Application Note

Pharmaceutical & Biotech

Pharmaceutical & Biotech analysis

Aminoglycosides
- Amikacin
- Framycetin Sulphate
- Gentamicin Sulphate
- Kanamycin Sulphate
- Lincomycin
- Neomycin
- Spectinomycin
- Tobramycin

PET imaging tracer
- FDG

Macrolide antibiotics
- Azithromycin
- Azaerythromycin
- Clarithromycin
- Erythromycin
- Roxithromycin

Bioanalysis of pharmaceutics
- Artemisinin
- Dihydro-artemisinin
- Artemether
- Etoposide
- 8-OH-DPAT
- mesna BNP7787
- Vincristine

Heparin Sodium - USP method

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

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. Anion Exchange HPLC in combination with Pulsed Amperometric Detection (PAD) is used in the U.S. Pharmacopoeia to determine the organic impurities in Heparin [35]. Using this method the presence of galactosamine in hydrolysed Heparin samples can be determined with high sensitivity.

Heparin Sodium - USP method

Summary

The Heparin analysis was evaluated on an Antec ALEXYS LC-EC analyzer according the official 2014 Heparin Sodium USP mono-graph 37-NF32 [5].

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

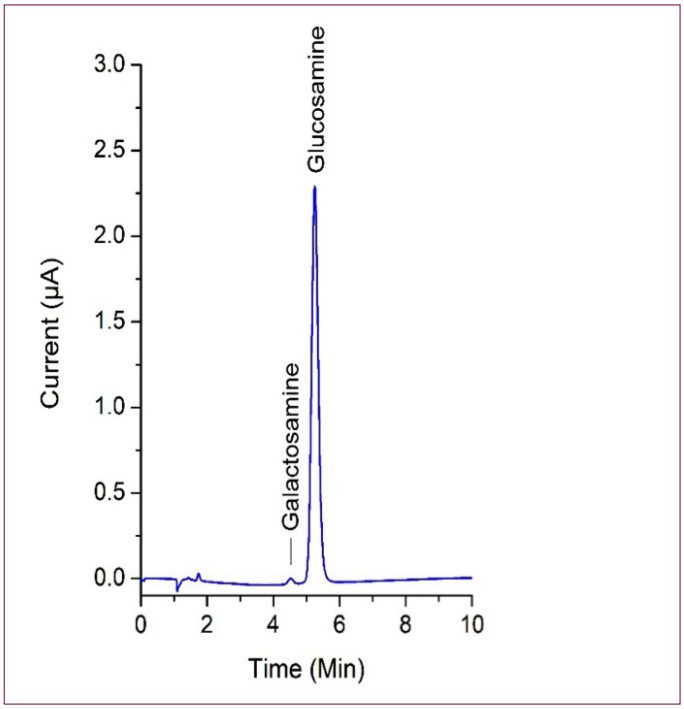

Figure 1: 10 µL injection of an acid-hydrolyzed standard solution of 8 µg/mL Glucosamine and 80 ng/mL Galactosamine in 50 mM HCl (hydrolyzed standard solution as described in the USP monograph).

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. The presence of galactosamine is a measure for the degree of contamination of Heparin with oversulfated 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.

Separation

Separation of GlcN and GalN is achieved using an anion-exchange column and elution with an alkaline mobile phase (14 mM potassium hydroxide).

Table 1

LC-EC Conditions	
HPLC	ALEXYS LC-EC Analyzer with electrically-actuated 1/8" low-pressure valve in the pump inlet line (mobile phase selector)
Column	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, 5µm, packing L69
Mobile phase	14 mM potassium hydroxide
Column cleaning	100 mM potassium hydroxide
Flow rate	0.5 mL/min
Vinjection	10 µL
Temperature	30°C for separation and detection
Flow cell	VT-03 with Au WE and HyREF (Pd/H_2) RE, spacer 50 µm
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
I-cell	ca. 1.5 µA
ADF	0.5 Hz
Range	5 µA

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, 5µm, packing L69. The following Thermo Scientific columns which matches this criteria were chosen for the method evaluation:

- AminoTrap column, 3x30mm (p/n 060146)
- CarboPac PA20 Guard, 3 x 30 mm (p/n 060144)
- CarboPac PA20 Analytical, 3x150 mm (p/n 060142)

The columns are installed in series, in the exact order, as listed above. The AminoTrap column is not required in case no amino acids are present in the sample. In such situation remove the AminoTrap column in the system for optimal performance. The analysis is based on a step-gradient, see table 2.

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

The LC-EC system was equipped with only one pump and a Vici electrically-actuated low pressure (LP) valve in the pump LP suction line to switch between mobile phase and the solution for column clean-up. 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 sonificated 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.

Detection

For the detection of the hexoamines an Antec VT-03 electrochemical flow cell is used for this evaluation. This flow cell has an Au working electrode (WE), HyREF (Pd/H_2) reference electrode (RE) and stainless steel auxiliary electrode (AE). 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 2 below.

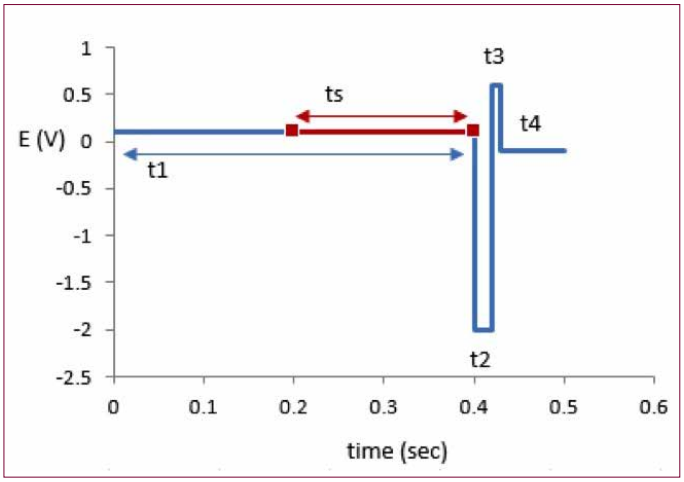

Figure 2: 4-step PAD potential waveform for the detection of GalN and GlcN as described in the Heparin sodium USP monograph.

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.

An ALEXYS LC-EC system with the new DECADE Elite electrochemical detector is required to be able to analyze Heparin with the 4-step potential waveform described in the USP monograph.

Sample preparation

Sample digestion was achieved in the following way:

- Transfer 12 mg of Heparin into 7 mL screw cap vial.
- Add 5 mL of 5N HCl solution and vortex the solution.
- Hydrolyze 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 5N HCl.
- 16 µg/mL Galactosamine stock solution: dissolve 160 mg Galactosamine in 100 mL 5N HCl. Subsequently add 100 µL of the 1.6 mg/mL solution to 99.9 mL 5N HCl.
- 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.

Heparin Sodium - USP method

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 HCl (hydrolyzed standard solution as described in the USP monograph) is show in figure 1. The retention times for Galactosamine and Glucosamine were 4.5 and 5.3, respectively.

In the USP monograph for Heparin Sodium the following system suitability requirements are specified:

- *Resolution*: minimum 2.0 between the Glucosamine and Galactosamine peak.
- *Column efficiency*: minimum 2000 theoretical plates for the Glucosamine peak.
- *Tailing factor*: between 0.8 and 2.0 for both the Glucosamine and the Galactosamine peak.

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

Table 3

USP System Suitability Requirement		
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 AminoTrap column has a negative effect on the chromatographic performance parameters. Without the AminoTrap 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 AminoTrap column for best performance.

Linearity, Repeatability & 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.

Table 4

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

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.

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 3 on the next page.

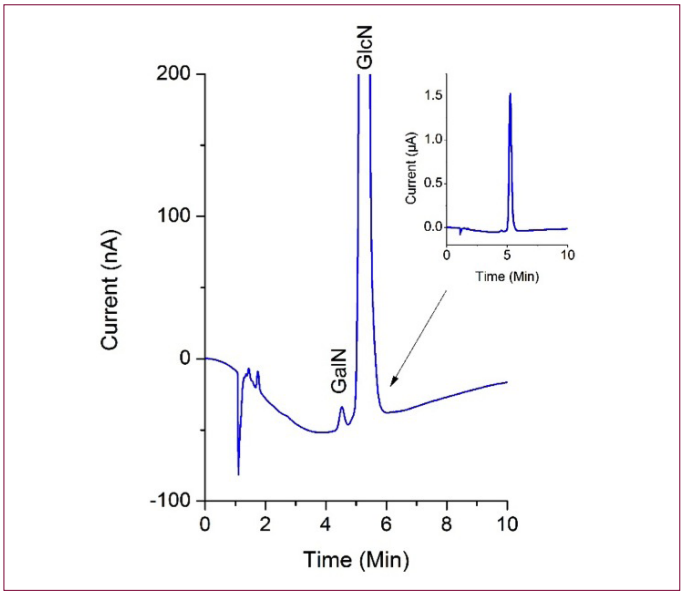

Figure 3: 10 µL injection of hydrolyzed sample 051M1130V (zoom-in on Galactosamine peak).Top-right: full scale chromatogram.

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 follows:

(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 is calculated in the following way:

(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 hexo- samines 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 in total hexosamine in Heparin sample		
Sample	*USP criteria %GalN*	*Measured %GalN*
Sample 051M1160V	< 1	0.6

The contents of Galactosamine in the analyzed Heparin sample was within the specified limits of the USP monograph.

Conclusion

The ALEXYS Analyzer including the new DECADE Elite detector 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.

References

1. Public Health Update: Recall of Heparin Sodium for Injection (2/28/2008), FDA web site: http://www.fda.gov/Drugs/DrugSafety/PostmarketDrugSafetyInformationforPatientsandProviders/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
6. *Analysis of Heparin using a 3-step potential waveform*, Antec application note, **217_034**

PART NUMBERS AND CONFIGURATIONS

180.0057C	ALEXYS Heparin analyzer, including columns & flow cell

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