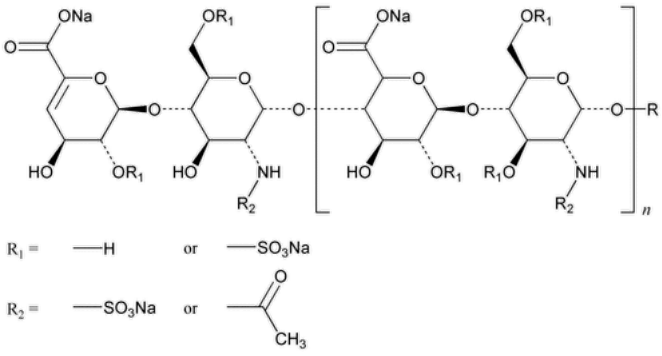
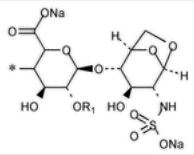
Status: Currently Official on 14-Feb-2025
Official Date: Official as of 01-Dec-2023
Document Type: USP Monographs
DocId: GUID-48AF7421-9DAB-487C-B132-3B704CAAD512_6_en-US
DOI: https://doi.org/10.31003/USPNF_M29290_06_01
DOI Ref: 1r2nq

Enoxaparin Sodium

Change to read:



R =	X = 15 - 25%		*n* = 0 to 20
	100-X	H	*n* = 1 to 21

CAS RN®: ▲679809-58-6.▲ (IRA 1-Dec-2023)

DEFINITION

Enoxaparin Sodium is the sodium salt of a depolymerized heparin. It is obtained by alkaline depolymerization of heparin benzyl ester. The starting material, heparin, is obtained exclusively from porcine intestinal mucosa. Heparin source material used in the manufacture of Enoxaparin Sodium complies with the compendial requirements stated in the Heparin Sodium monograph. Enoxaparin Sodium consists of a complex set of oligosaccharides that have not yet been completely characterized. The majority of the components have a 4-enopyranose uronate structure at the nonreducing end of their chain. About 20% of the materials contain a 1,6-anhydro derivative on the reducing end of the chain, the range being between 15% and 25%. The weight-average molecular weight of Enoxaparin Sodium is 4500 Da, the range being between 3800 and 5000 Da; about 16% have a molecular weight of less than 2000 Da, the range being between 12.0% and 20.0%; about 74% have a molecular weight between 2000 and 8000 Da, the range being between 68.0% and 82.0%. NMT 18.0% have a molecular weight higher than 8000 Da. When prepared as a solution, the solution is analyzed for clarity and degree of color using a validated method. The degree of sulfation is NLT 1.8 per disaccharide unit. It has a potency of NLT 90 and NMT 125 Anti-Factor Xa International Units (IU)/mg, and NLT 20.0 and NMT 35.0 Anti-Factor IIa IU/mg, calculated on the dried basis. The ratio of Anti-Factor Xa activity to Anti-Factor IIa activity is between 3.3 and 5.3.

IDENTIFICATION

• **A. Spectroscopic Identification Tests (197), *Ultraviolet-Visible Spectroscopy*:** 197U

Medium: 0.01 N hydrochloric acid

Sample solution: 500 µg/mL

Acceptance criteria: The spectra exhibit maxima at 231 ± 2 nm.

• **B. ^{13}C NMR Spectrum**

(See *Nuclear Magnetic Resonance Spectroscopy* (761).)

Standard solution: Dissolve 200 mg of USP Enoxaparin Sodium RS in a mixture of 0.2 mL of deuterium oxide and 0.8 mL of water. Add 0.05 mL of deuterated methanol to serve as an internal reference.

Sample solution: Dissolve 200 mg of Enoxaparin Sodium in a mixture of 0.2 mL of deuterium oxide and 0.8 mL of water. Add 0.05 mL of deuterated methanol.

Analysis: Transfer the *Standard solution* and the *Sample solution* to NMR tubes of 5-mm diameter. Using a pulsed (Fourier transform) NMR spectrometer operating at NLT 75 MHz for ^{13}C, record the ^{13}C NMR spectra of the *Standard solution* and the *Sample solution* at 40°.

Acceptance criteria: The spectra are similar.

• **C.** The ratio of the numerical value of the Anti-Factor Xa activity, in Anti-Factor Xa IU/mg, to the numerical value of the Anti-Factor IIa activity, in Anti-Factor IIa IU/mg, as determined by the *Assay* (*Anti-Factor Xa Activity*) and the test for *Anti-Factor IIa Activity*, respectively, is NLT 3.3 and NMT 5.3.

Change to read:

• **D. Molecular Weight Distribution and Weight-Average Molecular Weight**

▲(See *Low Molecular Weight Heparin Molecular Weight Determinations ⟨209⟩*.)

System suitability solution: 5 mg/mL of USP Enoxaparin Sodium RS in *Mobile phase*. Filter using a nylon membrane of 0.45-µm pore size.

System suitability

Sample: *System suitability solution*

Suitability requirements

Weight-average molecular weight (M_w): Take the mean of the calculated M_w from the duplicate injections of the *System suitability solution*, and round to the nearest 50 Da. The *Chromatographic system* is suitable if the M_w is within 150 Da of the labeled M_w value as stated in the USP certificate for USP Enoxaparin Sodium RS.

Analysis: Take the mean of the calculated M_w from the duplicate injections of the *Sample solution*, and round to the nearest 50 Da. Average the calculated percentage of enoxaparin sodium chains with molecular weight lower than 2000, (M_{2000}), percentage of enoxaparin sodium chains with molecular weight in the range of 2000–8000, ($M_{2000-8000}$) and percentage of enoxaparin sodium chains with molecular weight greater than 8000, (M_{8000}) from the duplicate injections of the *Sample solution*, express to the nearest 0.5%.▲ (IRA 1-Dec-2023)

Acceptance criteria

▲**For M_w:** 3800–5000 Da

For M_{2000}: 12.0%–20.0%

For $M_{2000-8000}$: 68.0%–82.0%

For M_{8000}: NMT 18.0%▲ (IRA 1-Dec-2023)

• **E. Identification Tests—General ⟨191⟩, *Chemical Identification Tests, Sodium*:** Meets the requirements

ASSAY

• **Anti-Factor Xa Activity**

Acetic acid solution: Glacial acetic acid and water (42:58)

pH 7.4 polyethylene glycol 6000 buffer: Dissolve 6.08 g of tris(hydroxymethyl)aminomethane and 8.77 g of sodium chloride in 500 mL of water. Add 1.0 g of polyethylene glycol 6000, adjust with hydrochloric acid to a pH of 7.4, and dilute with water to 1000 mL.

pH 7.4 buffer: Dissolve 6.08 g of tris(hydroxymethyl)aminomethane and 8.77 g of sodium chloride in 500 mL of water. Adjust with hydrochloric acid to a pH of 7.4, and dilute with water to 1000 mL.

pH 8.4 buffer: Dissolve 3.03 g of tris(hydroxymethyl)aminomethane, 5.12 g of sodium chloride, and 1.40 g of edetate sodium in 250 mL of water. Adjust with hydrochloric acid to a pH of 8.4, and dilute with water to 500 mL.

Human antithrombin III solution: Reconstitute a vial of antithrombin III (see *Reagents, Indicators, and Solutions—Reagent Specifications*) in water to obtain a solution containing 5 Antithrombin III Units/mL. Dilute this solution with *pH 7.4 polyethylene glycol 6000 buffer* to obtain a solution having a concentration of 1.0 Antithrombin III Unit/mL.

Factor Xa solution: Reconstitute a weighed quantity of bovine factor Xa (see *Reagents, Indicators, and Solutions—Reagent Specifications*) in *pH 7.4 polyethylene glycol 6000 buffer* to obtain a solution that gives an increase in absorbance value at 405 nm of NMT 0.20 absorbance units/min when assayed as described below but using as an appropriate volume, *V*, the volume in µL of *pH 7.4 buffer* instead of *V* µL of the enoxaparin solution.

Chromogenic substrate solution: Prepare a solution of a suitable chromogenic substrate for amidolytic test (see *Reagents, Indicators, and Solutions—Reagent Specifications*) for factor Xa in water to obtain a concentration of about 3 mM. Dilute with *pH 8.4 buffer* to obtain a solution having a concentration of 0.5 mM.

Standard solutions: Reconstitute the entire contents of an ampul of USP Enoxaparin Sodium for Bioassays RS with water, and dilute with *pH 7.4 buffer* to obtain four dilutions in the concentration range between 0.025 and 0.2 Anti-Factor Xa IU/mL.

Sample solutions: Proceed as directed for the *Standard solutions* to obtain concentrations of Enoxaparin Sodium similar to those obtained for the *Standard solutions*.

Analysis

Samples: *Acetic acid solution*, *pH 7.4 buffer*, *Human antithrombin III solution*, *Factor Xa solution*, *Chromogenic substrate solution*, *Standard solutions*, and *Sample solutions*

Label 18 suitable tubes: B1 and B2 for blanks; T1, T2, T3, and T4 each in duplicate for the dilutions of the *Sample solutions*; and S1, S2, S3, and S4 each in duplicate for the dilutions of the *Standard solutions*. [Note—Treat the tubes in the order B1, S1, S2, S3, S4, T1, T2, T3, T4, T1, T2, T3, T4, S1, S2, S3, S4, B2.] To each tube add the same volume, *V* (20–50 µL), of *Human antithrombin III solution* and an equal volume, *V*, of either the blank (*pH 7.4 buffer*) or an appropriate dilution of the *Sample solutions* or the *Standard solutions*. Mix, but do not allow bubbles to form. Incubate at 37° for 1.0 min. Add to each tube 2*V* (40–100 µL) of *Factor Xa solution*, and incubate for 1.0 min. Add a 5*V* (100–250 µL) volume of *Chromogenic substrate solution*. Stop the reaction after 4.0 min with a 5*V* (100–250 µL) volume of *Acetic acid solution*. Measure the absorbance of each solution at 405 nm, using a suitable spectrophotometer (see *Ultraviolet-Visible Spectroscopy ⟨857⟩*) against blank B1. The reading of blank B2 relative to blank B1 is NMT ± 0.05 absorbance unit.

Calculations: For each series, calculate the regression of the absorbance against log concentrations of the *Sample solutions* and of the *Standard solutions*, and calculate the potency of the Enoxaparin Sodium in IU of Anti-Factor Xa activity/mL, using statistical methods for parallel-line assays. The four independent log relative potency estimates are then combined to obtain the final geometric mean. Its confidence limits are calculated. Express the Anti-Factor Xa activity of Enoxaparin Sodium/mg.

Acceptance criteria: The potency is NLT 90 and NMT 125 Anti-Factor Xa IU/mg on the dried basis.

OTHER COMPONENTS

• **Benzyl Alcohol Content**

Mobile phase: Acetonitrile, methanol, and water (3:1:16)

Standard solution: 0.1 mg/mL of USP Benzyl Alcohol RS in water

Sample solution: Weigh 0.5 g of Enoxaparin Sodium into a 10-mL volumetric flask, and dissolve in 5.0 mL of 1 N sodium hydroxide. Allow to stand at room temperature for about 1 h. Add 1.0 mL of glacial acetic acid, dilute with water to volume, and mix.

Chromatographic system

(See *Chromatography ⟨621⟩, System Suitability*.)

Mode: LC

Detector: UV 256 nm

Column: 4.6-mm × 15-cm stainless steel; packing L7

Flow rate: 1.0 mL/min, maintained constant to ±10%

Injection volume: 20 µL

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of benzyl alcohol in the portion of Enoxaparin Sodium taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of benzyl alcohol from the *Sample solution*

r_S = peak area of benzyl alcohol from the *Standard solution*

C_S = concentration of benzyl alcohol in the *Standard solution* (mg/mL)

C_U = concentration of Enoxaparin Sodium in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.1%

• **Nitrogen Determination ⟨461⟩, *Method II*:** 1.8%–2.5% on the dried basis

• **Sodium Content**

(See *Atomic Absorption Spectroscopy ⟨852⟩*.)

Cesium chloride solution: 1.27 mg/mL of cesium chloride in 0.1 N hydrochloric acid

Standard solution A: 0.0025% of sodium chloride in *Cesium chloride solution*

Standard solution B: 0.0050% of sodium chloride in *Cesium chloride solution*

Standard solution C: 0.0075% of sodium chloride in *Cesium chloride solution*

Sample solution: Transfer 50.0 mg of Enoxaparin Sodium to a 100-mL volumetric flask, and dissolve in and dilute with *Cesium chloride solution* to volume.

Analysis

Samples: *Cesium chloride solution*, *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Concomitantly determine the absorbances of the *Cesium chloride solution* (blank), *Sample solution*, and *Standard solutions* at 330.3 nm, using a sodium hollow-cathode lamp and an air–acetylene flame. Using the absorbances of *Standard solutions A–C*, determine the sodium content in the *Sample solution* after an appropriate blank correction.

Acceptance criteria: 11.3%–13.5% on the dried basis

SPECIFIC TESTS

• **pH ⟨791⟩:** 6.2–7.7 for a 10.0% solution in water

• **Loss on Drying ⟨731⟩**

Sample: 1 g

Analysis: Dry the *Sample* in a vacuum at 70° for 6 h.

Acceptance criteria: NMT 10.0%

• **Specific Absorbance**

(See *Ultraviolet-Visible Spectroscopy ⟨857⟩*.)

Sample solution: 0.5 mg/mL of Enoxaparin Sodium in 0.01 N hydrochloric acid

Analysis: Obtain the UV spectra of the *Standard solution* and the *Sample solution* between 200 and 300 nm against a 0.01 N hydrochloric acid blank.

Calculate the specific absorbance at the wavelength of maximum absorbance at 231 ± 2 nm, with reference to the dried substance:

$$\text{Result} = A \times 100 \times 1000/[M \times l \times (100 - E)]$$

A = absorbance at the wavelength of maximum absorbance

M = weight of Enoxaparin Sodium in the *Sample solution* (mg)

l = path length (typically 1 cm)

E = loss on drying (%)

Acceptance criteria: 14.0–20.0 on the dried basis

- **Bacterial Endotoxins Test ⟨85⟩:** It contains NMT 0.01 USP Endotoxin Unit/IU of Anti-Factor Xa activity.
- **Anti-Factor IIa Activity**

Acetic acid solution, pH 7.4 polyethylene glycol 6000 buffer, pH 7.4 buffer, pH 8.4 buffer, and **Human antithrombin III solution:** Proceed as directed in the *Assay* for *Anti-Factor Xa Activity*, except that the concentration of the *Human antithrombin III solution* is 0.5 Antithrombin III Unit/mL.

Thrombin human solution: Reconstitute thrombin human (see *Reagents, Indicators, and Solutions—Reagent Specifications*) in water, and dilute in *pH 7.4 polyethylene glycol 6000 buffer* to obtain a solution having a concentration of 5 Thrombin Units/mL.

Chromogenic substrate solution: Prepare a solution of a suitable chromogenic substrate for an amidolytic test (see *Reagents, Indicators, and Solutions—Reagent Specifications*) for thrombin in water to obtain a concentration of about 3 mM. Immediately before use, dilute with *pH 8.4 buffer* to 0.5 mM.

Standard solutions: Reconstitute the entire contents of an ampul of USP Enoxaparin Sodium for Bioassays RS with water, and dilute with *pH 7.4 buffer* to obtain four dilutions having concentrations in the range between 0.015 and 0.075 IU of Anti-Factor IIa activity/mL.

Sample solutions: Proceed as directed under *Standard solutions* to obtain concentrations of Enoxaparin Sodium similar to those obtained for the *Standard solutions*.

Analysis: Proceed as directed in the *Assay* for *Anti-Factor Xa Activity*, except use *Thrombin human solution* instead of *Factor Xa solution* and use *Human antithrombin III solution* as described above.

Calculations: For each series, calculate the regression of the absorbance against log concentrations of the *Sample solutions* and of the *Standard solutions*, and calculate the potency of the Enoxaparin Sodium in IU of Anti-Factor IIa activity/mg, using statistical methods for parallel-line assays. The four independent dilution estimates are then combined to obtain the final weighted mean. Then calculate the confidence limits. Express the Anti-Factor IIa activity of Enoxaparin Sodium/mg.

Acceptance criteria: It has a potency of NLT 20.0 and NMT 35.0 Anti-Factor IIa IU/mg on the dried basis.

- **Molar Ratio of Sulfate to Carboxylate**

Mobile phase: Carbon dioxide-free water

Sample solution: 5 mg/mL of Enoxaparin Sodium in *Mobile phase*

Chromatographic system

(See *Chromatography ⟨621⟩, System Suitability*.)

Mode: LC

Detector: Ion

Columns: One 1.5-cm × 2.5-cm column, packed with an anion-exchange resin packing L64; and one 1.5-cm × 7.5-cm column, packed with a cation-exchange resin packing L65. The outlet of the anion-exchange column is connected to the inlet of the cation-exchange column.

Flow rate: 1 mL/min

Analysis

Sample: *Sample solution*

[Note—Regenerate the anion-exchange column and the cation-exchange column with 1 N sodium hydroxide and 1 N hydrochloric acid, respectively, between two injections.]

Inject the *Sample solution* into the anion-exchange column, and collect the eluate from the cation-exchange column in a beaker at the outlet until the ion detector reading returns to the baseline value. Quantitatively transfer the eluate to a titration vessel containing a magnetic stirring bar, and dilute with *Mobile phase* to about 60 mL. Position the titration vessel on a magnetic stirrer, and immerse the electrodes. Note the initial conductivity reading, and titrate with approximately 0.1 N sodium hydroxide added in 100-µL portions. [Note—Prepare the sodium hydroxide solution in *Mobile phase*.] Record the buret reading and the conductivity meter reading after each addition of the sodium hydroxide solution.

Calculations: Plot the conductivity measurements on the *y*-axis against the volumes of sodium hydroxide added on the *x*-axis. The graph will have three linear sections—an initial downward slope, a middle slight rise, and a final rise. For each of these sections draw the best-fit straight lines, using linear regression analysis. At the points where the first and second straight lines intersect and where the second and third lines intersect, draw perpendiculars to the *x*-axis to determine the volumes of sodium hydroxide taken up by the sample at those points. The point where the first and second lines intersect corresponds to the volume of sodium hydroxide taken up by the sulfate groups (V_S). The point where the second and third lines intersect corresponds to the volume of sodium hydroxide consumed by the sulfate and the carboxylate groups together (V_T).

Calculate the molar ratio of sulfate to carboxylate:

$$\text{Result} = V_S/(V_T - V_S)$$

Acceptance criteria: The molar ratio of sulfate to carboxylate is NLT 1.8.

ADDITIONAL REQUIREMENTS

• **Packaging and Storage:** Preserve in tight containers, and store below 40°, preferably at room temperature.

Change to read:

• **USP Reference Standards** ⟨11⟩

USP Benzyl Alcohol RS

USP Enoxaparin Sodium RS

▲▲ (IRA 1-Dec-2023)

USP Enoxaparin Sodium for Bioassays RS

Auxiliary Information - Please check for your question in the FAQs before contacting USP.

Topic/Question	Contact	Expert Committee
ENOXAPARIN SODIUM	Jennifer Tong Sun Senior Scientist II	BIO32020 Biologics Monographs 3 - Complex Biologics and Vaccines

Chromatographic Database Information: Chromatographic Database

Most Recently Appeared In:
Pharmacopeial Forum: Volume No. 48(3)

Current DocID: GUID-48AF7421-9DAB-487C-B132-3B704CAAD512_6_en-US

DOI: https://doi.org/10.31003/USPNF_M29290_06_01

DOI ref: 1r2nq