammonium salt was treated with dimethyl formamide (1.0 mL) and vortexed for 5 min. A magnetic stir bar was added along with sulfur trioxide pyridine complex (159 mg) and the reaction vial was capped and heated to 40 °C for 1 h. The reaction was quenched with MilliQ water (2 mL) then poured into a centrifuge tube containing 35 mL of EtOH saturated with sodium acetate. The tube was

placed in a refrigerator at 5 °C for 5 h, then the supernatant was removed after centrifugation and the precipitate dissolved in MilliQ water and exhaustively dialyzed with MilliQ water through a 1000 MWCO filter membrane. The dialyzed material was dried by vacuum centrifugation in a Speedvac system overnight at 40 °C prior to preparation of samples for analysis by ^{1}H NMR, SAX-HPLC and biological assays.

2.2.2. Partial sulfation of chondroitin sulfate A

A test tube was charged with CSA 2TBA (85 mg, 0.1 mmol, prepared by ion-exchange chromatography as described above) and dimethyl formamide (1.0 mL). The mixture was vortexed for 5 min to afford a clear colorless solution. A magnetic stir bar was added and the solution was cooled to 0 °C via ice-H_2O bath (or left at room temperature for the room temperature conditions). Sulfur trioxide pyridine complex (735 mg, 4.6 mmol, 15 equiv./OH) was added in a single portion, and the cloudy white reaction was capped and stirred at 0 °C (or room temperature) for 15 min. The reaction was quenched with MilliQ water (2 mL), and the mixture was adjusted to ~pH 8.0 (Whatman pH strips) using sodium hydroxide. The mixture was then concentrated using a Speedvac. The residue was re-dissolved in MilliQ water and exhaustively dialyzed overnight with MilliQ water through a 1000 MWCO filter membrane. The dialyzed material was dried by vacuum centrifugation in a Speedvac system overnight at 40 °C prior to preparation of samples for analysis by ^{1}H NMR, SAX-HPLC and biological assays.

2.3. HPLC separations

SAX-HPLC separations were performed on a Dionex IonPac® AS11-HC (250 mm × 4 mm) column (Dionex, Sunnyvale, CA, USA). The AS11-HC column characteristics are: bead diameter of 9 μm with a 2000 Å pore size, particles made of a divinylbenzene/ethylvinylbenzene polymer cross-linked at 55%, coated with microporous latex (DVB/EVB 6% cross-linked) 70 nm particles with hydroxyalkyl quaternary ammonium functional groups, and capacity of 290 μequiv./4 mm × 250 mm column. A column temperature of 35 °C was used. The mobile phase was MilliQ water (buffer A) and 2.5 M NaCl with 20 mM TRIS adjusted to pH 3.0 by addition of phosphoric acid (buffer B). The gradient was 0–2 min at 95% A with 5% B, followed by a linear gradient to 100% B at 26 min, a hold at 100% B until 31 min, a linear gradient to 95% A with 5% B at 32 min and a hold until end of run at 40 min. The flow rate was constant at 0.8 mL/min. The UV detector was set at 215 nm. A 40 μL injection volume was used. The liquid chromatography system consisted of Agilent HPLC with a G1314A variable wavelength detector, G1322A degasser, G1311A quaternary pump, column thermostat and G1313A autosampler.

2.4. ^{1}H NMR analysis

All samples were analyzed using a Varian Inova 500 instrument at the Washington University Chemistry Department NMR Facility operating at 499.893 MHz for ^{1}H-nuclei. Samples were run with the probe air temperature regulated at 25 °C. Spectral parameters include: a spectral window of 8000 Hz centered on residual water at 4.77 ppm, 16 transients co-added, a 90° pulse width, acquisition time of 1.892 s and a relaxation delay of 20 s. The total acquisition time per sample was 5.84 min. These acquisition parameters typically gave S/N values measured around the *N*-acetyl methyl proton signals at 2.045 ppm of approximately 1000–2000:1 on the 20 mg heparin samples. The concentration of the crude heparin in the NMR tube was 27 mg/mL (20 mg/700 μL). All samples were made *ca.* 3 mM in 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) as an internal chemical shift reference.

2.5. Anticoagulation time assay

Assays were based on published methods [24] and the USP Pharmacopeial Forum Vol. 35(2), March–April 2009. Phosphate-buffered saline containing 1 mg/mL bovine serum albumin was used to dissolve or dilute factor Xa, thrombin and AT. Factor Xa was made and aliquoted as 4.1 unit/10 μL and stored at −80 °C. Factor Xa was diluted 100 times to 4 unit/mL before using. Thrombin solution was made and aliquots of 12.5 unit/10 μL and stored at −80 °C and diluted to 12.5 unit/mL before using. AT was prepared at 12.5 μg/10 μL and kept at −80 °C and diluted to 12.5 μg/1 mL immediately before use. Tris–EDTA (50 mM Tris, 7.5 mM EDTA, and 175 mM NaCl, pH 8.4) was used to dilute heparin samples. Both substrates were made up at 1 mM in water. Briefly, 25 μL of the AT dilution was added to 25 μL of a serial dilution of heparin standard or samples (containing 2.5, 5, 10, 15, 20 and 25 ng heparin) in a 96-well plate. The reaction was incubated at 37 °C for 75 s, 25 μL of factor Xa or thrombin dilution was added. After incubating at 37 °C for 200 s, 25 μL of S-2765 or S-2238 was added. The absorbance at 405 nm was read every minute for 10 min on a plate reader (Bio-Tek Instruments, Winooski, VT). Normally, the absorbance at 2 or 3 min was used for calculation. The log of the absorbance values of the standard solutions and the sample solutions versus heparin amount was plotted. Separated data fit to a linear model using least-squares linear regression analyses for the sample and standard solutions were constructed and the slopes for sample (S_T) and USP standard (S_S) were used to calculate the anti-factor Xa or anti-factor IIa ratio.

2.6. Sample preparation

2.6.1. Spiked sample set

Two separate API heparin samples were selected for low levels of GAG impurities from available heparin samples. Thirty-six 20 ± 1 mg aliquots of each API were weighed into 1.5 mL low retention microcentrifuge tubes (Fisherbrand). Two hundred mg samples of the native impurities (*i.e.* CSA, CSB and HS) or amounts of oversulfated synthetic materials available from syntheses (*i.e.* OS-Hep, OS-CSA, OS-CSB, OS-HS, partially sulfated CSA #1 (PS-CSA #1) and PS-CSA #2) were weighed, diluted to 100 mL and loaded into a 200 mL pressure dialysis device with a 1000 molecular weight cut-off filter (Amicon, Millipore, Billerica, MA, USA). The samples were dialyzed overnight with 1–1.5 L of MilliQ water from a reservoir at a 1 mL/min flow rate. The final volume was reduced to <20 mL and the remaining solution loaded into tared glass test tubes for drying in a SpeedVac vacuum system at 40 °C overnight. Fifteen mg aliquots of the dialyzed and dried samples were weighed for each heparin or heparin analog, placed into a 5 mL glass volumetric flask and diluted with D_2O to volume. A 0.7 mL aliquot of this solution contains *ca.* 2.1 mg of each spiking material and added to the 20 mg aliquots of API heparin to make an approximately 10.0% weight percent spiked solution. The 5.0% and 1.0% weight percent spiking solutions were made by serial dilution of the 10.0% spiking solution into 5.0 mL glass volumetric flasks with glass pipettes. After dissolution the of the heparin sample in the spiking solution the solutions were placed into a 5 mm NMR tube (Wilmad Glass WG-1000-7, Buena, NJ). In addition, as a control three 20 mg samples of each of the two APIs (6 samples in total) were weighed and dissolved in un-spiked D_2O. Ten microliters of a D_2O solution containing *ca.* 3 mM DSS when diluted was added prior to acquiring the NMR data. After the NMR spectra were recorded the contents of the NMR tube was transferred to glass 2 mL injection vials (Fisher Scientific, 03-391-9) for the SAX-HPLC analysis. Then aliquots from the HPLC vials containing the 10.0% spike level were taken for factor IIa or factor Xa assay. Separately portions of the native and oversulfated GAGs were dried overnight under P_2O5, packaged and sent

Table 1
Chemical shifts in API heparin spectra with observable intensity changes when spiked with 1.0%, 5.0% or 10.0% weight percent of native or synthetic GAG contaminants.

GAG or oversulfated GAG	*N*-Acetyl methyl proton shifts (ppm)	Chemical shifts of intensity differences with spiked GAG in the 3.0–6.0 ppm spectral region						
Heparin	2.046 ± 0.001[a]							
CSA	2.02	3.38	3.58		4.02			
CSB	2.08		3.54	3.87	4.03		4.68	4.87
HS	2.04		3.67	3.86	4.02			
OS-Heparin	2.04							
OS-CSA	2.15				4.16	4.48		4.97, 5.01
OS-CSB	2.09, 2.12				4.27			4.93
OS-HS	2.04							
PS-CSA #1	2.02 (wk shld)[b] 2.04 2.08 (wk shld)				4.26		4.60	
PS-CSA #2	2.02 (wk shld)[b] 2.04 2.08 (wk shld)				4.26		4.60	

[a] $n = 72$ for heparin, $n = 6$ for other shifts.
[b] See Fig. 1c for visual representation of these signal patterns.

for weight percent sulfur determination at Galbraith Laboratories (TN, USA).

3. Results and discussion

3.1. NMR spectroscopy

The ^{1}H NMR spectrum of heparin at 500 MHz is a complex pattern of overlapping signals, the bulk of which fall between 3.0 and 6.0 ppm. In addition there are signals from the methyl protons of the *N*-acetyl methyl groups resonating around a chemical shift of *ca.* 2 ppm. As the possible impurity and contaminant GAGs often share similar molecular components to that of heparin, signals from their protons for the most part overlap with those of heparin. In this work we examine changes in the pattern of intensity of the heparin NMR spectra with 1.0%, 5.0% and 10.0% weight percent spikes of GAGs and synthetic oversulfated GAGs to identify signals that indicate the presence of potential impurities and contaminants in heparin APIs.

Table 1 summarizes the chemical shift values where intensity changes are observable in overlaid spectra. For example, Fig. 1a shows the overlaid spectra from 1.0%, 5.0% and 10.0% CSA and the same spiked levels of OS-CSA. In Fig. 1a, a shoulder at 2.02 ppm to the right of the main heparin *N*-acetyl methyl proton signal at 2.046 ppm increases in intensity with added CSA while OS-CSA (made from CSA) has a signal which increases in intensity at 2.15 ppm with added OS-CSA. Fig. 2a shows the 3.0–6.0 ppm region of the same overlaid spectra used to make Fig. 1a. The arrows at 3.38, 3.58 and 4.02 indicate signals with intensity changes that are most apparent with added CSA while the asterisks indicate signals associated with increasing OS-CSA sample content.

Of these signals, the OS-CSA *N*-acetyl methyl proton resonance is the most sensitive indicator of the presence of this molecule. Thus, Beyer et al. used the *N*-acetyl methyl proton OS-CSA NMR signal in contaminated heparin APIs and reported a 0.1% weight percent detection limit at 400 MHz [18]. Similarly we have shown the same 0.1% LOD value as Beyer obtained using the 2.15 ppm signal in crude heparins spiked with OSCS by 500 MHz ^{1}H NMR spectroscopy [20]. While the OS-CSA methyl proton signal is baseline resolved, the changes in the pattern when CSA is spiked in heparin all occur in regions where they overlap to some degree with heparin signals and as such are not ideal for measurement of weight percent amounts of CSA in heparin by NMR methods.

Fig. 1b shows the overlaid spectra from 1.0%, 5.0% and 10.0% CSB and the same spiked levels of OS-CSB. In Fig. 1b a signal at 2.08 ppm is observed to increases in intensity with added CSB while for OS-CSB (made from the CSB) two signals (at 2.09 and 2.11 ppm) appear to the left of the heparin methyl proton signal. Fig. 2b shows the 3.0 to 6.0 ppm region of the same overlaid spectra used to make Fig. 1b. The arrows at 3.54, 3.87, 4.03, 4.68 and 4.87 indicate signals with intensity changes that are most apparent with added CSB while the asterisks indicate the increase in the intensity of the signals at 4.27 and 4.93 ppm which are associated with increasing OS-CSB sample content.

The CSB and OS-CSB are readily identified by their *N*-acetyl methyl proton signals found to the left of the heparin API resonance. The weight percent sulfur content of the synthetic OS-CSB (Table 2) of 12.5% indicates that the oversulfation reaction did not fully sulfate the starting material (see discussion below). In a separate OS-CSB synthesis which yielded a %S value of 16.0% (*ca.* 4 sulfurs/disaccharide), the *N*-acetyl methyl proton signal was found at 2.12 ppm (data not shown). Similarly, Guerrini et al. reported a value of 2.12 ppm for their persulfonated OS-CSB [5]. Based on these data we attribute the signal at 2.11 ppm to the persulfonated regions of CSB and the 2.09 ppm signal to a partially sulfated intermediate form of CSB. In the 3.0–6.0 ppm region of these spectra (Fig. 2b) there are five distinct signals whose intensities increase relative to heparin signals with added CSB (arrows) while the presence of OS-CSB (asterisks) is more difficult to identify from changes in observed intensities in this region.

As part of a project to further characterize intermediate sulfation states of GAGs and their chromatographic and spectroscopic

Table 2
SAX-HPLC characterization of %GAG spiked in heparin at 1%, 5% and 10% levels and weight percent sulfur content determined by pyrolysis.

GAG	Elution time (min)[a]	FWHH (min)	%S[b]
Heparin	20.42 ± 0.03	1.9	10.91 ± 0.02
CSA	15.80 ± 0.10	1.3	5.38
CSB	16.20 ± 0.08	2.1	6.28
HS	15.35 ± 0.49	3.7	5.01
OS-Heparin	22.28 ± 0.15	1.1	14.94
OS-CSA	24.30 ± 0.10	0.7	14.41
OS-CSB	22.51 ± 0.05	0.6	12.51
OS-HS	22.74 ± 0.14	1.8	10.48
PS-CSA #1	23.56 ± 0.08	1.6	11.01
PS-CSA #2	23.56 ± 0.06	1.5	11.14

[a] SAX-HPLC elution times were determined for all GAGs spiked separately into two heparin sodium APIs ($n = 6$ except for heparin where $n = 72$).
[b] Weight percent sulfur was determined by pyrolysis by procedure ASTM D4239. The heparin %S assay was done in triplicate while all other values are a single measurement with reported %RSD of 2.6% and a 98% recovery.

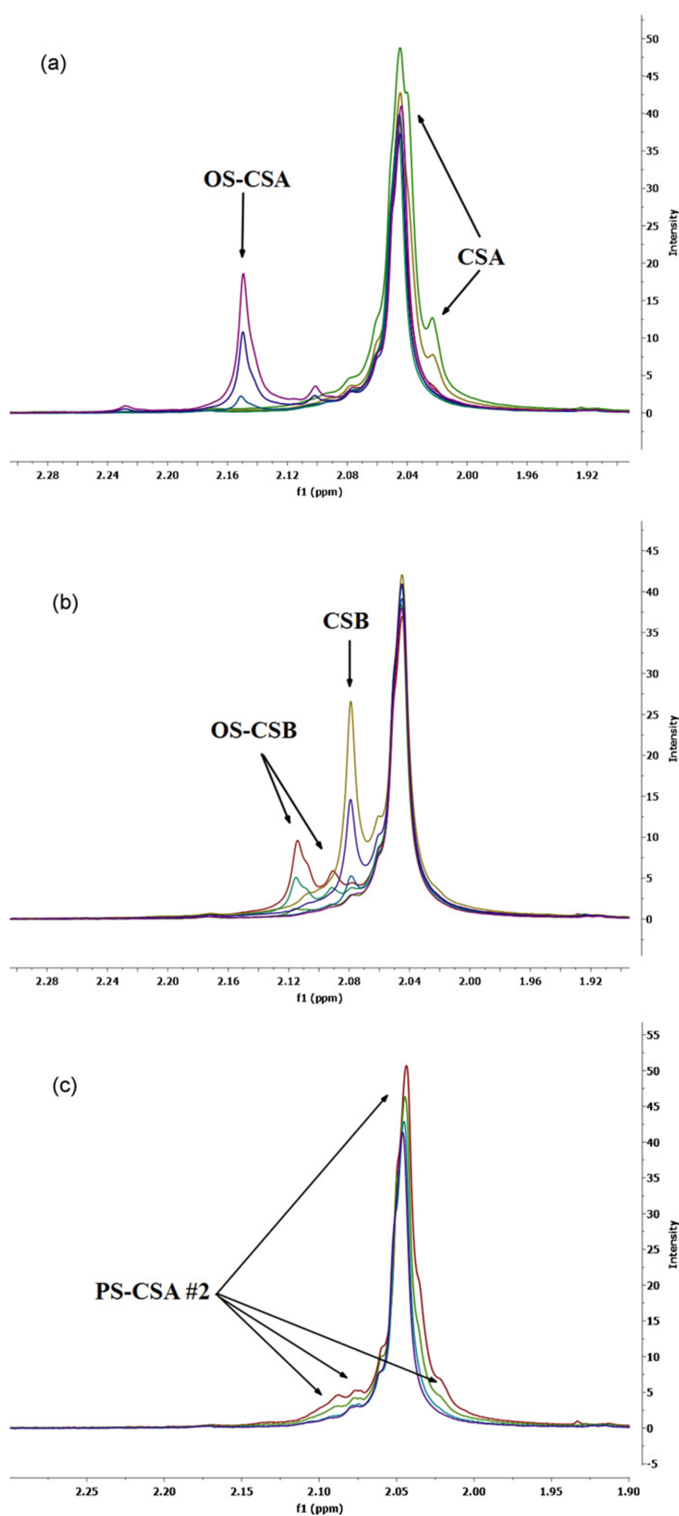

Fig. 1. A plot the 1.90–2.30 ppm region of an overlay of the 500 MHz ^{1}H NMR spectra of 20 mg samples of a heparin sodium API alone or spiked with 1.0%, 5.0% or 10.0% weight percent of CSA or OS-CSA (Panel A), and the same API alone or containing 1.0%, 5.0% or 10.0% of CSB or OS-CSB (Panel B), and the same API alone or containing PS-CSA #2 at the three spiked levels (Panel C). Signals associated with the presence of the spiked GAG components are denoted with arrows.

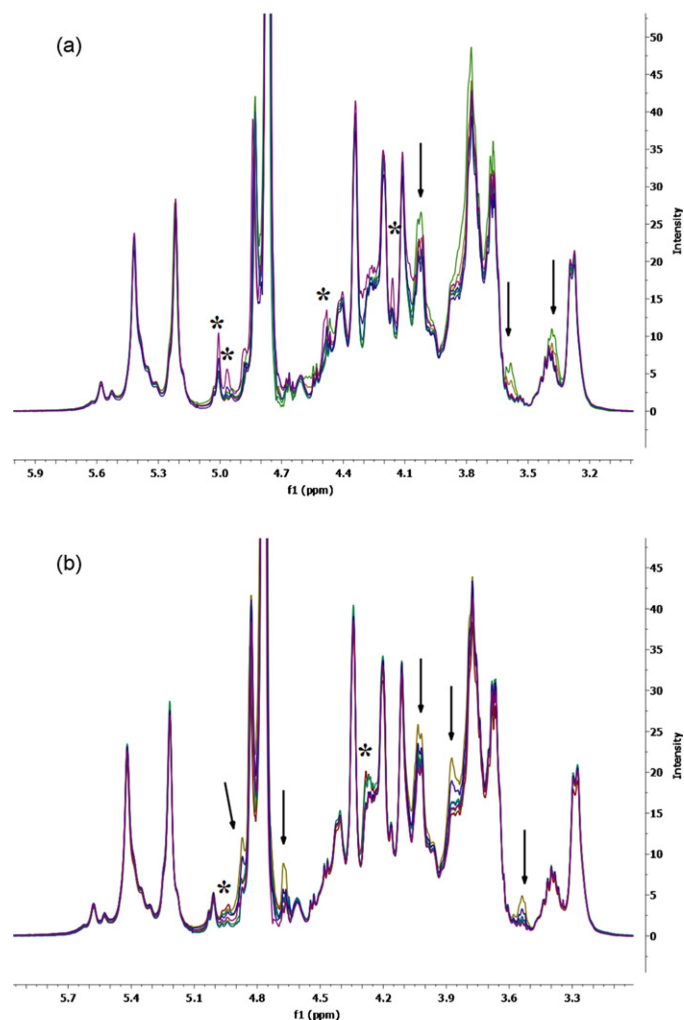

Fig. 2. A plot of the 6.00–3.00 ppm region of an overlay of the 500 MHz ^{1}H NMR spectra of 20 mg samples of heparin sodium API alone or spiked with 1.0%, 5.0% or 10.0% weight percent of CSA or OS-CSA (Panel A) or the same API alone or containing 1.0%, 5.0% or 10.0% of CSB or OS-CSB (Panel B). Signals that change in intensity with increasing amounts of spiked contaminants associated with the presence of CSA or CSB are denoted with arrows while those signals associated with OS-CSA or OS-CSB are denoted with asterisks.

properties we manipulated the time and temperature of the CSA oversulfation reaction to synthesize partially sulfated forms. The two partially sulfated CSA forms presented here as PS-CSA #1 and PS-CSA #2 were made by using 0 °C or room temperature reactions, respectively, with each reaction quenched after 15 min. These reaction conditions resulted in partially sulfated CSA forms with a weight %S of 11%. By contrast the starting material CSA and the persulfonated CSA have 5.4 and 14.4%S sulfur content values, respectively (Table 2). Although these partially oversulfated CSA analogs had similar net sulfur content to heparin they both eluted later than heparin on SAX-HPLC (see below).

By contrast, the 40 °C for 1 h protocol did not fully sulfate CSB or HS based on the %S values of 12.5% and 10.5% observed, respectively, for these reaction conditions. Thus, we determined that standard oversulfation reaction conditions for CSA (15 mequiv. $PyrSO_3$/-OH at 40 °C for 1 h) that lead to fully sulfated CSA, do not lead to complete oversulfation of CSB, HS or heparin. Again although these partially oversulfated forms share similar net sulfur content to heparin they elute later than heparin in SAX-HPLC chromatograms.

The NMR spectra of the partially oversulfated CSA forms spiked into heparin are much more difficult to distinguish from regular heparin based on new signals or differences in relative intensities. For example, Fig. 1c shows the *N*-acetyl methyl proton region of PS-CSA #2 spiked into heparin API at three levels. The presence of the oversulfated CSA chains presents as several broad shoulders and small increases in signal intensity at 4.26 and 4.60 ppm (Table 1).

Similarly, the presence of HS, OS-HS or OS-Heparin are difficult to definitively identify based solely on changes in the NMR spectra with added GAG (Supplemental Data). For HS the intensity of the *N*-acetyl methyl proton signal relative to other signals increases because HS has a greater *N*-acetyl methyl group content than heparin (55% NAc content in HS compared to 11% in heparin [25]). In addition, because HS is 66% glucuronic (G) acid (as opposed to 15% in heparin [25]) the G-H4 proton signal intensity at 3.86 ppm increases with added HS. Similarly as CSB contains primarily G type saccharides the 3.86 ppm signal pattern of the spectrum is altered with spiked CSB content. For OS-Heparin or OS-HS a difference in *N*-acetyl methyl proton signal intensities at 2.04 ppm was observed with added contaminant (Supplemental Data). Importantly, in the absence of spiked data these changes would be difficult to spot without careful measurement of NMR signal intensity ratios.

At the FDA the ^{1}H NMR data has been used primarily as an orthogonal assay from that of SAX-HPLC to identify the presence or absence of impurities or contaminants in heparin. SAX-HPLC has been used to quantify levels of CSB or OSCS instead of the NMR approach because the HPLC method is a more sensitive method for measurement of the levels of these GAGs in heparin [17,20]. Of the nine impurities or contaminants tested in this work, CSB, OS-CSA or OS-CSB spectra contain NMR signals which are sufficiently resolved from those of heparin to use in quantitative analysis of their levels. By contrast, NMR signals sufficiently resolved from those of heparin for CSA, HS, OS-Heparin, OS-HS or partially sulfated forms of CSA were not available for use in the analysis of the levels of these GAGs in the presence of heparin. Thus the NMR data was used to identify signals associated with the presence of specific impurities or contaminants but not used to quantify their levels. As described below, the SAX-HPLC data did have sufficiently resolved signals for all of the GAGs in this study and these HPLC peak areas were used to quantify their levels in the presence of heparin.

3.2. SAX-HPLC

The API sample set analyzed by NMR was also investigated by SAX-HPLC. The SAX-HPLC protocol applied to the spiked samples separated all of the native and synthetic compounds spiked into heparin API from heparin based on differences in the degree of sulfation and structure. For example, Fig. 3 shows the overlaid chromatograms of a heparin API spiked with CSB or OS-CSB at the 1.0%, 5.0% or 10.0% level. The CSB, heparin, and OS-CSB components elute at 16.2, 20.4 and 22.5 min, respectively, and their weight percent sulfur contents were measured as 6.3, 10.9 and 12.5%, respectively. Furthermore, Fig. 4 shows the overlaid SAX-HPLC chromatograms of heparin with a 10.0% spike level of HS, CSA, CSB, OS-CSB, OS-HS, PS-CSA #2 and OS-CSA (in order of elution). The elution time in the salt gradient is plotted versus the weight %S determined by pyrolysis for each GAG or oversulfated GAG in Fig. 5, and when linear regression is applied to these data an R^2 value of 0.885 is obtained. This correlation is consistent with the overall negative charge of these molecules being mainly due to (with the exception of the carboxylic acid groups) the presence of increasing amounts of sulfate groups as the sulfation reaction proceeds. However, other factors including chain length and sulfation pattern must also play a roles as several compounds with similar %S content elute at different times in SAX-HPLC.

Because heparin and heparin analogs are polydisperse mixtures of varying chain lengths, they elute from chromatography columns as broad peaks. The relative full-width at half-height (FWHH) of each peak eluting from the SAX-HPLC column is a measure of the heterogeneity of each GAG. For example, CSA, heparin, CSB and HS have FWHHs of 1.3, 1.9, 2.1 and 3.7 min, respectively (Table 2). When these materials are oversulfated using identical reaction con-

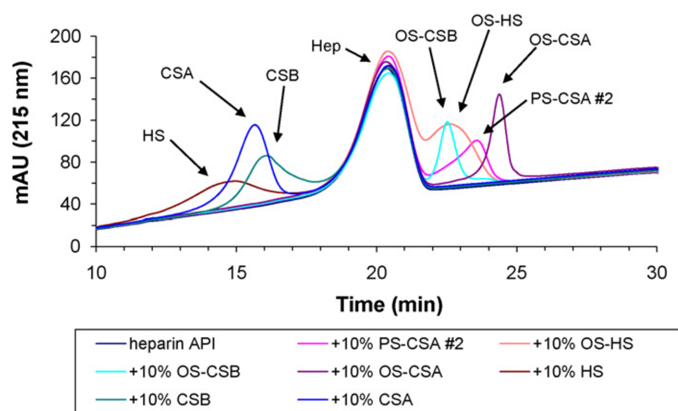

Fig. 4. Overlaid plots of the 10–30 min portion of SAX-HPLC chromatograms derived from injections of a heparin API spiked with selected impurities and contaminants. The chromatograms from the heparin API alone or 10% spiked solutions of CSA, CSB, HS, OS-CSA, OS-CSB, OS-HS or PS-CSA-#2 are shown.

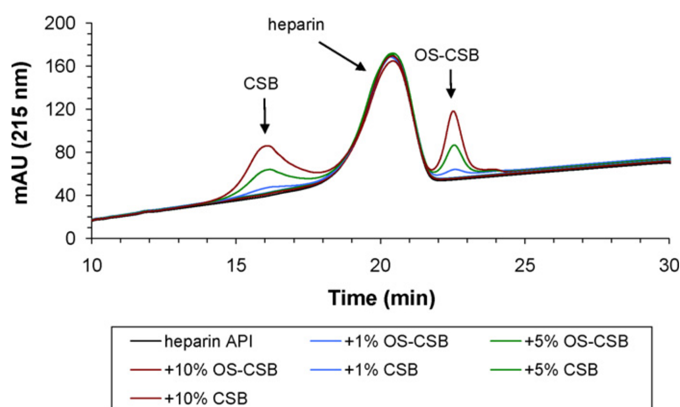

Fig. 3. Overlaid plots of the 10–30 min portion of SAX-HPLC chromatograms derived from injections of a heparin API alone or spiked with 1.0%, 5.0% or 10.0% CSB and the same heparin API alone or spiked with 1.0%, 5.0% or 10.0% OS-CSB.

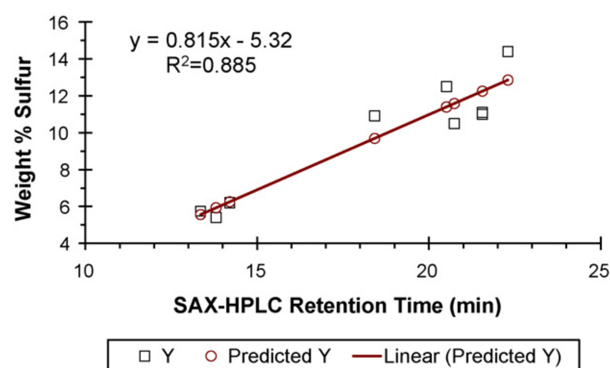

Fig. 5. A plot of the weight percent sulfur content determined via pyrolysis versus the SAX-HPLC gradient elution time. Actual elution time values (squares) are the mean of measurements on two different APIs spiked with 1.0%, 5.0% or 10.0% of each contaminant ($n = 6$). The predicted %S values (circles) are derived from the linear regression analysis of these data. The equation of the trendline and coefficient of determination (R^2) are shown.

Table 3
Linear regression analysis of SAX-HPLC data obtained on 1, 5 and 10% GAG spiked separately into two heparin APIs samples.

GAG	Linear regression fit[a]	Slope S.E.[b]	*y*-Int. S.E.[b]	*y*-Int. *P*-value[b]	*F*-Value[b]	Prob. from *F*-value[b]	LOD[c]
CSA	$y=63157x+532$	1660	118	0.14	1448	0.02	0.09
CSB	$y=52554x+564$	1723	118	0.13	921	0.02	0.11
HS	$y=35335x+579$	836	58	0.06	1788	0.02	0.08
OS-Heparin	$y=10980x-71$	413	25	0.22	706	0.02	0.12
OS-CSA	$y=37122x-87$	602	43	0.29	3791	0.01	0.05
OS-CSB	$y=23760x-46$	486	33	0.04	2392	0.01	0.07
OS-HS	$y=63552x-311$	916	63	0.13	4808	0.01	0.05
PS-CSA #1	$y=38098x-100$	727	50	0.29	2743	0.01	0.06
PS-CSA #2	$y=19719x-44$	321	22	0.30	3774	0.01	0.05

[a] R^2 values ranging from 0.9985 to 0.9998 were obtained in the linear regression analyses of the HPLC data.
[b] ANOVA of the regression gave *F*-values that were used to calculate the probability (95%) that the null hypothesis is true (*i.e.* that the slope is equal to zero). Based on the probabilities of observing an *F*-value this high we reject the null hypothesis and find that the most probable value for the slopes are the ones given by the regression lines. The standard errors of the intercepts are given. We conclude that the slopes and intercepts given by the regression analyses are the most probable values of the calibration line.
[c] LOD = $3.3\sigma/S$, where σ is the standard deviation of the response and *S* is the slope of the calibration curve (as per the ICH Harmonized Tripartite Guideline: Validation of Analytical Procedures: Methodology Q2B).

ditions (15 mequiv./-OH, 40 °C and 1 h) their SAX-HPLC FWHHs become 0.7, 1.1, 0.6 and 1.8 min, respectively. The approximate halving of the linewidths indicates the decrease in the polydispersity associated with more uniform sulfation of the GAG chains. In addition, because of the narrower linewidths, the oversulfated analogs have greater peak heights and in the case of OS-CSA and OS-CSB were better resolved from each other or heparin than their respective starting materials. By contrast, based on SAX-HPLC FWHHs, the partially sulfated CSA analogs were more heterogeneous than the CSA starting material (1.5–1.6 min versus 1.3 min). The increased micro-heterogenicity with partial sulfation is consistent with previously reported band broadening observed in PAGE data for partially *O*-sulfonated GAGs [23].

LOD values were calculated based on the slope of the line determined from a plot of the SAX-HPLC peak area versus 1%, 5% and 10% GAG spikes performed on two heparin APIs ($n=6$), and the standard deviation of the response determined from a linear regression analysis (Table 3). This approach is defined as valid method for the determination of the detection or quantitation limit of an analyte as described in the current International Conference on Harmonisation of Technical Requirements For Registration of Pharmaceuticals for Human Use, ICH Harmonized Tripartite Guideline: Validation of Analytical Procedures: Methodology Q2B. The equation of the lines determined for each spiked compound is shown in Table 3, and all had R^2 values greater than 0.9985. In addition ANOVA analysis of these data yielded standard errors and F-values for the regression line fits which gave good probabilities that the slopes and the intercepts accurately reflect the calibration line (Table 3).

The range of weight percent LOD values from the SAX-HPLC analysis of a 20 mg heparin sample for spiked heparin analogs was 0.05–0.12% (Table 3). The values observed in this work are consistent with the SAX-HPLC LODs obtained for CSB and OS-CSA in heparin of 0.1% or 0.03%, respectively, using the method of successive dilutions [17]. For comparison the LODs reported here for CSB or OS-CSA in heparin APIs were 0.11% or 0.05%, respectively. Based on these results the SAX-HPLC protocol can detect contamination in heparin by the GAGs studied in this work down to the 0.1% weight percent level.

The LOD values reported here are an improvement to the reported range of LOD values obtained from 10 mg NMR (0.5–10% LODs) or 100 μg PAGE (0.5–5% LODs) for determination of CSA, CSB, HS, OS-CSA and OS-CSB in heparin reported by Zhang et al. [21]. In addition, the SAX-HPLC approach is more sensitive to these same impurities studied by 2D-HSQC which reported an estimated weight percent LOD range of >3–5% [5].

3.3. Inactivation of factor IIa and factor Xa

To test the sensitivity of the anticoagulation activity assay to presence of oversulfated components in heparin we performed a modified USP anti-IIa or anti-Xa assay on a heparin API or on the same API spiked at the 10.0% level with the various native and oversulfated GAGs reported in this study. We observed that all of the samples fell within the USP acceptable limits for anti-Xa/anti-IIa activity with values between 0.9 and 1.1 (data not shown). Thus this assay was not sensitive to 10.0% levels of these native and oversulfated GAGs.

To assess what weight percent spiked OS-CSA levels in heparin would cause the anti-Xa or anti-IIa values to fall below the 0.9 USP specification we evaluated anticoagulation activity of heparin spiked with 2–75% of OS-CSA (Table 4). All values were determined by comparison to USP “Assay” standard heparin. A *ca.* 15% OS-CSA value was determined to be necessary to cause the Xa or IIa ratio value to fail the USP test. This value was obtained by plotting the Xa or IIa ratio versus %OS-CSA values (Table 4) and interpolating to determine where the Xa or IIa value fell below the 0.9 specification.

Table 4
Effect of OSCS spike levels on the factor Xa or IIa activity or potency[a].

Weight percent of OSCS spiked heparin	Xa ratio	Anti-Xa activity	IIa ratio	Anti-IIa Potency	Xa/IIa ratio
0	1	226.4	1	225.7	1
2%	1.03 ± 0.05	233.4 ± 11.6	0.92 ± 0.02	208.6 ± 5.3	1.12 ± 0.08
5%	0.98 ± 0.03	220.8 ± 7.3	0.91 ± 0.03	204.7 ± 6.7	1.08 ± 0.02
10%	0.93 ± 0.04	211.1 ± 8.4	0.95 ± 0.04	213.4 ± 9.8	0.99 ± 0.08
25%	0.78 ± 0.03	176.3 ± 5.7	0.84 ± 0.07	189.5 ± 16.5	0.94 ± 0.08
50%	0.47 ± 0.02	106.1 ± 4.1	0.60 ± 0.14	134.4 ± 30.8	0.81 ± 0.17
75%	0.26 ± 0.01	58.9 ± 3.0	0.37 ± 0.09	83.1 ± 19.2	0.73 ± 0.14
100%	0.00 ± 0.00	0.4 ± 0.01	0.07 ± 0.00	16.5 ± 0.4	0.03 ± 0.00

[a] All values are derived from $n=3$ assays with comparison to USP standard “Assay” heparin except the 100% OSCS values where $n=2$. The USP monograph for heparin sodium specifies that the factor Xa/IIa ratio should fall between 0.9 and 1.1.

4. Conclusion

Although the source of OS-CSA found in heparin products associated with adverse reactions in 2008 was not identified, OS-CSA synthesized from animal cartilage was inexpensively and readily obtainable in the marketplace as a nutraceutical used to self-treat osteoarthritis at the time of the crisis [26]. In addition, OS-CSA and other oversulfated hepariniods can have anticoagulant activity up to 50% of that observed for heparin [21]. Thus the economic motivation to add these compounds to heparin was present because one could maintain the anticoagulation activity of the product with less heparin content and concomitantly a greater profit margin.

In this work, as part of a larger study by the FDA to identify possible economically motivated additives to heparin, we synthesized and characterized several oversulfated GAGs whose starting materials could be available to manufacturers. Others have postulated that GAG waste byproducts of heparin purification could be used to make oversulfated GAGs inexpensively [5] for use as EMAs. The GAG waste products produced from heparin purification have been shown to contain mainly CSB with lesser amounts of heparin, CSA and heparan [5]. Of the synthetic contaminants studied here, only OS-CSA NMR and HPLC properties matched those observed in the contaminated heparin samples associated with adverse events. By contrast, OS-CSB properties did not match those found in the contaminated samples. Because GAG waste is primarily CSB and, concomitantly, OS-CSB would be a major component of GAG waste subjected to oversulfation, we can only conclude that GAG waste was not the source of the contaminated heparin lots found in the marketplace in early 2008.

In this work we performed NMR, SAX-HPLC and anticoagulation time assays on heparin samples spiked with one GAG at a time. The elution time of these GAGs with the polymer SAX-HPLC column were stable (*e.g.* heparin elutes at 20.42 ± 0.03 min in 66 spiked samples and 6 un-spiked samples with two different heparin APIs, Table 2). In heparin samples with a mixture of more than one impurity or contaminant which have similar %S content we would observed broad overlapping peaks with peak elution times varying depending on the composition of each co-eluting component. In this case the identification and quantification of the impurity or contaminant requires additional purification. We have had success with collection of fractions from injection of heparin API solutions over semi-preparative SAX-HPLC columns to obtain samples enriched in impurities, followed by desalting and NMR analysis steps to identify the GAGs present (data not shown) in peaks eluting earlier or later than heparin.

Importantly, all of the oversulfated components (partial or fully oversulfated) made for this study and the potential GAG impurities were readily distinguished from USP grade heparin in the SAX-HPLC analyses. The ^{1}H NMR or anticoagulation time assays had some limitations in terms of their sensitivity or specificity for the compounds tested in this work. Taken together, the application of the ^{1}H NMR and SAX-HPLC assays to the GAGs studied in this work would flag samples containing a possible economically motivated additive or an impurity with out of specification levels as potentially dangerous.

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Disclaimer: The findings and conclusions in this article have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination or policy.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2010.02.019.

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