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⟨207⟩ TEST FOR 1,6-ANHYDRO DERIVATIVE FOR ENOXAPARIN SODIUM

The following procedure is used to determine the levels of 1, 6-anhydro forms in enoxaparin sodium. [NOTE—The test for the 1,6-anhydro derivative is conducted only where specified in the individual monograph.]

INTRODUCTION

The disaccharides specified in this general chapter are listed by name and structure in [Appendix 1](#); the oligosaccharides are listed in [Appendix 2](#).

Depolymerization of heparin into enoxaparin sodium produces a partial but characteristic conversion of glucosamines at the reducing termini of oligosaccharide chains with terminal glucosamine 6-O sulfate, yielding 1,6-anhydro derivatives (see [Figure 1](#)).

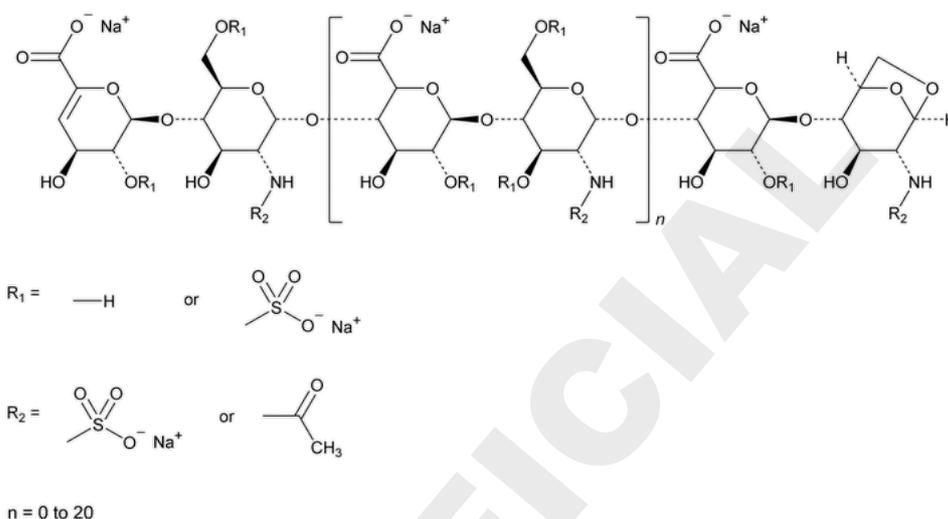


Figure 1. Structure of enoxaparin sodium containing a 1,6-anhydro derivative on the reducing end of the chain.

The percentage of oligosaccharide chains that are cyclized in a 1,6-anhydro ring is a characteristic of enoxaparin sodium.

PROCEDURES

• DEPOLYMERIZATION OF ENOXAPARIN SODIUM BY HEPARINASES AND RESULTING OLIGOSACCHARIDES

The assay involves HPLC analysis of a depolymerized enoxaparin sodium solution by a mixture of heparinases. After enzymatic depolymerization, the main 1,6-anhydro residues of enoxaparin sodium observed are 1,6-anhydro Δ IIS and 1,6-anhydro Δ IIS^{epi}, and 1,6-anhydro Δ IS and 1,6-anhydro Δ IS–IS^{epi} (see [Appendix 2](#)).

The 1,6-anhydro Δ IS–IS^{epi} tetrasaccharide (2-O-sulfated mannosamine form) is not completely cleaved by the heparinases. The two disaccharides (1,6-anhydro Δ IIS and 1,6-anhydro Δ IIS^{epi}), which generally co-elute, are poorly resolved with respect to Δ IIA (see [Appendix 1](#)), especially because the latter occurs as two anomers: α and β . To allow quantitation of 1,6-anhydro Δ IIS and 1,6-anhydro Δ IIS^{epi}, the enoxaparin sodium sample already depolymerized by heparinases is then reduced by sodium borohydride (see [Figure 2](#)).

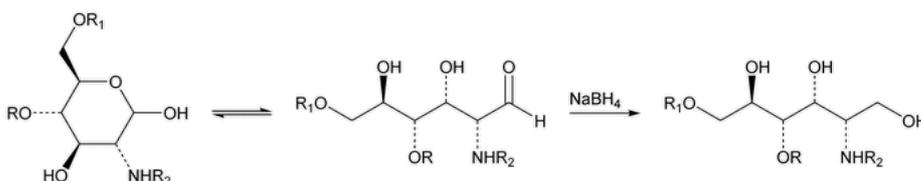


Figure 2. Reduction of oligosaccharides by sodium borohydride

The sodium borohydride reduction eliminates the $\alpha \leftrightarrow \beta$ anomeric effect by opening the terminal oligosaccharide ring. The four 1,6-anhydro derivatives (see [Appendix 2](#).) are not reduced by sodium borohydride because the ring opening is blocked by the 1,6-anhydro bridge. The reduction of the oligosaccharides decreases their retention time, whereas the retention time of the 1,6-anhydro derivatives remains unchanged. Thus, it is possible to separate the two compounds—1,6-anhydro Δ IIS and 1,6-anhydro Δ IIS^{epi}—from the reduced Δ IIA disaccharide peak.

[NOTE—1,6-Anhydro Δ IIS and 1,6-anhydro Δ IIS^{epi} are eluted as two nonresolved peaks and are quantitated together as a single compound, 1,6-anhydro Δ IIS. Therefore, for the purpose of simplification, the epimeric form is not referred to in the remaining text.]

Change to read:

• PROCEDURE

Solutions

Solution A: Dissolve 0.280 g of monobasic sodium phosphate in 950 mL of water, adjust with phosphoric acid to a pH of 3.0, and dilute with water to 1000 mL.

Solution B: Dissolve 140 g of sodium perchlorate in 950 mL of *Solution A*, adjust with phosphoric acid to a pH of 3.0, and dilute with *Solution A* to 1000 mL.

Mobile phase: Use variable mixtures of filtered and degassed *Solution A* and *Solution B* as directed in *Chromatographic system*.

Sodium/Calcium acetate pH 7.0 solution: Dissolve 10 mg of bovine serum albumin and 32 mg of calcium acetate in 60 mL of water. Add 580 μ L of glacial acetic acid, and adjust with 2 M sodium hydroxide to a pH of 7.0. Transfer to a 100-mL volumetric flask, and dilute with water to volume. Pass the solution through a filter having a porosity of 0.45 or 0.22 μ m.

Potassium phosphate pH 7.0 buffer: Dissolve 68 mg of monobasic potassium phosphate and 10 mg of bovine serum albumin in 30 mL of water in a 50-mL volumetric flask. Adjust with potassium hydroxide, if necessary, to a pH of 7.0, and dilute with water to volume. Pass the solution through a filter having a porosity of 0.45 or 0.22 μ m.

Sodium borohydride solution: Dissolve 12 mg of sodium borohydride in 400 μ L of water, and mix on a vortex mixer.

[NOTE—Prepare fresh *immediately* before use.]

Heparinase 1 solution: Dissolve heparinase 1 (see *Reagent Specifications* under *Reagents, Indicators, and Solutions*) [reference: heparin lyase I, EC 4.2.2.7] in *Potassium phosphate pH 7.0 buffer* to obtain a solution having an activity of 0.4 IU per mL. Store the solution at -20° until ready to use. [NOTE—Heparinase solutions can be stored for 3 months at -20° .]

Heparinase 2 solution: Dissolve heparinase 2 (see *Reagent Specifications* under *Reagents, Indicators, and Solutions* [no EC number] in *Potassium phosphate pH 7.0 buffer* to obtain a solution having an activity of 0.4 IU per mL. Store the solution at -20° until ready to use.

Heparinase 3 solution: Dissolve heparinase 3 (see *Reagent Specifications* under *Reagents, Indicators, and Solutions* [reference: heparitinase I, EC 4.2.2.8] in *Potassium phosphate pH 7.0 buffer* to obtain a solution having an activity of 0.4 IU per mL. Store the solution at -20° until ready to use.

Heparinases 1, 2, 3, solution: Prepare a 1:1:1 (v:v:v) mixture of *Heparinase 1 solution*, *Heparinase 2 solution*, and *Heparinase 3 solution*.

Peak identification solutions

[NOTE—The depolymerized test solutions and Standard solutions must be prepared at the same time. Depolymerized test solutions are stable for 1 month at -20° . Also, the reduced test solutions and Standard solutions must be prepared at the same time. Reduced solutions are also stable for 1 month at -20° .]

Disaccharide solutions: Separately prepare a 0.25 mg per mL solution of each disaccharide¹ Δ IA, Δ IIA, Δ IIIA, Δ IVA, Δ IS, Δ IIS, Δ IIIS, Δ IVS (see [Appendix 1](#)). Chromatograph each disaccharide solution, and record the peak responses.

Reduced disaccharide solutions: To 60 μ L of each *Disaccharide solution*, add 10 μ L of freshly prepared *Sodium borohydride solution*. Mix on a vortex mixer, and allow to stand at room temperature for at least 4 hours. Chromatograph each solution, and record the peak response.

Blank solution: Prepare a mixture of 20 μ L of water, 70 μ L of *Sodium/Calcium acetate pH 7.0 solution*, and 100 μ L of the *Heparinases 1, 2, 3 solution*. Mix gently by inversion, and allow to stand for at least 48 hours in a 25 $^\circ$ water bath. Prepare a mixture of 60 μ L of this depolymerized solution with 10 μ L of freshly prepared *Sodium borohydride solution*. Homogenize, and allow to stand at room temperature for at least 4 hours. Chromatograph the resulting solution, and record the peak responses.

Test solution 1: Prepare two solutions, each containing 20 mg of enoxaparin sodium in 1 mL of water.

Standard solution 1: Prepare one solution containing 20 mg of [USP Enoxaparin Sodium RS](#) in 1 mL of water.

Test solution 2: For each solution, prepare a mixture of 20 μ L of *Test solution 1*, 70 μ L of *Sodium/Calcium acetate pH 7 solution*, and 100 μ L of *Heparinases 1, 2, 3 solution*. Mix gently by inversion, and allow to stand for at least 48 hours in a 25 $^\circ$ water bath. After 48 hours of depolymerization, chromatograph the solution, and record the peak responses.

Standard solution 2: Prepare a mixture of 20 μ L of *Standard solution 1*, 70 μ L of *Sodium/Calcium acetate pH 7 solution*, and 100 μ L of *Heparinases 1, 2, 3 solution*. Mix gently, and allow to stand for at least 48 hours in a 25 $^\circ$ water bath. After 48 hours of depolymerization, chromatograph the solution, and record the peak responses.

Test solution 3: For each depolymerized test solution, prepare a mixture of 60 μ L of *Test solution 2* and 10 μ L of freshly prepared *Sodium borohydride solution*. Homogenize, and allow to stand loosely capped at room temperature for at least 4 hours before injecting into the chromatograph. *Test Solution 3* is stable for 48 hours at room temperature.

Standard solution 3: Prepare a mixture of 60 μ L of *Standard solution 2* and 10 μ L of freshly prepared *Sodium borohydride solution*.

Homogenize and mix on a vortex mixer, and allow to stand loosely capped at room temperature for at least 4 hours before injecting into the chromatograph. *Standard solution 3* is stable for 48 hours at room temperature.

Chromatographic system

(See [Chromatography \(621\)](#).)

The liquid chromatograph is equipped with a 234-nm detector and a 3-mm × 25-cm column that contains 5-μm packing L14. A guard column packed with the same material should also be used. The flow rate is 0.45 mL per minute, the column temperature is maintained at 50°, and the injection volume is 10 μL. The chromatograph is programmed as follows.

Time (min)	Solution A (%)	Solution B (%)	Elution
0–20	97→65	3→35	Linear gradient
20–50	65→0	35→100	Linear gradient
50–60	0	100	Isocratic
60–61	0→97	100→3	Linear gradient for re-equilibration
61–79	97	3	Isocratic for re-equilibration

Chromatograph the reduced *Test solution 3* and the reduced *Standard solution 3*, and record the peak responses as directed for *Procedure*.

Depolymerization suitability test: The ratio of the peak area of 1,6-anhydro-ΔIS-IS to that of 1,6-anhydro ΔIS is not more than 1.15 for the depolymerized *Standard solution 2*.

Column performance suitability test: Identify the peaks corresponding to reduced ΔIA and 1,6-anhydro-ΔIS for the *Standard solution 3*: the retention time of reduced ΔIS is between 27 and 33 minutes for the depolymerized and reduced *Standard solution 3*; and the resolution, *R*, between reduced ΔIA and 1,6-anhydro-ΔIS is not less than 1.5.

Reduction suitability test: The ratio of the peak area of ΔIS disaccharide to that of reduced ΔIS in the depolymerized and reduced *Standard solution 3* and *Test solution 3* is not more than ▲0.02.▲ (ERR 1-Oct-2018)

Procedure/Calculation: Separately inject equal volumes of the reduced *Test solutions 3* and the reduced *Standard solution 3* into the chromatograph. Use the normalized area percentage method for calculation. Each peak is integrated from the dwell volume peak to the last detected peak. Measure the area of each analyte peak after excluding solvent peaks at the beginning of the chromatogram and in the *Blank solution*. Using the previously obtained chromatograms of the *Reduced disaccharide solutions*, identify peaks belonging to the eight reduced disaccharides in the chromatograms for *Test solution 3* and *Standard solution 3*. The peaks belonging to 1,6-Anhydro ΔIS, 1,6-Anhydro ΔIIS, and 1,6-Anhydro ΔIS-ISEPI are identified from the relative retention times provided in [Table 1](#) and the Reference chromatogram provided with [USP Enoxaparin Sodium RS](#). Once the peaks have been identified, use the values in [Table 1](#) to calculate the (w/w) percentage of the three main 1,6-anhydro derivatives obtained after depolymerization of enoxaparin sodium using the following formula:

$$\% \text{ 1,6-anhydro } i \text{ (w/w)} = (100 \times MW_i \times A_i) / \Sigma(MW_x \times A_x)$$

MW_i = molecular weight of the 1,6-anhydro peak *i*

A_i = area of the 1,6-anhydro peak *i*

MW_x = molecular weight of either the peak *X* or the zone *X* specified by its retention time

A_x = area of either the peak *X* or the zone *X* specified by its retention time

[NOTE—Once the method is established, the peaks belonging to the different di- and tetrasaccharides can be easily identified using the [USP Enoxaparin Sodium RS](#) chromatogram. Thus, the use of the disaccharide Standards is only needed during the method-implementation stage.]

Calculate the molar percentage of components containing a 1,6-anhydro structure at the reducing end of their chain in the enoxaparin sodium test sample according to the following formula:

$$\%1.6anhydro = 100 \times \frac{MW}{\Sigma MW_x \times Area_x} \times (Area\Delta Is1.6anhydro + Area\Delta IIs1.6anhydro + Area\Delta Is - Is1.6anhydro)$$

in which *MW* is the mass-average molecular mass (see *Identification test D* under *Enoxaparin Sodium*); MW_x and $Area_x$ are the molecular weight and the area, respectively, of either the peak *X* or the range *X* specified by its retention time. The molar percentage of components having a 1,6-anhydro structure at the reducing end of their chain is between 15% and 25%. Typical retention times and molecular masses attributed to different oligosaccharide structures are provided in [Table 1](#).

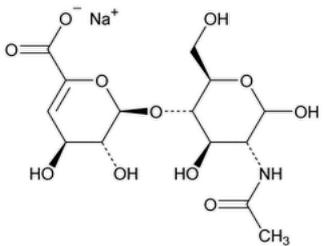
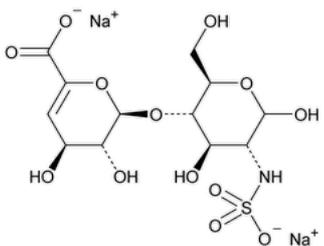
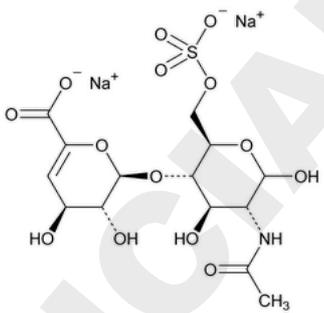
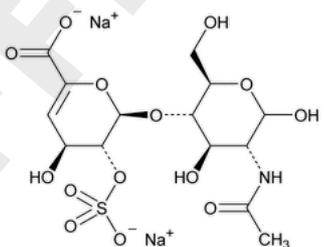
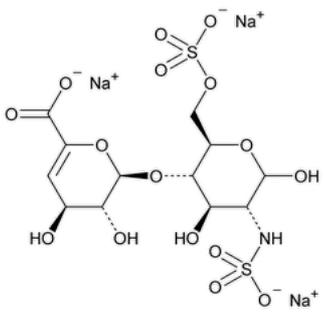
Table 1. Typical Relative Retention Times (t_{RR}) and Molecular Masses Attributed to Different Compounds*

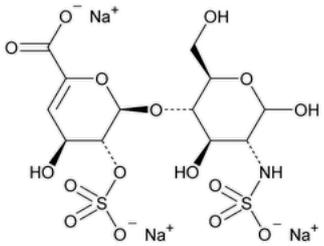
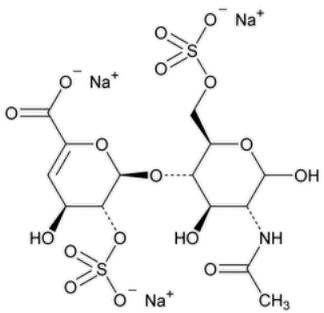
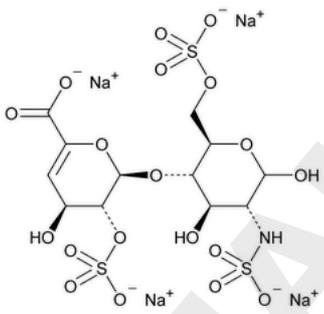
Compound	t_{RR}	Molecular Mass (Daltons)
–	< 0.25	741
Reduced Δ IVA	0.25	401
–	$0.25 < t_{RR} < 0.51$	741
Reduced Δ IVS	0.51	461
–	$0.51 < t_{RR} < 0.55$	483
Reduced Δ IIA	0.55	503
–	$0.55 < t_{RR} < 0.59$	503
1,6-Anhydro Δ IIS	0.59	443
–	$0.59 < t_{RR} < 0.64$	503
Reduced Δ IIIA	0.64	503
–	$0.64 < t_{RR} < 0.72$	533
Reduced Δ IIS	0.72	563
–	$0.72 < t_{RR} < 0.80$	563
Reduced Δ IIIS	0.80	563
–	$0.80 < t_{RR} < 0.88$	583
Reduced Δ IA	0.88	605
–	$0.88 < t_{RR} < 0.90$	635
1,6-Anhydro Δ IS	0.90	545
–	$0.90 < t_{RR} < 0.98$	635
Reduced Δ IIA- <u>IVSglu</u>	0.98	1066
–	$0.98 < t_{RR} < 1.00$	635
Reduced Δ IS	1.00	665
–	$1.00 < t_{RR} < 1.04$	665
Δ IS	1.04	665
–	$1.04 < t_{RR} < 1.10$	1228
Reduced Δ IIA- <u>IISglu</u>	1.10	1168
–	$1.10 < t_{RR} < 1.27$	1228
1,6-Anhydro Δ IS-IS	1.27	1210
–	$t_{RR} > 1.27$	1228

* Relative retention times were obtained with a depolymerized and reduced batch of enoxaparin sodium. They are expressed relative to the retention time of the main peak corresponding to reduced Δ IS. Note that according to the quality of the column, relative

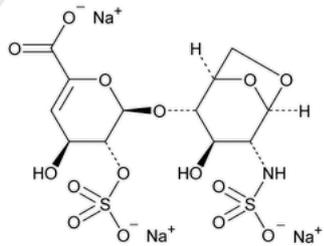
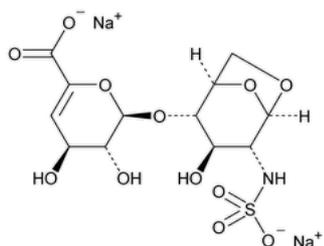
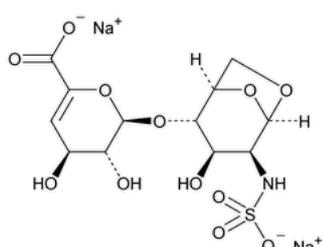
retention times can change slightly.

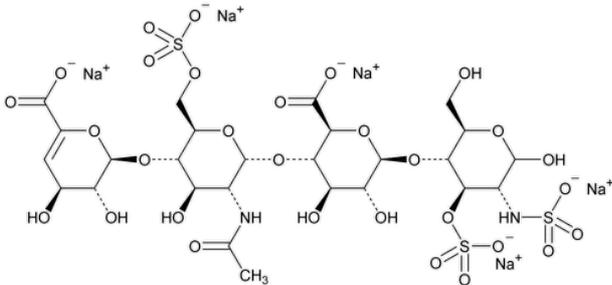
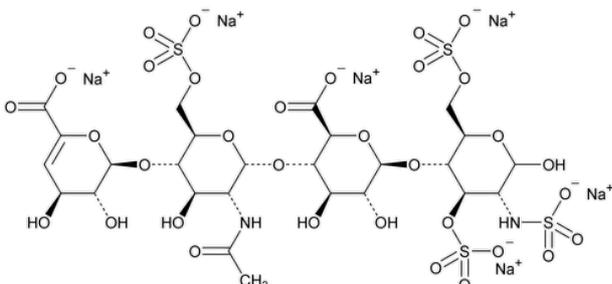
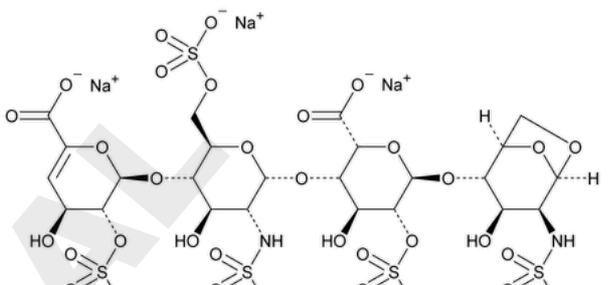
ADDITIONAL REQUIREMENTS• **USP REFERENCE STANDARDS (11)**[USP Enoxaparin Sodium RS](#)• **APPENDIX 1: STANDARD DISACCHARIDE STRUCTURES**

<p style="text-align: center;">ΔIVA</p>		<p style="text-align: center;">ΔUA-(1→4)α-GlcNAc</p>
<p style="text-align: center;">ΔIVS</p>		<p style="text-align: center;">ΔUA-(1→4)α-GlcN(NS)</p>
<p style="text-align: center;">ΔIIA</p>		<p style="text-align: center;">ΔUA-(1→4)α-GlcNAc(6S)</p>
<p style="text-align: center;">ΔIIIA</p>		<p style="text-align: center;">ΔUA-2S-(1→4)α-GlcNAc</p>
<p style="text-align: center;">ΔIIS</p>		<p style="text-align: center;">ΔUA-(1→4)α-GlcN (NS,6S)</p>

<p>ΔIIS</p>		<p>ΔUA-2S-(1→4)α-GlcN (NS)</p>
<p>ΔIA</p>		<p>ΔUA-2S-(1→4)α-GlcNAC(6S)</p>
<p>ΔIS</p>		<p>UA-2S-(1→4)α-GlcN (NS,6S)</p>

• APPENDIX 2: OLIGOSACCHARIDE STRUCTURES

<p>1,6-Anhydro ΔIS or 1,6-Anhydro ΔIS glucose</p>	
<p>1,6-Anhydro ΔIIS or 1,6-Anhydro ΔIIS glucose</p>	
<p>1,6-Anhydro ΔIIS epi or 1,6-Anhydro ΔIIS mannose</p>	

<p>ΔIIA-IVSglu</p>	
<p>ΔIIA-III Sglu</p>	
<p>1,6-Anhydro ΔIS-IS epi or 1,6-Anhydro ΔIS-IS mannose</p>	

¹ Suitable disaccharides are available from Grampian Enzymes (GE-H1001, GE-G1002, GE-H1003, GE-H1004, GE-H1005, GE-H1006, GE-H1007, GE-H1008), Nisthouse, Harray, Orkney, KW17 2LQ, United Kingdom, Tel: 01856 771771, Scottish Local Authority: Orkney Islands.

Auxiliary Information - Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
<207> TEST FOR 1,6-ANHYDRO DERIVATIVE FOR ENOXAPARIN SODIUM	Jennifer Tong Sun Senior Scientist II	BIO32020 Biologics Monographs 3 - Complex Biologics and Vaccines

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