

# Evaluation of the Two Ion Chromatography Methods in the Proposed Revision of the United States Pharmacopeia (USP) Heparin Sodium Monograph



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## INTRODUCTION

In late 2007 and early 2008, a contamination of the drug heparin led to adverse reactions in some patients, and to the death of other patients. The contamination occurred from over-sulfated chondroitin sulfate (OSCS) and was not detected by the existing USP and European Pharmacopeia monograph methods for sodium heparin. To correct this deficiency, the USP published a revision bulletin on June 18, 2008 for the heparin sodium monograph. Based on comments received concerning that publication, the USP further revised the heparin sodium monograph in March 2009. Two methods in the proposed revision are ion chromatography (IC) methods. One method for heparin identification uses an IonPac<sup>®</sup> AS11 column for separation and UV detection. The second IC method is an impurity test that measures the percent galactosamine of the total hexosamine; this test uses a CarboPac<sup>®</sup> PA20 column for separation and pulsed amperometric detection (PAD). The authors evaluated both methods for reproducibility and ruggedness. The identification test separates dermatan sulfate and OSCS from heparin. This test was evaluated by measuring the effect of eluent concentration, eluent pH, sample load, and the column on method reproducibility and ruggedness; it was determined that eluent concentration and sample load impact the method's ability to meet the USP acceptance criteria. The impurity test measures galactosamine and glucosamine after acid hydrolysis. The authors evaluated this test by measuring the effect of guard column removal, AminoTrap<sup>™</sup> column removal, and use of manually prepared eluent versus eluent prepared by an eluent generator. To evaluate method accuracy, The authors performed spike recovery experiments. Our studies revealed the method to be both rugged and accurate. This presentation will review the two IC methods and the results of these studies.

## EXPERIMENTAL

### Method 1 (USP Heparin Sodium Monograph Chromatographic Identification)

Dionex ICS-3000 Chromatography system consisting of:

SP Single Pump or DP Dual Pump module with a GM-4 (2 mm) Gradient Mixer column

TC Thermal Compartment with a 6-port injection valve

VWD Variable Wavelength Detector with PEEK<sup>™</sup> semi-micro flow cell, 2.5  $\mu$ L, 7 mm

AS Autosampler with Sample Tray Temperature Controlling option, 100  $\mu$ L sample syringe and 1.5 mL sample tray

Chromeleon<sup>®</sup> 6.8 Chromatography Data System

Bottles, 1 L or 2 L (two each), glass coated, GL45 for mobile phase solutions

Vial Kit, 1.5 mL glass with caps and septa or 0.3 mL polypropylene sample vials with caps and slit septa

### Method 2 (USP Heparin Sodium Monograph Organic Impurities)

Dionex ICS-3000 consisting of:

SP Single Pump or DP Dual Pump module

(Gradient pump required if manually prepared eluents are used)

EG Eluent Generator module (unless using only manually prepared eluents)

EluGen<sup>®</sup> EGC II KOH cartridge

Continuously-Regenerated Anion Trap Column, CR-ATC

Now sold under the  
Thermo Scientific brand

**Thermo**  
SCIENTIFIC

DC Detector/Chromatography module (single or dual temperature zone configuration)  
 AS Autosampler  
 ICS-3000 ED Electrochemical detector  
 Electrochemical cell  
 Disposable gold electrode, carbohydrate certified  
 Reference electrode  
 10 µL PEEK Sample injection loop  
 EG Vacuum Degas Conversion Kit  
 Chromeleon 6.8 Chromatography Data System  
 0.3 mL polypropylene injection vials with caps  
 For information on standards, standard and sample preparation, eluent preparation, and system configuration and setup, see Dionex Application Notes 233 (IC Method #2) and 235 (IC Method #1).<sup>1,2</sup>

## CONDITIONS

### Method 1

Column: IonPac AG11 Guard, 2 × 50 mm  
 IonPac AS11 Analytical, 2 × 250 mm  
 Flow Rate: 0.22 mL/min  
 Mobile Phases: A: 2.6 mM Sodium phosphate monobasic, pH = 3.0  
 B: 1.0 M Sodium perchlorate in 2.6 mM sodium phosphate monobasic, pH = 3.0  
 Gradient: See Table 1  
 Column Temp.: 30 °C  
 Tray Temp.: 10 °C  
 Inj. Volume: 10 µL  
 Detection: UV absorbance at 202 nm  
 Typical System  
 Backpressure: 1100–1300 psi  
 Run time: 75 min

### Method 2

Columns: AminoTrap column, 3 × 30 mm  
 CarboPac PA20 Analytical, 3 × 150 mm  
 -or-  
 AminoTrap column, 3 × 30 mm,  
 CarboPac PA20 Guard, 3 × 30 mm  
 CarboPac PA20 Analytical, 3 × 150 mm  
 (manual eluent only)\*

\* A July 2009 bulletin from the USP no longer recommends this 3 column format.

Eluent: 14 mM KOH from 10–0 min,  
 14 mM KOH from 0–10 min,  
 100 mM KOH from 10–20 min  
 Eluent Source: EGC II KOH with CR-ATC  
 -or-  
 200 mM KOH, manually prepared  
 Flow Rate: 0.5 mL/min  
 Temperature: 30 °C  
 Inj. Volume: 10 µL  
 Detection: PAD, Au (disposable)  
 Background: 5–25 nC (using the carbohydrate waveform)  
 System  
 Backpressure: ~2625 psi (using the AminoTrap 3 × 30 mm and CarboPac PA20 3 × 150 mm columns)  
 -or-  
 ~3010 psi (using the AminoTrap 3 × 30 mm, CarboPac PA20 3 × 30 mm guard, and CarboPac PA20 3 × 150 mm analytical columns as originally described by the USP)

**Table 1: Gradient Conditions**

| Time (min) | Mobile Phase A (%) | Mobile Phase B (%) | Elution                                                                   |
|------------|--------------------|--------------------|---------------------------------------------------------------------------|
| 0          | 80                 | 20                 | Start linear gradient.                                                    |
| 60         | 10                 | 90                 | End linear gradient. Start the linear gradient to the initial conditions. |
| 61         | 80                 | 20                 | Equilibrate at the initial conditions.                                    |
| 75         | 80                 | 20                 | End run.                                                                  |

## RESULTS

### Method 1

In the initial experiments it was determined that when using anhydrous salts, as described in the proposed USP monograph, the retention times for the three analytes (dermatan sulfate (DS), heparin, and OSCS) were shorter than reported. After consulting with the USP switched to monohydrated salts were used to achieve appropriate resolution and improved retention. Figure 1 shows the separation of 20 mg/mL USP heparin using these eluents.

Due to the heterogeneity of chain length in the heparin sample, the peak is broad. There is a rise in the baseline due to the gradient. This can be corrected by doing a post-acquisition baseline subtraction of a water injection. This subtraction was used for the chromatograms in Figure 2.

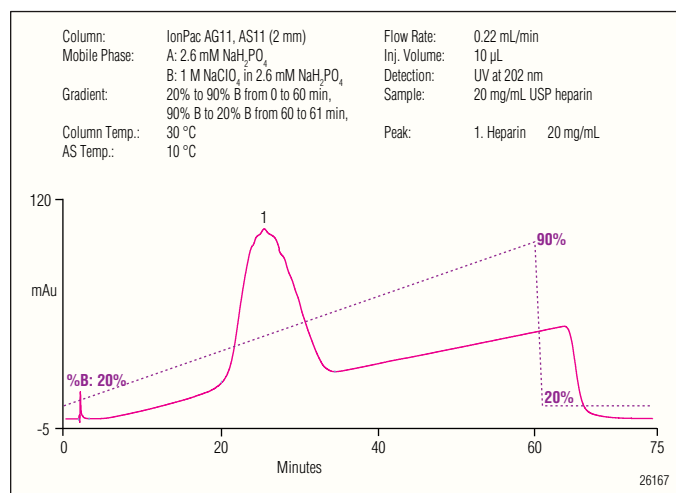


Figure 1. 20 mg/mL USP heparin identification standard.

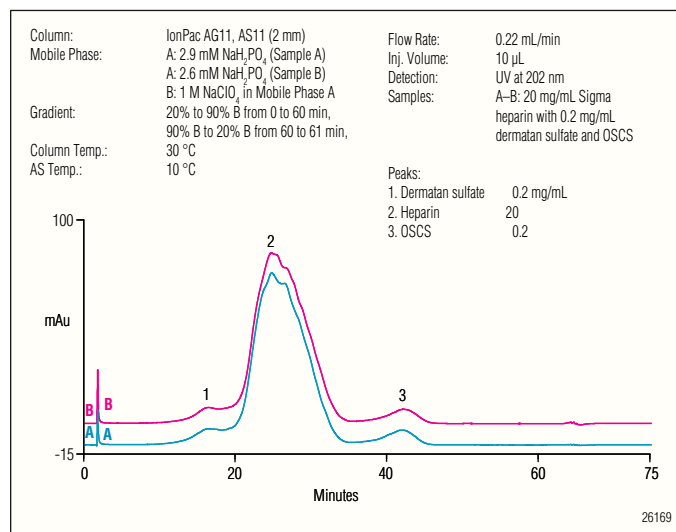


Figure 2. Comparison of 1% System Sustainability sample separated with mobile phase A prepared with A) monohydrate and B) dihydrate salts of sodium phosphate monobasic.

Figure 2 shows heparin is resolved from DS and OSCS using either the monohydrated or dihydrated monobasic sodium phosphate to prepare the mobile phase. The figure also shows that 1% DS and 1% OSCS are easily identified in a 20 mg/mL heparin sample. The separations in Figure 2 both exceeded the USP resolution requirements of not less than (NLT) 1.0 for DS and heparin ( $R_s = 1.1 \pm 0.1$ ) and NLT 1.5 for heparin and OSCS ( $R_s = 1.8 \pm 0.1$ ).

To evaluate method ruggedness, the authors tested small pH differences ( $\pm 0.1$ ) of the mobile phase and a change of column. Neither change significantly impacted the results, though for some samples the  $R_s$  of NLT 1.0 was not met ( $0.93 \pm 0.06$  for all samples) (see AN 235 for more details). Not meeting the  $R_s$  of 1.0 did not prevent identification of the OSCS.

The authors also evaluated ruggedness by injecting 310 samples over 27 consecutive days. By design, this study included multiple mobile phase changes and an injection of the standard sample shown in Figure 2 every 15 sample injections, with additional injections made at the beginning and end of the study. Retention times were stable throughout the study (Figure 3) and both resolution criteria were met on average for all standards ( $R_s$  for DS and heparin was  $1.04 \pm 0.07$ , and for heparin and OSCS  $1.76 \pm 0.12$ ), though as in the mobile phase pH study; the  $R_s$  of NLT 1.0 was not always met. The  $R_s$  values for the last three standard injection sets were higher than the first three, demonstrating no significant impact on the chromatography after 310 heparin injections.

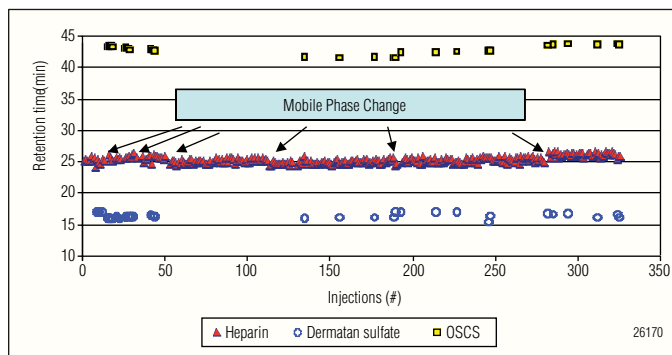


Figure 3. Retention time stability of 20 mg/mL heparin, 0.2 mg/mL dermatan sulfate, and 0.2 mg/mL OSCS over 27 days.

## Method 2

Heparin is composed of a glucosamine and a uronic acid. Therefore, after acid hydrolysis, a monosaccharide analysis should only yield glucosamine. Chondroitin sulfate and its synthetic derivative, OSCS, both contain a galactosamine. After acid hydrolysis, its monosaccharide analysis will yield galactosamine. This is also true for DS. Therefore an acid hydrolysis of a contaminated heparin lot will reveal galactosamine in addition to the expected glucosamine. This is the basis of the USP Heparin Sodium Monograph Organic Impurities Method.

Figure 4 shows the separation of galactosamine and glucosamine on a high-performance anion-exchange (HPAE) column, the CarboPac PA20, and an AminoTrap guard. These two amino sugars are easily resolved using either 14 mM KOH eluent prepared by an eluent generator or prepared manually. They can also be resolved with a manually prepared 14 mM NaOH eluent. The sugars are detected with high sensitivity by pulsed amperometric detection (PAD). HPAE-PAD is a commonly used technique for carbohydrate analysis, including monosaccharides.

This HPAE-PAD method easily detects a 1% adulteration of heparin by DS (Figure 4B).

The authors evaluated heparin and heparin spiked with 1% DS using the method shown in Figure 4 against the USP criteria. Each USP criterion for method performance was easily met. Resolution values ranged from 3.1 to 3.6 for all variations of the method, well over the USP limit of NLT 2. Asymmetry ranged from 1.1 to 1.4, well within the USP limit of 0.8 to 2.0, and the minimum efficiency was 4441, much greater than the USP limit of NLT 2000. Table 2 shows that this method easily identified the contamination with DS, and could detect as little as 0.04% galactosamine.

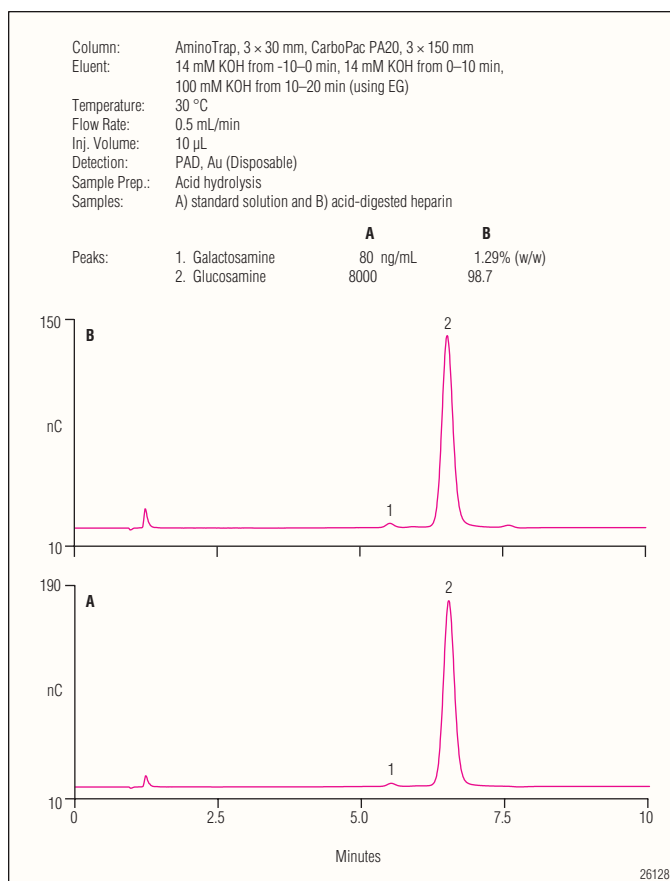


Figure 4. Separation of A) the standard solution and B) a digested heparin sample on the CarboPac PA20 column.

**Table 2. Comparison of Triplicate Heparin Analysis Results to USP Criteria When Using an EG Eluent\***

| Day | Sample                         | % GaIN (USP limit <1%) | RSD for % GaIN Determined | Standard Solution Response Factor |
|-----|--------------------------------|------------------------|---------------------------|-----------------------------------|
| 1   | Standard solution              | N/A                    | N/A                       | 1.16                              |
|     | Heparin, sample A              | 0.04                   | 3.3                       |                                   |
|     | 1% DS-spiked heparin, sample A | 1.29                   | 0.17                      |                                   |
| 2   | Standard solution              | N/A                    | N/A                       | 1.19                              |
|     | Heparin, sample A              | 0.04                   | 9.3                       |                                   |
|     | 1% DS-spiked heparin sample A  | 1.28                   | 0.07                      |                                   |
| 3   | Standard solution              | N/A                    | N/A                       | 1.16                              |
|     | Heparin, sample A              | 0.04                   | 9.0                       |                                   |
|     | 1% DS-spiked heparin, sample A | 1.40                   | 0.79                      |                                   |

\* AminoTrap and CarboPac PA20 analytical columns used.

In the initial publication, the method in the revised USP heparin sodium monograph used both an AminoTrap and a CarboPac PA20 guard in front of the CarboPac PA20 analytical column. When these three columns are used in series, the backpressure may exceed 3000 psi; this makes it difficult to use an eluent generator with a 3000 psi backpressure limit. Our results in Figure 4B and Table 2 suggest the extra guard is unnecessary, and in a July 2009 bulletin the USP no longer recommends the use of the extra guard column (PA20 guard). Before that announcement, the authors evaluated the effect of manually preparing either a KOH or NaOH eluent, and using a second CarboPac PA20 column on the effectiveness of this method (Table 3). In these experiments, the three columns were used in series. The authors determined that all configurations easily identified the contamination with DS, and all USP criteria were met.

**Table 3: Comparison of Triplicate Heparin Analysis Results to USP Criteria When Using Manually Prepared KOH or NaOH Eluents\***

| Instrumental Conditions                                               | Sample                         | % GaIN (USP limit <1%) | Standard Solution Response Factor |
|-----------------------------------------------------------------------|--------------------------------|------------------------|-----------------------------------|
| Manually prepared KOH proportioned to 14 mM CarboPac PA20<br>Column 1 | Standard solution              | N/A                    | 1.15                              |
|                                                                       | Heparin, sample A              | 0.03                   |                                   |
|                                                                       | 1% DS-spiked heparin, sample A | 1.40                   |                                   |
|                                                                       | Heparin, sample B              | 0.55                   |                                   |
| Manually prepared KOH proportioned to 14 mM<br>Column 2               | Standard solution              | N/A                    | 1.12                              |
|                                                                       | Heparin, sample A              | 0.03                   |                                   |
|                                                                       | 1% DS-spiked heparin, sample A | 1.30                   |                                   |
|                                                                       | Heparin, sample B              | 0.52                   |                                   |
| Manually prepared NaOH proportioned to 14 mM<br>Column 2              | Standard solution              | N/A                    | 1.12                              |
|                                                                       | Heparin, sample A              | 0.04                   |                                   |
|                                                                       | 1% DS-spiked heparin, sample A | 1.27                   |                                   |
|                                                                       | Heparin, sample B              | 0.53                   |                                   |

\*AminoTrap, CarboPac PA20 guard, and CarboPac PA20 analytical columns used.

## CONCLUSIONS

- The USP Heparin Sodium Monograph Chromatographic Identification method using the IonPac AS11 column proved to be rugged for 310 sample injections over 27 days (Figure 3) and was tolerant to small mobile phase pH differences and a change of column.
- The USP Heparin Sodium Monograph Organic Impurities method using the CarboPac PA20 column proved to be rugged to change of column, use of different guard column configurations, and different eluent preparation methods (manual or automatic, with an eluent generator) (Table 3).

## REFERENCES

1. Dionex Corporation. *Determination of Galactosamine Containing Organic Impurities in Heparin by HPAE-PAD Using the CarboPac PA20 Column*, Application Note 233, LPN 2286, Sunnyvale, CA, 2009.
2. Dionex Corporation. *Determination of Oversulfated Chondroitin Sulfate and Dermatan Sulfate in Heparin Sodium Using Anion-Exchange Chromatography with UV Detection*, Application Note 235, LPN 2306-01, Sunnyvale, CA, 2009.

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