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Determination of Galactosamine Containing Organic Impurities in Heparin by HPAE-PAD Using the CarboPac PA20 Column

INTRODUCTION

In early 2008, unusual reactions to heparin, including hypotension, swelling of the larynx, and in some cases death, prompted a recall of the product.^{1,2} The lots of heparin that led to these anaphylactic reactions were tested using existing reported methods and no additional components were found. After extensive investigation, it was determined that the heparin in question had been contaminated with oversulfated chondroitin sulfate.³

At the time of the recall, existing heparin assay methods could not detect chondroitin sulfates in heparin. For this reason, the U.S. Pharmacopeia (USP) has proposed a revision to the heparin sodium monograph that includes chromatographic methods for the identification of heparin and the determination of organic impurities in heparin.⁴ This USP monograph is scheduled to become official on August 15, 2009. The heparin chromatographic identity portion of the monograph, which determines oversulfated chondroitin and dermatan sulfate in heparin, will not be discussed in this Application Note (AN), but is available elsewhere.⁵

The organic impurities section of the heparin monograph relies on hydrolyzing the polysaccharide and determining the relative amounts of galactosamine and glucosamine in the sample digests. Heparin is composed

of glucosamine and uronic acid. Acid hydrolysis of heparin samples releases glucosamine, which is easily determined by electrochemical detection. In comparison, chondroitin sulfates are composed of galactosamine and uronic acid. In these compounds, acid hydrolysis releases galactosamine, which can also easily be determined by electrochemical detection. The USP method measures the ratio of galactosamine/glucosamine as an indication of the heparin purity and to identify heparin samples that may be contaminated or adulterated with chondroitin sulfate compounds.

In this AN, the organic impurities in heparin are determined by the HPAE-PAD method using the CarboPac[®] PA20 column following the USP monograph method. This method was repeated using manually prepared eluents and an electrolytically generated eluent, with both eluent preparation options providing data that exceeds the system suitability requirements in the monograph. In addition, this document includes deliberate variation of several chromatographic parameters to evaluate method ruggedness. The HPAE-PAD method provides sensitive determination of galactosamine in acid-hydrolyzed heparin samples, enabling the identification of heparin that has been contaminated with chondroitin sulfates.

EQUIPMENT

Dionex ICS-3000 Reagent-Free™ IC system with eluent generation (RFIC-EG™) system consisting of:
SP Single Pump or DP Dual Pump module
(Gradient pump required if manual eluent is used)
EG Eluent Generator module
DC Detector/Chromatography module (single or dual temperature zone configuration)
AS Autosampler
EluGen® EGC II KOH cartridge (Dionex P/N 058900)
Continuously-Regenerated Anion Trap Column, CR-ATC (Dionex P/N 060477)
ICS-3000 ED Electrochemical detector (Dionex P/N 079831)
Electrochemical cell (Dionex P/N 061757)
Disposable gold electrode, carbohydrate certified (Dionex P/N 060139)
Reference electrode (Dionex P/N 061879)
10 µL PEEK™ Sample injection loop (Dionex P/N 042949)
EG Vacuum Degas Conversion Kit (Dionex P/N 063353)
Chromeleon® 6.8 Chromatography Data System
0.3 mL polypropylene injection vials with caps (Dionex P/N 055428)
Nalgene® 1000 mL 0.2 µm nylon filter units (VWR P/N 28198-514)
7 mL polypropylene screw cap tubes (Sarstedt P/N 60.550)
500 mL PMP volumetric flasks, Class A (Vitalab P/N 67504)
Dry block heater (VWR P/N 13259-005)

REAGENTS AND STANDARDS

Deionized water (DI), Type I reagent grade, 18 MΩ-cm resistivity or better
Hydrochloric acid, Ultrex II, (JT Baker P/N 6900-05)
Potassium hydroxide, 45% (w/w) (Fisher P/N SP236-500)
Sodium hydroxide, 50% (w/w) (Fisher P/N SS254-500)
Glucosamine hydrochloride (Sigma-Aldrich P/N G-4875)
Galactosamine hydrochloride (Pfanstiehl Laboratories P/N RGG-104)

SAMPLES

Chondroitin sulfate B, sodium salt (β-heparin, dermatan sulfate, sodium salt) (Sigma-Aldrich P/N C3788)
Sample A: Heparin, sodium salt, grade 1-A (Sigma-Aldrich P/N H3393)
Sample B: Heparin, sodium salt (Sigma-Aldrich P/N H4784)

CONDITIONS

Columns: AminoTrap™ column
3 × 30 mm (P/N 060146)
CarboPac PA20 Analytical,
3 × 150 mm (P/N 060142)
-or-
AminoTrap column,
3 × 30 mm (P/N 060146)
CarboPac PA20 Guard,
3 × 30 mm (P/N 060144)
CarboPac PA20 Analytical, 3 × 150 mm
(P/N 060142) (manual eluent only)
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: Pulsed amperometric,
disposable gold working electrode
Background: 5–25 nC (using the carbohydrate waveform)
Noise: 20–50 pC
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 described
by the USP)

Carbohydrate 4-Potential Waveform for the ED

Time(s)	Potential(V)	Gain Region*	Ramp*	Integration
0.00	+0.1	Off	On	Off
0.20	+0.1	On	On	On
0.40	+0.1	Off	On	Off
0.41	-2.0	Off	On	Off
0.42	-2.0	Off	On	Off
0.43	+0.6	Off	On	Off
0.44	-0.1	Off	On	Off
0.50	-0.1	Off	On	Off

*Settings required in the ICS-3000, but not used in older Dionex systems.

Reference electrode in Ag mode (Ag/AgCl reference). See Technical Note 21 for more information.⁶

PREPARATION OF SOLUTIONS AND REAGENTS

Eluent Solutions

Generate the potassium hydroxide (KOH) eluent online by pumping high-quality degassed, DI water through the EGC II KOH cartridge. The Chromeleon software will track the amount of KOH used and calculate the remaining lifetime.

The method can be executed with manually prepared eluents. Prepare 1 L of 200 mM KOH from 45% w/w KOH concentrate by adding 17 mL of 45% KOH to 983 g of degassed, DI water. If desired, NaOH can be used in place of KOH. To prepare 1 L of 200 mM NaOH, add 10.4 mL of 50% NaOH to 989.6 mL of degassed, DI water.

Proportion the 200 mM hydroxide solution with DI water to produce either a 14 mM NaOH or KOH eluent for the sample elution or a 100 mM NaOH or KOH eluent for column cleaning. See Tech Note 71 for detailed information on manual eluent preparation.⁷

5 N Hydrochloric Acid for Sample Digestion

Dilute 102 g (88 mL) of 33% hydrochloric acid to a total of 211 g with DI water.

Standard Stock Solutions

Glucosamine

Prepare a 1.6 mg/mL stock solution of glucosamine hydrochloride by dissolving 0.1600 g of glucosamine hydrochloride in 100 mL of 5 N hydrochloric acid. This stock will be used to prepare the standard solution for digestions.

Galactosamine

Prepare a 16 mg/mL solution of galactosamine hydrochloride by dissolving 0.0320 g of galactosamine hydrochloride in 2.00 mL of DI water. Further dilute this concentrate by adding 100 μ L to 99.9 mL of 5 N hydrochloric acid to prepare a 16 μ g/mL stock solution of galactosamine.

Glucosamine and galactosamine stock solutions were stored at 4 °C.

Standard Solution

Prepare the standard solution by transferring 2.5 mL of glucosamine stock solution into a 7 mL screw cap vial containing 2.5 mL of galactosamine stock solution. This solution contains 8 μ g/mL of galactosamine and 800 μ g/mL of glucosamine (1% w/w galactosamine with respect to glucosamine). Freshly prepare the standard solution before each digestion.

DIGESTION OF SAMPLES

Prepare samples for digestion by adding 12 mg of heparin to a 7 mL screw cap vial. Add 5 mL of 5 N HCl to the vial and vortex the solution to mix. Heat the samples and the standard solution at 100 °C for 6 h to hydrolyze the samples into glucosamine and galactosamine. After digestion of the samples, allow the samples to cool to ambient temperature, quantitatively transfer the contents of the vial to a 500 mL PMP volumetric flask, and fill to the mark on the flask with DI water.

PRECAUTIONS AND EXPERIMENTAL CONSIDERATIONS

Labware

Glass volumetric flasks should not be used for dilution of samples and standards after digestion. Peak heights may be reduced if glass is used. For this application, Class A PMP flasks were used, although polypropylene would be acceptable. Similarly, polypropylene, rather than glass, digestion vials and injection vials should be used.

When using PMP or polypropylene labware, it is important to remove bubbles from the surface of the plastic labware. This can be accomplished by gently swirling the solution in the volumetric flask while it is approximately one-half full. The final dilution should be made by gently adding water down the side of the flask. Bubbles on the walls of the flask can lead to dilution errors. Similarly, bubbles in injection vials should be tapped out before the samples are loaded in the AS to ensure consistent injection volumes.

Use of Sodium Hydroxide for Manual Eluent Preparation

Sodium hydroxide can be substituted for potassium hydroxide when manually preparing eluents. Glucosamine peak asymmetry and resolution between galactosamine and glucosamine pass the USP requirements.

Equilibration of the Column and Retention Time Precision

To optimize retention time precision, each sequence should start with 3–5 injections of a 50 mM HCl blank. When using EG, equilibration of the system with three blank acid injections led to retention time precision RSDs ranging from <0.001 to 0.58. If greater retention time precision is needed, additional blank injections can be performed to stabilize the system or the equilibration time prior to sample injection can be increased. When using manually prepared eluents, this effect is magnified demonstrating RSDs for glucosamine ranging between <0.01 and 3.1.

Guard Column Considerations

When implementing the method using an EG eluent, the AminoTrap column should be used in place of the CarboPac PA20 guard column. If both the CarboPac PA20 Guard and Analytical column are installed, excessive backpressure can occur and the EG cartridge may be damaged. To prevent such damage, the Chromeleon software will automatically turn off the pump at a pressure of 3000 psi.

When implementing the method using manually prepared eluents, the two guard columns and the CarboPac PA20 column can be used as described in the USP monograph. However, only one guard is necessary. If no amino acids are expected in the samples, the CarboPac guard column should be used without the AminoTrap column.

When using the AminoTrap column, particular care should be taken to avoid flowing deionized water through the trap column. If the column is damaged by water, fronting of the glucosamine and galactosamine peaks may be observed. This will reduce the resolution between glucosamine and galactosamine and decrease the measured column efficiency. If either peak fronting or a sudden decrease in resolution is observed, replace the AminoTrap column.

RESULTS AND DISCUSSION

Separation

Figure 1 shows the separation of hydrolyzed glucosamine/galactosamine standard solution when using the AminoTrap and CarboPac PA20 analytical columns with eluent generation. The galactosamine (GalN) peak is well resolved (USP resolution = 3.2) from the glucosamine (GlcN) peak and clearly identified at a concentration of 1% of the GlcN concentration. The average retention times for GlcN and GalN are 6.51 and 5.51 min, respectively. Equivalent chromatography is obtained if manual eluents are used with the three columns specified in the USP monograph. However, due to the addition of the CarboPac PA20 guard column, the retention times for GlcN and GalN increase to 7.07 and 5.97 min respectively.

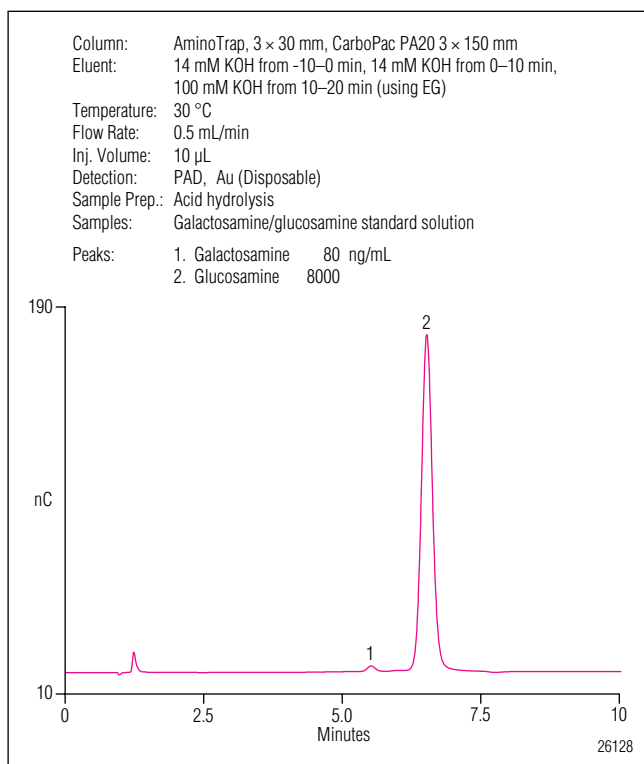


Figure 1. Separation of the standard solution on the CarboPac PA20.

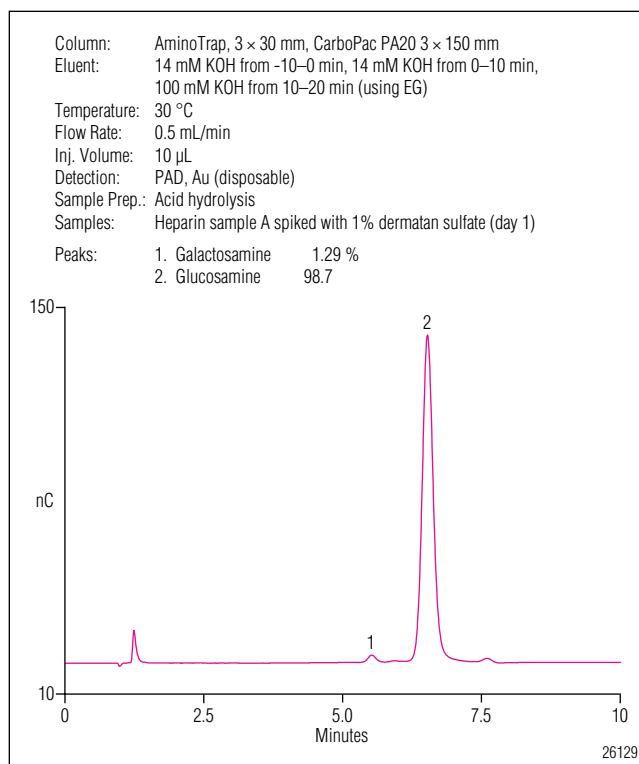


Figure 2. Separation of acid-hydrolyzed heparin spiked with 1% dermatan sulfate on the CarboPac PA20.

Sample Analysis

Calculations: The percentage of GalN in the heparin digests is calculated by comparison against the standard solution, which contains 1% (w/w) of GalN/GlcN in 5 N HCl. For each set of digestions a 5 mL aliquot of standard solution was also digested and the relative response of GalN/GlcN was calculated by the following formula:

Response ratio:

$$(\text{GalN}_R) = (\text{GalN}_B) / (\text{GalN}_W) * (\text{GlcN}_W) / (\text{GlcN}_B)$$

Where:

GalN_B = the galactosamine peak area from the hydrolyzed standard solution

GalN_W = the weight of galactosamine in the standard solution

GlcN_W = the weight of glucosamine in the standard solution

GlcN_B = the glucosamine peak area from the hydrolyzed standard solution

The response ratio of GalN/GlcN determined for the standard solutions ranged from 1.03 to 1.19 during four weeks of sample analysis.

The response factor was used to calculate the percentage of galactosamine in the heparin digests according to the formula below:

$$\% \text{GalN} = [\text{GalN}_U / \text{GalN}_R] / [(\text{GalN}_U / \text{GalN}_R) + \text{GlcN}_U] \times 100$$

Where:

GalN_U = the galactosamine peak area from the hydrolyzed heparin sample

GalN_R = the response ratio

GlcN_U = the glucosamine peak area from the hydrolyzed heparin sample

Figure 2 shows the separation of heparin sample A spiked with 1% (w/w) of dermatan sulfate (chondroitin sulfate B). The GalN peak is well resolved from GlcN (USP resolution = 3.2) and 1.29% GalN was determined in the sample. In unspiked samples, 0.04% galactosamine was determined in the hydrolysate.

Table 1. Comparison of Triplicate Heparin Analysis Results to USP Criteria When Using an EG Eluent*

Day	Sample	% GalN (USP limit <1%)	RSD for % GalN Determined	Standard Solution Response Factor	Resolution (USP limit NLT 2)	Efficiency (USP limit NLT 2000)	Asymmetry (USP limit 0.8–2.0)
1	Standard solution	N/A	N/A	1.16	3.2	5292	1.1
	Heparin, sample A	0.04	3.3		3.2	4955	1.2
	1% Dermatan-spiked heparin, sample A	1.29	0.17		3.2	5053	1.2
2	Standard solution	N/A	N/A	1.19	3.2	5326	1.1
	Heparin, sample A	0.04	9.3		3.3	5224	1.1
	1% Dermatan-spiked heparin sample A	1.28	0.07		3.2	5330	1.1
3	Standard solution	N/A	N/A	1.16	3.2	5320	1.1
	Heparin, sample A	0.04	9.0		3.3	4441	1.4
	1% Dermatan-spiked heparin, sample A	1.40	0.79		3.1	4602	1.4

* AminoTrap and CarboPac PA20 analytical columns used.

Precision and Reproducibility when Using Eluent Generation

Table 1 displays the USP criteria and the experimental results for three days of triplicate testing of the standard solution, heparin sample A, and the dermatan-spiked heparin. As shown in Table 1, all USP criteria are met. The between-day sample analysis had an RSD of 0.6, although the intraday precision RSDs ranged from 3.2 to 9.3. This precision is excellent considering the low concentrations of galactosamine present in the digested heparin. The value of 0.04% GalN is at the limit of detection and is therefore an extreme measure of reproducibility. Spiked heparin showed an average of 1.3% galactosamine with a between-day precision RSD of 4.3. The differences observed in the spiked samples are likely due to slight variations in spiked amounts.

Method Ruggedness

Manually Prepared Eluents

Manual potassium hydroxide and manual sodium hydroxide eluents were prepared to compare analysis results against sequences generated using an EG eluent. Manually prepared 200 mM potassium or sodium hydroxide was prepared and proportioned at 7% and 50% with DI water to deliver 14 mM hydroxide and 100 mM hydroxide, respectively. Additionally, the CarboPac

PA20 guard column was installed to match the column set specified by the proposed method. Table 2 shows the analysis results for the standard solution, heparin sample A, dermatan-spiked heparin sample A, and heparin sample B using both manually prepared KOH and NaOH. In both cases, the USP criteria are met and the determined percentages of GalN are consistent. Comparison of these percentages with those found while using EG (Table 1) show that both EG and manual eluent preparation are suitable for the method described in the USP monograph.

Column Reproducibility

For comparison, a second column was tested using manually prepared KOH eluent. The efficiency of the column was slightly lower than the original column used, but results still greatly exceed the USP criteria and analysis of samples led to equivalent results. Table 2 shows the results of sample analysis using the same batch of manually prepared KOH on two different columns.

Guard Column Use (Manual Eluents)

As a further test, the AminoTrap column was removed. When sample digests were analyzed with this column set and manual KOH eluent, the % GalN determined was 0.11%, 0.57%, and 1.40% in heparin sample A, heparin sample B, and dermatan-spiked heparin

Table 2: Comparison of Triplicate Heparin Analysis Results to USP Criteria When Using Manually Prepared KOH or NaOH Eluents*

Instrumental Conditions	Sample	% GalN (USP limit <1%)	Standard Solution Response Factor	Resolution (USP limit NLT 2)	Efficiency (USP limit NLT 2000)	Asymmetry (USP limit 0.8–2.0)
Manually prepared KOH proportioned to 14 mM CarboPac PA20 Column 1	Standard solution	N/A	1.15	3.3	5736	1.1
	Heparin, sample A	0.03		3.6	6136	1.2
	1% Dermatan-spiked heparin, sample A	1.40		3.6	6326	1.1
	Heparin, sample B	0.55		3.6	6359	1.1
Manually prepared KOH proportioned to 14 mM Column 2	Standard solution	N/A	1.12	3.1	5120	1.2
	Heparin, sample A	0.03		3.3	5304	1.2
	1% Dermatan-spiked heparin, sample A	1.30		3.2	5353	1.2
	Heparin, sample B	0.52		3.2	5382	1.2
Manually prepared NaOH proportioned to 14 mM Column 2	Standard solution	N/A	1.12	3.2	5121	1.2
	Heparin, sample A	0.04		3.3	5209	1.2
	1% Dermatan-spiked heparin, sample A	1.27		3.2	5206	1.2
	Heparin, sample B	0.53		3.2	5217	1.2

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

sample A, respectively. All USP criteria are exceeded with the resolution, column efficiency, and peak asymmetry being equivalent to results while using the AminoTrap column. A slight increase in the sensitivity at low concentrations of GalN is observed, but otherwise results are equivalent to using all three columns. If samples are not expected to contain amino acid contaminants, only the CarboPac PA20 guard is necessary.

CONCLUSION

In this AN, the organic impurities in two research grade heparin samples were determined by the HPAE-PAD system using the CarboPac PA20 column, following the organic impurities method in the proposed revision of the heparin sodium USP monograph. The method ruggedness was shown by comparing results when using EG or manual hydroxide eluents, evaluating the guard columns for both EG and manual eluent system configurations, and by performing sample analysis on two different CarboPac PA20 columns. The HPAE-PAD system allows reliable determination of galactosamine in acid-hydrolyzed heparin samples, thereby providing a method to easily identify heparin that has been contaminated with chondroitin sulfates.

LIST OF SUPPLIERS

VWR, 1310 Goshen Parkway, West Chester, PA 19380, USA. Tel: 800-932-5000.

<http://www.vwr.com>

Fisher Scientific, One Liberty Lane, Hampton, NH, 03842, USA. Tel: 800-766-7000

<http://www.fishersci.com>

Sigma-Aldrich, P.O. Box 14508, St. Louis, MO 63178, USA. Tel: 800-325-3010.

<http://www.sigma-aldrich.com>

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LPN 2286 PDF 06/09
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