Application Note 233

Determination of Galactosamine-Containing Organic Impurities in Heparin by HPAE-PAD

Deanna Hurum and Jeffrey Rohrer
Thermo Fisher Scientific, Sunnyvale, CA, USA

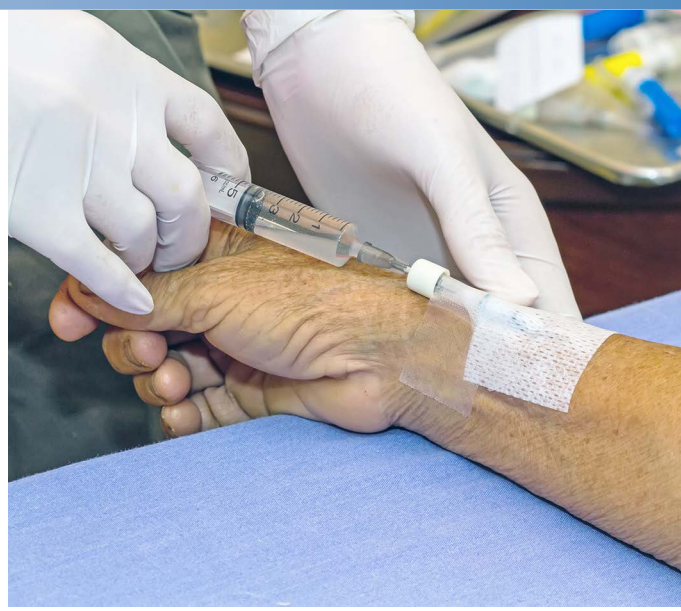
Key Words
AminoTrap, Chondroitin Sulfates, Electrochemical Detection , Eluent Generation, RFIC-EG, USP

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 high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) method using the Thermo Scientific™ Dionex™ 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.

Thermo SCIENTIFIC

Equipment

- Thermo Scientific™ Dionex™ ICS-3000 Reagent-Free™ IC system with eluent generation (RFIC-EG™) system consisting of the following:
  - 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
- Thermo Scientific™ Dionex™ EluGen™ EGC II KOH cartridge (P/N 058900)
- Thermo Scientific™ Dionex™ CR-CTC Continuously Regenerated Cation Trap Column (P/N 060477)
- Thermo Scientific™ Dionex™ ICS-3000 ED Electrochemical detector (P/N 079831)
- Electrochemical cell (P/N 061757)
- Disposable gold electrode, carbohydrate certified (P/N 060139)
- Reference electrode (P/N 061879)
- 10 µL PEEK Sample injection loop (P/N 042949)
- EG Vacuum Degas Conversion Kit (P/N 063353)
- Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System
- 0.3 mL polypropylene injection vials with caps (P/N 055428)
- Thermo Scientific™ Nalgene™ 1000 mL 0.2 µm nylon filter units (P/N 164-0020)
- 7 mL polypropylene screw cap tubes (Sarstedt P/N 60.550)
- 500 mL PMP volumetric flasks, Class A (Vitlab 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 Scientific™ P/N SP236-500)
- Sodium hydroxide, 50% (w/w) (Fisher Scientific P/N SS254-500)
- Glucosamine hydrochloride (Sigma-Aldrich® P/N G-4875)
- Galactosamine hydrochloride (Pfanstiehl® P/N RGG-104)

Samples

- Chondroitin sulfate B, sodium salt (Ω-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:	Thermo Scientific™ Dionex™ AminoTrap™ column 3 × 30 mm (P/N 060146) Dionex CarboPac PA20 analytical, 3 × 150 mm (P/N 060142) -or- Dionex AminoTrap column, 3 × 30 mm (P/N 060146) Dionex CarboPac PA20 guard, 3 × 30 mm (P/N 060144) Dionex 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:	Dionex 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 Dionex AminoTrap 3 × 30 mm and Dionex CarboPac PA20 3 × 150 mm columns) -or- ~3010 psi (using the Dionex AminoTrap 3 × 30 mm, Dionex CarboPac PA20 3 × 30 mm guard, and Dionex 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 Dionex ICS-3000 system, but not used in older Dionex systems.

Reference electrode in Ag mode (Ag/AgCl reference).

See Dionex 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 Dionex 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 Dionex 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 µL to 99.9 mL of 5 N hydrochloric acid to prepare a 16 µg/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 µg/mL of galactosamine and 800 µg/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 Dionex AminoTrap column should be used in place of the Dionex CarboPac PA20 guard column. If both the Dionex 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 Dionex 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 Dionex CarboPac guard column should be used without the Dionex AminoTrap column.

When using the Dionex 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 Dionex AminoTrap column.

Results and Discussion

Separation

Figure 1 shows the separation of hydrolyzed glucosamine/galactosamine standard solution when using the Dionex AminoTrap and Dionex 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 Dionex CarboPac PA20 guard column, the retention times for GlcN and GalN increase to 7.07 and 5.97 min, respectively.

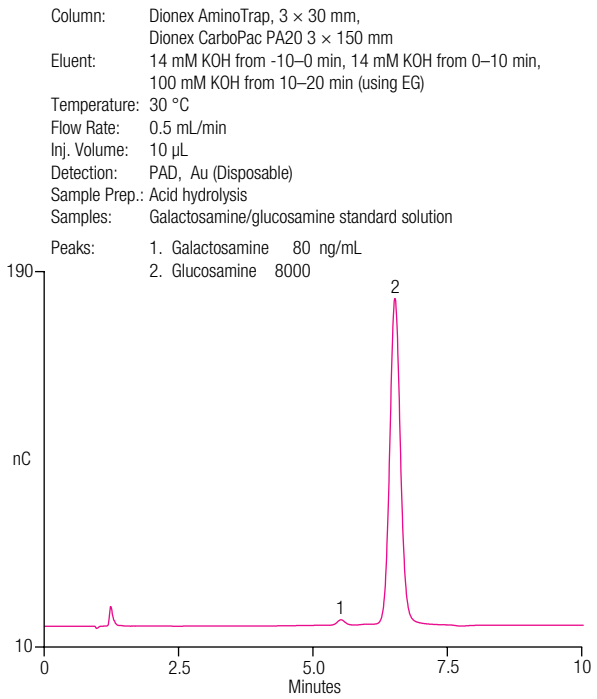


Figure 1. Separation of the standard solution on the Dionex CarboPac PA20. Concentrations expressed as galactosamine HCl and glucosamine HCl.

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:

$$(GalN_R) = (GalN_B)/(GalN_W) \times (GlcN_W)/(GlcN_B)$$

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:

$$\% \ GalN = [GalN_U/GalN_R] / [(GalN_U/GalN_R) + GlcN_U] \times 100$$

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.

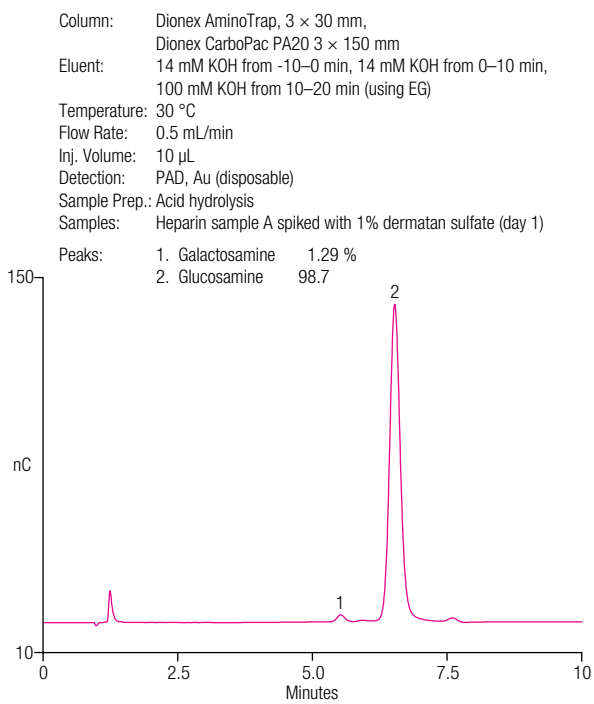


Figure 2. Separation of acid-hydrolyzed heparin spiked with 1% dermatan sulfate on the Dionex CarboPac PA20.

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 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 Dionex 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 Dionex 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 sample A, respectively. All USP criteria are exceeded with the resolution, column efficiency, and peak asymmetry being equivalent to results while using the Dionex 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 Dionex CarboPac PA20 guard column is necessary.

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

* Dionex AminoTrap and Dionex CarboPac PA20 analytical columns used.

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 Dionex CarboPac PA20 column 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

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

Conclusion

In this AN, the organic impurities in two research-grade heparin samples were determined by the HPAE-PAD system using the Dionex 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 Dionex 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

References

1. Contaminant Detected in Heparin Material of Specified Origin in the USA and in Germany; Serious Adverse Events Reported; Recall Measures Initiated. World Health Organization Alert No. 118, March 7, 2008. http://www.who.int/medicines/publications/drugalerts/Alert_118_Heparin.pdf. Last accessed 05/04/09.
2. Recall of Heparin Sodium Injection and Heparin Lock Flush Solution (Baxter) FDA Public Health Update, February 2, 2008. http://www.fda.gov/cder/drug/infopage/heparin/public_health_update.htm. Last accessed 05/18/09.
3. Guerrini, M.; Beccati, D.; Shriver, Z.; Naggi, A.; Viswanathan, K.; Bisio, A.; Capila, I.; Lansing, J.C.; Guglieri, S.; Fraser, B.; Al-Hakim, A.; Gunay, N.S.; Zhang, Z.; Robinson, L.; Buhse, L.; Nasr, M.; Woodcock, J.; Langer, R.; Venkataraman, G.; Linhardt, R.J.; Casu, B.; Torri, G.; Sasisekharan, R.; Oversulfated Chondroitin Sulfate is a Contaminant in Heparin Associated with Adverse Clinical Events, *Nature Biotechnology* **2008,** *26,* 669–675.
4. Heparin Sodium, *Pharmocopeial Forum* **2009,** *35,* 1–10.
5. Dionex Corporation. *Determination of Oversulfated Chondroitin Sulfate, Dermatan Sulfate, and Heparin Sodium Using Anion-Exchange Chromatography with UV Detection (IC-UV),* Application Note 235, LPN 2306. Sunnyvale, CA, 2009. http://www.dionex.com/en-us/webdocs/78398-AN235-IonPac-Heparin-12Nov2010-LPN2306-03.pdf
6. Dionex Corporation. *Optimal Settings for Pulsed Amperometric Detection of Carbohydrates Using the Dionex ED40 Electrochemical Detector,* Technical Note 21, LPN 034889-03. Sunnyvale, CA, 1998. http://www.dionex.com/en-us/webdocs/5050-TN21_LPN034889-03.pdf
7. Dionex Corporation. *Eluent Preparation for High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection,* Technical Note 71, LPN 1932-01. Sunnyvale, CA, 2007. http://www.dionex.com/en-us/webdocs/58087-TN71-HPAE-PAD-Eluent-Prep-TN70669_E.pdf

Africa +43 1 333 50 34 0
Australia +61 3 9757 4300
Austria +43 810 282 206
Belgium +32 53 73 42 41
Brazil +55 11 3731 5140
Canada +1 800 530 8447
China 800 810 5118 (free call domestic)
400 650 5118
Denmark +45 70 23 62 60
Europe-Other +43 1 333 50 34 0
Finland +358 10 3292 200
France +33 1 60 92 48 00
Germany +49 6103 408 1014
India +91 22 6742 9494
Italy +39 02 950 591
Japan +81 6 6885 1213
Korea +82 2 3420 8600
Latin America +1 561 688 8700
Middle East +43 1 333 50 34 0
Netherlands +31 76 579 55 55
New Zealand +64 9 980 6700
Norway +46 8 556 468 00
Russia/CIS +43 1 333 50 34 0
Singapore +65 6289 1190
Sweden +46 8 556 468 00
Switzerland +41 61 716 77 00
Taiwan +886 2 8751 6655
UK/Ireland +44 1442 233555
USA +1 800 532 4752

Thermo SCIENTIFIC
A Thermo Fisher Scientific Brand

AN71937-EN 0916S