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A Faster Solution with Increased Resolution for Determining Chromatographic Identity and Absence of OSCS in Heparin Sodium

INTRODUCTION

Heparin is a complex highly sulfated glycosaminoglycan that has been used since the early 20th century as an anticoagulant for the treatment of thrombosis.¹ It is estimated that more than 10 million Americans receive heparin annually and more than 70 million vials are sold for cardiac surgery, dialysis, and a variety of other uses. In 2008, researchers detected a contaminant now known as oversulfated chondroitin sulfate (OSCS) in heparin samples that was associated with life-threatening allergic reactions in hundreds of patients. The samples were also determined to contain the impurity dermatan sulfate (DS), which is commonly found in heparin due to incomplete purification.²

To address the immediate health concerns, nuclear magnetic resonance (NMR) and capillary electrophoresis (CE) methods were rapidly developed to detect OSCS in the suspected heparin samples.³ Although CE was able to detect the contaminant, OSCS and DS were not fully resolved from heparin. Due to these and other inherent challenges of implementing CE in a quality control environment, the United States Pharmacopeia (USP) published a chromatographic method that resolved DS and OSCS from heparin.⁴ The current USP Heparin Sodium monograph contains a chromatographic identity method (Identification B) that prescribes the separation of DS and OSCS from heparin using a USP type L61 column (IonPac® AS11) with a sodium perchlorate gradient followed by absorbance detection at 202 nm.⁵ Additional information on this method can be found in Dionex Application Note 235.⁶

While this method is able to achieve the objective of resolving the target compounds from heparin, the resolution between DS and heparin often just meets the USP specification of not less than (NLT) 1.0. In addition, the analysis run time is 75 min, and using sodium perchlorate as the eluent raises environmental and human health concerns. Therefore, there is significant opportunity to improve this analytical method for the determination of DS and OSCS in heparin.

Alternative chromatographic methods have been investigated to improve the current USP method for the determination of heparin. For example, weak anion-exchange (WAX) stationary phases were evaluated to provide alternative columns to the USP method, which specifies a strong anion-exchange column. Although one publication demonstrated improvement in the method by increasing sample throughput by significantly decreasing run times, the separation still required perchlorate to resolve DS and OSCS from heparin.⁷ Larive et al. also demonstrated that a WAX column could be used to resolve these compounds.⁸ In this example, a mildly alkaline mobile phase was used, but the sensitivity of OSCS was inferior to the current USP method.

In 2009, researchers at the United States Food and Drug Administration (US FDA) published a method using the IonPac AS11-HC column with a sodium chloride in Tris gradient to resolve the target compounds from heparin.⁹ This method provides several benefits over the current USP method, such as eliminating the need for perchlorate in the mobile phase, reducing the analysis time from 75 min to 40 min, and improving the resolution between DS and heparin.

The testing here demonstrates an improved method for resolving DS and OSCS in heparin with an IonPac AS11-HC (2 × 250 mm) column using a NaCl gradient followed by absorbance detection at 215 nm, which is similar to the US FDA method previously described. The higher capacity of the IonPac AS11-HC column, relative to the IonPac AS11 column described in the current USP method, minimizes the possibility of column overload and provides improved resolution between DS and heparin. The microbore column format was chosen to reduce eluent consumption and thereby reduce the time and labor required for manual eluent preparation. This study demonstrates the method's ruggedness and precision, and the ability to detect <1% (w/w) DS and OSCS in heparin.

EQUIPMENT

Dionex ICS-3000 system^a including:

SP Single Pump module with a GM-4 gradient mixer
TC Thermal Compartment with a 6-port injection valve

VWD Variable Wavelength Detector 3400 with PEEK™ semi-micro flow cell (2.5 µL, 7 mm, (P/N 6074-0300)

AS Autosampler with Sample Tray Temperature Controlling option, 100 µL sample syringe (P/N 055064), and 1.5 mL sample tray

Chromeleon® 7.1 Chromatography Data System (CDS) software

Vial Kit, 1.5 mL glass with caps and septa (P/N 055427) or 0.3 mL polypropylene sample vials with caps and slit septa (P/N 055428)

Nalgene® Media-Plus with 90 mm, 0.45 µm nylon filter (Nalge Nunc International P/N 164-0020) or equivalent nylon filter

Vacuum pump

10 µL PEEK sample loop (P/N 042949)

pH Meter with pH electrode

Magnetic stirrer

^aThis application can be performed on a Dionex ICS-5000 system.

REAGENTS AND STANDARDS

Deionized (DI) water, Type 1 reagent-grade, 18.2 MΩ-cm resistivity

Use only ACS reagent-grade chemicals for all reagents and standards

Heparin, Grade 1A from porcine mucosa (Sigma-Aldrich P/N H-3393)

Heparin Sodium Identification Standard (9.3 mg, USP P/N 1304038)

Heparin Sodium System Suitability Standard (>90% heparin, <10% oversulfated chondroitin sulfate, 50 mg, USP P/N 1304049)

Chondroitin sulfate B (dermatan sulfate, sodium salt), sodium salt from porcine intestinal mucosa, >90% lyophilized powder (Sigma-Aldrich P/N C3788)

Sodium chloride (FW 58.44, VWR International P/N JT3624-19)

Tris hydrochloride (Tris[hydroxymethyl]aminomethane hydrochloride, FW 157.1, VWR International P/N EM1.08219.9025)

pH 7 buffer (VWR International P/N BDH5046)

pH 4 buffer (VWR International P/N BDH5018)

Phosphoric acid, 85–87% (H₃PO₄, VWR International P/N JT0260)

CONDITIONS

Column: IonPac AS11-HC Guard, 2 × 50 mm (P/N 052963)

IonPac AS11-HC Analytical, 2 × 250 mm (P/N 052961)

Mobile Phases: A: DI water
B: 2.5 M Sodium chloride, 20 mM Tris (pH 3)

Gradient: See Table 1.

Flow Rate: 0.20 mL/min

Column Temp.: 40 °C

Sample Volume: 10 µL

Detection: UV, 215 nm

Background: 0–15 mAU over the gradient

Noise: <100 µAU

System

Backpressure: 1550 psi

Run Time: 40 min

Table 1. Gradient Conditions		
Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0	95	5
2	95	5
26	5	95
31	5	95
32	95	5
40	95	5

PREPARATION OF SOLUTIONS AND REAGENTS

General Tips

Use high quality, Type 1, 18.2 MΩ-cm resistivity DI water to prepare mobile phase solutions and standards. Prepare 1 L of degassed DI water weekly for the AS Autosampler flush solution using vacuum filtration. Use glass containers for storing and preparing mobile phase solutions to minimize leachable compounds from plastic containers that can result in increased chromatography noise and contaminant peaks.

3 M Phosphoric Acid for pH Adjustment

Add 1 mL of 85% phosphoric acid to 4 mL DI water and mix thoroughly. This solution will be used to adjust the pH of Mobile Phase B. Dispense 500 µL aliquots of 3 M phosphoric acid using a micropipettor with a filtered pipette tip to prevent the acid from damaging the micropipettor.

Mobile Phase Solutions

Mobile Phase A: Deionized Water

Degas 2 L of DI water. Transfer the DI water to a 2 L eluent bottle, connect the bottle to the Mobile Phase A line, and place the mobile phase under 5 psi of inert gas, such as nitrogen. Prime the pump with the new mobile phase.

Mobile Phase B: 2.5 M Sodium Chloride, 20 mM Tris (pH = 3)

Prepare 1 L of Mobile Phase B by weighing 146.1 g of sodium chloride and 3.14 g of Tris hydrochloride (FW 157.1) into a 1 L volumetric flask and dissolve the reagent in ~800 mL of deionized water. Stir with a magnetic stirrer until the reagents are fully dissolved. Remove the stir bar, dilute to the 1 L mark, and mix by inverting the flask. Carefully re-add the stir bar and stir while adjusting to pH 3.0 with 3 M phosphoric acid. Typically, 5–40 µL is used to adjust a 1 L solution to pH 3.0. Filter and degas using vacuum filtration with applied vacuum for 10 min. Transfer the mobile phase to a separate 2 L glass bottle, immediately cap the bottle, connect it to the corresponding mobile phase line, and place the mobile phase solutions under ~4–5 psi of nitrogen or other inert gas. Prime the pump with the new mobile phase solution and equilibrate the column for 1 h at the starting conditions prior to use.

Standard Solutions

Prepare DS, Heparin System Suitability RS, heparin stock standard, and 1% (v/v) System Suitability working solutions according to the instructions described in AN 235.⁵

Note: A 300 µL volume is sufficient for 6 full-loop injections of 10 µL (2 × the sample loop volume plus 25 µL used for small loops) or 15 partial-loop injections of 10 µL from a 25 µL loop with a 5 µL cut volume (2 × cut volume plus the injection volume).

SYSTEM PREPARATION AND CONFIGURATION

To configure the system, follow the instructions in AN 235 and the system and column product manuals. Use red PEEK (0.005 in i.d.; 0.013 mm i.d.) tubing for all liquid lines from the injection valve to the detector. Assemble the UV semi-micro PEEK cell according to the instructions in AN 235.

Note: The semi-micro PEEK cell was selected for the short flow path and suitability of the PEEK material for the acidic high-salt mobile phases used here.

RESULTS AND DISCUSSION

In this study, the separation of DS from heparin was evaluated using similar conditions published in the literature⁷⁻⁹ with the objectives of improving the resolution of DS from heparin, avoiding a perchlorate-based mobile phase, and reducing the analysis time. The initial investigation evaluated the feasibility of using the ProPac® WAX-10 column, the ProSwift® WAX-1S column, and the ProSwift SAX-1S monolith column for this application. The target analytes were separated using either conditions similar to those described in the current USP monograph, a NaCl gradient in phosphate buffer at pH 3, or a NaCl gradient in phosphate buffer at pH 6–10 with absorbance detection at 202 or 215 nm. For the ProPac WAX-10 column, the separation of DS and heparin required perchlorate to elute the analytes from the column. In addition, the resolution between these compounds did not meet the USP specification of NLT 1. At higher mobile phase pH (6–10), the compounds were not retained on the column and therefore eluted at or near the void. For the ProSwift WAX-1S and SAX-1S columns, DS and heparin could not be resolved using a NaCl mobile phase with either Tris or phosphate at pH 3.

Researchers at the US FDA demonstrated that the IonPac AS11-HC column could separate DS and OSCS from heparin using a NaCl gradient in Tris at pH 3 that met the objectives described above.⁹ Therefore, further investigation and minor modifications to this approach were made to produce an improved method for determining the target compounds in heparin sodium. The modifications included using a 2 mm IonPac AS11-HC column and increasing the column temperature from 35 to 40 °C, which produced slightly better results.

Figure 1 demonstrates the separation of 0.2 mg/mL DS and OSCS from 20 mg/mL heparin. To displace heparin from the column, a significantly higher salt concentration (2.5 M NaCl) is required relative to the perchlorate concentration in the current USP method. However, this was expected due to the weaker eluting effect of chloride relative to perchlorate. In addition, the IonPac AS11-HC column has more than six times the capacity of the IonPac AS11 column and, therefore, requires greater eluent strength.

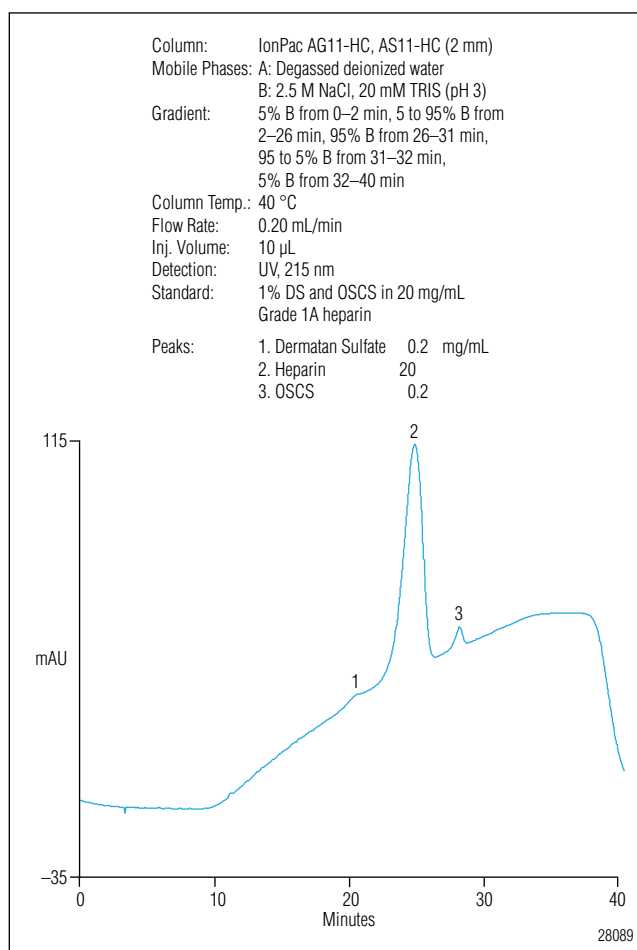


Figure 1. Heparin standard separation prior to baseline subtraction.

Using the conditions described in this study, the resolution between DS and heparin was 1.9, which is nearly double the USP specification and therefore a significant improvement relative to the current monograph. All analytes eluted from the column in about 40 min, which is nearly half the time required for the current USP method. To correct for the increase in absorbance during the gradient, the baseline was subtracted from a DI water injection using the Chromeleon CDS processing method (Figure 2). The baseline subtraction improves peak integration and therefore the method's accuracy and precision.

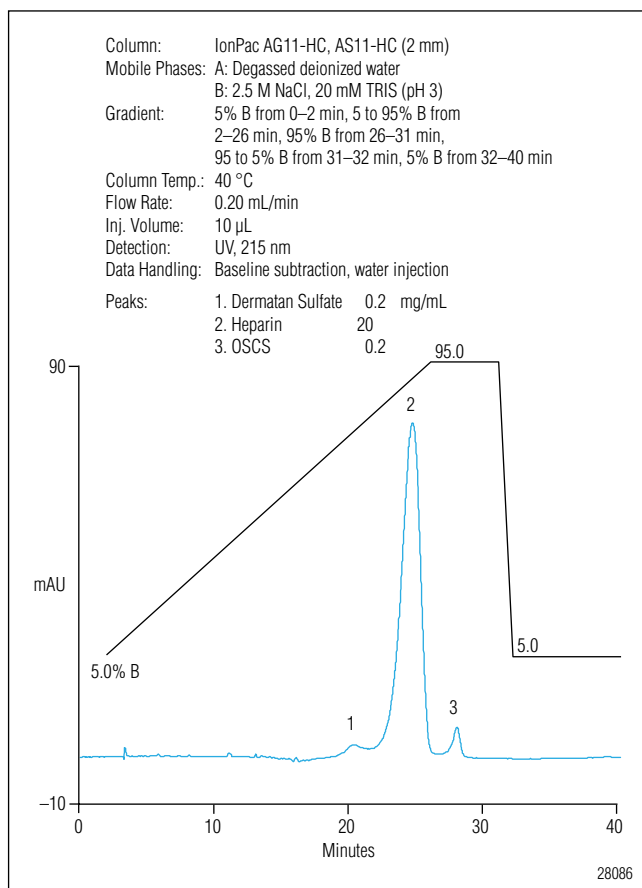


Figure 2. Separation of 1% dermatan sulfate and OSCS in 20 mg/mL heparin on an IonPac AS11-HC column using a salt in Tris gradient, UV detection, and baseline subtraction.

The method performance was evaluated by determining the between-day precision from 82 injections over three days, the effect of daily mobile phase preparation, and differences between a column temperature of 35 and 40 °C. In addition, two different IonPac AS11-HC column lots were investigated to evaluate any potential variability in the separation.

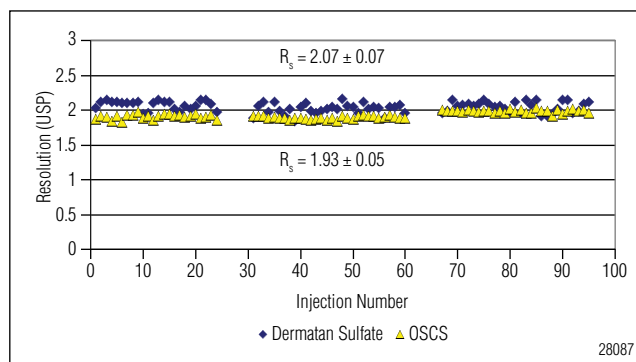


Figure 3. Resolution reproducibility over three days.

The between-day retention time and peak area precisions (RSDs) for the three compounds were <0.5 and ≤6.0, respectively. As shown in Table 2, the peak area precision for heparin was <0.6, which is well within the USP specification of NMT 2. The resolution between DS and heparin had an average R_s value of 2.0 ± 0.07 and the resolution between heparin and OSCS had an average R_s value of 1.93 ± 0.05 over the three-day study. In comparison, the DS/heparin and heparin/OSCS resolutions using the conditions described in the current USP method were determined to be 1.04 ± 0.07 and 1.76 ± 0.12 , respectively in AN 235. Therefore, this method significantly improves the resolution between these compounds, while avoiding sodium perchlorate in the mobile phase.

Figure 3 demonstrates the stability of the resolution between DS and OSCS from heparin for 82 injections over three days, which indicates no loss in column capacity. Column temperatures of 35 and 40 °C were also evaluated as part of this study. Although the results for the target compounds were not significant between the two temperatures, a column temperature of 40 °C provided a slight improvement in peak response for OSCS and therefore was used for this method.

Table 2. Summary of Reproducibility Experiments Over Three Days^a

Analyte	Retention Time (min)	RSD	Peak Area (mAU-min)	RSD	Resolution from Heparin (R_s)	RSD
Dermatan Sulfate	20.3 ± 0.08	0.37	1.59 ± 0.10	6.0	2.04 ± 0.07^c	3.7
Heparin	24.6 ± 0.04	0.17	119.3 ± 0.65	0.55 ^b		
OSCS	27.9 ± 0.12	0.43	4.14 ± 0.17	4.1	1.93 ± 0.05^d	2.5

^an = 82

^bUSP requirement is NMT 2 for n = 3

^cUSP requirement is NLT 1

^dUSP requirement is NLT 1.5

In comparing the separation between two different columns lots, the retention times, peak area responses, and resolutions were nearly identical. These results demonstrate the method robustness and therefore the ability of quality control laboratories to use this method for routine analysis.

CONCLUSION

This study demonstrates an improved separation of 1% DS and OSCS from heparin using an IonPac AS11-HC column with a NaCl/Tris (pH 3) mobile phase and UV absorbance detection at 215 nm. The method takes advantage of the high capacity and strong anion-exchange properties of the column to improve the resolution of the two contaminants in heparin. In addition, the method eliminates the need for sodium perchlorate in the mobile phase, but still reduces the analysis time nearly 50% compared to the current USP method. This study also demonstrates good column stability based on consistent resolution between heparin and the critical contaminants over three days. In addition, the higher capacity of the IonPac AS11-HC column relative to the IonPac AS11 column reduces the possibility of column overload and should improve the detection of DS and OSCS in heparin. This study provides a faster more sensitive method to detect OSCS and DS contamination in the worldwide heparin supply, thus preventing future OSCS-related fatalities.

SUPPLIERS

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VWR International, Inc., Goshen Corporate Park West,
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LPN 2715 PDF 09/16
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