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The use of SAX-HPLC–CD as a heparin screening strategy

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ABSTRACT

Heparin, a heterogeneous polysaccharide, has been widely used as an anticoagulant for decades. Recently, however, international events involving the sudden onset of allergic-type reactions following heparin administration led to numerous fatalities, and demanded the use of multiple laborious, time consuming techniques to identify an economically motivated adulterant. Using these methods cooperatively, the semi-synthetic molecule known as oversulfated chondroitin sulfate (OSCS), was found to be present at significant concentrations. Since the discovery of this adulterant, several analytical methods have been put forth or updated to advance the process of screening pharmaceutical heparins; of these, strong anion exchange high performance liquid chromatography (SAX-HPLC) methods have now become routine. In this preliminary work, we report the use of circular dichroism (CD) detection in conjunction with existing SAX-HPLC methods to quantitate various sulfated polysaccharides. The proposed strategy exploits the selectivity associated with CD detection of heparin and heparin-like polysaccharides, while taking advantage of the method's insensitivity to the use of mobile phase additives and programmed gradients. The limit of detection of heparin by CD was found to be ~0.22 mg/mL, whereas traditional UV/Vis detection yielded a detection limit of ~1.09 mg/mL. The success of CD detection varied for other polymers, however no significant modifications were made to the separations method to capitalize on the advantages of CD detection.

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1. Introduction

Heparin, a highly heterogeneous glycosaminoglycan, has been used as an anticoagulant for over seventy years [1]. Recently, however, severe allergic-type reactions to heparin were reported, many with fatal results; these occurrences were eventually reported in approximately a dozen countries [2,3]. Given heparin's extensive clinical track record, these events quickly led to the investigation of possible contamination and a non-naturally occurring molecule, oversulfated chondroitin sulfate (OSCS), was discovered in suspect lots of pharmaceutical heparin [2]. The highly sulfated nature of this semi-synthetic, heparin-like compound allowed it to defeat traditional screening assays (i.e., those based on anticoagulant activity) [2,3].

Because of the complexity of heparin and heparin-like polysaccharides, OSCS was determined only through the use of expensive instrumentation and various laborious techniques, such as multidimensional NMR and capillary electrophoresis (CE). Since 2008, several methods have been put forth in an effort to simplify the process of accounting for OSCS in pharmaceutical heparins [4–8]. One of these methods, SAX-HPLC, has proven successful in separating heparin from the adulterant OSCS as well as a naturally occurring impurity, dermatan sulfate (DS) [4,9]; low levels of DS were also found in compromised samples, and must now be accounted for in the pharmaceutical validation methods. SAX-HPLC is capable of accounting for low levels of OSCS and DS in heparin samples and has now replaced CE as the USP's recommended method for screening heparin [10].

Here, we report the use of CD detection in conjunction with SAX-HPLC to screen heparin for OSCS and DS. CD detection capitalizes on the preferential absorption of circularly polarized light (right-handed or left-handed) that arises from structural asymmetry within a chiral molecule [11]. In general, circular dichroism can be observed for any chiral analyte, so long as the sample is enantiomerically enriched; hence, CD is well suited for the analysis of many biological compounds (e.g., nucleic acids and proteins) [12]. CD has previously been used to investigate heparins and related polysaccharides, but such studies frequently made use of synchrotron radiation sources [13–19]. Recently, however, similar methods were reported for the screening of heparin for heparin-like species using traditional CD spectropolarimetry [20]. The extension of this work to the SAX-HPLC screening platform promises several desirable advantages, relative to the use of traditional UV/Vis detection. These advantages include: (1) increased specificity (i.e., analytes must be optically active), (2) reduced sensitivity to absorbing mobile phase additives, (3) reduced sensitivity to changing mobile phase conditions (i.e., the use of a gradient).

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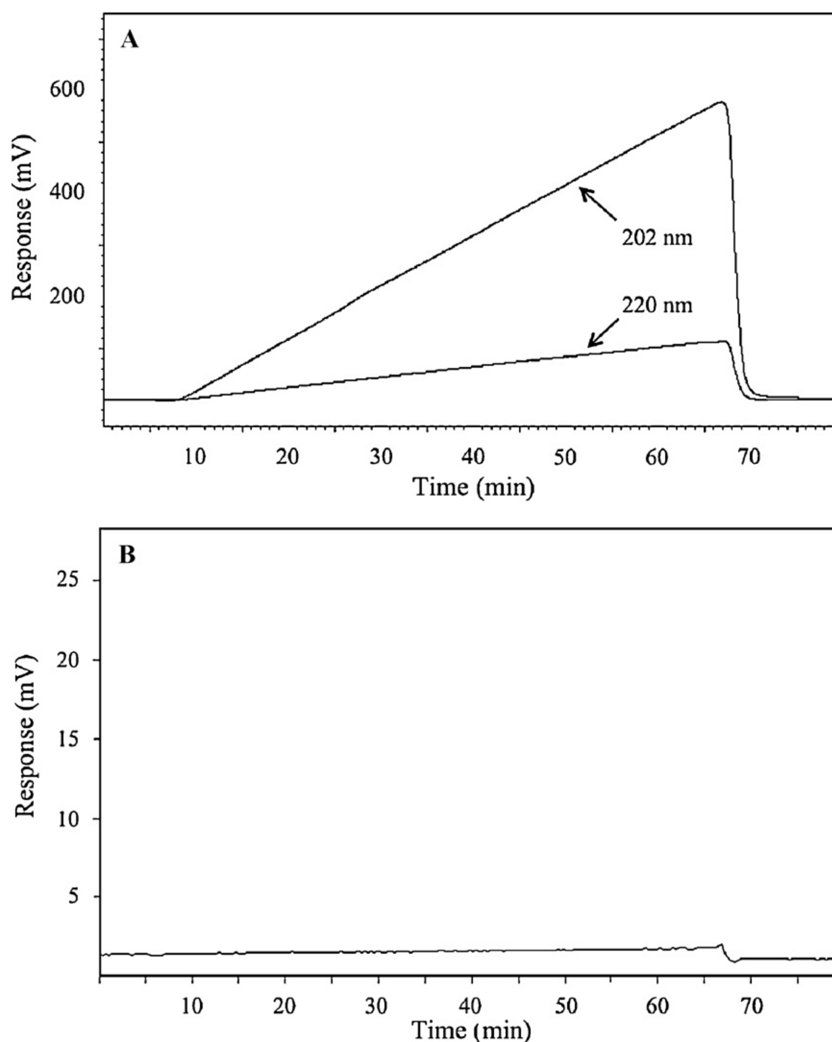

Fig. 1. Chromatograms obtained by (A) UV–Vis detection (202 nm and 220 nm) and (B) CD detection (220 nm) for blank samples.

2. Materials and methods

2.1. Materials

Heparin sodium salt from porcine intestinal mucosa and oversulfated chondroitin sulfate were donated by from Baxter Healthcare (Deerfield, IL). Dermatan sulfate was purchased from Calbiochem (San Diego, CA). Monobasic sodium phosphate dihydrate and phosphoric acid were purchased from Fisher Scientific (Fair Lawn, NJ). Sodium perchlorate monohydrate was purchased from G Frederick Smith Chemical Company (Columbus, OH). Nalgene PTFE syringe filters (0.45 μm, 13 mm) were obtained from Nalge Nunc International (Rochester, NY).

2.2. HPLC instrumentation

Separations were carried out using a Shimadzu (Columbia, MD) HPLC system consisting of a DGU-14A degasser, an SIL-10AF auto injector, two LC-10AT pumps, a CTO-10AC column heater and an SPD-10A UV-Visible detector (λ = 202 nm and 220 nm) equipped with a 1 cm path length cell. These components were controlled using a Shimadzu SCL-10A system controller and a PC equipped with Shimadzu EZStart software (v. 7.3 SP1). HPLC–CD detection was carried out using a Jasco (Easton, MD) CD-1595 detector (λ = 220 nm), equipped with a 2.5 cm cell, that was placed in-line between the column and the UV/Vis detector; the CD detector was processed by a second PC equipped with a Chrom and Spec for Windows software package (v. 1.52).

2.3. Sample preparation

All samples were prepared fresh daily by measuring out appropriate amounts of each polysaccharide for dissolution into DI water up to a total sample volume of 1 mL; this was carried out using a 1 mL volumetric flask. Once dissolved, the samples were filtered using Nalgene PTFE syringe filters (0.45 μm, 13 mm). Lastly, the samples were transferred to the HPLC auto injector tray in preparation for analysis.

2.4. SAX-HPLC separations

All separations were carried out at 40 °C on a Dionex (Sunnyvale, CA) IonPac AS11 Analytical column (2 mm × 250 mm) donated by the Dionex Corporation.

The SAX-HPLC investigations used a binary gradient program (flow rate = 0.22 mL/min) and 20 μL sample injections. Mobile phase A consisted of 2.6 mM monobasic sodium phosphate in DI water adjusted to pH 3 using 2.4 M phosphoric acid; Mobile phase B consisted of 1.0 M sodium perchlorate and 2.6 mM monobasic sodium phosphate in DI water adjusted to pH 3

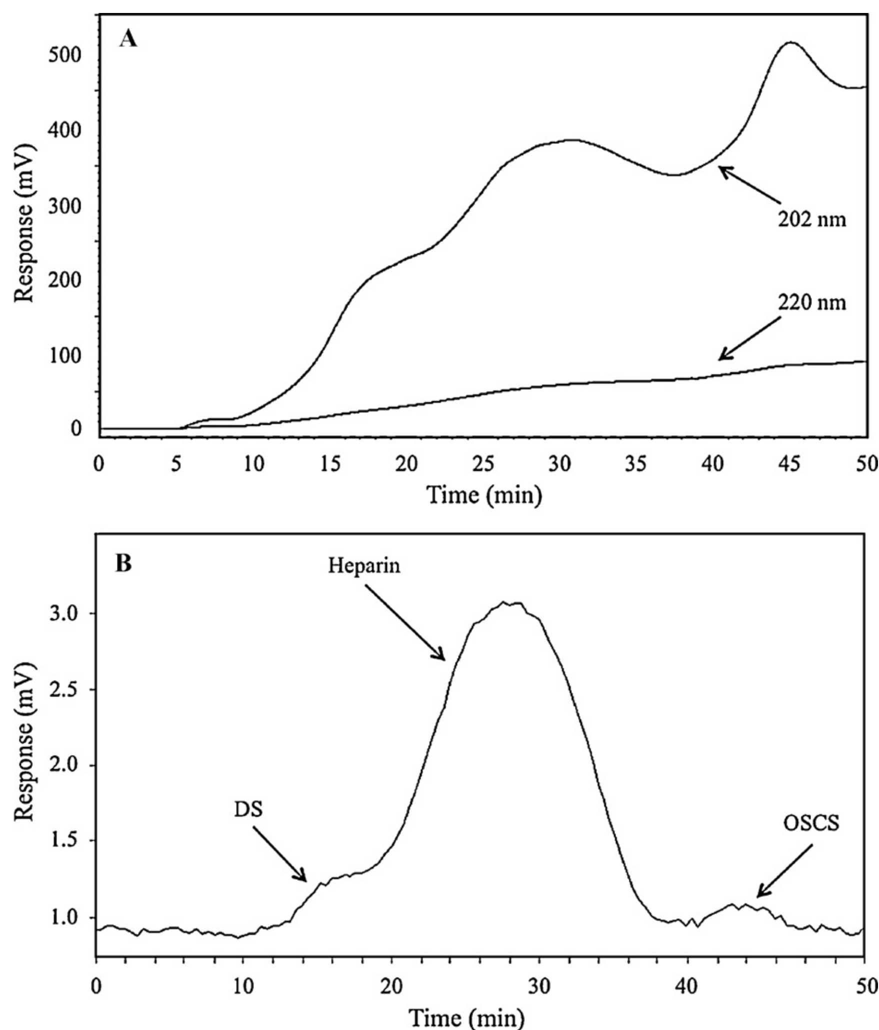

Fig. 2. Chromatograms obtained by (A) UV–Vis detection (202 nm and 220 nm) and (B) CD detection (220 nm) for the separation of 2 mg/mL DS, 20 mg/mL heparin, and 2 mg/mL OSCS.

using 2.4 M phosphoric acid. Samples were injected into a mobile phase composed of 80% A and 20% B at the start of each run. A linear gradient was applied between 0 and 60 min that ended with a mobile phase composition of 10% A and 90% B. At 60 min, the mobile phase composition was returned to 80% A and 20% B and the analysis was continued for 20 additional minutes.

The limits of detection associated with the separation results were calculated using the 3× standard deviation method as found in Harris' [21].

3. Results and discussion

3.1. Investigating the impact of method conditions on UV–Vis and CD detection

The SAX-HPLC experiments performed during these investigations were monitored by both UV–Vis and CD detection; the CD detection scheme was selected based on previous work investigating heparin adulteration [20]. As shown in Fig. 1A, the employed sodium perchlorate gradient causes a significant rise in baselines collected by UV detection at 202 nm. These findings are consistent with previous reports that have recommended monitoring at higher wavelengths to reduce the negative impact of absorbing eluents [4]; additional experiments at higher wavelength (220 nm) reduced, but did not eliminate, the negative impact of eluent absorption. In contrast to these results, CD detection (Fig. 1B) shows minimal drift over a similar time period as the results discussed above. As such, the proposed method appears to circumvent the issue of baseline drift that is associated with UV–Vis detection in conjunction with SAX-HPLC method [10].

Following sample introduction, DS, heparin, and OSCS were observed, in that order, in less than 1 h using previously established separations procedures; these results are illustrated in Fig. 2A [9]. As expected, results collected by UV–Vis (Fig. 2A) at 202 nm provided the greatest response to each of the polymers of interest, but were also significantly impacted by a substantial rise in the chromatogram baseline as a function of the sodium perchlorate concentration. Additional results collected at the higher wavelength (220 nm) are also shown and clearly demonstrate the significant losses in sensitivity that occur for each of the analytes as a function of decreased absorptivity. In contrast, CD detection at 220 nm, shown in Fig. 2B, yielded minimal baseline drift and an improved response profile relative to that observed by UV–Vis at the same wavelength.

It is important to note, given the less than ideal quality of the above separations, that these results are meant solely to introduce a SAX-HPLC scheme that is insensitive to mobile phase conditions (i.e., gradients, additives, etc.); additional advances in the separation of heparin and similar species are greatly needed and the removal of buffer phase incompatibilities may greatly assist in these efforts. Furthermore, the peak broadening observed above

Table 1
Summary of the results obtained for the detection heparin, DS, and OSCS by CD and UV–Vis detection in conjunction with SAX-HPLC.

	CD detection (λ = 220 nm)		UV detection (λ = 220 nm)	
	Limit of detection (mg/mL)	Response factor	Limit of detection (mg/mL)	Response factor
Heparin	~0.22	~73.01	~1.09	~344 000
DS	~1.12	~73.91	~1.02	~635 000
OSCS	~3.06	~27.98	~0.66	~683 000

may arise partially from the large cell path length (2.5 cm) of the CD detector. Work with a shorter path length flow cell would likely lead to improvements in the quality of these separations albeit with a reduction in sensitivity. While this loss in sensitivity could be offset by detection at 202 nm, the CD detector is limited to detection between 220 and 420 nm.

3.2. Investigating SAX-HPLC–CD detection of heparin, OSCS, and DS

As the utility of the current separation method has been extensively explored in terms of analyte resolution [4,9], and enhanced separation is not the central theme of the present work, the investigations discussed below were carried out using single analyte samples for the sake of simplicity. In the case of heparin, increasingly concentrated samples were analyzed by SAX-HPLC–CD to investigate the method's sensitivity. As shown in Fig. 3, heparin samples of increasing concentration yielded progressively larger CD responses that were found to grow linearly as a function of heparin concentration (R^2 = 0.999); the average error associated with these measurement was determined to be ~2.9%. Using these results, the heparin limit of detection was calculated, according to the three times standard deviation method [21], to be ~0.22 mg/mL using CD detection. Interestingly, decreased analyte retention was observed at higher heparin concentrations indicating that column overload is occurring. It is expected that overload will likely interfere with the detection of DS in pharmaceutical heparins; however, previous research [4,9] has shown that this issue does little to undermine the utility of the SAX-HPLC method.

Investigations similar to those conducted for heparin were carried out for both DS and OSCS. As with heparin, linear increases in CD response were observed as a function of increasing polymer concentration (DS, R^2 = 0.995; OSCS, R^2 = 0.982). The limits of detection for DS and OSCS were calculated to be ~1.12 mg/mL and ~3.06 mg/mL, respectively. These results are summarized in Table 1, along with those obtained for heparin. Interestingly, the

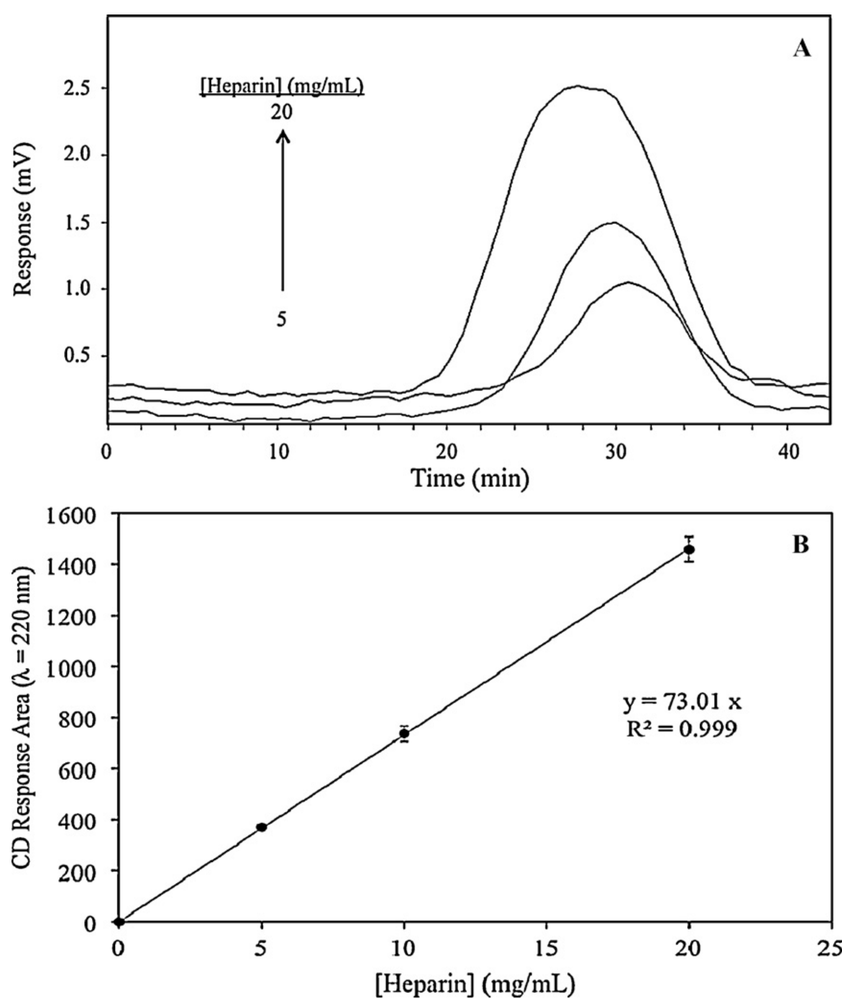

Fig. 3. (A) Overlay of chromatograms obtained by CD detection for samples containing increasing concentrations of heparin. (B) Plotted results obtained by SAX-HPLC–CD for increasing concentrations of heparin (*N* = 3).

highest LOD determined was that of OSCS. As the peak for OSCS is typically the sharpest of three, the rationale behind this LOD may be spectroscopic in nature; recent investigations suggest OSCS should be the most strongly detected [20]. However, the conditions employed in this study differ significantly from the previous work and may play a role in the reduced LOD obtained for OSCS.

Comparison of the results obtained by CD detection to those obtained by UV–Vis, also summarized in Table 1, highlights the potential of the proposed method. As shown, the response factors obtained for each polymer were significantly larger for UV–Vis detection than for CD detection, yet the calculated limits of detection are comparable. In fact, CD detection appears to be superior in the case of heparin detection, even once the differences in detector cell path lengths are accounted for. The similar detection limits, despite the different response factors, likely arises from the reduced impact of eluent absorption, the inherent selectivity of CD detection towards the analytes, and the relatively reduced error associated with the CD measurements made in this work; for example, the average standard deviation associated the measurement of heparin by CD detection was ~2.9%, whereas average standard deviation for UV–Vis detection was ~5.0%.

It should be noted that the UV–Vis detection limits obtained in this work differ from those previously reported for the use of SAX-HPLC; presumably this is largely a function of differences in wavelength selection. Furthermore, the results shown here do not support the use of SAX-HPLC–CD as a means of accounting for sub-percent levels of DS and OSCS in pharmaceutical heparins. However, the current SAX-HPLC method has not been optimized to capitalize upon the advantages of CD detection and additional method refinement will likely be of significant value.

4. Conclusions

The use of CD detection in conjunction with SAX-HPLC is reported in this work for the investigation of heparin, DS, and OSCS. As expected, heparin was separated from both dermatan sulfate and OSCS, with better resolution being observed for the latter; this separation was observable by both CD and UV–Vis detection. Interestingly, however, CD detection appears to be significantly less impacted by the mobile phase conditions than the more commonly used UV–Vis detector. Additionally, the limits of detection obtained for DS, heparin, and OSCS individually appear to be comparable for the use of CD and UV–Vis detection using the current instrumentation. As such, the present use of CD detection points to possible advantages that will be useful in furthering heparin-screening methods by offering reduced sensitivity towards mobile phase conditions and greater selectivity towards optically active analytes.

Acknowledgements

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