Application Note

Drugs & Pharmaceuticals

Heparin Sodium - USP Method

- **U.S. Pharmacopeia 37NF32 (2014)**
- **Analysis of galactosamine impurities in heparin**
- **Reproducible & robust**

Summary

The heparin analysis was evaluated on an Antec ALEXYS® Carbohydrate Analyzer according the official 2014 Heparin Sodium USP monograph 37-NF32 [5].

In this application note typical results obtained with the ALEXYS® Carbohydrate Analyzer are reported, demonstrating its performance for the analysis of organic impurities in heparin products.

The most reliable LC-EC applications for Drugs & Pharmaceuticals analysis

Antipsychotic drugs
Clozapine
Olanzapine
Risperidone

PET imaging tracer
Fluorodeoxyglucose (FDG)
FDG impurities

Pharmaceuticals, API
Acetaminophen
Artemether
Artemisinin
Dihydro- artemisinin
Betadex sulfobutyl ether sodium
Etoposide
Epinephrine
Heparin
mesna BNP7787
8-OH-DPAT
Vincristine

Sulfides
Glutathione
Aminothiols
Disulfides

Aminoglycoside drugs
Amikacin
Framycetin sulphate
Gentamicin sulphate
Kanamycin
Netilmycin
Neomycin sulfate
Spectinomycin
Lincomycin
Tobramycin

Heparin sodium - USP method

Introduction

Heparin is a highly sulfated glycosaminoglycan widely used as an injectable anticoagulant. Pharmaceutical grade heparin is derived from mucosal tissues of slaughtered meat animals such as porcine (pig) intestines or bovine (cattle) lungs.

In March 2008 a major recall of contaminated heparin was announced in the US due to reported adverse reactions (hypotension, allergic reactions) leading in some cases to death [1, 2]. Upon investigation it became evident that the heparin was contaminated with over sulfated chondroitin sulfate, a closely related substance which mimics heparin closely. In 2009 the USP revised the heparin monograph as a result of the adulteration problem. High Performance Anion Exchange Chromatography followed by Pulsed Amperometric Detection (HPAEC-PAD) is used in the U.S. Pharmacopoeia to determine the organic impurities in heparin [35]. Using this method the presence of galactosamine in hydrolyzed heparin samples can be determined with high sensitivity.

Method

The U.S. Pharmacopoeia method to determine the amount of organic impurities is based on the acid hydrolysis of heparin into glucosamine (GlcN) residues and hexuronic acid. Over-sulfated chondroitin sulfate in adulterated samples on the contrary consist of galactosamine (GalN) moieties and hexuronic acid which will also be released upon hydrolysis. Both galactosamine and glucosamine can be detected by pulsed amperometric detection on an Au working electrode.

Figure 1: ALEXYS Carbohydrate Analyzer

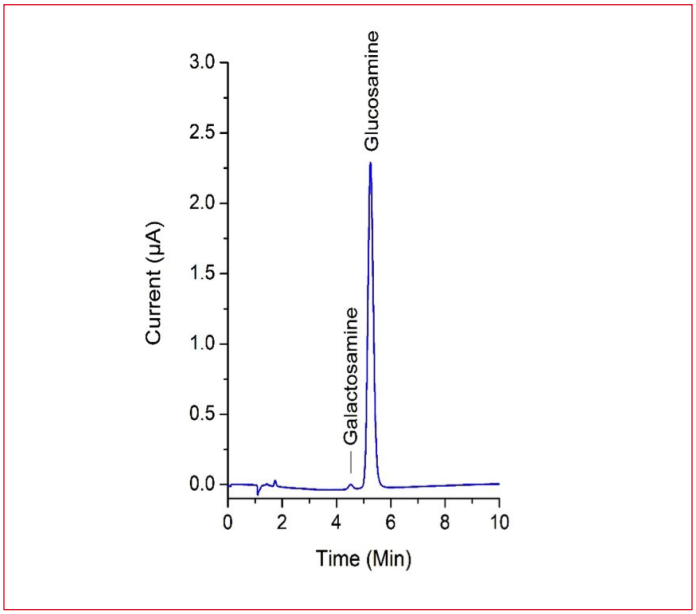

Figure 2: 10 µL injection of an acid-hydrolyzed standard solution of 8 µg/ mL glucosamine and 80 ng/mL galactosamine in 50 mM HCl (hydrolyzed standard solution as described in the USP monograph).

The presence of galactosamine is a measure for the degree of contamination of heparin with over-sulfated chondroitin sulfate. The USP acceptance criteria for heparin is that not more than 1% galactosamine is present relative to the total amount of hexosamine (GlcN & GalN) in a hydrolyzed sample solution.

Table 1

LC-ECD conditions (EP)	
HPLC	ALEXYS Carbohydrate Analyzer
Columns (in series)	AminoTrap™ Column, 30 x 3 mm ID, 6.5 µm CarboPac™ PA20 IC columns, 30 x 3 mm ID, 6 µm + 150 x 3 mm ID, 6 µm All columns: Thermo Scientific™ Dionex™
Mobile phase	14 mM potassium hydroxide
Column cleaning	100 mM potassium hydroxide
Flow rate	0.5 mL/mL
Temperature	30 °C for separation and detection
$V_{injection}$	10 µL
Pump piston wash	Water (refresh weekly)
Flow cell*	SenCell™ with 2 mm Au and HyREF (Pd/H_2), AST pos. 2
Potential waveform (4-step)	E1, E2, E3, E4: +0.1, -2.0, +0.6, -0.1 V ts, t1, t2, t3, t4: 0.2, 0.4, 0.02, 0.01, 0.07 s
Range	5 µA/V
ADF	0.5 Hz
I-cell	About 1.5 µA

* Original data recorded with a VT-03 3 mm Au and HyREF, spacer 50 µm

Separation

Separation of GlcN and GalN is achieved using an anion-exchange column and elution with an alkaline mobile phase (14 mM potassium hydroxide). The analysis is based on a step-gradient, see Table 2.

In the monographs the use of the following column types is described: 3 mm ID x 3 cm amino acid trap column in series with a 3 mm ID x 3 cm guard column and a 3 mm ID x 15 cm, 5 µm, packing L69. We chose to apply the columns listed in Table 1. The trap column is not required in case no amino acids are present in the sample. In such situation we recommend to remove the trap column from the system for optimal performance.

The ALEXYS Carbohydrate Analyzer (Fig. 1) is equipped with a Solvent Selection Valve (SSV), which is used to switch between the mobile phase and the column clean-up solution. The eluents were carefully prepared manually using a commercial 45% KOH solution (< 0.3% K_2CO_3). The diluent was deionized water (resistivity >18 MΩ-cm), which was sonicated and sparged with Helium 5.0 prior to use. The bottles with mobile phase and column clean-up solution were blanketed with helium during the analysis to minimize the build-up of carbonate ions in the mobile phase and to assure a reproducible analysis.

Table 2

Step- gradient program

Time (min)	*Mobile phase*	*Description*
-10 - 0	14 mM KOH	Pre-stabilization with mobile phase
0 - 10	14 mM KOH	Elution & detection
10 - 20	100 mM KOH	Column clean-up/regeneration

Detection

For the detection of the hexoamines, an Antec electrochemical flow cell is used for this evaluation. This flow cell has an Au working electrode, HyREF (Pd/H_2) reference electrode and stainless steel auxiliary electrode. A 4-step potential waveform is used as described in the USP monograph to detect the hexoamines on the Au working electrode, see Table 1 and Figure 3.

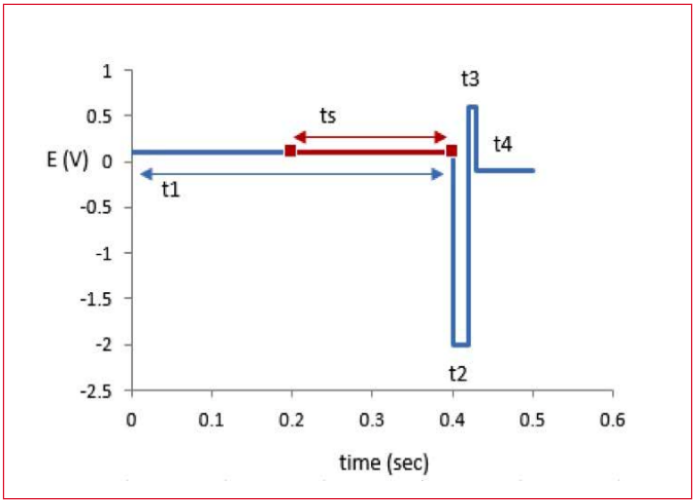

Figure 3: 4-step PAD potential waveform for the detection of GalN and GlcN as described in the Heparin sodium USP monograph.

The cell current was typical about 1.5 µA with these PAD settings. This particular 4-step waveform with a pulse duration of 500 ms has been claimed to have as benefits: (1) a consistent long-term peak area response and (2) minimal electrode wear [4]. The temperature for separation and detection was set to 30°C.

Sample preparation

Sample digestion was achieved in the following way:

- Transfer 12 mg of heparin into a 7 mL screw cap vial.
- Add 5 mL of 5 N HCl solution and vortex the solution.
- Hydrolyze the sample for 6 hours at 100 °C.
- Cool to ambient and dilute the sample 1:100 with water.

Preparation of the hydrolyzed standard solution:

- 1.6 mg/mL glucosamine stock solution: dissolve 160 mg glucosamine in 100 mL 5 N HC_l.
- 16 µg/mL galactosamine stock solution: dissolve 160 mg galactosamine in 100 mL 5 N HC_l. Subsequently add 100 µL of the 1.6 mg/mL solution to 99.9 mL 5 N HC_l.
- Mix equal volumes of the stock solutions (5 mL) to prepare the standard solution.
- Transfer 5 mL of the standard solution into a 7 mL screw cap vial.
- Hydrolyze the solution for 6 hours at 100 °C
- Cool to ambient and dilute the sample 1:100 with water.

Results

System suitability

A chromatogram of a 10 µL injection of an acid-hydrolyzed standard solution of 8 µg/mL glucosamine and 80 ng/mL galactosamine in 50 mM HC_l (hydrolyzed standard solution as described in the USP monograph) is show in Figure 2. The retention times for galactosamine and glucosamine were 4.5 and 5.3 min, respectively.

The system suitability is evaluated using the chromatogram obtained with hydrolyzed standard solution. The results are listed in Table 3, and t is evident that the system suitability requirements are met for all performance parameters.

Table 3

USP system suitability requirements

Parameter	*USP criteria*	*Measured*
Resolution between GalN and GlcN	> 2.0	2.1
Column efficiency (GlcN)	>2000	3016
Tailing factor (GalN)	0.8-2.0	1.1
Tailing factor (GlcN)	0.8-2.0	1.2

It was observed that the presence of the trap column has a negative effect on the chromatographic performance parameters. Without the trap column installed in the system (so with guard + analytical column only) the efficiency was > 5000 (GlcN) and resolution > 3.0. So in case no amino acids are expected in the sample it is advisable to work without the trap column for best performance.

Linearity, repeatability and LOD

The linearity for both glucosamine and galactosamine were investigated in the concentration range of 0.05 µg/mL – 1 µg/mL and 1 – 8 µg/mL, see Table 4. The method shows good linearity.

The relative standard deviation (RSD) in peak area was determined for 45 replicate injections of the standard solution. The RSD in peak area was 0.5% and 0.4% for GalN and GlcN, respectively. The sensitivity of the method was excellent and a Limit of Detection (LOD) for galactosamine of 1.6 ng/mL was achieved, which corresponds to approximately 0.03% GalN.

Table 4

Linearity

Component	*Concentration range (µg/mL)*	R^2
Galactosamine	1 - 8	0.9957
Glucosamine	1 - 8	0.9986
Galactosamine	0.05 - 1	0.9995
Glucosamine	0.05 - 1	0.9996

Sample analysis

As an example, a commercial sample was analyzed from Sigma Aldrich: Heparin sodium salt from porcine intestinal mucosa (p/n H4784, batch 051M1130V). The sample is abbreviated as sample 051M1130V from this point onwards. The chromatogram obtained from the hydrolyzed sample is shown in Figure 4.

The percentage of GalN in the hydrolyzed Heparin sample is calculated compared to that of the hydrolyzed standard solution.

The relative response ratio (GalNR) of GalN/GlcN in the hydrolyzed standard solution was calculated as:

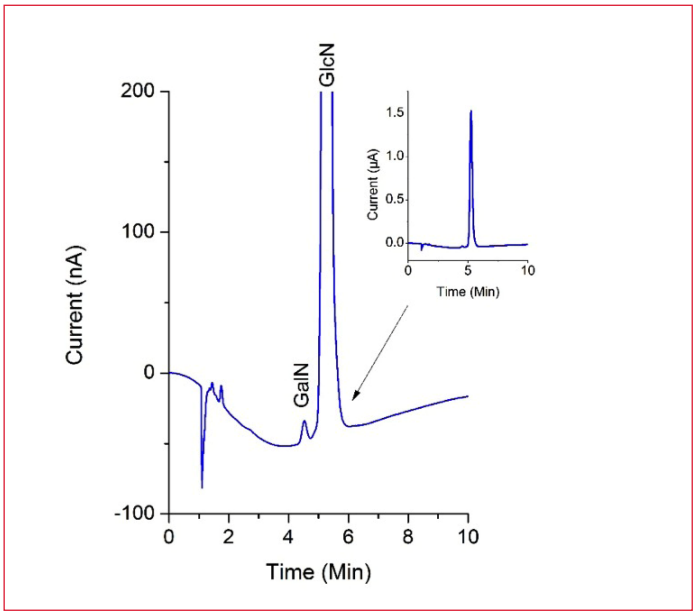

Figure 4: 10 µL injection of hydrolyzed sample 051M1130V (zoom-in on galactosamine peak). Top-right: full scale chromatogram.

(**1**) GalNR = (GalNB/GalNW) x (GlcNW/GlcNB)

where:
GalNB = Peak area of GalN from hydrolyzed standard solution
GalNW = Weight of GalN for the standard solution
GlcNW = Weight of GlcN for the standard solution
GlcNB = Peak area of GlcN from hydrolyzed standard solution

The percentage of galactosamine in the sample was calculated as:

(**2**) %GalN = [(GalNU/GalNR)] / [(GalNU/GalNR) + GlcNU] x 100

where:
GalNU = Peak area of GalN from hydrolyzed sample solution
GalNR = Response ratio of GalN (1)
GlcNU = Peak area of GlcN from hydrolyzed sample solution

The USP acceptance criteria for heparin is that not more than 1% galactosamine is present relative to the total amount of hexosamines in a hydrolyzed sample solution. The result for sample 051M1130V is listed in Table 5. The calculated %GalN is the average of a triplicate analysis of the heparin sample.

Table 5

Limit of galactosamine for total hexosamine in heparin sample

Sample	*USP limit %GalN*	*Measured %GalN*
051M1160V	<1	0.6

References

1. Public Health Update: Recall of Heparin Sodium for Injection (2/28/2008), FDA web site: http:// www.fda.gov/Drugs/ DrugSafety/ PostmarketDrugSafetyInformationforPatient-sandProviders/ucm112665.htm
2. 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 2008, WHO website: http://www. who.int/medicines/publications/ drugalerts/Alert_118_ Heparin.pdf
3. W.R. LaCourse, “Pulsed Electrochemical Detection in High Performance Liquid Chromatography”, John Wiley & Sons, New York, 1ed, 1997.
4. R.D. Rocklin, A.P. Clarke, M. Weitzhandler, Anal. Chem, 70, (1998), 1496 1501
5. Heparin Sodium, United States Pharmacopoeia (USP), USP37-NF32, 3222 3226

Conclusion

The ALEXYS Carbohydrate Analyzer provides a reliable solution for the analysis of galactosamine containing organic impurities in commercial heparin samples following the official USP method. The system suitability requirements are met for all performance parameters

Heparin sodium - USP method

Figure 5. Recommended instrument configuration for this application: the ALEXYS Carbohydrate Analyzer
The system consists of a P6.1L pump with integrated Solvent Switch Valve (SSV) capable of running step gradients, an AS6.1L autosampler, an ET 210 Eluent tray for helium blanketing, and the DECADE Elite electrochemical detector. A CT 2.1 column oven with broad temperature range can be added optionally. The ALEXYS Carbohydrate Analyzer can be fully controlled by different Chromatography Data System (CDS) software, namely DataApex™ Clarity™ CDS (version 8.3 and up) or Thermo Scientific™ Chromeleon™ CDS (version 7.2 SR 5 and up).

Ordering information

Detector only	
176.0035A	DECADE Elite SCC electrochemical detector
116.4321	SenCell 2 mm Au HyREF
Recommended ALEXYS analyzer + parts	
180.0055W	ALEXYS Carbohydrate Analyzer - with Solvent Switch Valve
116.4321	SenCell 2 mm Au HyREF
Software	
195.0035#	Clarity CDS single instr. incl LC, AS module

#) optional: Antec ECD drivers for use with Chromeleon CDS , OpenLAB CDS or OpenLAB Chemstation CDS are available.

For research purpose only. The information shown in this communication is solely to demonstrate the applicability of the ALEXYS system and DECADE Elite detector. The actual performance may be affected by factors beyond Antec's control. Specifications mentioned in this application note are subject to change without further notice.

Antec Scientific (USA)
info@AntecScientific.com
www.AntecScientific.com
T 888 572 0012

Antec Scientific (worldwide)
info@AntecScientific.com
www.AntecScientific.com
T +31 71 5813333