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Analysis of crude heparin by ^{1}H NMR, capillary electrophoresis, and strong-anion-exchange-HPLC for contamination by over sulfated chondroitin sulfate

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ABSTRACT

We previously published a strong-anion-exchange-high performance liquid chromatography (SAX-HPLC) method for the detection of the contaminant over sulfated chondroitin sulfate (OSCS) in heparin sodium active pharmaceutical ingredient (API). While APIs have been processed to remove impurities, crude heparins contain insoluble material, chondroitin sulfates, heparan sulfate, and proteins that may interfere with the recovery and measurement of OSCS. We examined 500 MHz ^{1}H NMR, capillary electrophoresis (CE), and SAX-HPLC to quantify OSCS in crude heparin. Using our standard API protocol on OSCS spiked crude heparin samples; we observed a weight percent LOD and LOQ for the NMR approach of 0.1% and 0.3%, respectively, while the SAX-HPLC method gave values of 0.03% and 0.09%, respectively. CE data was not amenable to quantitative measurement of OSCS in crude heparin. We developed a modified HPLC sample preparation protocol using crude dissolved at the 100 mg/mL level with a 2.5 M NaCl solution. This SAX-HPLC approach gave a weight percent LOD of 0.02% and a LOQ of 0.07% and had better performance characteristics than that of the protocol used for APIs.

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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 and anti-thrombotic agent [3,4]. Because heparin is purified from an animal source, formulations of the processed heparin can contain low levels (normally less than 1%) of several natural contaminants (*e.g.* heparan sulfate or chondroitin sulfate A, B, or C) that are not associated with adverse health effects. Heparin active pharmaceutical ingredients (APIs) are purified from crude heparin that is prepared from scraped mucosa or whole intestine by closely held proprietary processes. Because of the differences in these proprietary processes, the source of the intestine, and the large scale (only 1–2% of the porcine intestinal mucosa is heparin), the level of chondroitin sulfates, heparan sulfate, insoluble material, and proteins varies widely from batch to batch of the crude unrefined heparin. A robust assay to screen crude heparin for native or added components prior to further processing would identify contaminants at an earlier stage of the drug formulation process.

In 2008 heparin raw materials and finished drug products entering the United States from overseas were found to contain non-native contaminants that put U.S. consumers at risk [5]. Increased reports of adverse events associated with heparin products led to a collaborative study involving researchers from the FDA, industry, and academia which identified over sulfated chondroitin sulfate A (OSCS) as the heparin contaminant whose presence in heparin was associated with anaphylactic reactions in certain patients [6,7]. OSCS was found in crude heparin and remained in the API and final formulations after the purification processes. In addition, samples of heparin were found to contain greater than 1% by weight of the impurity dermatan sulfate (*i.e.* chondroitin sulfate B) which is an indication of a poor quality purification of the crude material.

In response to this contamination, the FDA required nuclear magnetic resonance (NMR) spectroscopy [6] and capillary electrophoresis (CE) analysis [8] for detection of OSCS in API formulations. In addition, new proposed U.S. Pharmacopeia (www.usp.org/hottopics/heparin.html) assays include expansion of the current NMR technique and a new strong-anion-exchange-high performance liquid chromatography (SAX-HPLC) protocol.

Heparin is challenging for analytical chromatography because the drug is a polydisperse mixture containing chains of alternating iduronic acid and amino sugars that vary in molecular weight

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from 5000 to 40,000 Da [1]. In addition, there is a significant level of sequence heterogeneity with variation in N-acetyl, N-sulfation, O-sulfation, and iduronic acid versus glucuronic acid content. However, because on average these properties are similar, heparin and other glycosaminoglycans elute as broad peaks on HPLC and these peaks can be used to measure the levels of the various native contaminants in heparin when they are resolved by chromatography.

Chromatographic and capillary electrophoresis methods have been developed for the analysis of heparin APIs [9–15]. Most often chemical- or enzyme-induced heparin depolymerization is employed to simplify the assay and eliminate interference from components of the sample susceptible to acid or enzymatic hydrolysis (*e.g.* [11,14]). Trehy et al. developed a robust SAX-HPLC protocol that does not require digestion and separates intact OSCS and dermatan sulfate (DS) from heparin with weight percent LOQs of 0.1% and 0.8% for OSCS and DS, respectively [16].

NMR has primarily been used as an orthogonal technique to identify the presence or absence of contaminants in heparin rather than as a quantitative tool because heparin, OSCS, and dermatan sulfate do not have defined molecular weights due to variations in starting material, synthesis, or processing. To quantify these polymers by NMR, an average molecular weight is required because while concentration can easily be determined by NMR using an internal standard, the corresponding concentration cannot be converted to weight percent without a known molecular weight for each component [17]. To work around this issue 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 [9]. These authors used spectral subtraction and deconvolution of the standard addition data and reported an OSCS LOD of 0.1% for their NMR method.

While these methods have been used with heparin APIs they have not been evaluated for assay of OSCS in crude heparin. Because pre-screening of crude heparin can detect contaminants present prior to further processing, a reliable method for assaying these samples would save time, resources, and expense in the formulation of safe heparin products. Thus we examined the standard protocols used by the FDA for APIs in the assay of crude heparins and present an assay with improved characteristics for OSCS detection and measurement. This method was developed to provide a robust method for the analysis of crude heparin using routinely available laboratory instrumentation.

2. Materials and methods

2.1. Chemicals

Batches of crude heparin were collected from international markets by the FDA. Chondroitin sulfate A sodium salt from bovine trachea and Trizma™ base (TRIS) were purchased from Sigma (St. Louis, MO, USA). OSCS was synthesized by sulfation of chondroitin sulfate A following the literature procedure [18]. The OSCS was characterized by SAX-HPLC, CE [16], and NMR spectroscopy [6]. HPLC elution time (SAX 24.9 min and CE 5.3 min), NMR N-acetyl methyl proton chemical shift value (2.16 ± 0.03 ppm), and other chemical shift values observed for the synthetic OSCS matched OSCS values reported in the literature. In addition, the percent sulfur content analysis was performed by pyrolysis (Galbraith Laboratories, Memphis, TN) and found to be consistent with the presence of 4 sulfur atoms per disaccharide unit. The contaminant in heparin responsible for adverse events and referred to as OSCS in previous publications is the fully sulfated compound containing on average 4 sulfurs per disaccharide unit. For the CE analysis, monobasic sodium phosphate, monohydrate, ACS grade, and phosphoric acid 85%, N.F. Food Grade, were obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). OmniSolv acetonitrile was purchased from EMD Chemicals. Phosphoric acid, 85%, was purchased from Mallinckrodt. Sodium chloride, >99.5% purity, was purchased from Sigma. Ultra-pure water was obtained using a Milli-Q system (Millipore, Billerica, MA, USA). Micro-Spin filter tubes, 0.2 μm cellulose acetate membrane filters were purchased from Alltech Associates (Deerfield, IL, USA).

2.2. HPLC separations

SAX-HPLC separations of crude heparin, OSCS, or other contaminants 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 μeq/4 mm × 250 mm column. A column temperature of 35 °C was used. The mobile phase was Milli-Q 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 an Agilent HPLC with a G1314A variable wavelength detector, G1322A degasser, G1311A quaternary pump, column thermostat, and G1313A auto-sampler.

System suitability test: To insure the HPLC system response over time was consistent a system suitability test sample consisting of 1.0 mg/mL of OSCS was made and characterized. With a new Dionex Ion Pac AS-HC polymer column, six replicate 40 μL injections were performed. The OSCS peak eluting at 24.9 min had values for peak area, symmetry, theoretical plates, and USP tailing factor of 1085.9 ± 9.4 (%RSD 0.9), 1.36 ± 0.04 (%RSD 2.8), 11,857 ± 121 (%RSD 1.0), and 1.43, respectively. For comparison, a column that had been used extensively for heparin analysis for over a year gave values of 960, 0.71, 9165, and 2.35 for peak area, symmetry, theoretical plates, and USP tailing factor, respectively. Based on these data, we established system suitability specifications for reproducibility (6 replicate injections) of not more than 2.0%, tailing factor of not more than 2.0, and theoretical plates not less than 8000. In cases where the column characteristics did not pass these criteria, we found that the column could be regenerated by pumping a 25% methanol solution containing 50 g of NaCl per liter at a flow rate of 0.5 mL/min for 60 min followed by a water rinse for 30 min.

2.3. ^{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 25 mg heparin samples. The concentration of the crude heparin in the NMR tube was 35 mg/mL (25 mg/700 μL). An aliquot of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) was added as an internal chemical shift reference.

The inversion recovery pulse sequence was used to determine the T_1s of the N-acetyl methyl proton signals of OSCS, dermatan sul-

fate, heparin, and DSS as 1.34 ± 0.02, 1.90 ± 0.01, 2.11 ± 0.10, and 3.67 ± 0.04 s, respectively. Thus the 20 s relaxation delay insures more than five times the longest T_1 of the signals of interest so that complete relaxation occurs between pulses. All NMR data were collected on the standard protocol sample set on the same day.

2.4. Capillary electrophoresis

The CE analysis was conducted on a Hewlett Packard 3D-CE instrument equipped with a diode array detector set at a wavelength of 200 nm (band width 10 nm). The separations were performed in a bare fused silica capillary, with an internal diameter of 50 μm, a 64.5 cm-total length (a 56 cm-effective length), and a column temperature of 25 °C. The polarity was negative with a voltage of 30 kV. The samples were prepared as described below. The sample solutions were injected using hydrodynamic pressure at 50 mbar for 10 s. The electrolyte solution was 36 mM phosphate buffer (pH 3.5) filtered with a 0.2 μm cellulose acetate syringe filter (Grace, Deerfield, IL, USA). The capillary column was preconditioned at the beginning of each day by flushing with water and prior to running each sample by flushing with water for 2 min and electrolyte solution for 2 min. The concentration of the CE samples was 10 mg/mL of crude heparin.

2.5. Sample preparation

2.5.1. Crude heparin sample set prepared by API method

Three separate crude heparin samples and one API were prepared by transferring 25 ± 1 mg into each of four glass centrifuge tubes (16 tubes total). Separately, a stock solution of 35.6 mg of OSCS in 10.0 mL of D_2O was made in a glass 10.0 mL volumetric flask. When 700 μL of this solution is added to 25 mg of crude heparin the amount of OSCS present represents 10.0% by weight of the crude heparin weight. Then, 5 mL of the 10.0% solution was transferred to a second 10.0 mL volumetric flask with a glass pipette and diluted to 10.0 mL to make a 5.0% OSCS spiking solution in D_2O. Finally, 2.0 mL of the 5% solution were transferred via a glass pipette to a third 10.0 mL glass volumetric and diluted to 10.0 mL of D_2O (1% solution). One aliquot each of the three crudes and the API control were separately dissolved in 700 μL of D_2O, 1.0% spike solution, 5.0% spike solution, and the 10.0% spike solution. The samples were vortexed for approximately 10 s (crude heparins often contain components insoluble in aqueous buffers) and then centrifuged for 5 min at 3000 rpm in a Jouan GR422 centrifuge with a swinging bucket rotor. The supernatant was removed with a glass Pasteur pipette and filtered with 0.2 μm cellulose acetate spin filters (Micro-Spin filter tubes, Alltech Associates, Deerfield, IL, USA) prior to analysis. The final sample volume was determined by difference in tared NMR tubes using the weight of the solution and the density of D_2O at 25 °C (1.1044 g/mL). Fifty microliters of a 41.2 mM DSS solution was added to each tube as an internal chemical shift reference and a concentration standard (approximately 3.2 mM). In addition a set of 6 empty glass test tubes with (3 tubes) and without (3 tubes) filtering were analyzed by the same protocol as a control for possible OSCS losses during the sample preparation procedure. After NMR spectra were acquired on these samples, aliquots of 4 mg in 0.2 mL (20 mg/mL) and 2 mg in 0.2 mL (10 mg/mL) were removed from the NMR tubes and used for SAX-HPLC and CE, respectively. Low volume HPLC vials were used with plastic inserts (Xpertek 250 μL bottom spring inserts) inside amber 2.0 mL HPLC injection vials (Fisher Scientific, 03-391-9) and blue 9 mm screw caps (Fisherbrand, 03-391-14). For CE 250 μL polypropylene vials were used (Agilent Technologies, 9301-0978).

2.5.2. Crude heparin sample set prepared for modified SAX-HPLC protocol

A new sample preparation method was developed due to variable recovery with different crude samples. Specifically we observed poor recovery of OSCS in assays at weight percents lower than 1.0%. We attributed the poor recovery to the fact that most of the crude samples contain insoluble matter which may associate with OSCS and prevent its detection. By contrast, API samples completely dissolve in water. Several parameters were investigated to mitigate the observed losses attributed to sorption on 8 different crude heparins, including; an ethanol precipitation step, and various sonication steps, and different salt concentrations. The 2.5 M NaCl SAX-HPLC eluent buffer used to elute heparin and OSCS from the SAX column was found to be the best means to free OSCS from the insoluble matter present in crude heparin.

In addition, 1 g samples of crude heparin dissolved in 10.0 mL of buffer B (100 mg/mL) were used in the method development to sample a larger portion of the heterogeneous sample and allow loading of more OSCS on column at a given weight percent contamination level. To conserve the use of synthetic OSCS used to spike the crude samples in this study, 200 mg aliquots of crude heparins dissolved in 2.0 mL of spiked standard solutions (100 mg/mL) were used in the LOD and LOQ determination experiments.

Therefore, six 200 mg aliquots each of three different crude heparins were weighed into separate 50 mL plastic centrifuge tubes (18 tubes total). Separately, a stock standard solution containing 2.0 mg (as is) synthetic OSCS/mL was prepared in extraction solution (mobile phase B). Dilutions from this stock standard solution were prepared in extraction solution at 1.0, 0.5, 0.1, and 0.05 mg OSCS/mL. These OSCS standard solutions were used for the SAX-HPLC standard curve in the absence of the crudes and for dissolution of the crude heparin samples. One aliquot each of the three crudes were separately dissolved in plain Milli-Q water, 2.00%, 1.00%, 0.50%, 0.10%, or 0.05% weight percent OSCS spike solutions. The samples were then vortexed until completely suspended, sonicated for 15 min, centrifuged for 30 min at 4000 rpm, an aliquot filtered through a Millex®-GN syringe driven 2.0 μm filter (Millipore, Billerica, MA, USA) into an auto-sampler vial, and analyzed by the SAX-HPLC method.

3. Results and discussion

3.1. Capillary electrophoresis

CE electropherograms of the API protocol spiked samples of three crudes and one API were analyzed by the CE method (Fig. 1a and b). In the absence of heparin, OSCS is observed at 5.3 min. In the presence of API heparin, a 5.5 min migration time is observed for OSCS while heparin elutes at 6.0 min. OSCS at a weight of 1.0% of heparin is observed as a slight shoulder on the leading edge of the heparin peak. We also observed that the presence of 10% by weight of OSCS decreases the intensity of the API heparin signal eluting at 5.9 min by over 40%. Similar changes in the observed peak intensities with added OSCS were noted in the electropherograms of all three crudes. We can only attribute the decreased intensity in CE peaks other than OSCS with spiked OSCS to changes in response factors caused by association or aggregation of OSCS with other components in crude heparins.

An example of a crude heparin electropherogram and 1%, 5%, and 10% spiked solutions of the same crude are shown in Fig. 1b. Similar to the API heparin electropherograms, the intensity pattern of the peaks change with added OSCS and the OSCS peak at 5.3 min elutes on the leading edge of the broad heparin peak at 5.7 min. The peak at approximately 7 min in Fig. 1b is ascribed to dermatan sulfate, chondroitin sulfate A, other GAGs, or proteins. Because of the incomplete resolution of OSCS from heparin and the variability in

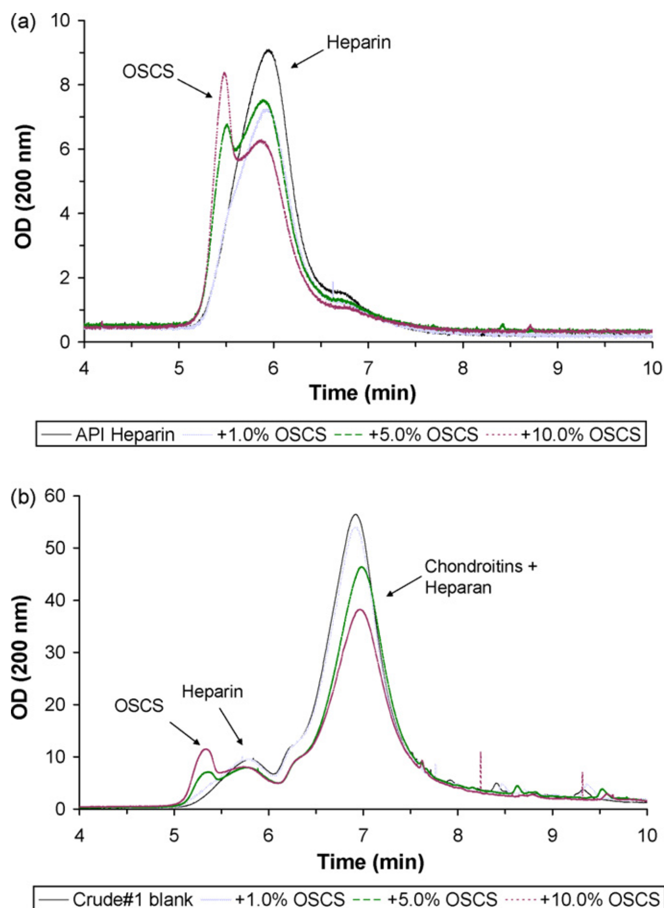

Fig. 1. A plot of the CE electropherograms from 4 to 10 min of (a) an API heparin (solid line) and the same API containing 1.0%, 5.0%, or 10.0% OSCS and (b) crude heparin #1 and the same crude containing the same spiked percentages as shown in (a). In electropherograms the migration order is OSCS then heparin followed by chondroitins and heparan sulfate.

the migration time and peak intensity with added OSCS, we elected not to use CE to quantitate components in crude heparins.

3.2. NMR spectroscopy

The OSCS signal at 2.16 ppm is well resolved at 500 MHz from the N-acetyl methyl proton signals of heparin (2.04 ppm), dermatan sulfate (2.08 ppm), CSA (2.02 ppm), and heparan sulfate (2.04 ppm). An example of change in the 1.90–2.40 ppm N-acetyl methyl proton resonance region with the addition of 1.0%, 5.0%, and 10.0% OSCS is shown in Fig. 2. The spiked OSCS sample peaks and the blank crude sample without added OSCS were integrated, the blank subtracted, and the mean area obtained from three crudes used to plot the area response to varying levels of OSCS (Fig. 3 and Table 1).

Linear regression analysis of the integrated peak area versus weight percent OSCS gave a best fit line described by the equation $y = 69x + 5$. An R^2 value of 0.9990 was obtained and the 2.12 standard deviation of the slope was used to calculate a LOD and LOQ of 0.1% and 0.3%, respectively. ANOVA of the regression gave an F value of 1060. The probability of observing an F-value this high or higher is 0.0196 if the null hypothesis (*i.e.* that the slope is equal to zero) is true. Thus we reject the null hypothesis and find that the most probable value for the slope is the value given by the regression line. The standard error of the intercept was 13.7, giving a P-value of 0.78. Thus at the 95% confidence level we conclude that the slope and intercept given by the regression equation are the most probable values of the calibration line. Importantly, the LOD for the NMR

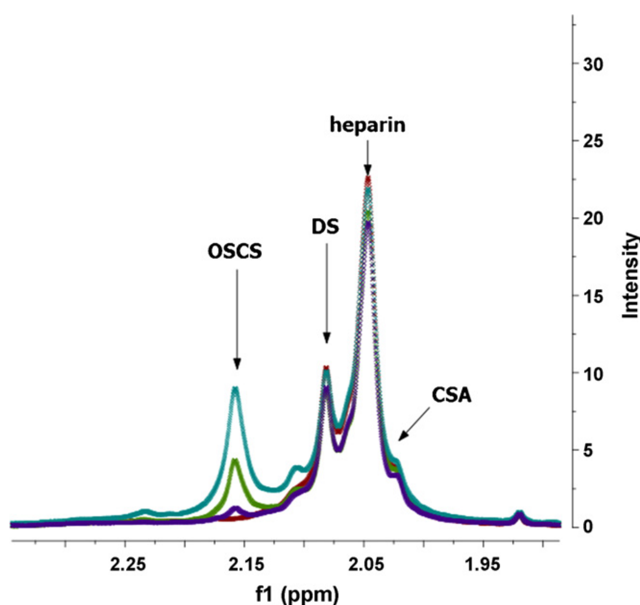

Fig. 2. A plot of the 1.90–2.30 ppm region of the 500 MHz ^{1}H NMR spectra of crude heparin #1 superposed on the spectra of crude #1 containing 1.0%, 5.0%, or 10.0% OSCS. Arrows designate the N-acetyl methyl proton resonances for OSCS at 2.16 ppm, dermatan sulfate at 2.08 ppm, heparin at 2.046 ppm, and chondroitin sulfate A at 2.02 ppm.

approach of 0.1% derived here was the same value determined by Beyer et al. in experiments that spiked OSCS into heparin APIs with successive dilutions to obtain the LOD [9].

However, low recoveries were obtained (70–78%) and the percent RSDs of the areas of the 1.0% and 5.0% level spiked sample were large (67% and 13%, respectively) over the three crudes tested (Table 1). Although the NMR spectra of heparins contain a wealth of signals that can be used to identify and determine relative levels of impurities, our focus in this work was on the determination of the most sensitive approach for measuring levels of OSCS in crude heparins (*i.e.* HPLC).

3.3. SAX-HPLC

The API protocol sample set analyzed by CE and NMR was also investigated by SAX-HPLC. The SAX-HPLC protocol on the 25 mg/mL samples gave improved percent recoveries, percent RSDs, and LOD and LOQ values compared to the NMR method (Table 1). Linear regression analysis of the peak area versus weight percent OSCS

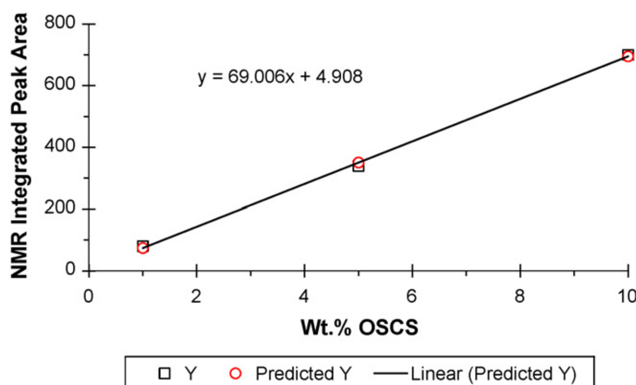

Fig. 3. A plot of the integrated NMR peak area of the OSCS resonance at 2.16 ppm versus the percent OSCS. Actual area values (squares) are the mean of measurements on three different crudes. The predicted area values (circles) are derived from the linear regression analysis of these data. The equation of the trendline is shown.

Table 1
Comparison of recovery, area of peaks, LOD and LOQ values obtained from the NMR and HPLC using the standard API heparin protocol and a modified protocol for crude heparin.

	NMR[a]	%RSD	SAX[a]	%RSD	SAX-2[b]	%RSD
%Rec: 200 mg crude heparin + 0.05% OSCS					96.1	4.2
%Rec: 200 mg crude heparin + 0.10% OSCS					95.2	5.9
%Rec: 200 mg crude heparin + 0.50% OSCS					86.9	5.8
%Rec: 200 mg crude heparin + 1.00% OSCS					88.4	0.7
%Rec: 200 mg crude heparin + 2.00% OSCS					90.6	3.9
%Rec: 25 mg crude heparin + 1.0% OSCS	78.0	9.4	90.2	9.5		
%Rec: 25 mg crude heparin + 5.0% OSCS	76.3	15.8	86.0	5.3		
% Rec: 25 mg crude heparin + 10.0% OSCS	70.8	18.6	82.9	6.4		
Area: 200 mg crude heparin + 0.05% OSCS					30	16.9
Area: 200 mg crude heparin + 0.10% OSCS					67	10.7
Area: 200 mg crude heparin + 0.50% OSCS					411	5.8
Area: 200 mg crude heparin + 1.00% OSCS					878	3.4
Area: 200 mg crude heparin + 2.00% OSCS					1844	1.8
Area: 25 mg crude heparin + 1.0% OSCS	80	67	206	8.2		
Area: 25 mg crude heparin + 5.0% OSCS	339	12.8	966	7.2		
Area: 25 mg crude heparin + 10.0% OSCS	700	4.6	1886	7.2		
LOD[c] (wt.% OSCS/crude heparin)	0.10		0.03		0.02	
LOQ[c] (wt.% OSCS/crude heparin)	0.30		0.09		0.07	

[a] NMR and SAX method values are derived from data collected on 3 crudes with 3 spike levels using the standard API heparin protocol.
[b] SAX-2 values are derived from data collected on 3 crudes with 5 spike levels using the modified SAX-HPLC protocol developed in this work for crude heparin.
[c] LOD = $3.3\sigma/S$, LOQ = $10\sigma/S$, where σ is the standard deviation of the response and S is the slope of the calibration curve.

data gave a best fit line described by the equation of $y = 187x + 24$ (data not shown). An R^2 value of 0.9999 was obtained and the 1.76 standard deviation of the slope was used to calculate a LOD and LOQ of 0.03% and 0.09%, respectively. ANOVA of the regression gave a F value of 11,262. The probability of observing an F-value this high or higher is 0.0060 if the null hypothesis (*i.e.* that the slope is equal to zero) is true. The standard error of the intercept was 11.4, giving a P-value of 0.28.

The LOQ value of 0.03% derived from regression analysis of these data is the same as that reported in our earlier work [9], where five replicate injections of spiked API heparin sodium were made at the LOQ. The SAX-HPLC approach gave 86.4 ± 3.7% recoveries of OSCS with 5–9% RSDs. For comparison, the control recovery of 1.0–10.0% solutions of OSCS in the absence of heparin was 95.9 ± 6.5%.

During the repeat injections of crude heparins, a broad carry-over peak was observed eluting at approximately 15 min. This carry-over does not interfere with the measurement of OSCS content. However, because this peak is close to the elution region of the dermatan sulfate, heparan sulfate, CSA, and the protein content in crude heparins, care should be taken in analysis of the levels of these components by this method.

To test the standard API method for crudes at weight percent spike levels near the LOD and LOQ values of the SAX-HPLC protocol, we prepared 25 mg samples spiked at levels less than 2.0% OSCS. Problems with poor recovery of OSCS were observed in these samples and were attributed to sorption of the OSCS on insoluble crude components or glassware. We found that these sorption issues could be minimized by using the eluent buffer B containing 2.5 M sodium chloride as the extractant.

The final experimental protocol utilizes a 100 mg/mL crude heparin concentration for the SAX-HPLC analysis. This approach has several advantages, including: (1) better sampling of heterogeneous crude samples with 100 mg/mL aliquot, (2) more OSCS injected on-column at a given weight percent with the larger aliquot, and (3) better recovery of OSCS at low weight percents with buffer B being used as the dissolution solvent.

A plot of the overlay of SAX-HPLC chromatograms obtained on separate 200 mg aliquots of crude heparin #2 without added OSCS and at OSCS weight percent spike levels of 0.05%, 0.10%, 0.50%. 1.00% and 2.00% is shown in Fig. 4a. The 22–28 min regions of these data are shown in Fig. 4b. The relative retention time value for OSCS compared to heparin in these chromatograms is 1.22.

Using the improved protocol, data obtained from three crude heparins were used to derive the values presented in Table 1 (see column labeled SAX-2). Linear regression analysis of the peak area versus weight percent OSCS data gave a best fit line described by the equation of $y = 940x - 27$ (Fig. 5). An R^2 value of 0.9998 was obtained and the 6.93 standard deviation of the slope was used to calculate a LOD and LOQ of 0.02% and 0.07%, respectively. ANOVA of the regression gave a F value of 18,357. The probability of observing an F-value this high or higher is 8.86×10^{-7} if the null hypothesis (*i.e.* that the slope is equal to zero) is true. The standard error of the intercept was 7.02, giving a P-value of 0.03.

Based on these data and the comparisons between published LOD values determined by successive dilutions and LOD values

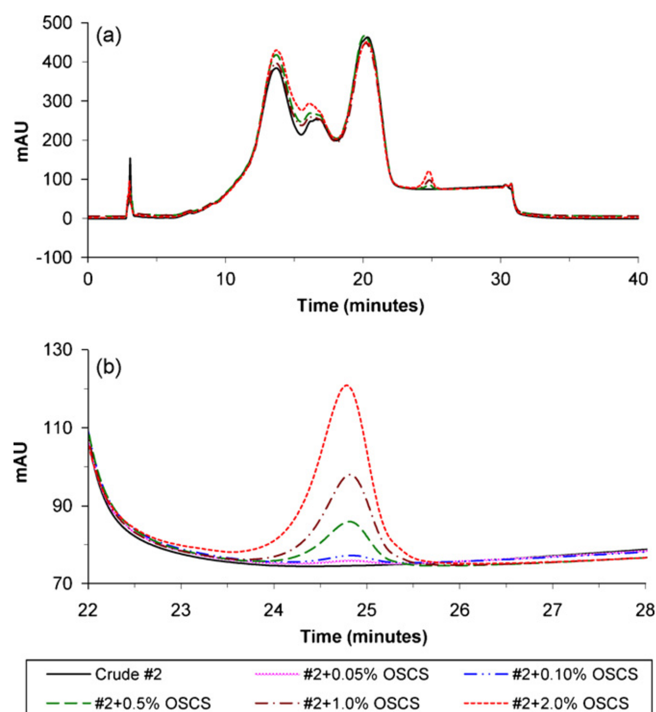

Fig. 4. Overlaid plots of the SAX-HPLC chromatograms of (a) crude #2 alone and crude #2 spiked at 0.05%, 0.10%, 0.50%, 1.00% and 2.00% OSCS levels and (b) expansion of the 22–28 min OSCS elution region of the chromatograms shown in (A).

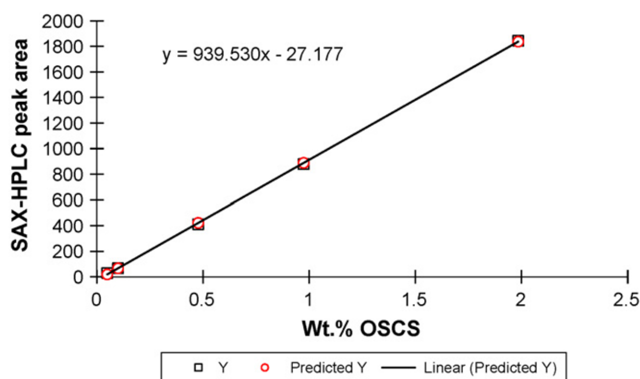

Fig. 5. A plot of the integrated SAX-HPLC peak area of the OSCS peak at 24.8 min versus the percent OSCS added. Actual area values (squares) are the mean of measurements on three different crudes. The predicted area values (circles) are derived from the linear regression analysis of these data. The equation of the trendline is shown.

determined by the standard deviation of the response and the slope discussed above, the 0.02% LOD value for OSCS in crude heparin should be considered a good estimate of the actual value. The new 100 mg/mL SAX-HPLC approach for crude heparin gave 90.9 ± 3.8% recovery of 0.05–2 weight percent OSCS with 1–6% RSDs. These data show that the 100 mg/mL SAX-HPLC method is more sensitive for assay of OSCS in crude than the NMR or original SAX-HPLC method for assay of OSCS in heparin APIs.

One caveat of loading additional material on-column was that more of the crude components that lead to the carry-over peak observed at ca. 15–16 min in the SAX-HPLC gradient are also loaded. After extended sequences of crude heparin injections the carry-over peak often remained present after seven blanks, making running blanks in between crude injections impractical. We observed that the carry-over components could be removed after all of the crude injections had been completed by pumping a 25% methanol solution containing 50 g of NaCl per liter at a flow rate of 0.5 mL/min for 60 min followed by a water rinse for 30 min. Extensive flushing with water overnight was also applied prior to running extended sequences of samples. The presence of carry-over peak in crude heparin chromatography highlights the necessity of running the system suitability test to insure the integrity of the column prior to analysis for contaminants in crude heparin. Importantly, the carry-over peak does not interfere with the analysis of OSCS in crude heparin.

4. Conclusions

Currently no OSCS is allowed in heparin API and finished dose products according to USP specifications. Although no USP criteria exist for crude heparin, firms may want to screen their crude for OSCS before use, and the FDA needs a validated method to screen crude entering the country for contamination. Therefore, we presented CE (10 mg/mL), 500 MHz ^{1}H NMR (35 mg/mL), and SAX-HPLC (20 mg/mL) data obtained on a set of crude heparin samples spiked with 1–10 weight percent OSCS. Comparisons of these data show that SAX-HPLC is more sensitive than the NMR approach. In addition, we conclude that USP CE method for heparin API is not appropriate for quantitative analysis of OSCS in crude heparins. We presented a SAX-HPLC method using a 100 mg/mL concentration of crude heparin in a 2.5 M NaCl solution and compare these data to those obtained at the 20 mg/mL level dissolved in water alone. We showed improved recovery, percent relative standard deviations, and sensitivity for assay of OSCS levels in crude heparins using this approach. Importantly, the NMR, CE, and SAX-HPLC methods provide orthogonal methods that can detect not only OSCS but other possible oversulfated glycosaminoglycans and non-sugar based impurities in crude and API heparins.

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