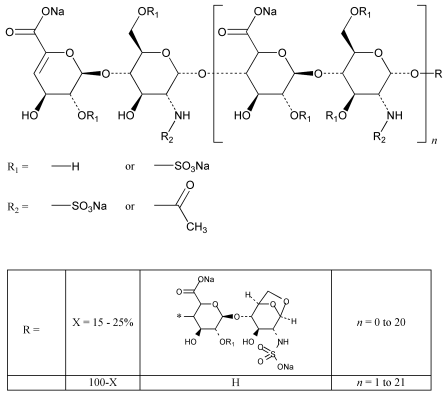
Add the following:

■Enoxaparin Sodium



[*9041-08-1*].

Change to read:

» 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.•(RB 01-Dec-2008) 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 non-reducing end of their chain. About 20 percent of the materials contain a 1,6-anhydro derivative on the reducing end of the chain, the range being between 15 and 25 percent. The weight-average molecular weight of Enoxaparin Sodium is 4,500 Da, the range being between 3,800 and 5,000 Da; about 16 percent have a molecular weight of less than 2,000 Da, the range being between 12.0 and 20.0 percent; about 74 percent have a molecular weight between 2,000 and 8,000 Da, the range being between 68.0 and 82.0 percent. Not more than 18.0 percent have a molecular weight higher than 8,000 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 not less than 1.8 per disaccharide unit. It has a potency of not less than 90 and not more than 125 Anti-Factor X_a International Units (IU) per mg, and not less than 20.0 and not more than 35.0 Anti-Factor II_a IU per mg, calculated on the dried basis. The ratio of anti-factor X_a activity to anti-factor II_a activity is between 3.3 and 5.3.

Packaging and storage—Preserve in tight containers, and store below 40°, preferably at room temperature.

USP Reference standards ⟨11⟩**—***USP Benzyl Alcohol RS. USP Endotoxin RS. USP Enoxaparin Sodium RS. USP Enoxaparin Sodium Solution for Bioassays RS. USP Enoxaparin Sodium Molecular Weight Calibrant A RS. USP Enoxaparin Sodium Molecular Weight Calibrant B RS.*

Identification—

A: *Ultraviolet Absorption* ⟨197U⟩—

Solution: 500 µg per mL.

Medium: 0.01 N hydrochloric acid. The spectra exhibit maxima at 231 ± 2 nm.

B: *^{13}C 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.

Test 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.

Procedure—Transfer the *Standard solution* and the *Test solution* to NMR tubes of 5-mm diameter. Using a pulsed (Fourier transform) NMR spectrometer operating at not less than 75 MHz for ^{13}C, record the ^{13}C NMR spectra of the *Standard solution* and the *Test solution* at 40°. The spectra are similar.

C: The ratio of the numerical value of the anti-factor X_a activity, in Anti-Factor X_a IU per mg, to the numerical value of the anti-factor II_a activity, in Anti-Factor II_a IU per mg, as determined by the *Assay (anti-factor X_a activity)* and the *Anti-factor II_a activity*, respectively, is not less than 3.3 and not more than 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 having a porosity of 0.45 µm or less, and degas with helium.

Standard solution—Dissolve about 10 mg of USP Enoxaparin Sodium RS, accurately weighed, in 1 mL of *Mobile phase*.

Test solution—Dissolve about 10 mg of Enoxaparin Sodium, accurately weighed, in 1 mL of *Mobile phase.*

Chromatographic system (see *Chromatography* ⟨621⟩)—The high performance size exclusion chromatograph is equipped with a differential refractive index detector, a 6- × 40-mm guard column and two 7.8- × 300-mm analytical columns in series, both analytical and guard columns prepacked with packing L59, and used at room temperature. The flow rate is about 0.6 mL per minute maintained constant to±1.0%.

Procedure—Reconstitute one vial each of USP Enoxaparin Sodium Molecular Weight Calibrant A RS and USP Enoxaparin Sodium Molecular Weight Calibrant B RS in 1 mL of *Mobile phase.* Separately inject 20 µL of USP Enoxaparin Sodium Molecular Weight Calibrant A RS and USP Enoxaparin Sodium Molecular Weight Calibrant B RS, record the chromatograms, and measure the retention times. Inject in duplicate 20 µL of each of the *Standard solution* and the *Test solution*, and record the chromatograms for a length of time to ensure complete elution, including salt and solvent peaks. Calculate the total area under each of the *Standard solution* and *Test solution* chromatograms, excluding salt and solvent peaks at the end.

Calibration curve—Plot the retention times on the *x*-axis against the peak molecular weights on the *y*-axis for the peaks in the chromatograms of USP Enoxaparin Sodium Molecular Weight Calibrant A RS and USP Enoxaparin Sodium Molecular Weight

Calibrant B RS, and fit the data to a third-order polynomial using a suitable gel permeation chromatography (GPC) software.

Calculations—Compute the data, using the same GPC software and determine the weight-average molecular weight, M_W, for each of the duplicate chromatograms of the *Standard solution* and the *Test 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 of USP Enoxaparin Sodium RS is within 150 Da of the labeled M_W value. The M_W for the *Test solution* is between 3,800 and 5,000 Da. Using the same software, determine for each of the duplicate *Test 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 to 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%. M_{2000} is between 12.0% and 20.0%, $M_{2000–8000}$ is between 68.0% and 82.0%, and M_{8000} is not more than 18.0%.

E: It responds to the test for *Sodium* ⟨191⟩.

Specific absorbance (see *Spectrophotometry and Light-Scattering* ⟨851⟩)—

Test solution—Dissolve 50.0 mg of Enoxaparin Sodium in 100 mL of 0.01 N hydrochloric acid.

Procedure—Obtain the UV spectra of the *Standard solution* and the *Test solution* between 200 nm and 300 nm against 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, using the following formula:

$$A \times 100 \times 1000 / [\, M \times l \times (100 - E)]$$

in which A is the absorbance at the wavelength of maximum absorbance; M is the weight, in mg, of Enoxaparin Sodium in the *Test solution;* l is the pathlength (typically l = 1 cm); and E is the loss on drying, in percent. The specific absorbance is between 14.0 and 20.0, calculated on the dried basis.

Bacterial endotoxins ⟨85⟩**—**It contains not more than 0.01 USP Endotoxin Unit per IU of anti-factor X_a activity.

pH ⟨791⟩**:** between 6.2 and 7.7 of a 10.0% solution in water.

Loss on drying ⟨731⟩**—**Dry 1 g in a vacuum at 70° for 6 hours: it loses not more than 10.0% of its weight.

Nitrogen content, *Method II* ⟨461⟩**:** between 1.8% and 2.5%, calculated on the dried basis.

Heavy metals, *Method I* ⟨231⟩**—**Prepare a 5% solution in water: the limit is not more than 0.0030%.

Sodium content (see *Spectrophotometry and Light-Scattering* ⟨851⟩)—

Cesium chloride solution—Prepare a solution of cesium chloride in 0.1 N hydrochloric acid containing 1.27 mg per mL.

Standard solutions—Dissolve an accurately weighed quantity of sodium chloride in *Cesium chloride solution* to obtain a solution having a known concentration of about 0.2% sodium. Dilute accurately measured volumes of this solution with *Cesium chloride solution* having known concentrations of 0.0025%, 0.0050%, and 0.0075% of sodium.

Test solution—Transfer an accurately weighed quantity of about 50.0 mg of Enoxaparin Sodium to a 100-mL volumetric flask, and dissolve in and dilute with *Cesium chloride solution* to volume.

Procedure—Concomitantly determine the absorbances of the *Cesium chloride solution* (blank), *Test 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,* determine the sodium content in the *Test solution* after appropriate blank correction. The sodium content, calculated on the dried basis, is between 11.3% and 13.5%.

Molar ratio of sulfate to carboxylate (see *Chromatography* ⟨621⟩)—

Mobile phase: carbon dioxide-free water.

Test solution—Dissolve an accurately weighed quantity of about 50 mg of Enoxaparin Sodium in 10 mL of carbon dioxide-free water.

Chromatographic system—The liquid chromatographic system consists of two peristaltic pumps, a six-port injection valve, an ion detector, and two columns—one 1.5- × 2.5-cm column packed with an anion-exchange resin L64 packing and one 1.5- × 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. The flow rate is about 1 mL per minute.

Procedure—[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 *Test 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 burette 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 downwards 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 by the formula:

$$V_S / (V_T - V_S)$$

The molar ratio of sulfate to carboxylate is not less than 1.8.

Benzyl alcohol content—

Mobile phase—Prepare a filtered and degassed mixture of water, acetonitrile, and methanol (80 : 15 : 5 v/v).

Standard solution—Dissolve 100 mg of USP Benzyl Alcohol RS in 200 mL of water. Transfer 5 mL of this solution to a 25-mL volumetric flask, and dilute with water to volume.

Test 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 hour. Add 1.0 mL of glacial acetic acid, dilute with water to volume, and mix.

Chromatographic system (see *Chromatography* ⟨621⟩)—The liquid chromatograph is equipped with a 256-nm detector and a 4.6-mm × 15-cm stainless steel column that contains L7 packing. The flow rate is about 1.0 mL per minute maintained constant to ±10%.

Procedure—Separately inject equal volumes (about 20 µL) of the *Standard solution* and the *Test solution,* record the chromatograms, and measure the peak responses.

Calculation—Calculate the percentage of benzyl alcohol in Enoxaparin Sodium taken by the formula,

$$(A_T \times C_S)/(A_S \times C_T)$$

in which A_T is the benzyl alcohol peak area in the *Test solution;* C_S is the concentration, in mg per mL, of benzyl alcohol; A_S is the area of the benzyl alcohol peak in the *Standard solution;* and C_T is the

concentration, in mg per mL, of Enoxaparin Sodium. The percentage of benzyl alcohol is not more than 0.1%.

Anti-factor II_a 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 under *Assay (anti-factor X_a activity),* except that the concentration of the *Human antithrombin III solution* is 0.5 Antithrombin III Unit per mL.

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

Chromogenic substrate solution—Prepare a solution of a suitable chromogenic substrate for an amidolytic test (see *Reagent Specifications* in the section *Reagents, Indicators, and Solutions*) 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—Dilute USP Enoxaparin Sodium Solution for Bioassays RS with *pH 7.4 Buffer* to obtain four dilutions having concentrations in the range between 0.015 and 0.075 IU of anti-factor II_a activity per mL.

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

Procedure—Proceed as directed under *Assay (anti-factor X_a activity),* except to use *Thrombin human solution* instead of *Factor X_a solution* and to use the *Human antithrombin III solution* as described above.

Calculations—For each series, calculate the regression of the absorbance against log concentrations of the *Test solutions* and of the *Standard solutions,* and calculate the potency of the enoxaparin sodium in IU of anti-factor II_a activity per 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 II_a activity of Enoxaparin Sodium per mg, calculated on the dried basis. It has a potency of not less than 20.0 and not more than 35.0 anti-Factor II_a IU per mg.

Assay (anti-factor X_a activity)—

Acetic acid solution—Transfer 42 mL of glacial acetic acid to a 100-mL volumetric flask, dilute with water to volume, and mix.

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 *Reagent Specifications* in the section *Reagents, Indicators, and Solutions*) in water to obtain a solution containing 5 Antithrombin III Units per 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 per mL.

Factor X_a solution—Reconstitute an accurately weighed quantity of bovine factor X_a (see *Reagent Specifications* in the section *Reagents, Indicators, and Solutions*) in *pH 7.4 Polyethylene glycol 6000 buffer* to obtain a solution that gives an increase in absorbance value at 405 nm of not more than 0.20 absorbance units per minute when assayed as described below but using as an appropriate volume (*V,* 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 *Reagent Specifications* in the section *Reagents, Indicators, and Solutions*) for factor X_a 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 preparations—Dilute USP Enoxaparin Sodium Solution for Bioassays RS with *pH 7.4 Buffer* to obtain four dilutions in the concentration range between 0.025 and 0.2 USP Anti-Factor X_a IU per mL.

Assay preparations—Proceed as directed for *Standard preparations* to obtain concentrations of Enoxaparin Sodium similar to those obtained for the *Standard preparations*.

Procedure—Label 18 suitable tubes: B1 and B2 for blanks; T1, T2, T3, and T4 each in duplicate for the dilutions of the *Assay preparations;* and S1, S2, S3, and S4 each in duplicate for the dilutions of the *Standard preparations*. [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 to 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 *Assay preparations* or the *Standard preparations*. Mix, but do not allow bubbles to form. Incubate at 37° for 1.0 minute. Add to each tube volume 2*V* (40 to100 µL) of *Factor X_a solution,* and incubate for 1.0 minute. Add 5*V* (100 to 250 µL) volume of *Chromogenic substrate solution*. Stop the reaction after 4.0 minutes with 5*V* (100 to 250 µL) volume of *Acetic acid solution*. Measure the absorbance of each solution at 405 nm using a suitable spectrophotometer (see *Spectrophotometry and Light-Scattering* ⟨851⟩) against blank B1. The reading of blank B2 relative to the blank B1 is not more than ±0.05 absorbance units.

Calculations—For each series, calculate the regression of the absorbance against log concentrations of the *Assay preparations* and of the *Standard preparations,* and calculate the potency of the enoxaparin sodium in IU of anti-factor X_a activity per 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 X_a activity of Enoxaparin Sodium per mg, calculated on the dried basis. The potency is not less than 90 and not more than 125 Anti-Factor X_a IU per mg.■2S (*USP31*)