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Determination of impurities in heparin by capillary electrophoresis using high molarity phosphate buffers

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ABSTRACT

Oversulfated chondroitin sulfate (OSCS), an impurity found in some porcine intestinal heparin samples was separated from intact heparin by capillary electrophoresis (CE) using a 600 mM phosphate buffer, pH 3.5 as the background electrolyte in a 56 cm × 25 μm i.d. capillary. This method was confirmed in two separate labs, was shown to be linear, reproducible, robust, easy to use and provided the highest resolution and superior limits of detection compared to other available CE methods. Glycosoaminoglycans such as dermatan sulfate and heparan sulfate were separated and quantified as well during a single run. The heparin peak area response correlated well to values obtained using the official assay for biological activity. A high speed, high resolution version of the method was developed using 600 mM lithium phosphate, pH 2.8 in a 21.5 cm × 25 μm i.d. capillary which provided limits of detection for OSCS that were below 0.1%.

1. Introduction

Heparin is the most commonly used anticoagulant in medicine. In January 2008, an increase in the number of adverse reactions associated with the administration of heparin was observed [1]. In February 2008 a voluntary drug recall was initiated. Shortly thereafter, reports of similar adverse reactions began to come in from different countries, threatening the worldwide heparin supply.

An intense effort was undertaken by the affected parties to develop biological and analytical methods to determine the cause of the adverse events. A capillary electrophoresis (CE) method developed at Baxter Healthcare later appeared on the Food and Drug Administration (FDA) web site [2]. As illustrated in Fig. 1, this method showed a crude separation of a then unknown heparin-like compound. This emergency method in conjunction with an NMR method was successful in reducing contaminated heparin from the world supply and the number of adverse reactions showed an immediate decline.

The contaminant was soon found to be oversulfated chondroitin sulfate (OSCS), a heparin-like compound [3]. The source of the contamination has not been identified but it was theorized that it was a by-product of attempting to increase the potency of heparin by sulfation [4]. Exposure to certain porcine viruses was known to trigger production of minimal amounts of OSCS [4] but some heparin samples had as much as 20% of their content as OSCS.

Heparin is a naturally occurring polydisperse glycosoaminoglycan (GAG) with the highest negative charge density of any known biological molecule. This and other GAGs are found in many animal tissues and are potential contaminates. Structural differences between GAGs are often determined by measuring the disaccharide or oligosaccharide content of each material [5,6]. Despite their polydispersity, it is still desirable to perform separations on intact GAGs. Stefansson and Novotny [7] showed a partial separation of polydisperse heparins using a buffer containing the ion-pairing agent ethylenediamine. This work proved that the broad peak found for heparin was not a wall effect but reflected the reality of a very complex mixture of similar compounds.

Fig. 2 shows the major repeating units of each of these molecules. Chondroitin sulfate normally has a sulfate group in either the 4 position (chondroitin A) as shown or the 6 position (chondroitin C). If one or more of the R groups are sulfated, the chondroitin sulfate is considered oversulfated or OSCS. If an OSCS contains sulfate groups at all of the R positions, it is considered fully sulfated or FSCS.

Malsch et al. [8] reported separations of low molecular mass heparins using 20 mM phosphate buffer, pH 3.5. Patel et al. used 50 mM phosphate buffer, pH 3.5 to compare separations of stressed and unstressed low molecular weight heparin [9]. The latter two separations employed countermigration CE with reversed polarity. Neither paper showed separations of other GAGs such as dermatan

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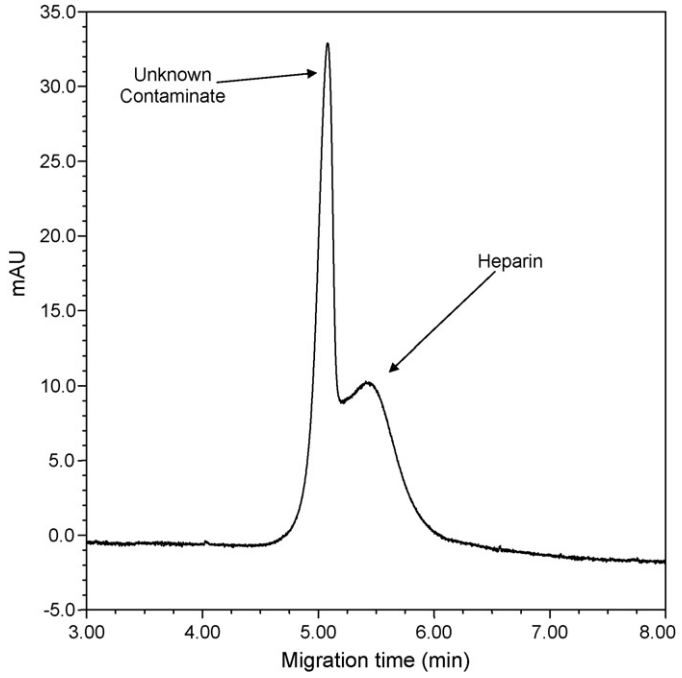

Fig. 1. 10 mg/mL contaminated heparin API, buffer: 36 mM phosphate pH 3.5, capillary: 50 μm i.d. × 56 cm effective × 64.5 cm total, temperature 25 °C, −30 kV and injection 500 mbs cathodic.

sulfate (DS) nor heparan sulfate (HS) that might also be found in heparin material.

The limit of detection (LOD) of the method found on the FDA website for OSCS site was estimated to be between 1 and 5% of total heparin [10]. While this may have been satisfactory for an emergency method, it is far higher then the state of the art where LODs of 0.1% or less are desired. In addition, the method does not have sufficient resolution to accurately quantitate DS or HS. The FDA method was adapted and validated by the United States Pharmaceopia (USP) and an official method was posted on their web site on 18 June 2008 [11]. The buffer concentration specified in the CE monograph test was 36 mM.

In order to achieve very low LODs, it is ideal to maximize the heparin concentration. Heparin is extremely soluble in water, so soluble that it can become the solvent and water the solute. To take advantage of this extreme solubility, high concentration or high conductivity buffers are very important. These buffers provide for sample stacking thereby allowing large injections of high concentration heparin solutions to be introduced into the instrument. In order to control the current, it is necessary to use capillaries with inner diameters of 25 μM. The buffer must be UV transparent in the low UV and in addition, allow the electroosmotic flow (EOF) and solute mobilities to be manipulated. In this paper, the experiments are described that permit the separation of numerous GAG impurities found in heparin, with high reproducibility, ease of use and with very low LODs for key impurities, in particular OSCS.

2. Materials and methods

2.1. Materials

Sodium heparin active pharmaceutical ingredient (API) was supplied by Scientific Protein Laboratories, LLC (Changzhou, China). Sodium heparin USP grade, DS and HS were purchased from Sigma–Aldrich (St. Louis, MO, USA). OSCS was synthesized internally. Ortho-phosphoric acid was from Spectrum Chemicals (Gardena, CA, USA) or Sigma Chemical and sodium phosphate monobasic monohydrate was from Mallinckrodt Chemicals (Phillipsburg, NJ, USA) or the anhydrous form was from Fisher Biotech (Fairlawn, NJ, USA). Lithium phosphate, 99% was from Aldrich (Milwaukee, WI, USA). Buffers and sample solutions were prepared from Milli-Q water or Agilent CE water and were filtered through a 0.21 μm cellulose acetate filter prior to use.

2.2. Instrumentation

Agilent Technologies CE instruments (Waldbronn, Germany), equipped with a deuterium UV lamps and diode array detectors

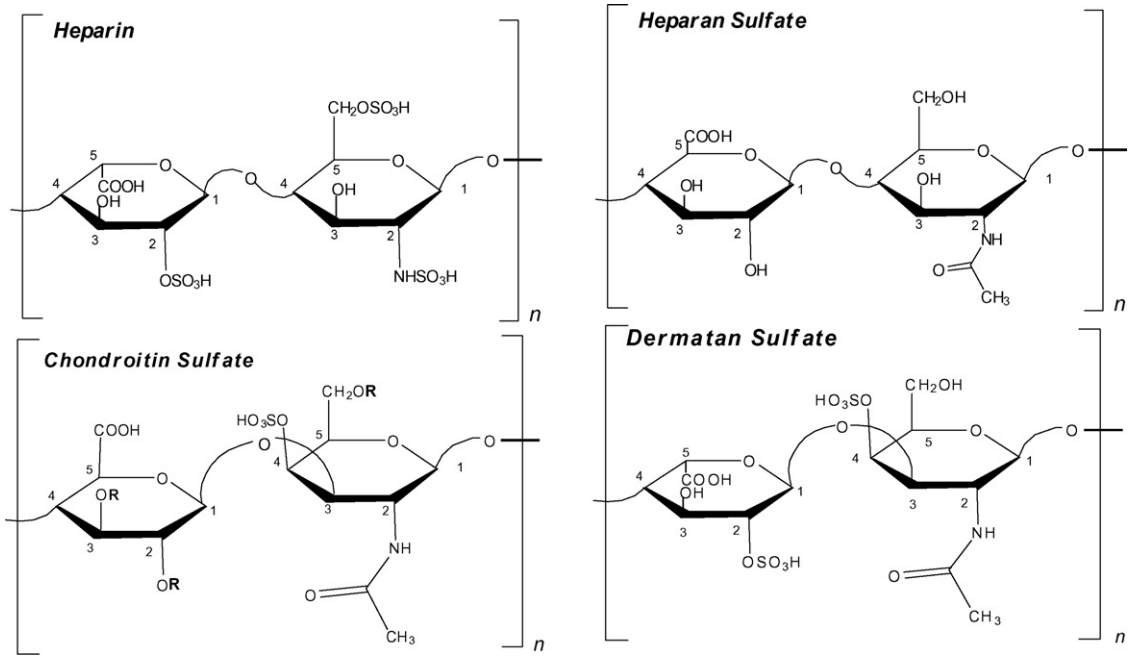

Fig. 2. Chemical structures of heparin, heparan sulfate, chondroitin sulfate A and dermatan sulfate major repeating units. Oversulfated chondroitin sulfate, will have at least one but possibly up to all three (fully sulfated) R groups as sulfate groups.

(190–600 nm) were used for all CE investigations. Instrument control was carried out using Agilent Chemstation software. Data analysis was performed using either the Agilent Chemstation or Dionex Corporation (Sunnyvale, CA, USA) Chromeleon software. Extended pathlength bare fused silica capillaries were from Agilent Technologies and used as received. A detection wavelength of 200 nm (bandwidth 10 nm), reference 450 nm (bandwidth 80 nm) and a capillary temperature of 35 °C were used unless otherwise noted.

2.3. Buffer preparation

Equimolar concentrations of sodium phosphate and phosphoric acid were prepared separately as follows. Sodium phosphate monobasic phosphate electrolyte (600 mM) was prepared using 8.28 g (137.99 g/mole) of sodium phosphate monobasic monohydrate in 100 mL with water. Phosphoric acid (600 mM) was prepared by transferring 6.9 g (4.1 mL) of 85% phosphoric acid (98.0 g/mole) and diluting to volume with water. Using a pH meter, the pH was adjusted to 3.5 by titrating an aliquot of phosphate solution with phosphoric acid. Preparation of phosphate buffers of different salts, concentrations and pH values were performed using a similar protocol.

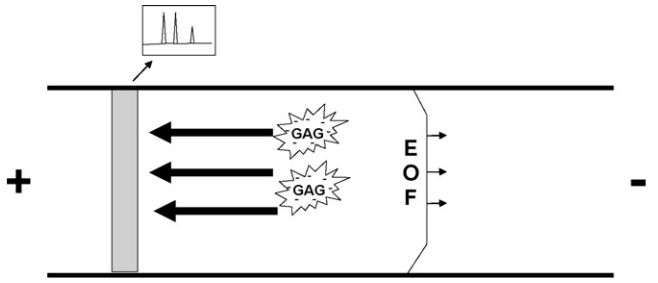

Fig. 3. Counter-migration reversed polarity CE. The migration of the highly negatively charged GAG is in the opposite direction of the slower EOF.

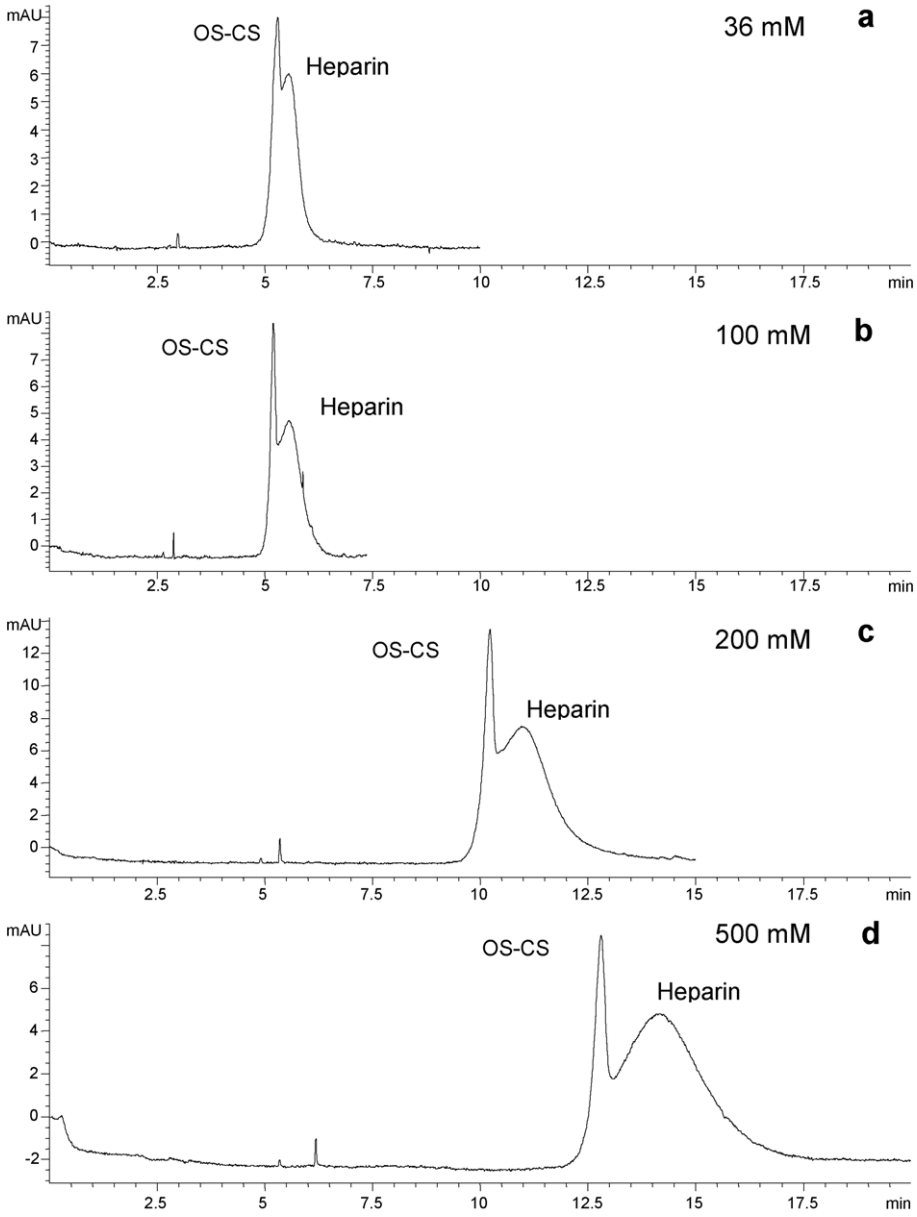

Fig. 4. Impact of buffer concentration on the migration time and resolution of OSCS and heparin. Buffer pH: 3.5, temperature: 25 °C—(a and b) capillary 50 μm × 56 cm × 64.5 cm, voltage: −30 kV, injection: 500 mbs and (c and d) capillary 25 μm × 72 cm × 80.5 cm, voltage: −30 kV, injection: 2000 mbs.

2.4. Capillary electrophoresis analysis

Unless otherwise noted, the following protocol was used. Between each run, the capillary was flushed for 5 min with buffer. The samples were introduced into the cathodic end of the capillary by hydrodynamic injection for 40 s at 50 mbar, 2000 millibarseconds (mbs). The capillary inlet was dipped with water prior to and after injection to minimize carryover. The separation was performed in the reverse polarity mode with a constant voltage of −18.5 kV.

The high-speed version of the method employed 600 mM lithium phosphate, pH 2.8 in a 21.5 cm × 25 μm i.d. capillary. The voltage was set at −14 kV. At the beginning of each day, the capillary was flushed with buffer for between 5 and 15 min. Buffer vials were replaced after 10 injections with fresh buffer. New capillaries were flushed for at least 15 min with buffer prior to use. Many of the experiments were verified in the author's, separate laboratories.

2.5. Heparin solution preparation and calibration

Unless otherwise specified, heparin was prepared to a final concentration of 30 mg/mL with water. A heparin solution was divided in two and sufficient OSCS was added to a concentration of 10 mg/mL. Dilutions of the OSCS/heparin solution with heparin were used to produce the calibration curve.

2.6. Intermediate precision

A heparin sample was spiked with approximately 0.85% (w/w) each of OSCS and DS in relation to heparin. This sample was injected 10 times a day on 3 separate days. Fresh running buffer vials and a fresh aliquot of the spiked heparin were replenished at the beginning of each day's analysis. The corrected peak areas were measured and the percent relative standard deviations were calculated.

2.7. Heparin activity

Solutions of porcine and bovine intestinal heparin with known USP activity values were prepared in water to a final concentration of 30 mg/mL. The resulting heparin peak area was recorded for each injection and was plotted against the labeled USP activity.

3. Results and discussion

3.1. Countermigration CE

In order to achieve precise migration times, the EOF must be well controlled. Under many conditions, dynamic coatings are required to obtain precision of the highest order [12]. One exception to that rule is when low pH buffers, particularly phosphate buffers, are utilized. The EOF is naturally low in the low pH region and phosphate buffer is known to coat the capillary wall [13].

When the EOF and the solute migration are in the same direction, this is known as co-migration. This occurs naturally when cations are separated since both the EOF and solute migrations are directed toward the cathode or negative electrode. When the EOF and solute migrations are in opposite directions, this is known as countermigration. Countermigration is advantageous in CE since the solute has to swim upstream against the EOF. This prolongs the length of time the solute spends on capillary allowing the more time for solute resolution. It is more efficient than increasing the capillary length because field strength is not lost.

Countermigration is frequently applied at high pH. Under these conditions, anions attempt to migrate upstream to the positive electrode but are swept to the negative electrode by the unrelenting EOF. At low pH, the situation is different. As Fig. 3 illustrates, the negatively charged GAGs outrun the slow EOF and reach the positive electrode. This means that the system must be operated using reversed polarity and injection is made at the cathodic end of the capillary by the negative electrode. These nomenclatures are not

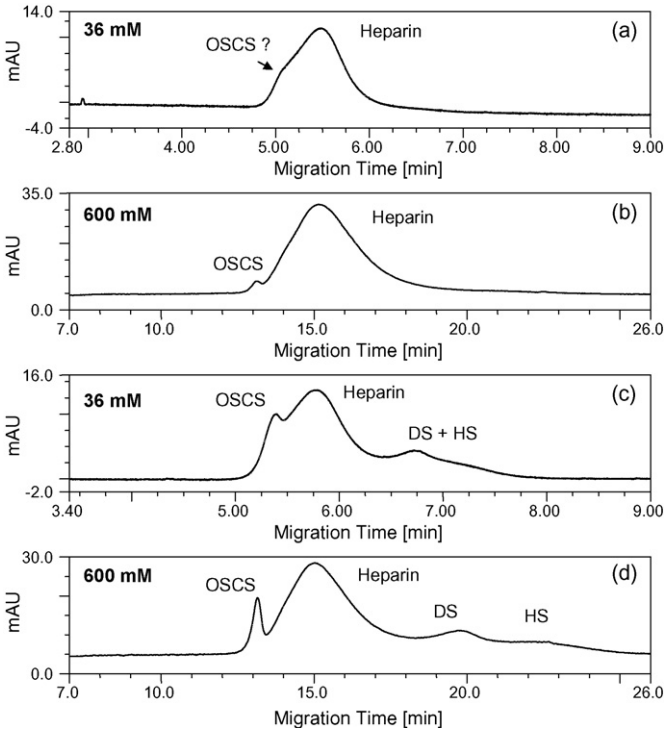

Fig. 5. (a and c) Official method. Conditions: capillary: 50 μm i.d. × 56 cm effective × 64.5 cm total length, temperature: 25 °C, voltage: −30 kV and injection: 500 mbs at cathodic end. (b and d) Enhanced method. Conditions: capillary: 25 μm i.d. × 56 cm effective length × 64.5 cm total length, temperature: 35 °C, voltage: −18.5 kV and injection: 2000 mbs cathodic.

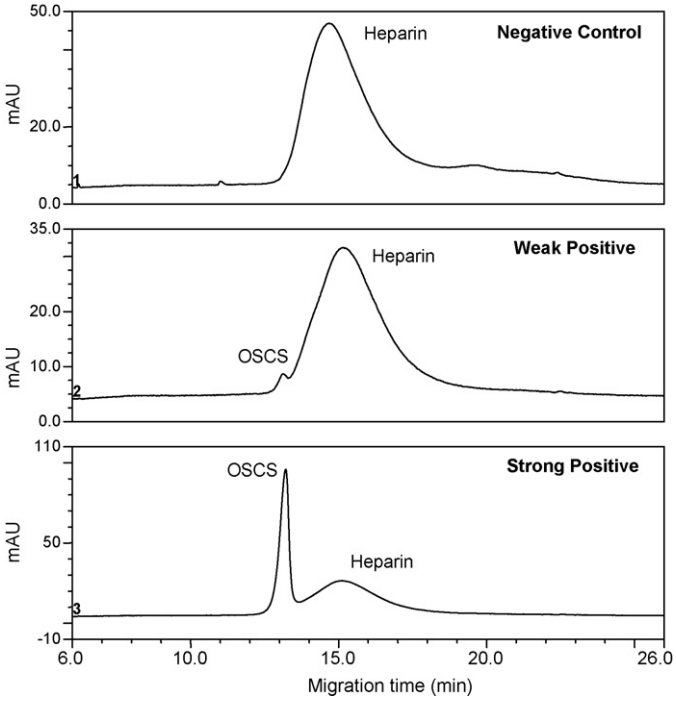

Fig. 6. 600 mM phosphate buffer, pH 3.5 phosphate, 25 μm × 56 cm effective length × 64.5 cm total length capillary, temperature 35 °C, voltage −18.5 kV and injection: 2000 mbs cathodic.

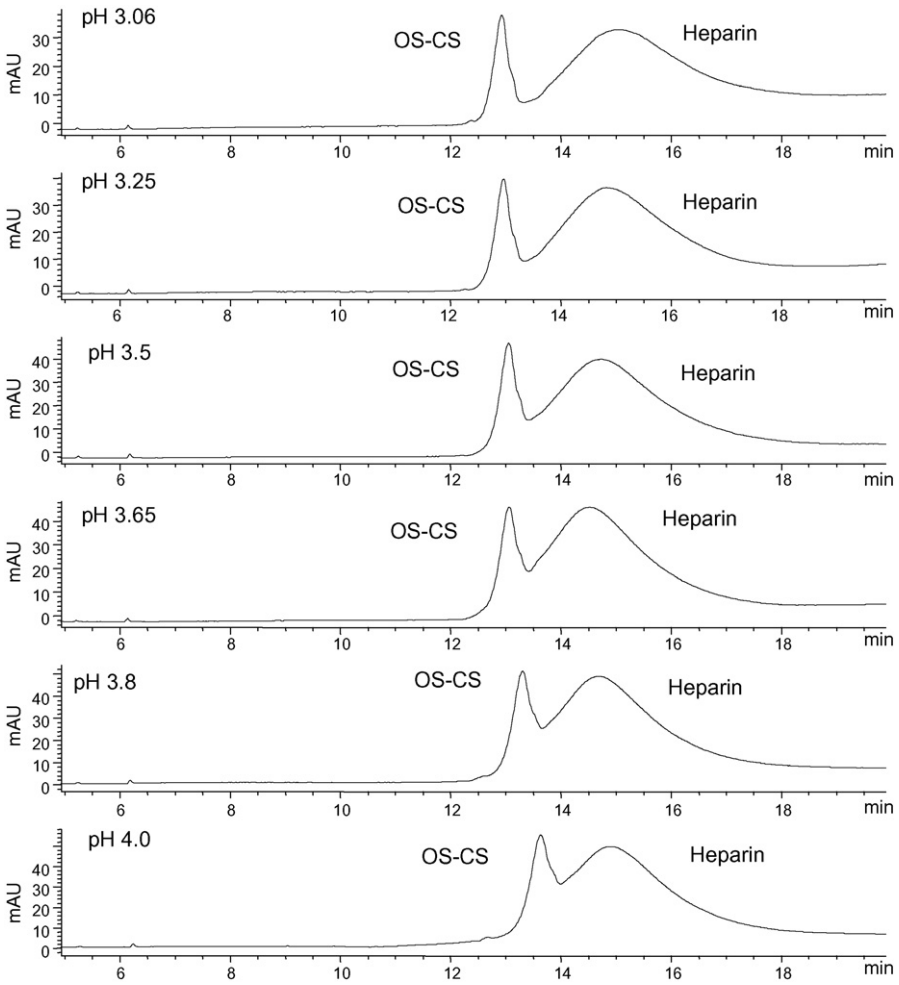

Fig. 7. Effect of pH on resolution. 600 mM phosphate buffer, 25 μm × 56 cm effective length × 64.5 cm total length capillary, temperature 35 °C, voltage −18.5 kV and injection 2000 mbs cathodic.

completely understood by some practitioners in the field causing conflicting terminology to appear in some methods.

3.2. Impact of buffer concentration

Phosphate buffer was the buffer of choice for heparin separations. Its favorable properties include optical clarity in the low ultraviolet region of the spectrum, good buffer capacity at low pH and ready availability with numerous counterions.

The impact of buffer concentration on the separation of heparin from OSCS is shown in Fig. 4. When the buffer concentration was increased from 36 to 100 mM, the migration times of the two solutes were not changed. The peak height for OSCS increased while the peak width decreased. This was due to more effective sample stacking from the more concentrated buffer. The steadiness of the migration times was unusual. Since solute mobility decreases with increasing buffer concentration, the EOF must decrease equally as well by coincidence.

Next, the buffer concentration was increased to 200 mM. In order to compensate for high current as a result of the high buffer concentration, a 25 μm capillary was used. The capillary length was 28% longer as well. This, coupled with the reduced field strength due to added capillary length, should have produced a migration time for OSCS of 8 min; however, a time of 10 min was observed. This means that the higher buffer concentration reduced solute mobility to a degree that was greater than the reduction of EOF. Increasing the buffer concentration to 500 mM confirmed that observation. The migration times continued to increase and the OSCS began to move down the front of the heparin peak indicating improved resolution. The current under these conditions was an acceptable −53.8 μA. Had a 50 μm i.d. capillary been used under comparable conditions, the current would be at least 215 μA. The optimal buffer concentration for this enhanced method was 600 mM. At higher buffer concentrations resolution began to decrease probably due to Joule heating effects.

The advantages of such high concentration buffers go beyond improving solute resolution. Sample stacking was dramatically improved. This meant that large injections of highly concentrated heparin samples could be used thereby improving the limits of detection of the impurities. Injections of 2000 mbs were used without any noticeable loss of resolution compared to smaller injections. Typical CE injections are much smaller, even when a 25 μm i.d. capillary is employed. Heparin concentrations as high as 60 mg/mL were used, although 20–30 mg/mL were the usual concentrations used for these studies. Typical solute concentrations in CE are often only 1–2 mg/mL.

The high concentration buffer greatly improved the resolution of the OSCS and heparin but the separation time doubled from 15 to 30 min and a 25 μm i.d. capillary was required. High-resolution methods were required to resolve ambiguities between the FDA's CE and NMR methods. We observed several cases where the NMR method gave positive results for OCSC whereas the official CE method produced negative results. The high resolution CE method

resolved the differences between the two methods as both now produced positive tests for OSCS.

Data for one of these ambiguous lots of heparin is shown in Fig. 5. Fig. 5a, which was produced with the official method only showed a slope change on the front of the heparin peak that would not be recognized as a positive result. The enhanced method (Fig. 5b) unambiguously established the presence of OSCS.

Other GAGs such as dermatan sulfate and heparan sulfate find their way through the heparin purification process and are known to occur in finished product. As shown in Fig. 5c (official method) one or more unresolved peaks migrate on the tailing end of the heparin peak. The enhanced method (Fig. 5d) better separates and distinguishes these materials compared to the official method. The later eluting peaks were later determined to be DS and HS.

Fig. 6 shows a comparison of a strong positive sample (signified by OSCS intensity) with a weak positive sample and a sample known to be free of OSCS by the NMR method, thereby establishing the specificity of the enhanced method.

3.3. Impact of buffer pH

In their 2008 paper, Patel et al. [8] found pH 3.5 to be optimal. They found poor signal and peak shape at pH 2.0 and suggested that heparin might partially degrade under acidic conditions. These data were gathered at a buffer concentration of 50 mM. Since we observed very different behaviors when high concentration buffers were employed, we revisited the impact of pH.

Fig. 7 shows separations of heparin and OSCS covering a pH range of approximately 3–4. As the pH was progressively lowered, the separation between the two solutes continued to improve. The optimal pH was found to be 2.8 (data not shown). At that pH, OSCS is separated into three separate peaks that are partially resolved from OSDS and OSHS. A prolonged run time might be required to better separate these solutes. Since they are all adulterated impurities, we determined that it was best not to separate them, at least during scouting runs.

The lower pH also resulted in prolonged run times and broad peaks for dermatan sulfate and heparan sulfate. Since it was also important to separate these solutes, pH 3.5 was selected as a compromise for most studies. The aforementioned heparin degradation which we may have observed at pH 1.8 was not present at pH 2.8 (data not shown).

3.4. System operation with 25 μm i.d. capillaries

Needless to say, it is important to filter all buffers and samples using 0.21 μm syringe filters. Cellulose acetate filters were used in these studies and no loss of solutes was noted due to filtration. Lack of buffer filtration did not necessarily cause clogging but an increase in baseline noise was noted in at least one instance.

No special capillary preparation was required. It was not necessary to pre-condition the capillary with sodium hydroxide, as is often a common practice. No inter-sample wash other than a 5 min buffer flush was required. While the system could be operated almost immediately after installation of a new capillary, the baseline became more stable after hours of use. It was best to fill the capillary with buffer and let it equilibrate overnight prior to use. While not performed on these studies, it was later found that an inter-sample wash of 1 min with 0.1N sodium hydroxide and 1 min with 600 mM phosphoric acid reduced the OSCS blank to non-detectable.

The capillary was best stored in buffer. If the instrument was to be idle for more than a few days it was prudent to flush the buffer out of the capillary. Dry storage of the capillary was not always successful, as the restored capillary did not come back the same way. Storage of the capillary in water was successful during intermittent use for at least 1 month. Since capillaries were inexpensive, fresh capillaries were used rather than attempt regeneration of an air-stored capillary.

To optimize the LOD of the system, 25 μm capillaries with a "bubble-cell" were used. These commercially available capillaries provided a 5× increase in the optical pathlength of the capillary without incurring significant bandbroadening. The system was found sufficiently sensitive that a straight capillary still provided acceptable results.

3.5. Quantitative analysis

Quantitation was confounded by the lack of certified reference standards. Normalized area percent could be employed but this introduces inaccuracy if the molar absorptivity of the solutes are different. In fact OSCS and DS had greater molar absorptivities compared to heparin. DS and HS were purchased from a company specifying purity of greater than 90%. With this assumption, at

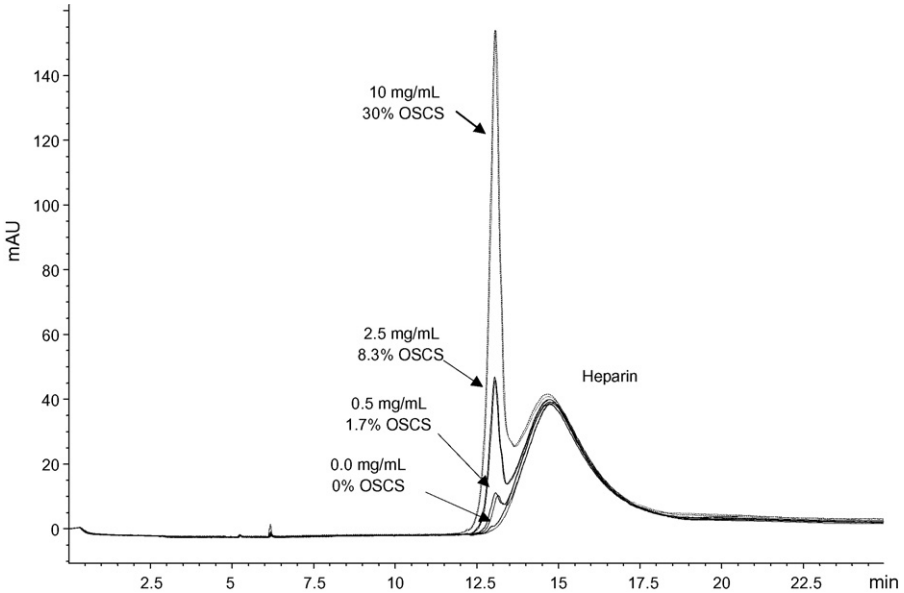

Fig. 8. 600 mM phosphate, pH 3.5, capillary: 25 μm i.d. × 56 cm effective × 64.5 cm total, temperature 35 °C, voltage: −18.5 kV and injection: 2000 mbs cathodic.

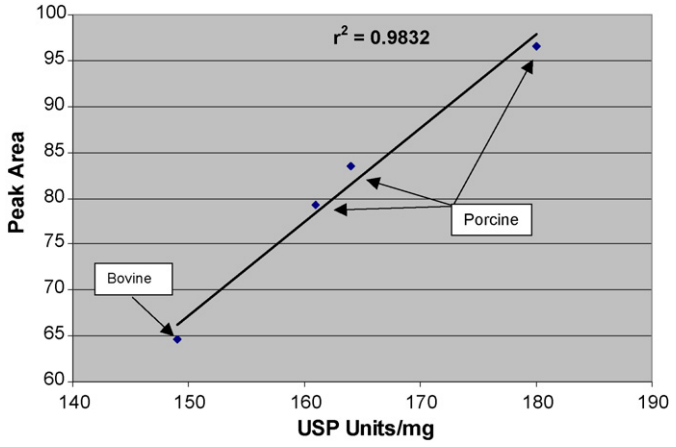

Fig. 9. Porcine and bovine intestinal heparin peak area versus USP activity. 600 mM phosphate, pH 3.5, capillary: 25 μm i.d. × 56 cm effective × 64.5 cm total, temperature 35 °C, voltage: −18.5 kV and injection: 2000 mbs cathodic.

worst the impurity concentrations are overestimated by 10% so the method errs on the side of safety.

A standard curve for OSCS, run in duplicate, is shown in Fig. 8. The standard curve was linear and passed near the origin when a tangential skim is used for peak area integration. In actual practice, the origin was forced and the correlation coefficient equaled 0.9994. Since the migration times were so precise, it was not necessary to use corrected peak areas. The LOD was easily less than 0.1% of total heparin. Calibration data for DS and HS were also linear (data not shown). The LOD for DS was estimated to be about 0.5% of total heparin. The peak for HS was broader than the other impurities and gave an estimated LOD of 2% of total heparin. In practice, it will not be necessary to calibrate the system using external standards once accurate molar absorptivities are known for heparin and its impurities. When that occurs, calculations based on normalized peak area % will prove sufficient.

Intermediate precision was assessed by performing 10 runs each day over 3 days. The method is very repeatable, even for a sample fortified with only 0.85% OSCS and DS. The run-to-run peak area %R.S.D. ranged from 1.7 to 1.8% for OSCS, 3.7 to 5.5% for heparin and 1.6 to 1.7% for DS.

3.6. Correlation of the CE method to the heparin bioassay

The USP activity of heparin is assigned based on the result of a multi-step anti-factor X_a activity assay [11]. Since the enhanced CE method appears specific, the relationship between the heparin peak area and biological activity was tested using four lots of heparin (three porcine intestinal and one bovine intestinal). The data, illustrated in Fig. 9 show a linear relationship between CE peak area and USP bioactivity even in samples with detectable amounts of dermatan sulfate. These results can provide an additional data point for heparin potency above and beyond the bioassay and at some point offer an alternative approach to the bioassay.

3.7. High speed, high resolution separation

While we were quite pleased with the performance of this method, we then considered approaches to reduce the separation time. The data shown in Fig. 7 indicated that the resolution between OSCS and heparin are improved at lower pHs. This means that a shortened capillary can be used.

The phosphate buffer counter-ion was changed from sodium to lithium. Lithium has a lower mobility compared to sodium because of its large hydration sphere. That means lithium buffers have lower conductivity compared to sodium buffers and as a result, the field strength can be increased.

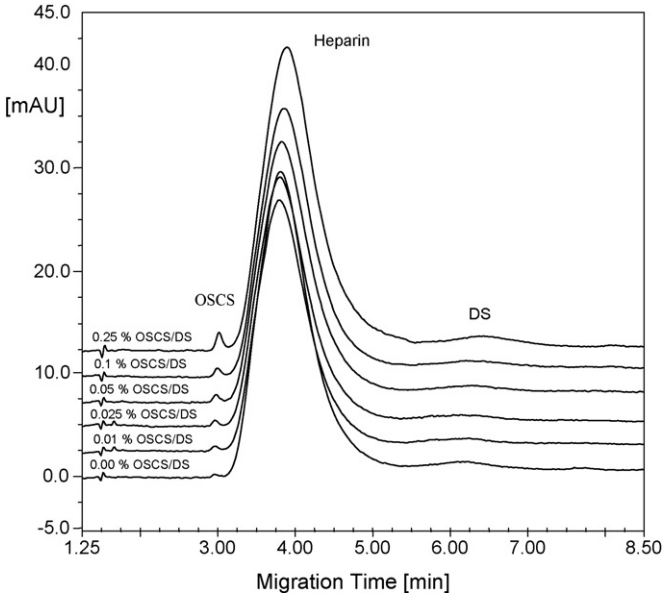

Fig. 10. High-speed method, 600 mM Lithium phosphate pH 2.8, capillary: 25 μm i.d. × 21.5 cm effective length × 30 cm total length, temperature: 20 °C, injection: 600 mbs and voltage: −14 kV.

When the shortened capillary was combined with lithium phosphate buffer, the separation was dramatically improved and the run time was very short. These results are illustrated in Fig. 10 for a low concentration calibration run. Baseline resolution was obtained between OSCS and heparin. The LOD is well below 0.1% (w/w of total heparin concentration) for OSCS despite the appearance of a small peak in the blank. The method is being fine tuned and validated and these results will be presented in a future paper. Separations of other GAGs are also being studied using this new method.

4. Conclusion

The data presented in this paper demonstrates substantial improvements in resolution and LOD for impurities found in heparin compared to existing CE methodology. The method is sensitive, selective, robust, reproducible, and capable of identifying and quantifying OSCS, DS and HS in a single analysis without sample pretreatment such as depolymerization. The method does not require expensive columns, is simple to implement and run, is very repeatable and easily transferred from lab to lab and also has the potential of measuring the USP activity of a sample.

Acknowledgments

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