

Change to read:**Heparin Sodium****DEFINITION**

Heparin Sodium is the sodium salt of sulfated glycosaminoglycans present as a mixture of heterogeneous molecules varying in molecular weights that retains a combination of activities against different factors of the blood clotting cascade. It is present in mammalian tissues and is usually obtained from the intestinal mucosa or other suitable tissues of domestic mammals used for food by man. The sourcing of heparin material must be specified in compliance with applicable regulatory requirements. The manufacturing process should be validated to demonstrate clearance and inactivation of relevant infectious and adventitious agents (e.g., viruses, TSE agents). See *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* (1050) for general guidance on viral safety evaluation. It is composed of polymers of alternating derivatives of α -D-glucosamido (*N*-sulfated, *O*-sulfated, or *N*-acetylated) and *O*-sulfated uronic acid (α -L-iduronic acid or β -D-glucuronic acid). The component activities of the mixture are in ratios corresponding to those shown by the USP Heparin Sodium for Assays RS. Some of these components have the property of prolonging the clotting time of blood. This occurs mainly through the formation of a complex of each component with the plasma proteins antithrombin and heparin cofactor II to potentiate the inactivation of thrombin (factor IIa). Other coagulation proteases in the clotting sequence, such as activated factor X (factor Xa), are also inhibited. The ratio of anti-factor Xa activity to anti-factor IIa potency is between 0.9 and 1.1. The potency of Heparin Sodium, calculated on the dried basis, is NLT 180 USP Heparin Units in each mg.

IDENTIFICATION**A. ¹H NMR SPECTRUM**

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

Standard solution: NLT 20 mg/mL of USP Heparin Sodium Identification RS in deuterium oxide with 0.02% (w/v) deuterated trimethylsilylpropionic (TSP) acid sodium salt

System suitability solution: Prepare 1% (w/w) USP Oversulfated Chondroitin Sulfate RS in *Standard solution*.

Sample solution: NLT 20 mg/mL of Heparin Sodium in deuterium oxide with 0.02% (w/v) deuterated TSP

Analysis

Samples: *Standard solution* and *System suitability solution*

Using a pulsed (Fourier transform) NMR spectrometer operating at NLT 500 MHz for ¹H, acquire a free induction decay (FID) using NLT 16 scans using a 90° pulse and 20 s delay. Record the ¹H NMR spectra of the *Standard solution* and *System suitability solution* at 25°. Collect the ¹H NMR spectrum with a spectral window of at least 10 to –2 ppm and without spinning. The number of transients should be adjusted until the signal-to-noise ratio of the *N*-acetyl heparin signal in the *Standard solution* is at least 1000/1 in the region near 2 ppm. The *Standard solution* shall be run at least daily when *Sample solutions* are being run. For all samples, the TSP methyl signal should be set to 0.00 ppm. The chemical shift for the *N*-acetyl resonance of heparin and oversulfated chondroitin sulfate in the *System suitability solution* should be observed at 2.05 ± 0.02 and 2.16 ± 0.03 ppm, respectively. Record the ¹H NMR spectrum of the *Sample solution* at 25°. Draw a baseline from 8.00 ppm to 0.10 ppm. The ppm values for H1 of GlcNAc/GlcNS, 6S (signal 1), H1 of IdoA2S (signal 2), the H2 of GlcNS (signal 3), and the methyl of GlcNAc (signal 4) of heparin are present at 5.42, 5.21, 3.28 (doublet centered at 3.28 ppm), and 2.05 ppm, respectively.¹ The ppm values of these signals do not differ by more than ±0.03 ppm. Measure the signal heights above the baseline of signal 1 and signal 2, and calculate the mean of these signal heights. Other signals of

¹ GlcNAc, *N*-acetylated glucosamine; GlcNS, *N*-sulfated glucosamine; S, Sulfate; IdoA, iduronic acid; GlcN, glucosamine; GalN, galactosamine.

variable heights and ppm values, attributable to heparin and HOD, may be seen between signal 2 and 4.55 ppm. Residual solvent signals may be observed in the 0.10–3.00 range. Heparin Sodium must meet the requirements stated in *Residual Solvents* (467).

Acceptance criteria: No unidentified signals greater than 4% of the mean of signal height of 1 and 2 are present in the following ranges: 0.10–2.00, 2.10–3.20, and 5.70–8.00 ppm. No signals greater than 200% signal height of the mean of the signal height of 1 and 2 are present in the 3.35–4.55 ppm for porcine heparin.

B. CHROMATOGRAPHIC IDENTITY

Solution A: Dissolve 0.8 g of monobasic sodium phosphate dihydrate in 2 L of water and adjust with phosphoric acid to a pH of 3.0. Filter the solution through a 0.45- μ m membrane and degas prior to use.

Solution B: Dissolve 0.8 g of monobasic sodium phosphate dihydrate and 280 g of sodium perchlorate monohydrate in 2 L of water and adjust with phosphoric acid to a pH of 3.0. Filter the solution through a 0.45- μ m membrane and degas prior to use.

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)	Elution
0	80	20	Equilibration
60	10	90	Linear gradient
61	80	20	Linear gradient
75	80	20	Re-equilibration

Standard solution: NLT 20 mg/mL of USP Heparin Sodium Identification RS in water

System suitability solution: Prepare 1% (w/w) USP Oversulfated Chondroitin Sulfate RS and 1% (w/w) USP Dermatan Sulfate RS in *Standard solution*.

Sample solution: NLT 20 mg/mL of Heparin Sodium in water

Chromatographic system

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

Mode: LC

Detector: UV 202 nm

Column: 2-mm × 25-cm; packing L61

Guard column: 2-mm × 50-mm; packing L61

Column temperature: Maintain columns at 40°

Flow rate: 0.22 mL/min

Injection size: 10 μ L

System suitability

Sample: *System suitability solution*

NOTE—The retention times for dermatan sulfate, heparin, and oversulfated chondroitin sulfate are about 20, 30, and 50 min, respectively.

Suitability requirements

Resolution: NLT 1.0 between the dermatan sulfate and the heparin peaks, and NLT 1.5 between the heparin and the oversulfated chondroitin sulfate

Relative standard deviation: NMT 2% for the heparin peak determined from three replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Record the chromatograms, and measure the retention times for the major peaks.

Acceptance criteria: The retention time of the major peak from the *Sample solution* corresponds to that from the *Standard solution*.

C. ANTI-FACTOR Xa TO ANTI-FACTOR IIa RATIO**Anti-factor Xa activity**

pH 8.4 buffer: Dissolve amounts of tris(hydroxymethyl)aminomethane, edetic acid, and sodium chloride in water containing 0.1% of polyethylene glycol 6000 to obtain a solution having concentrations of 0.050 M, 0.0075 M, and 0.175 M, respectively. Adjust, if necessary, with hydrochloric acid or sodium hydroxide solution to a pH of 8.4.

Antithrombin solution: Reconstitute a vial of antithrombin (see *Reagents, Indicators, and Solutions—Reagent Specifications*) as directed by the manufacturer, and further dilute with *pH 8.4 buffer* to obtain a solution having a concentration of 1.0 Antithrombin IU/mL.

Factor Xa solution: Reconstitute bovine factor Xa as directed by the manufacturer (see *Factor Xa* in *Reagents, Indicators, and Solutions—Reagent Specifications*), and further dilute in *pH 8.4 buffer* to obtain a solution that gives an absorbance value between 0.65 and 1.25 at 405 nm when assayed as described below but using 30 μ L of *pH 8.4 buffer* instead of 30 μ L of the *Standard solutions* or the *Sample solutions*. [NOTE—*Factor Xa solution* contains about 3 nanokatalytic units/mL, but can vary depending upon the manufacturer of factor Xa or the substrate used.]

Chromogenic substrate solution: Prepare a solution of a suitable chromogenic substrate for amidolytic test (see *Reagents, Indicators, and Solutions—Reagent Specifications*) specific for factor Xa in water to obtain a concentration of 1 mM.

Stopping solution: 20% (v/v) solution of acetic acid

Standard solutions: Reconstitute the entire contents of an ampule of USP Heparin Sodium for Assays RS with water, and dilute with *pH 8.4 buffer* to obtain at least 5 dilutions in the concentration range between 0.03 and 0.375 USP Heparin Units/mL.

Sample solutions: Dissolve or dilute an accurately measured quantity of Heparin Sodium in *pH 8.4 buffer*, and dilute with the same buffer to obtain solutions having activities approximately equal to those of the *Standard solutions*.

Analysis

NOTE—The procedure can also be performed using alternative platforms. Perform the test with each *Standard solution* and *Sample solution* in duplicate.

To each of a series of suitable plastic tubes placed in a water bath set at 37°, transfer 120 μ L of *pH 8.4 buffer*. Then separately transfer 30 μ L of the different dilutions of the *Standard solutions* or the *Sample solutions* to the tubes. Add 150 μ L of *Antithrombin solution*, prewarmed at 37° for 15 min, to each tube, mix, and incubate for 2 min. Add 300 μ L of *Factor Xa solution*, prewarmed at 37° for 15 min, to each tube, mix, and incubate for 2 min. Add 300 μ L of *Chromogenic substrate solution*, prewarmed at 37° for 15 min, to each tube, mix, and incubate for exactly 2 min. Add 150 μ L of *Stopping solution* to each tube, and mix. Prepare a blank for zeroing the spectrophotometer by adding the reagents in reverse order, starting with the *Stopping solution* and ending with the addition of 150 μ L of *pH 8.4 buffer*, and excluding the *Standard solutions* or the *Sample solutions*. Record the absorbance at 405 nm against the blank.

Calculations: Plot the log of the absorbance values of the *Standard solutions* and the *Sample solutions* versus heparin concentrations in USP Units. Calculate the activity of Heparin Sodium in USP Units/mg using statistical methods for slope ratio assays. Calculate the anti-factor Xa activity of Heparin Sodium by the formula:

$$A \times (S_T/S_S)$$

A = the potency of USP Heparin Sodium for Assays RS

S_T = slope of the line for the *Sample solutions*

S_S = slope of the line for the *Standard solutions*

Express the anti-factor Xa activity of the *Sample solution* as USP Heparin Units/mg, calculated on the dried basis. Calculate the ratio of anti-factor Xa activity against anti-factor IIa potency (see *Assay* below) by the formula:

anti-factor Xa activity/anti-factor IIa potency

Acceptance criteria: NLT 0.9 and NMT 1.1

• **D. IDENTIFICATION TESTS—GENERAL, SODIUM** (191): It meets the requirements of the flame test for sodium.

ASSAY

• ANTI-FACTOR IIa POTENCY

pH 8.4 buffer: Dissolve 6.10 g of tris(hydroxymethyl)aminomethane, 10.20 g of sodium chloride, 2.80 g of edetate sodium, and, if suitable, between 0 and 10.00 g of polyethylene glycol 6000 and/or 2.00 g of bovine serum albumin in 800 mL of water. [NOTE—2.00 g of human albumin may be substituted for 2.00 g of bovine serum albumin.] Adjust with hydrochloric acid to a pH of 8.4, and dilute with water to 1000 mL.

Antithrombin solution: Reconstitute a vial of antithrombin (see *Reagents, Indicators, and Solutions—Reagent Specifications*) in water to obtain a solution of 5 Antithrombin IU/mL. Dilute this solution with *pH 8.4 buffer* to obtain a solution having a concentration of 0.125 Antithrombin IU/mL.

Thrombin human solution: Reconstitute thrombin human (factor IIa) (see *Reagents, Indicators, and Solutions—Reagent Specifications*) in water to give 20 Thrombin IU/mL, and dilute with *pH 8.4 buffer* to obtain a solution having a concentration of 5 Thrombin IU/mL. [NOTE—The thrombin should have a specific activity of NLT 750 IU/mg.]

Chromogenic substrate solution: Prepare a solution of a suitable chromogenic thrombin substrate for amidolytic test (see *Reagents, Indicators, and Solutions—Reagent Specifications*) in water to obtain a concentration of 1.25 mM.

Stopping solution: 20% (v/v) solution of acetic acid

Standard solutions: Reconstitute the entire contents of an ampule of USP Heparin Sodium for Assays RS with water and dilute with *pH 8.4 buffer* to obtain at least four dilutions in the concentration range between 0.005 and 0.03 USP Heparin Unit/mL.

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

Analysis

[NOTE—The procedure can also be performed using alternative platforms.]

For each dilution of the *Standard solutions* and the *Sample solutions*, at least duplicate samples should be tested. Label a suitable number of tubes depending on the number of replicates to be tested: for example, if five blanks are to be used: B1, B2, B3, B4, and B5 for the blanks; T1, T2, T3, and T4 each at least in duplicate for the dilutions of the *Sample solutions*; and S1, S2, S3, and S4 each at least in duplicate for the dilutions of the *Standard solutions*. Distribute the blanks over the series in such a way that they accurately represent the behavior of the reagents during the experiments. [NOTE—Treat the tubes in the order B1, S1, S2, S3, S4, B2, T1, T2, T3, T4, B3, T1, T2, T3, T4, B4, S1, S2, S3, S4, B5.] Note that after each addition of a reagent, the incubation mixture should be mixed without allowing bubbles to form. Add twice the volume (100–200 μ L) of *Antithrombin solution* to each tube containing one volume (50–100 μ L) of either the *pH 8.4 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 at least 1 min. Add to each tube 25–50 μ L of *Thrombin human solution*, and incubate for at least 1 min. Add 50–100 μ L of *Chromogenic substrate solution*. Please note that all reagents, *Standard solutions*, and *Sample solutions* should be prewarmed to 37° just prior to use.

Two different types of measurements can be recorded:

1. **Endpoint Measurement:** Stop the reaction after at least 1 min with 50–100 μ L of *Stopping solution*. Measure the absorbance of each solution at 405 nm using a suitable spectrophotometer (see *Spectrophotometry and Light-Scattering* (851)). The RSD over the blank readings is less than 10%.
2. **Kinetic Measurement:** Follow the change in absorbance for each solution over 1 min at 405 nm using a suitable spectrophotometer (see *Spectrophotometry and Light-Scattering* (851)). Calculate the change in absorbance/min (Δ OD/min). The blanks for kinetic measurement are also expressed as Δ OD/min and should give the highest values as they are carried out in the absence of heparin. The RSD over the blank readings is less than 10%.

Calculations: The statistical models for *Slope ratio assay* or *Parallel-line assay* can be used depending on which model best describes the correlation between concentration and response.

Parallel-line assay: For each series, calculate the regression of the absorbance or change in absorbance/min against log concentrations of the *Sample solutions* and the *Standard solutions*, and calculate the potency of Heparin Sodium in USP Units/mL using statistical methods for parallel-line assays. Express the potency of Heparin Sodium/mg, calculated on the dried basis.

Slope ratio assay: For each series, calculate the regression of the log absorbance or the log change in absorbance/min against concentrations of the *Sample solutions* and of the *Standard solutions*, and calculate the potency of Heparin Sodium in USP Units/mL using statistical methods for slope ratio assays. Express the potency of Heparin Sodium/mg, calculated on the dried basis.

Acceptance criteria: The potency of Heparin Sodium, calculated on the dried basis, is NLT 180 USP Heparin Units in each mg.

INORGANIC IMPURITIES

- **RESIDUE ON IGNITION** (281): Between 28.0% and 41.0%
- **NITROGEN DETERMINATION, Method I, Nitrates and Nitrites Absent** (461): Between 1.3% and 2.5%, calculated on the dried basis
- **HEAVY METALS, Method II** (231): NMT 30 ppm

ORGANIC IMPURITIES

- **LIMIT OF GALACTOSAMINE IN TOTAL HEXOSAMINE** (a measure of dermatan sulfate and other galactosamine containing impurities)

Mobile phase: 14 mM potassium hydroxide

Glucosamine standard solution: 1.6 mg/mL of USP Glucosamine Hydrochloride RS in 5 N hydrochloric acid

Galactosamine standard solution: 16 µg/mL of USP Galactosamine Hydrochloride RS in 5 N hydrochloric acid

Standard solution: Mix equal volumes of *Glucosamine standard solution* and *Galactosamine standard solution*.

Hydrolyzed standard solution: Transfer 5 mL of the *Standard solution* to a 7-mL screw-cap test tube, cap, and heat for 6 h at 100°. Cool to room temperature, quantitatively transfer the solution to a 500-mL volumetric flask, and dilute with water to volume.

Sample solution: Transfer 12 mg of Heparin Sodium to a 7-mL screw-cap test tube, dissolve in 5 mL of 5 N hydrochloric acid, and cap.

Hydrolyzed sample solution: Heat the *Sample solution* for 6 h at 100°. Cool to room temperature and dilute with water (1 in 100).

Chromatographic system

(See *Chromatography* (621), *System Suitability*)

Mode: HPLC

Detector: Pulsed amperometric detector, set to the following waveform:

Step	Time (s)	Potential (V)	Integration
1	0.00	+0.1	—
2	0.20	+0.1	Begins
3	0.40	+0.1	Ends
4	0.41	-2.0	—
5	0.42	-2.0	—
6	0.43	+0.6	—
7	0.44	-0.1	—
8	0.50	-0.1	—

Column: 3-mm × 30-mm amino acid trap column in series with a 3- × 30-mm guard column and a 3-mm × 15-cm column that contains packing L69

Column temperature: Maintain columns at 30°

Flow rate: 0.5 mL/min

Pre-equilibration: At least 60 min with *Mobile phase*

Injection size: 10 µL

Elution: 10 min with *Mobile phase*

Column cleaning: At least 10 min with 100 mM potassium hydroxide

Equilibration: At least 10 min with *Mobile phase* before each injection

System suitability

Sample: *Hydrolyzed standard solution*

Suitability requirements

Resolution: NLT 2 between the galactosamine and glucosamine peaks

Column efficiency: NLT 2000 theoretical plates for glucosamine

Tailing factor: Between 0.8 and 2.0 for the galactosamine and glucosamine peaks

Analysis

Samples: *Hydrolyzed standard solution* and *Hydrolyzed sample solution*

Record the chromatograms, and measure the responses for the peaks at the retention time of galactosamine and glucosamine. Calculate the response ratio of galactosamine to glucosamine (GalN_R) in the *Hydrolyzed standard solution*:

$$(\text{GalN}_B/\text{GalN}_W) \times (\text{GlcN}_W/\text{GlcN}_B)$$

GalN_B = galactosamine peak area from the *Hydrolyzed standard solution*

GalN_W = weight of galactosamine for the *Standard solution*

GlcN_B = glucosamine peak area from the *Hydrolyzed standard solution*

GlcN_W = weight of glucosamine for the *Standard solution*

Calculate the percentage of galactosamine in the portion of total hexosamine taken:

$$\{[(\text{GalN}_U/\text{GalN}_R)]/[(\text{GalN}_U/\text{GalN}_R) + \text{GlcN}_U]\} \times 100$$

GalN_U = galactosamine peak area from the *Hydrolyzed sample solution*

GalN_R = galactosamine response ratio

GlcN_U = glucosamine peak area from the *Hydrolyzed sample solution*

Acceptance criteria: The percent galactosamine peak area of the total hexosamine of the *Hydrolyzed sample solution* must be NMT 1%.

• **NUCLEOTIDIC IMPURITIES:** *Biotechnology-Derived Articles—Total Protein Assay* (1057), *Method I* with the following modifications

Analysis: Dissolve 40 mg of Heparin Sodium in 10 mL of water. Measure the absorbance of this solution at 260 nm using the light scattering correction procedure of *Biotechnology-Derived Articles—Total Protein Assay* (1057), *Method I*.

Acceptance criteria: The absorbance of this solution at 260 nm is NMT 0.20.

• **ABSENCE OF OVERSULFATED CHONDROITIN SULFATE**

A: Proceed as directed in *Identification* test A. No features associated with oversulfated chondroitin sulfate are found between 2.12 and 3.00 ppm.

B: Proceed as directed in *Identification* test B. No peaks corresponding to oversulfated chondroitin sulfate should be detected eluting after the heparin peak.

• **PROTEIN IMPURITIES**

Standard stock solution: 0.100 mg/mL of bovine serum albumin in water

Standard solutions: Dilute portions of the *Standard stock solution* with water to obtain NLT 5 standard solutions having concentrations between 0.005 and 0.100 mg/mL of bovine serum albumin, the concentrations being evenly spaced.

Sample solution: 5 mg/mL of Heparin Sodium in water. Prepare in triplicate.

Blank: Water

Lowry reagent A: Prepare a solution of 10 g/L of sodium hydroxide in water and a solution of 50 g/L of sodium carbonate in water. Mix equal volumes (2V:2V) of each solution, and dilute with water to 5V.

Lowry reagent B: Prepare a solution of 29.8 g/L of disodium tartrate dihydrate in water. Prepare a solution of 12.5 g/L of cupric sulfate in water. Mix equal volumes of both solutions (2V:2V), and dilute with water to 5V.

Lowry reagent C: Mix 50 volumes of *Lowry reagent A* with 1 volume of *Lowry reagent B*.

Diluted Folin-Ciocalteu's phenol reagent: Dilute Folin-Ciocalteu's phenol reagent 2–4 times with water. The dilution should be chosen such that the pH of the samples (i.e., *Standard solution* and *Sample solution* after addition of *Lowry reagent C* and the *Diluted Folin-Ciocalteu's phenol reagent*) is 10.25 ± 0.25 .

Analysis

Samples: *Standard solutions*, *Sample solution*, and *Blank*

To 1 mL each of *Standard solution*, *Sample solution*, and *Blank*, add 5 mL of *Lowry reagent C*. Allow to stand at room temperature for 10 min. Add 0.5 mL of *Diluted Folin-Ciocalteu's phenol reagent* to each solution, mix immediately, and allow to stand at room temperature for 30 min. Determine the absorbance as directed in *Biotechnology-Derived Articles—Total Protein Assay* (1057), *Method 2*.

Calculations: See *Biotechnology-Derived Articles—Total Protein Assay* (1057), *Method 2*.

Acceptance criteria: NMT 1.0% (w/w) is found.

- **RESIDUAL SOLVENTS** (467): It meets the requirements.

SPECIFIC TESTS

- **pH** (791): 5.0–7.5, (1 in 100) solution
- **BACTERIAL ENDOTOXINS TEST** (85): NMT 0.03 USP Endotoxin Unit/USP Heparin Unit
- **STERILITY TESTS** (71): Where it is labeled as sterile, it meets the requirements.
- **LOSS ON DRYING** (731): Dry under vacuum at 60° for 3 h. It loses NMT 5.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store below 40°, preferably at room temperature.
- **LABELING:** Label it to indicate the tissue and the animal species from which it is derived.
- **USP REFERENCE STANDARDS** (11)
 - USP Oversulfated Chondroitin Sulfate RS
 - USP Dermatan Sulfate RS
 - USP Endotoxin RS
 - USP Galactosamine Hydrochloride RS
 - USP Glucosamine Hydrochloride RS
 - USP Heparin Sodium for Assays RS
 - USP Heparin Sodium Identification RS_s