Application Note

Drugs & Pharmaceuticals

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Artemisinin
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Etoposide
Epinephrine
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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 according to USP method

- **Pharmacopeia USP-NF 2024**
- **New USP L69 listed stationary phase**
- **Novel efficient amino acid trap, 5 µm particles**
- **Limit of galactosamine in total hexosamine**

Summary

Galactosamine impurities in heparin sodium hydrolyzates are analyzed on an ALEXYS carbohydrates analyzer using the method described in the official USP monograph 2024 [1,2]. The new SweetSep™ AEX20 column (USP L69 packing) is used in combination with a trap column to eliminate amino acids from the sample that may interfere with the analysis. The stationary phase of both the SweetSep™ AEX20 anion-exchange column and amino acid trap column are based on highly monodisperse 5 µm resin particles. The new AEX20 column in combination with an amino acid trap precolumn enables fast high-resolution analysis of galactosamine (GalN) impurities in hydrolyzed Heparin samples, with superior resolution between galactosamine and glucosamine peaks.

Application Note # 217_033_15

Heparin sodium

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 heparin was announced in the US due to reported adverse reactions (hypotension, allergic reactions) leading to fatalities in some cases [3, 4]. Upon investigation it became evident that the heparin products were adulterated with over-sulfated chondroitin sulfate, a closely related substance which resembles heparin. As a result, in 2009 the U.S. Pharmacopoeia (USP) revised the heparin monograph to address this specific adulteration issue and ensure the safety and quality of pharmaceutical-grade heparin.

In the USP-NF 2024 monograph a method based on High Performance Anion Exchange Chromatography followed by Pulsed Amperometric Detection (HPAEC-PAD) is described to analyze the organic impurities in heparin [1, 5]. Using this approach the presence of small amounts of galactosamine impurities in hydrolyzed heparin samples can be determined with high sensitivity. In this application note we present data of the impurity analysis of heparin following the USP monograph using the new SweetSep™AEX20 column and amino acid trap precolumn, based on 5 µm polymer particle technology.

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.

Figure 1. ALEXYS Carbohydrate Analyzer consisting of the ET210 eluent tray (for N_2 blanketing), a P6.1L quaternary LPG pump, AS6.1L autosampler, CT2.1 column thermostat, and the DECADE Elite electrochemical detector.

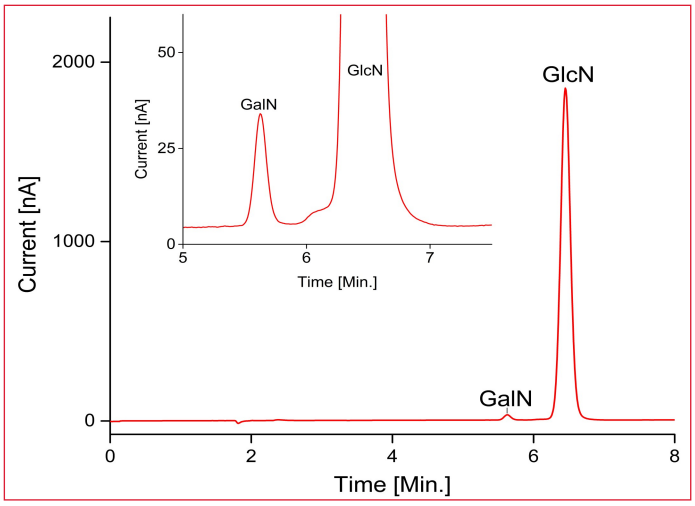

Figure 2. 10 µL injection of an acid-hydrolyzed standard solution of 8 µg/ mL glucosamine and 80 ng/mL galactosamine in 50 mM HCl (hydrolyzed standard solution as described in the USP monograph).

On the contrary, over-sulfated chondroitin sulfate in adulterated heparin samples consists 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 of the degree of contamination of heparin with over-sulfated chondroitin sulfate. The USP acceptance criteria for heparin is

Table 1

LC-ECD conditions	
HPLC	ALEXYS™ Carbohydrate Analyzer
Detector	DECADE Elite electrochemical detector
Columns	SweetSep™ AEX20, 4 × 200 mm column, 5 µm Amino acid trap, 4 × 50 mm column, 5 µm Borate ion trap, 4 x 50 mm column, 10 µm (Antec Scientific)
Mobile phase	A: 14 mM KOH (elution) B: 100 mM KOH (column clean-up & regeneration) Eluents prepared & blanketed with Nitrogen 5.0
Flow rate	0.7 mL/min
System backpressure	About 185 bar
Temperature	30 °C for separation and detection
Injection volume	10 µL
Pump piston wash	DI water (refresh weekly)
Flow cell	SenCell™ with 2 mm Au and HyREF Pd RE, 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 0.2 - 0.4 µA

that not more than 1% galactosamine is present relative to the total amount of hexosamine (GlcN & GalN) in a hydrolyzed sample solution.

Separation

The separation of GlcN and GalN is achieved using an ALEXYS carbohydrates analyzer equipped with an ET 210 eluent tray for Nitrogen blanketing, quaternary LPG pump, AS 6.1L auto sampler, CT 2.1 column thermostat and DECADE Elite electro-chemical detector with SenCell (Figure 1). The separation is based on elution on an anion-exchange column with an alkaline mobile phase (14 mM potassium hydroxide). The analysis is based on a step-gradient, consisting of elution step, followed by a column clean-up and equilibration step (Table 2).

Table 2

Step- gradient program

Time (min)	*Mobile phase*	*Description*
0 - 10	14 mM KOH (A)	Elution & detection
10 - 20	100 mM KOH (B)	Column clean-up/regeneration
20 - 40	14 mM KOH (A)	Equilibration to the starting condition

The eluents were carefully prepared manually using a commercial 45% KOH solution (< 0.3% K_2CO_3). The diluent was deionized water (resistivity >18.2 MΩ-cm, TOC < 5 ppb), which was sonicated and sparged with nitrogen 5.0 prior to use. The mobile phases were blanketed with nitrogen during the analysis to minimize the build-up of carbonate ions in the mobile phase and to ensure reproducible analysis.

Columns

In the USP Heparin monograph the use of the following column types are described: 30 x 3 mm ID amino acid trap column in series with a 30 x 3 mm ID guard column and a 150 x 3 mm ID cm analytical column. The guard and analytical column being packed with USP L69 phase. The L69 packing, as described by the USP, consists of an ethylvinylbenzene/divinylbenzene substrate agglomerated with quaternary amine functionalized 130 nm latex beads, about 6.5 µm in diameter.

AEX20 analytical column

Antec Scientific has introduced an innovative new stationary phase, AEX20, based on the same polymeric substrate as the L69 packing, featuring highly monodisperse 5 µm particles, a substantially higher degree of crosslinking (80% instead of 55%) and agglomerated with latex nano particles function-alized with quaternary amine exchange groups. The SweetSep AEX20 stationary phase is listed at the USP as L69 packing [2].

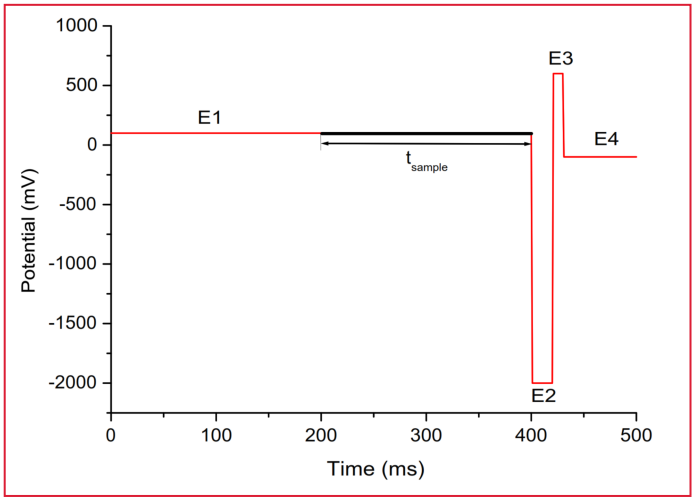

Figure 3. 4-step PAD potential waveform for the detection of GalN and GlcN as described in the Heparin sodium USP monograph.

and due to its smaller 5 µm particle size enables higher resolution separations of the amino sugars released from hydrolyzed heparin samples. An 200 x 4 mm ID AEX20 analytical column without guard column was used for this evaluation. A guard column is not necessary, because of the use of an amino acid trap. The trap column will function as a guard to prevent the accumulation of contaminations and particulate matter on the analytical column.

Amino acid trap column

The novel Antec scientific amino acid trap column is also based on a monodisperse 5 µm polymeric resin which efficiently retain amino acids and peptides that may be present in hydrolyzed heparin samples, without affecting the retention of galactosamine (GalN) and glucosamine (GluN) significantly. The small particle size and distribution of the trap resin will also assure better peak efficiencies and resolution of the sugars of interest on the analytical column.

Borate ion trap column

In carbohydrate analysis, the peak shape of certain sugars, such as mannose, sugar alcohols and fructose, are deteriorated when traces of borate are present in the mobile phase. A borate ion trap column (4 x 50 mm) was installed in the solvent line between the pump and autosampler as a precaution to eliminate borate ions from the mobile phase.

The USP (general 621 chromatography chapter) allows adjustments of the column length (L) and particle size (dp) as long as the L/dp ratio remains in the range of –25% to + 50% of the prescribed L/dp ratio. In this case the prescribed L/dp ratio is 27700 , based on the length of the analytical + guard column (180 mm). The L/dp ratio based on the 200 mm AEX20 column is 40000, which is allowed as it lays within the specified upper

limit of +50%. With respect to flow rate adjustments in connection with adjustments in particle size and column inner diameter the USP general chapter uses the following formula to correct the flow rate for such changes:

$F_2 = F_1 \times [dc_2^2 \times dp_1)/(dc_1^2 \times dp_2)]$

Where F_1 and F_2 are the flow rates for the original and modified conditions, respectively; dc_1 and dc_2 are the respective column diameters, and dp_1 and dp_2 the particle sizes. In addition, the flow rate can be adjusted by ± 50% (isocratic elution only).

The corrected flow rate based on the change in particle size, column length and inner diameter was calculated using the before mentioned formula. To compensate for the applied adjustments a flow rate of 1.16 mL/min was calculated. However, a lower flow rate of 0.7 mL/min (-40%) was chosen for the AEX20 column. This flow rate is optimal for this specific column with respect to plate height (van Deemter), and within the allowed limit for flow rate changes (± 50%) described in the USP general chromatography chapter.

Table 3

USP system suitability requirements

Parameter	*USP criteria*	*Measured*
Resolution between GalN and GlcN	> 2.0	3.5
Column efficiency (GlcN)	>2000	9194
Tailing factor (GalN)	0.8-2.0	1.09
Tailing factor (GlcN)	0.8-2.0	1.06

Table 4

Linearity

Compound	*Concentration range (µg/mL)*	*R*
Galactosamine	0.1 - 8	0.99997
Glucosamine	0.1 - 8	0.99999

Detection

For the PAD detection of the hexoamines the Antec SenCell™ electrochemical flow cell (Figure 1) is used [6]. This flow cell with wall-jet design consists of a Au working electrode, palladium hydrogen (HyREF) 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. The cell current was typically about 0.2-0.4 µA with these PAD settings. This particular 4-step waveform with a pulse duration of 500 ms has several benefits: (1) a consistent long-term peak area response and (2) minimal electrode wear [7]. The temperature for separation and detection was set to 30°C as specified in the monograph.

Sample preparation

Sample digestion was achieved in the following way:

- Transfer 12 mg of heparin into a 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 HCl.
- 16 µg/mL galactosamine stock solution: dissolve 160 mg galactosamine in 10 mL 5 N HCl. Subsequently, add 100 µL of the 16 mg/mL solution to 99.9 mL 5 N 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.

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 HCl is shown in Figure 2. The retention times for galactosamine and glucosamine were 5.6 and 6.5 min, respectively. The system suitability is evaluated using the chromatogram obtained with the hydrolyzed standard solution. The results are listed in Table 3, and it is evident that all performance parameters are well within the criteria of the USP system suitability requirements.

Linearity, repeatability, and LOD

The linearity for both glucosamine and galactosamine were investigated in the concentration range of 0.1 µg/mL – 8 µg/mL, see Table 4. The linear correlation coefficient was > 0.9999 for both sugars. The relative standard deviation (RSD) in peak area was determined for 10 replicate injections of the standard

solution. The RSD in peak area was 0.4% and 0.1% for GalN and GlcN, respectively. The Limit of Quantitation (LOQ) for GalN was determined based on the response obtained with a 0.1 µg/mL standard, and was about 10 ng/mL, which demonstrates the excellent sensitivity of the method.

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 sample was also spiked with the system suitability standard solution to ensure the identification of galactosamine, which may present in a small amount in the sample. The final concentration of the spike solution was 8 µg/mL glucosamine and 80 ng/mL galactosamine. The chromatograms obtained for the spiked sample and non-spiked hydrolyzed sample are shown in Figure 4.

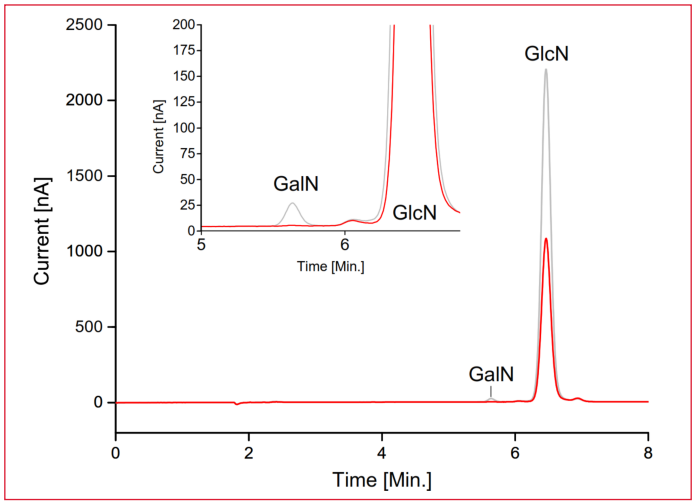

Figure 4. 10 µL injection of non-spiked hydrolyzed sample 051M1130V (red trace) and 10 µL injection of spiked hydrolyzed sample 051M1130V (grey trace). Inset shows the zoom-in on galactosamine and glucosamine peaks.

Table 5

Limit of galactosamine for total hexosamine in heparin sample

Sample	*USP limit %GalN*	*Measured %GalN*
051M1160V	<1	0.04

The percentage of GalN in the hydrolyzed heparin sample is calculated compared to that of the hydrolyzed standard solution. The relative response ratio ($GalN_R$) of GalN/GlcN in the hydrolyzed standard solution was calculated as:

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

where:
$GalN_B$ = Peak area of GalN from hydrolyzed standard solution
$GalN_W$ = Weight of GalN for the standard solution
$GlcN_W$ = Weight of GlcN for the standard solution
$GlcN_B$ = Peak area of GlcN from hydrolyzed standard solution

The percentage of galactosamine in the sample was calculated as:

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

where:
$GalN_U$ = Peak area of GalN from hydrolyzed sample solution
$GalN_R$ = Response ratio of GalN (1)
$GlcN_U$ = Peak area of GlcN from hydrolyzed sample solution

The USP acceptance criteria 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. The amount of galactosamine present in the sample 051M1130V is within the USP acceptance criteria for heparin.

References

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5. W.R. LaCourse, "Pulsed Electrochemical Detection in High Performance Liquid Chromatography", John Wiley & Sons, New York, 1ed, 1997.
6. H. Louw, H.J. Brouwer, N. Reinhoud, Electrochemical flowcell, (2016) US patent 9310330
7. R.D. Rocklin, A.P. Clarke, M. Weitzhandler, Anal. Chem, 70, (1998), 1496-1501

Conclusion

The presented method using an ALEXYS carbohydrate analyzer provides a reliable solution for the sensitive analysis of low level of GalN impurities in commercial heparin hydrolysate samples based on the official USP method.

The use of the new SweetSep™ AEX20 (USP L69 packing) in combination with the amino acid trap column resulted in separations with exceptionally good peak efficiencies, tailing factors and resolution between GalN & GlcN. The excellent performance ensures that the system suitability requirements are easily met and contribute to more robust and hassle-free analysis.

Heparin sodium

Ordering information

ALEXYS analyzer	
180.0057W	ALEXYS Carbohydrates Analyzer - gradient (quaternary LPG)
116.4321	SenCell 2 mm Au HyREF
186.ATC00	CT2.1 Column Thermostat
Columns	
260.0020	SweetSep™ AEX20, 4 x 200 mm column, 5 µm
260.0040	Amino acid trap, 4 × 50 mm column, 5 µm
260.0030	Borate ion trap, 4 x 50 mm column, 10 µm
260.0100*	Pre-column filter PEEK, 0.5 µm
Software#	
195.0035	Clarity CDS single instr. incl LC, AS module

*) In case samples might contain particulate matter it is advised to use a pre-column filter.

#) Antec ECD drivers are available for Chromeleon CDS, OpenLAB CDS and Empower CDS. The ALEXYS Carbohydrates Analyzer (full system) can also be controlled under Thermo Fisher Scientific Chromeleon™ CDS. Please contact Antec for more details.

Reagents, standards and sample prep accessories

Potassium hydroxide solution 45%	Fluka, pn 3564
DI water 18.2 MΩ.cm, TOC < 5 ppb	YoungIn Chromass Aquapuri Essence+ 393
D-(+)-Glucosamine hydrochloride	Sigma Aldrich, pn G4875
D-(+)-Galactosamine hydrochloride	Sigma Aldrich, pn G0500
Heparin Sodium salt from porcine intestinal mucosa	Sigma Aldrich, pn H3393-100KU
Hydrochloric acid 36.5-38%	Fisher scientific, pn 9535-01

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 and may be adjusted accordingly. Specifications mentioned in this application note are subject to change without further notice.

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