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Analysis of heparin sodium by SAX/HPLC for contaminants and impurities

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ABSTRACT

A chromatographic method was developed for the detection and quantification of the contaminant oversulfated chondroitin sulfate (OSCS) and the impurity dermatan sulfate in heparin active pharmaceutical ingredient (API). The HPLC analysis of heparin is carried out using a polymer-based strong anion exchange (SAX) column with gradient elution from 0.125 M sodium chloride to 2.5 M sodium chloride buffered mobile phase. The limit of detection (LOD) and limit of quantitation (LOQ) for the contaminant OSCS in heparin were determined to be 0.03% and 0.1%, respectively. The LOD and LOQ for dermatan sulfate, an impurity in heparin sulfate, were determined to be 0.1% and 0.8%, respectively. This manuscript is not a policy document and is not intended to replace either of the methods (capillary electrophoresis and NMR) currently required by the FDA.

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1. Introduction

Heparin, a complex sulfated glycosaminoglycan, is widely used as an anticoagulant [1–3]. Recently, contaminated lots of heparin were associated with an acute, rapid onset of a potentially fatal allergic-type reaction [4–7]. Oversulfated chondroitin sulfate (OSCS) was determined to be a contaminant in heparin samples associated with the adverse reaction [8,9]. Samples of heparin were also found to contain the impurity dermatan sulfate likely present as a result of incomplete purification. NMR [8] and capillary electrophoresis [10] methods for analysis of heparin samples were quickly developed to screen heparin lots for OSCS in order to prevent further exposure to patients by contaminated heparin. Descriptions of the NMR and CE procedures are available on the Internet [11,12]. The CE method does not fully resolve heparin from the OSCS, and quantification of dermatan would be easier with an HPLC method. Chromatographic and capillary electrophoresis methods have been developed for the analysis of heparin and heparin saccharides [13–16] but none for the determination of both OSCS and dermatan sulfate. Analytical methods previously reported were usually carried out on the hydrolyzed fragments of the glycosoaminoglycans because the glycosoaminoglycans including heparin are complex mixture of heterogeneous molecules of widely varying molecular weight. The glycosoaminoglycans are copolymers of alternating uronic and amino sugars residues, with sulfate esters. The ionized sulfate and carboxylate groups give these polymers their highly negative charge. Although the number of disaccharides per chain can vary significantly giving rise to a large spread in molecular weight, their average properties remain similar and elute as a single broad peak. In the current study, an HPLC method was developed and validated for the determination of the contaminant OSCS in intact heparin and can be used to determine the presence of other impurities which are also found in heparin samples such as dermatan sulfate. This method was developed to provide an additional rapid and sensitive method for the analysis of heparin sodium using routinely available laboratory instrumentation.

2. Materials and methods

2.1. Chemicals

Heparin sodium salt from porcine intestinal mucosa (>140 USP units/mg), chondroitin sulfate A sodium salt from bovine trachea, chondroitin sulfate B sodium salt (dermatan sulfate sodium salt) from porcine intestinal mucosa, >90%, lyophilized powder, Trizma base and TRIS were purchased from Sigma (St. Louis, MO, USA). Oversulfated chondroitin sulfate was synthesized by sulfation of chondroitin sulfate A following a literature procedure [17]. While chondroitin sulfate may be sulfated to various degrees, the product synthesized here is a fully sulfated compound in which each N-acetylgalactosamine-glucuronic acid disaccharide unit contains four sulfate groups. The contaminant in heparin responsible for adverse events and referred to as OSCS is the fully sulfated compound. Structure was confirmed by 600 MHz NMR and 2D NMR [8]. OmniSolv acetonitrile was purchased from EMD Chemicals.

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Phosphoric acid, 85%, was purchased from Mallinckrodt. Sodium chloride, 99.5% purity, was purchased from Fluka. 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.

2.2. HPLC separations

Strong anion exchange (SAX)–HPLC separations of heparin, OSCS, and dermatan sulfate were performed on a Dionex IonPac® AS11-HC (250 mm × 4 mm) column (Dionex, Sunnyvale, CA). The AS11-HC column characteristics are bead diameter of 9 μm 2000 Å pore size particle of divinylbenzene/ethylvinylbenzene polymer crosslinked at 55%, coated with microporous latex (DVB/EVB 6% crosslinked) 70 nm particles with hydroxyalkyl quaternary ammonium functional groups and capacity of 290 μeq/4 mm × 250 mm column. The mobile phase was Milli-Q water (solution A) and 2.5 M sodium chloride with 20 mM TRIS adjusted to pH 3 by addition of phosphoric acid (solution B). The gradient was 0–2 min 95% A with 5% B, linear gradient to 100% B at 26 min, hold at 100% B until 31 min, linear gradient to 95% A with 5% B at 32 min and hold until end of run at 40 min. A hold time of 5 min was used through out the method evaluation. No peaks were observed to elute during the hold time and hold at 100% B does not appear to be necessary. Flow rate was constant at 0.8 ml/min. 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. A column temperature of 35 °C was used.

2.3. Sample preparation

Samples were prepared by transferring approximately 20 mg of heparin sodium accurately weighed into a sample vial and adding approximately 1 g of Milli-Q water accurately weighed. Crude heparin samples were filtered through Micro-spin 0.2 μm cellulose acetate filters prior to analysis. Heparin injection solutions were analyzed undiluted.

2.4. Standards preparation

Limited quantities of the OSCS standard which had been synthesized in house were available. In order to minimize consumption of the standards, the standard solutions were prepared in the autosampler vials by diluting the stock standard in the autosampler vials. Stock standard was prepared by transferring approximately 8 mg of dermatan sulfate and approximately 1 mg of OSCS accurately weighed to a sample vial and adding approximately 1 g of Milli-Q water accurately weighed to the vial and mixing to dissolve. Working standards were prepared by transferring approximately 20 mg of heparin sodium accurately weighed to each of four sample vials and than adding approximately 20 mg, 80 mg, 120 mg, and 400 mg of stock standard accurately weighed to the separate vials containing heparin sodium and adding Milli-Q water to a total weight of approximately 1 g accurately weighed. The lowest standard prepared above is at the LOQ and contains approximately 0.1% OSCS and 0.8% dermatan sulfate. The signal-to-noise for this standard should be greater than 10 with a resolution of 1.0 of heparin sodium from dermatan sulfate and 1.8 of OSCS from heparin.

3. Results and discussion

An electropherogram of a contaminated sample analyzed by the CE method is shown in Fig. 1(A). Due in part to the incomplete resolution of OSCS and dermatan sulfate from heparin, work was initiated to find an HPLC method which would resolve these two components from heparin. Initial HPLC analysis employed either a weak anion exchange column (DEAE) stationary phase on silica or polymer or a SAX column on silica. Both weak anion exchange and SAX columns allow for determination of OSCS in heparin. However, neither of these separated dermatan sulfate from heparin. The SAX silica column gave the best separation of heparin from OSCS [18] but the retention times continually became shorter with column use and eventually after approximately 200 h of use the column would fail due to high pressure. Drifting elution times with silica based columns when using the high salt content eluent has been reported previously [19] and appears to be due to column degradation. The polymeric SAX Dionex AS11-HC column separated OSCS and dermatan sulfate from heparin under the elution conditions selected as shown in Fig. 1(B) and was selected for use in the analysis of heparin for OSCS. Fig. 2 shows a chromatogram for a heparin standard spiked at 4 wt% dermatan sulfate and 1 wt% OSCS. The analyte retention times are reproducible and column life appears to be good.

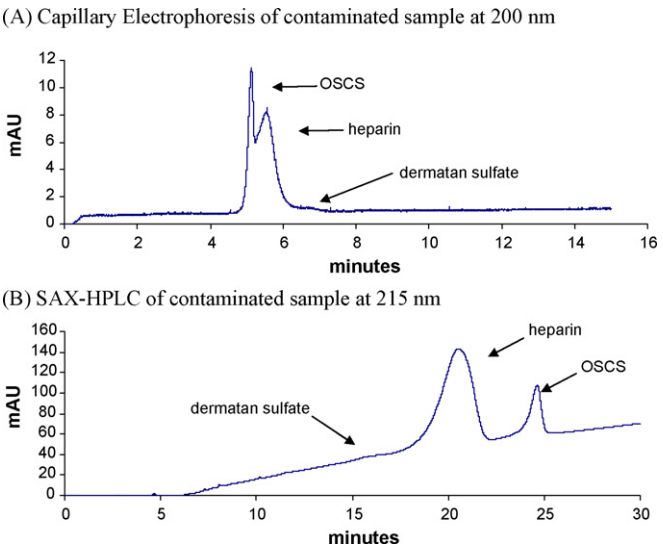

Fig. 1. Comparison of CE electropherogram to SAX–HPLC chromatogram. In electropherogram the elution order is OSCS then heparin followed by dermatan sulfate while in SAX–HPLC the elution order is dermatan sulfate, heparin then OSCS.

3.1. Selection of eluent

Various eluent compositions were evaluated for resolution and theoretical plates. The best results for these criteria were obtained with 2.5 M sodium chloride containing 20 mM TRIS adjusted to pH 3.0 with phosphoric acid as shown in Table 1. This composition was used for the remainder of analyses.

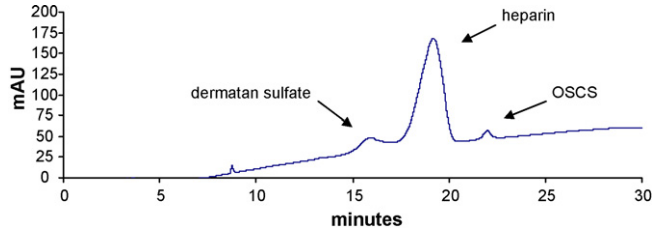

Fig. 2. Standard containing heparin at approximately 20 mg/g of solution and approximately 0.8 mg of dermatan sulfate/g and 0.2 mg of OSCS/g of solution. Retention times are 16.0 min for dermatan sulfate, 19.2 min for heparin, and 22.0 min for OSCS.

Table 1
Eluent composition versus resolution and column efficiency.

Eluent	Resolution dermatan/heparin	Column theoretical plates for heparin
pH 8, 2.5 M NaCl with 20 mM TRIS	1.3	1622
pH 3, 2.5 M NaCl pH adjusted with H_3PO_4	1.3	1116
pH 3, 2.5 M NaCl with 20 mM TRIS pH adjusted with H_3PO_4	1.4	2223

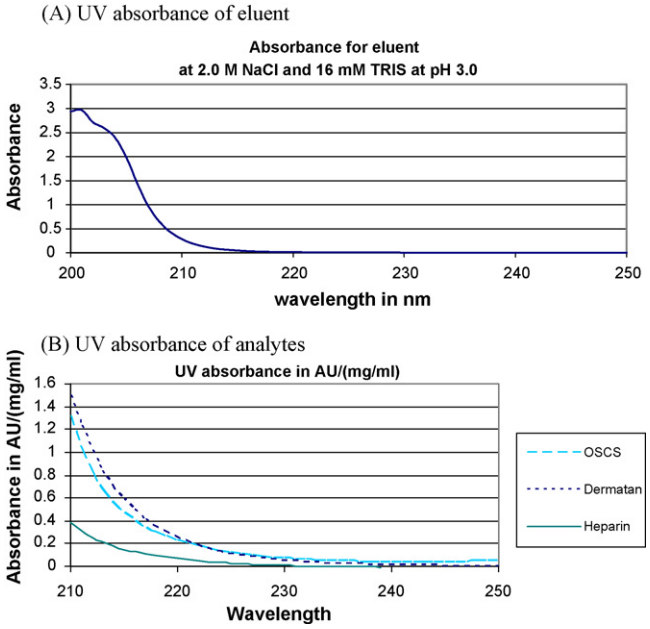

Fig. 3. (A) Absorbance of eluent (2.0 M NaCl with 16 mM TRIS adjusted to pH 3.0 with phosphoric acid) at the elution time for OSCS. (B) UV absorbance of OSCS, dermatan sulfate, and heparin in 20% mobile phase A and 80% mobile phase B.

3.2. Selection of wavelength

A wavelength of 232 nm is frequently employed for the SAX–HPLC analysis of the oligosaccharides produced by heparinase-catalyzed depolymerization of heparin [13,15,19]. The enzymatic reaction used for depolymerization results in an unsaturated uronic acid residue at the nonreducing terminal sugar in the oligosaccharide product [20]. Initial analyses for the intact heparin and OSCS were done at 232 nm. In order to determine the optimum wavelength for the analysis, the signal-to-noise and precision were determined over the range from 210 nm to 240 nm.

The eluent absorbs strongly from 200 nm to 210 nm as shown in Fig. 3(A). This problem is further accentuated by the use of a gradient from 0.125 M sodium chloride to 2.5 M sodium chloride causing a significant baseline increase at wavelengths below 220 nm. However, the response factor for OSCS, heparin, and dermatan sulfate increases as the wavelength decreases as shown in Fig. 3(B). These competing factors result in a compromise in choosing the optimum wavelength for analysis. In order to determine the optimum wavelength for the analysis, five replicate injections, equivalent to 0.5 μg OSCS on-column, of standard solution were analyzed at 210 nm, 215 nm, 220 nm, 225 nm and 232 nm and the percent relative standard deviation was determined. The optimum wavelength was determined to be 215–220 nm as shown in Table 2.

Table 2
Area % RSD for an injection of 0.5 μg OSCS, S/N, and shift in baseline from beginning of chromatogram to end for OSCS determination.

Wavelength (nm)	210	215	220	225	232
% RSD	17%	13%	13%	13%	25%
S/N	26	21	23	15	4
Baseline change (mAU)	290	59	17	3	1

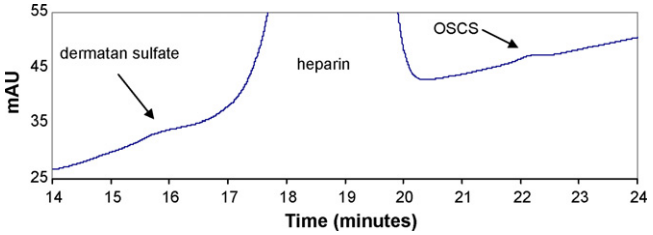

Fig. 4. Standard for OSCS at 0.096% and for dermatan sulfate at 0.36% in an aqueous heparin solution.

3.3. Linearity, LOQ and LOD for OSCS and dermatan sulfate

Table 3 below summarizes the results for linearity, LOD and LOQ for dermatan sulfate and OSCS in heparin. Standard solutions for dermatan sulfate at 23.2 μg/g, 84.2 μg/g, 157 μg/g, 900 μg/g, 1.76 mg/g, 3.92 mg/g and 9.54 mg/g and for OSCS at 6.2 μg/g, 22.5 μg/g, 42 μg/g, 241 μg/g, 470 μg/g, 1.05 mg/g and 2.55 mg/g were prepared in an aqueous heparin matrix containing approximately 20 mg of heparin per gram of solution. The standards were found to be linear over the range tested with a correlation coefficient of 0.9999 for both the dermatan sulfate and the OSCS standards. The slope for dermatan sulfate was 1798 with an intercept of −62 while the slope for OSCS is 1341 with an intercept of −14 measured over the 7 different concentrations listed above. Five replicate injections were made at the LOQ. The LOQ for dermatan sulfate was 157 μg/g equivalent to 0.75% in heparin with a relative standard deviation of 11%. The LOQ for OSCS was 22.5 μg/g equivalent to 0.09% OSCS in heparin with a relative standard deviation of 10.7%. A chromatogram of a standard prepared at 0.096% OSCS and 0.36% dermatan sulfate as a % of the heparin present at 19.45 mg/g of solution is shown in Fig. 4.

3.4. Recovery and precision

Dermatan sulfate was spiked into a 20 mg/g solution of heparin, such that dermatan sulfate was present over the range from 0.12 wt% to 35.45 wt% of the heparin. Recoveries of 96% to 103% were obtained from 0.75 wt% to 35.45 wt% dermatan sulfate in heparin with % RSDs of 0.6% to 11% when calculating the results using linear regression (Table 4).

Oversulfated chondroitin sulfate was spiked into a 20 mg/g solution of heparin such that the OSCS was present over the range from 0.03 wt% to 9.48 wt% of the heparin. Recoveries of 95% to

Table 3
Linearity, Limit of Detection (LOD), and Limit of Quantitation (LOQ) for fully sulfated chondroitin sulfate and dermatan sulfate in heparin solution containing 20 mg of heparin per gram of solution.

	Linearity	LOD	LOQ
OSCS	6.2 μg/g solution to 2.6 mg/g solution	6.2 μg/g solution (0.03% in heparin)	23 μg/g solution (0.1% in heparin)
Dermatan sulfate	23 μg/g solution to 9.5 mg/g solution	23 μg/g solution (0.1% in heparin)	157 μg/g solution (0.8% in heparin)

Table 4
Precision and accuracy for 20 mg of heparin per gram of solution spiked at the specified levels with dermatan sulfate and OSCS.

Dermatan sulfate			OSCS		
wt% in heparin[a]	% RSD	% of spiked value	wt% in heparin[a]	%RSD	% of spiked value
35.45	0.6	99.8	9.48	0.9	100.1
20.67	0.6	101.2	3.43	1.9	99.9
6.38	1.1	101.2	1.71	1.2	99.3
4.06	1.9	95.6	1.09	1.9	94.5
0.75	11.0	103.0	0.20	2.6	102.8
0.33	9.2	62.5	0.09	10.7	110.8%
0.12	32.4	173.8	0.03	27.5	−46%

[a] Calculated by linear regression.

Table 5
Relative retention times of impurities and contaminants relative to the retention time of heparin.

Compound	Compound R_t (min)/R_t heparin (min)
Heparin	1.000
Heparan sulfate	0.740
Chondroitin sulfate A	0.774
Dermatan sulfate (chondroitin sulfate B)	0.778
Oversulfated heperan sulfate	1.060
Oversulfated dermatan sulfate	1.083
Oversulfated chondroitin sulfate A (OSCS)	1.204

111% were obtained for 0.09 wt% OSCS to 9.48 wt% in heparin with a % RSD of 0.9% to 11% when calculating the results using linear regression (Table 4). Recoveries of spiked dermatan sulfate and OSCS at the LOD level, while calculated and included in the table, are poor due to difficulty in integrating very small peaks on a sloping baseline. Samples close to the limit of quantitation would be more accurately calculated using an average response factor for the standards and is the procedure chosen for routine analysis.

Preliminary analysis indicates that this method would also separate OSCS from possible interferences from heparan sulfate, chondroitin sulfate A, oversulfated heperan sulfate and oversulfated dermatan sulfate which could also be contaminants in heparin. The relative retention times for these materials are shown in Table 5.

4. Conclusions

The HPLC method is being used to analyze heparin active pharmaceutical ingredients and heparin products for the presence of OSCS but may also be used to screen crude heparin for OSCS prior to purification. The presence of the OSCS can easily be detected and quantitated at 215–220 nm when OSCS is present at 0.1%. The HPLC method provides low limits of detection for heparin containing contaminants, e.g., OSCS and impurities, dermatan sulfate employing instrumentation generally available in most laboratories. Alternative columns, such as weak anion exchange, could be used in conjunction with this procedure to verify heparin purity. This would reduce the likelihood of other partially sulfated contaminants coeluting with heparin on the AS11-HC column. The use of this HPLC technique in combination with other recommended techniques like capillary electrophoresis to detect high charge materials and high resolution NMR for structure verification may provide greater protection to the public from contaminated or impure heparin. The complex nature of heparin and the possible contaminants will likely require continued work in method development for detection of impurities. However, this manuscript is not a policy document and is not intended to replace either of the methods (capillary electrophoresis and NMR) currently required by the FDA.

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