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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 humans. 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. The heparin manufacturing process should also be validated to demonstrate clearance of lipids. 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 [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 Spectroscopy \(761\)](#).)

Standard solution: NLT 20 mg/mL of [USP Heparin Sodium Identification RS](#) in deuterium oxide with 0.002% (w/v) deuterated trimethylsilylpropionic (TSP) acid sodium salt

System suitability solution: Prepare 0.3% (w/w) [USP Oversulfated Chondroitin Sulfate RS](#) in the *Standard solution*.

Sample solution: NLT 20 mg/mL of Heparin Sodium in deuterium oxide with 0.002% (w/v) deuterated TSP. [NOTE—EDTA may be added to the *Sample solution* to NMT 12 μ g/mL. In the event that EDTA is added to the *Sample solution*, spectra should be recorded and compared both with and without addition of EDTA.]

Instrumental conditions

(See [Nuclear Magnetic Resonance Spectroscopy \(761\)](#).)

Mode: NMR, pulsed (Fourier transform)

Frequency: NLT 500 MHz (for ¹H)

Temperature: 20°–30°

System suitability

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, an acquisition time of NLT 2 s, and at least a 10-s delay. Record the ¹H NMR spectra of the *Standard solution* and the *System suitability solution* at a stable temperature between 20°–30°. 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 $\pm$ 0.02 and 2.16 $\pm$ 0.03 ppm, respectively. Record the ¹H NMR spectrum of the *Sample solution* at a stable temperature between 20°–30°. 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 chemical shifts of these signals do not differ by more than $\pm$ 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 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 0.10–3.75 range. Heparin Sodium must meet the requirements stated in [Residual Solvents \(467\)](#).

Suitability requirements

Number of transients: Adjust 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.

Chemical shift: The TSP methyl signal should be set to 0.00 ppm for all samples.

Chemical shifts (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

Analysis

Sample: *Sample solution*

Acceptance criteria: No unidentified signals greater than 4% of the mean of the height of signals 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% of the mean of the height of signals 1 and 2 are present in the 3.75–4.55 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. Pass the solution through a membrane filter with a 0.45-μm pore size, and degas before 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. Pass the solution through a membrane filter with a 0.45-μm pore size, and degas before use.

Mobile phase: See [Table 1](#).

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	80	20
30	10	90
31	80	20
45	80	20

Standard solution: NLT 20 mg/mL of [USP Heparin Sodium Identification RS](#) in water

System suitability solution: Prepare 0.1% (w/w) [USP Oversulfated Chondroitin Sulfate RS](#) and 0.5% (w/w) [USP Dermatan Sulfate RS](#) in the *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 L81²

Guard column: 2-mm × 5-cm; packing L61

Column temperature: Maintain columns at 40°

Flow rate: 0.22 mL/min

Injection volume: 20 μL

System suitability

Sample: *System suitability solution*

[NOTE—The retention times for dermatan sulfate, heparin, and oversulfated chondroitin sulfate are about 17, 22, and 30 min, respectively.]

Suitability requirements

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

Relative standard deviation: NMT 2% for the heparin peak area 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 of the *Sample solution* corresponds to that of the *Standard solution*.

• **C. ANTI-FACTOR Xa TO ANTI-FACTOR IIa RATIO**

[ANTI-FACTOR Xa AND ANTI-FACTOR IIa ASSAYS FOR UNFRACTIONATED AND LOW MOLECULAR WEIGHT HEPARINS\(208\)](#), [Anti-Factor Xa and Anti-Factor IIa Assays for Unfractionated Heparin](#)

Acceptance criteria: 0.9–1.1

• **D. MOLECULAR WEIGHT DETERMINATIONS**

1 M ammonium acetate solution: Accurately weigh 77.1 g of ammonium acetate, and dissolve in 1 L of water.

1% sodium azide solution: Dissolve 1 g of sodium azide in 100 mL of water.

Mobile phase: Transfer 100 mL of 1 M ammonium acetate solution to a 1-L volumetric flask, add 20 mL of 1% sodium azide solution, and dilute with water to volume. Filter using a nylon membrane with a 0.2-μm pore size prior to use.

Calibration solution: Prepare by dissolving 10 mg of the [USP Heparin Sodium Molecular Weight Calibrant RS](#) in 2 mL of Mobile phase, and filter using a nylon membrane with a 0.2-μm pore size.

System suitability solution: 5 mg/mL of [USP Heparin Sodium Identification RS](#) in Mobile phase. Filter using a nylon membrane with a 0.2-μm pore size.

Sample solution: Dissolve about 10 mg of Heparin Sodium sample in 2 mL of Mobile phase, and filter using a nylon membrane with a 0.2-μm pore size.

Chromatographic system

(See [Chromatography \(621\)](#), [System Suitability](#).)

[NOTE—The temperature of the refractive index detector must be set at the same temperature as the *Column temperature*.]

Mode: LC

Detector: Refractive index

Columns: One 7.8-mm × 30-cm, 8-μm packing L59 in series with a 7.8-mm × 30-cm, 5-μm packing L59³

Guard column: 6-mm × 4-cm; 7-μm packing L59

Column temperature: 30°

Flow rate: 0.6 mL/min ± 0.1%

Column equilibration: 0.6 mL/min for 2 h

Injection volume: 20 μL

System suitability

Samples: *Calibration solution* and *System suitability solution* (duplicate injections)

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 100 Da. The chromatographic system is suitable if the M_w of the *System suitability* sample is within 500 Da of the labeled value as stated in the USP Certificate for [USP Heparin Sodium Identification RS](#).

Peak molecular weights (M_p): The peak molecular weights (M_p) of the duplicate injections of the *System suitability solution* do not differ by more than 5% of the upper value.

Resolution: There is baseline resolution between the heparin and salt peaks.

Calibration curve: The linear regression coefficient of the calibration curve fitted to the Broad Standard Table values must be NLT 0.990, using a third-order polynomial equation.

Analysis

Samples: Inject 20 μL of the *System suitability solution* (duplicate injections), *Sample solution* (duplicate injection), and *Calibration solution* (single injection), and record the chromatograms for a length of time to ensure complete elution, including salt and solvent peaks (about 50 min). [NOTE—The calibrant, standard, or sample of heparin will give a broad heparin peak between about 20 and 40 min, followed by a later eluting narrow salt peak, as illustrated in the USP Certificate for [USP Heparin Sodium Molecular Weight Calibrant RS](#).]

Calculations: Calculate the total area under the heparin peak in the *Calibration solution* chromatogram, and the cumulative area at each point under the peak as a percent of the total. Do not include the salt peak. Using the Broad Standard Table provided in the USP Certificate for [USP Heparin Sodium Molecular Weight Calibrant RS](#), identify those points in the chromatogram for which the percent cumulative area is closest to the percent fractions listed in the Table, and assign the molecular weight (MW) in the Table to the corresponding retention time (RT) in the chromatogram. For the set of retention times and molecular weights identified, fit log(MW) vs. RT to a third-order polynomial function using suitable gel permeation chromatography (GPC) software [or: find values of a , b , c , and d such that $\log(MW) = a + b(RT) + c(RT)^2 + d(RT)^3$].

Using the same GPC software, for each of the duplicate chromatograms of the *System suitability solution* and the *Sample solution*, with the calibration function derived as described above, calculate M_w according to the following formula: