Enoxaparin Sodium

**Acceptance criteria:**

**Mode:**

- **B.** 13C NMR SPECTRUM
- **A.** ULTRAVIOLET ABSORPTION
- **•** IDENTIFICATION

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 0.2% 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 greater than 2000 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. Ultraviolet Absorption (197U)**
  - **Medium:** 0.01 N hydrochloric acid
  - **Sample solution:** 500 µg/mL
  - **Acceptance criteria:** The spectra exhibit maxima at 231 ± 2 nm.
- **B. 13C NMR Spectrum**
  - **(See Nuclear Magnetic Resonance (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 13C, record the 13C 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 Impurities (Anti-Factor IIa Activity), respectively, is NLT 3.3 and NMT 5.3.
- **D. Molecular Weight Distribution and Weight-Average Molecular Weight**
  - **Mobile phase:** Prepare a 0.5 M lithium nitrate solution. Pass through a membrane filter of 0.45-µm or smaller pore size, and degas with helium.
  - **Standard solution:** 10 mg/mL of USP Enoxaparin Sodium RS in Mobile phase
  - **Sample solution:** 10 mg/mL of Enoxaparin Sodium in Mobile phase

**Chromatographic system**

- **Mode:** Size exclusion LC
- **Detector:** Differential refractive index \([9041-08-1]\)
- **Temperature:** Room temperature
- **Flow rate:** 0.6 mL/min maintained constant to ± 1.0%
- **Analysis:** Reconstitute 1 vial each of USP Enoxaparin Sodium Molecular Weight Calibrator A RS and USP Enoxaparin Sodium Molecular Weight Calibrator B RS in 1 mL of Mobile phase. Separately inject 20 µL of USP Enoxaparin Sodium Molecular Weight Calibrator A RS and USP Enoxaparin Sodium Molecular Weight Calibrator B RS, record the chromatograms, and measure the retention times. Inject in duplicate 20 µL each of the Standard solution and the Sample solution, and record the chromatograms for a length of time to ensure complete elution, including salt and solvent peaks. Calculate the total peak areas under each of the Standard solution and Sample solution chromatograms, excluding salt and solvent peaks.
- **Calibration curve:** Plot the retention times on the x-axis against the peak molecular weights on the y-axis for the peaks from USP Enoxaparin Sodium Molecular Weight Calibrator A RS and USP Enoxaparin Sodium Molecular Weight Calibrator B RS, and fit the data to a third-order polynomial, using suitable gel permeation chromatography (GPC) software.
- **Calculations:** Compute the data, using the same GPC software; determine the weight-average molecular weight, \(M_w\), for each of the duplicate chromatograms of the Standard solution and the Sample solution; and take the average for each solution. Correct the mean value of \(M_w\) to the nearest 50. The Chromatographic system is suitable if \(M_w\) for USP Enoxaparin Sodium RS is within 150 Da of the labeled \(M_w\) value. The \(M_w\) for the Sample solution is between 3800 and 5000 Da. Using the same software, determine for each of the duplicate Sample solution chromatograms the percentage of Enoxaparin Sodium chains with molecular weights lower than 2000 Da, \(M_{2000}\), the percentage of Enoxaparin Sodium chains with molecular weights in the range 2000–8000 Da, \(M_{2000–8000}\), and the percentage of Enoxaparin Sodium chains with molecular weights greater than 8000 Da, \(M_{8000}\). Average the duplicate values, and express to the nearest 0.5%.
- **Acceptance criteria:** \(M_{2000}\) is between 12.0% and 20.0%, \(M_{2000–8000}\) is between 68.0% and 82.0%, and \(M_{8000}\) is NMT 18.0%.
ASSAY

E. Identification Tests—General, Sodium (191): Meets the requirements

ASSAY

Change to read:

• 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 mL of pH 7.4 buffer instead of V mL 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 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: Standard solution and Sample solution

Calculate the percentage of benzyl alcohol in the portion of 300 mg of Enoxaparin Sodium taken:

\[ \text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_U}{C_S} \right) \times 100 \]

where:

- \( r_U \) = peak area of benzyl alcohol from the Sample solution
- \( r_S \) = peak area of benzyl alcohol from the Standard solution
- \( C_U \) = concentration of benzyl alcohol in the Standard solution (mg/mL)
- \( C_S \) = concentration of benzyl alcohol in the Sample solution (mg/mL)

Acceptance criteria: NMT 0.1%

Other components

• Benzyl Alcohol Content

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

Standard solution: 0.1 mg/mL of USP Benzylo 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: Standard solution and Sample solution

Calculate the percentage of benzyl alcohol in the portion of the enoxaparin sodium taken:

\[ \text{Result} = \left( \frac{r_U}{r_S} \right) \times \left( \frac{C_U}{C_S} \right) \times 100 \]

where:

- \( r_U \) = peak area of benzyl alcohol from the Sample solution
- \( r_S \) = peak area of benzyl alcohol from the Standard solution
- \( C_U \) = concentration of benzyl alcohol in the Standard solution (mg/mL)
- \( C_S \) = concentration of benzyl alcohol in the Sample solution (mg/mL)

Acceptance criteria: NMT 0.1%

• Nitrogen Determination, Method II (461): 1.8%–2.5% on the dried basis

Sodium Content

(See Spectrophotometry and Light-Scattering (851).)

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: Standard solution A, Standard solution B, Standard solution C, Cesium chloride solution, 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

IMPURITIES

Change to read:

- **HEAVY METALS,** Method I (231): NMT 30 µg/g, using a 2.7% (w/v) hydroxide solution in water

SPECIFIC TESTS

- **pH** (731): 6.2–7.7 for a 10.0% solution in water
- **Loss on Drying** (731): Dry 1 g in a vacuum at 70° for 6 h; it loses NMT 10.0% of its weight.

- **Specific Absorbance** (See Spectrophotometry and Light-Scattering (851)).
- **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 nm 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:

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

- **A** = absorbance at the wavelength of maximum absorbance
- **M** = weight of Enoxaparin Sodium in the Sample solution (mg)
- **I** = 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.

Change to read:

- **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 (See 5-Dec-2011) 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 to use Thrombin human solution instead of Factor Xa solution and to 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 carbon dioxide-free water

Chromatographic system
(See Chromatography (621), System Suitability.)

Mode: LC
Detector: Ion
Column: Two columns: one 1.5-cm × 2.5-cm column, packed with an anion-exchange resin L64 packing; and one 1.5-cm × 7.5-cm column, packed with a cation-exchange resin L65 packing. 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 carbon dioxide-free water 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 carbon dioxide-free water.] 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 (V1). 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:

\[
\text{Result} = \frac{V_d}{V_t - V_d}
\]

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°C, preferably at room temperature.

**Change to read:**

- **USP Reference Standards (11)**
  - USP Benzyl Alcohol RS
  - USP Endotoxin RS
  - USP Enoxaparin Sodium RS
  - USP Enoxaparin Sodium Molecular Weight Calibrant A RS

USP Enoxaparin Sodium Molecular Weight Calibrant B RS
USP Enoxaparin Sodium RS for Bioassays RS