BRIEFING

Heparin Sodium, *USP 35* page 3403. Because of the suspected serious adverse events associated with the contamination of heparin with oversulfated chondroitin sulfate, USP has further revised the USP Heparin Sodium monograph. On the basis of comments received on the published and new methods submitted by the industry, it is proposed to make the following changes:

1. Procedural improvements are added to increase sensitivity of the ^{1}H *NMR Identification* test. The concentration of oversulfated chondroitin sulfate in the *System suitability solution* is lowered to 0.3% (w/w).
2. On the basis of public comments and supporting data, the strong ion-exchange HPLC method is optimized to shorten run time and to improve the resolution between heparin and chondroitin sulfate. The liquid chromatographic procedures are based on analyses performed with the Dionex brand of L61 guard and analytical columns, Ion-Pac AG11-HC and Ion-Pac AS11-HC, respectively. Typical retention times are about 17, 22, and 30 min for dermatan sulfate, heparin, and oversulfated chondroitin sulfate, respectively.
3. Another *Identification* test, *Molecular Weight Determinations*, is added. M_{24000} is NMT 20%, M_w is between 15,000 Da and 19,000 Da, and the ratio of $M_{8000–16000}$ to $M_{16000–24000}$ is NLT 1.0. The liquid chromatographic procedures are based on analyses performed with the Tosoh Biosciences brand of 7.8-mm × 30-cm (TSK 4000 SWXL) and 7.8-mm × 30-cm (TSK 3000 SWXL) columns in series.
4. The chromogenic anti-factor IIa assay and anti-factor Xa assay have been moved to the *USP* general chapter for heparin potency assays *Anti-Factor Xa and Anti-Factor IIa Assays for Unfractionated and Low Molecular Weight Heparins* ⟨ 208 ⟩.
5. A new impurity test to quantitatively measure nucleotidic impurities is added. The liquid chromatographic procedures are based on analyses performed with the Phenomenex brand of L1 column, Synergi Fusion RP, 4.6-mm × 15 cm. A lower limit of 0.1% (w/w) is proposed.
6. An improved residual proteins test with a step to remove interfering substances is added. A lower limit of 0.1% (w/w) is proposed.

(BIO1: A. Szajek.)
Correspondence Number—C79193

Comment deadline: January 31, 2013

Heparin Sodium

DEFINITION

Change to read:

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.

▲The heparin manufacturing process should also be validated to demonstrate clearance of lipids.▲*USP37*

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

Change to read:

- **A. ^{1}H 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%~~

  ▲0.002%▲*USP37*

  (w/v) deuterated trimethylsilylpropionic (TSP) acid sodium salt

  System suitability solution: Prepare ~~1%~~

  ▲0.3%▲*USP37*

  (w/w) USP Oversulfated Chondroitin Sulfate RS in the *Standard solution*.

Sample solution: NLT 20 mg/mL of Heparin Sodium in deuterium oxide with ~~0.02%~~ ▲0.002%▲*USP37* (w/v) deuterated TSP.

▲[NOTE—EDTA may be added to the *Sample solution* to NMT 12 µg/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.]▲*USP37*

Instrumental conditions

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

Mode: NMR, pulsed (Fourier transform)

Frequency: NLT 500 MHz (for ^{1}H)

Temperature: ~~25°~~ ▲20°–30°▲*USP37*

System suitability

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

Using a pulsed (Fourier transform) NMR spectrometer operating at NLT 500 MHz for ^{1}H, acquire a free induction decay (FID) using NLT 16 scans using a 90° pulse, ~~and 20-s delay~~ ▲an acquisition time of NLT 2-s, and at least a 10-s delay.▲*USP37*

Record the ^{1}H NMR spectra of the *Standard solution* and the *System suitability solution* at ~~25°~~ ▲a stable temperature between 20°–30°.▲*USP37*

Collect the ^{1}H 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 ^{1}H NMR spectrum of the *Sample solution* at ~~25°~~ ▲a stable temperature between 20–30°.▲*USP37*

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~~ ▲chemical shifts▲*USP37* 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 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~~ ▲3.75▲*USP37* 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*):▲*USP37* Should be observed at 2.05 ± 0.02 and 2.16 ± 0.03 ppm, respectively. ~~*System suitability solution*~~ ▲▲*USP37*

Analysis

Sample: *Sample solution*

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.

Change to read:

- **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 filter membrane 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 filter membrane with a 0.45-µm pore size, and degas before use.

Mobile phase: See ~~the gradient table below~~ ▲*Table 1*.▲*USP37*

▲**Table 1**▲*USP37*

Time (min)	Solution A (%)	Solution B (%)	~~Elution~~ ▲▲*USP37*
			~~Equilibration~~

0	80	20	▲ ▲*USP37*
~~60~~ ▲30 ▲*USP37*	10	90	~~Linear gradient~~ ▲ ▲*USP37*
~~61~~ ▲31 ▲*USP37*	80	20	~~Linear gradient~~ ▲ ▲*USP37*
~~75~~ ▲45 ▲*USP37*	80	20	~~Re-equilibration~~ ▲ ▲*USP37*

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

System suitability solution: Prepare ~~1%~~ ▲0.1% ▲*USP37* (w/w) USP Oversulfated Chondroitin Sulfate RS and ~~1%~~ ▲0.5% ▲*USP37* (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 L61 ▲[2] ▲*USP37*

Guard column: 2-mm × ~~50-mm~~ ▲5-cm; ▲*USP37* packing L61

Column temperature: Maintain columns at 40°

Flow rate: 0.22 mL/min

~~**Injection size:** 10 µL~~

▲**Injection volume:** 20 µL ▲*USP37*

System suitability

Sample: *System suitability solution*

[NOTE—The retention times for dermatan sulfate, heparin, and oversulfated chondroitin sulfate are about ~~20, 30, and 50~~ ▲17, 22, and 30 ▲*USP37* 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

Relative standard deviation: NMT 2% for the heparin peak ▲area ▲*USP37*

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 of the *Standard solution*.

Change to read:

- **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, 0.0075, 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 µL of *pH 8.4 buffer* instead of 30 µL 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 five dilutions in the concentration range between 0.03 and 0.375 USP Heparin Units/mL.~~

~~**Sample solutions:** Dissolve or dilute a 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 µL of *pH 8.4 buffer*. Then separately transfer 30 µL of the different dilutions of the *Standard solutions* or the *Sample solutions* to the tubes. Add 150 µL of *Antithrombin solution*, prewarmed at 37° for 15 min, to each tube, mix, and incubate for 2 min. Add 300 µL of *Factor Xa solution*, prewarmed at 37° for 15 min, to each tube, mix, and incubate for 2 min. Add 300 µL of *Chromogenic substrate solution*, prewarmed at 37° for 15 min, to each tube, mix, and incubate for exactly 2 min. Add 150 µL 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 µL 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 the 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:~~

$$\text{Result} = A \times (S_T/S_S)$$

~~A = 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 the *Assay*):~~

~~Result = anti-factor Xa activity/anti-factor IIa potency~~

▲**Anti-Factor Xa and Anti-Factor IIa Assays for Unfractionated and Low Molecular Weight Heparins, Anti-factor Xa Assay for Unfractionated Heparin** 〈 208 〉▲*USP37*

Acceptance criteria: 0.9–1.1

Add the following:

▲• **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.

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.

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.

Chromatographic system

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

[NOTE—The temperature of 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, 5-µm packing L59 in series with a 7.8-mm × 30-cm, 8-µm packing L59[3]

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

Sample: *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 up 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 *Calibrant* 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 $\log(M_w) = a + b(\mathrm{RT}) + c(\mathrm{RT})^2 + d(\mathrm{RT})^3$].

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:

$$M_w = \Sigma(RI_iM_i)/\Sigma RI_i$$

where the detector response at each point is defined as RI_i and the MW at each point as M_i. Round up the mean value of M_w to the nearest 100 Da.

Using the same GPC software, determine for each of the duplicate *Sample solution* chromatograms: the percentage of heparin with molecular weights lower than 8,000 Da, M_{8000}, the percentage of heparin with molecular weight in the range 8,000–16,000, $M_{8000–16000}$, the percentage of heparin with molecular weight in the range 16,000–24,000, $M_{16000–24000}$, and the percentage of heparin with molecular weight greater than 24,000, M_{24000}. Round the mean percentage values to the nearest 1%.

Acceptance criteria: M_{24000} is NMT 20%, M_w is between 15,000 Da and 19,000 Da, and the ratio of $M_{8000–16000}$ to $M_{16000–24000}$ is NLT 1.0.▲*USP37*

Change to read:

- **Đ**

▲**E.**▲*USP37*

IDENTIFICATION TESTS—GENERAL, *Sodium* 〈191〉**:** It meets the requirements of the flame test for sodium.

ASSAY

Change to read:

- **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 µL) of *Antithrombin solution* to each tube containing one volume (50–100 µL) of either the *pH 8.4 buffer* or an appropriate dilution of the *Standard solutions* or the *Sample solutions*. Mix, but do not allow bubbles to form. Incubate at 37° for at least 1 min. Add to each tube 25–50 µL of *Thrombin human solution*, and incubate for at least 1 min. Add 50–100 µL of *Chromogenic substrate solution*. Please note that all reagents, *Standard solutions*, and *Sample solutions* should be prewarmed to 37° just before use. Two different types of measurements can be recorded:

1. Endpoint measurement: Stop the reaction after at least 1 min with 50–100 µL 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 (ΔOD/min). The blanks for kinetic measurement are also expressed as ΔOD/min and should give the highest values because 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 *Standard solutions* and the *Sample 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 *Standard solutions* and the *Sample 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.

▲**Anti-Factor Xa and Anti-Factor IIa Assays for Unfractionated and Low Molecular**

Weight Heparins, Anti-factor Xa Assay for Unfractionated Heparin ⟨208⟩ ▲*USP37*

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

OTHER COMPONENTS

- **NITROGEN DETERMINATION,** *Method I* ⟨461⟩**:** 1.3%–2.5%, calculated on the dried basis, using the procedure for *Nitrates and Nitrites Absent*

IMPURITIES

- **RESIDUE ON IGNITION** ⟨281⟩**:** 28.0%–41.0%
- **HEAVY METALS,** *Method II* ⟨231⟩**:** NMT 30 ppm

Change to read:

- **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 the *Glucosamine standard solution* and the *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: HPIC

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

▲See *Table 2*.▲*USP37*

▲**Table 2**▲*USP37*

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~~ ▲3-mm × 3-cm ▲*USP37* guard column and a 3-mm × 15-cm column that contains packing L69

Column temperature: ~~Maintain columns at~~ ▲ ▲*USP37* 30°

Flow rate: 0.5 mL/min

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

Injection ~~size~~ ▲volume: ▲*USP37* 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*:

$$\text{Result} = (GalN_B/GalN_W) \times (GlcN_W/GlcN_B)$$

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

$GalN_W$ = weight of galactosamine for the *Standard solution*

$GlcN_W$ = weight of glucosamine for the *Standard solution*
$GlcN_B$ = peak area of glucosamine from the *Hydrolyzed standard solution*

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

$$\text{Result} = \{[(GalN_U/GalN_R)]/[(GalN_U/GalN_R) + GlcN_U]\} \times 100$$

$GalN_U$ = peak area of galactosamine from the *Hydrolyzed sample solution*
$GalN_R$ = response ratio of galactosamine
$GlcN_U$ = peak area of glucosamine 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%.

Change to read:

- **NUCLEOTIDIC IMPURITIES**

~~(See *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩, *Method 1*, 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 1*.~~
~~**Acceptance criteria:** The absorbance of this solution at 260 nm is NMT 0.20.~~

▲**Solution A:** Dissolve 3.08 g of ammonium acetate in 2 L of water, and adjust with glacial acetic acid to a pH of 4.4 ± 0.2. Degas for 2 min under vacuum with sonication before use.

Solution B: 100% acetonitrile. Degas for 1 min under vacuum with sonication before use.

Mobile phase: See *Table 3*.

Table 3

Time (min)	Solution A (%)	Solution B (%)
0	98	2
5.00	98	2
15.00	80	20
20.00	80	20
20.10	98	2
25.00	98	2

Nucleoside identification solution: Accurately weigh and transfer about 25 mg of uridine, guanosine, cytidine, thymidine, 2′-deoxyadenosine, 2′-deoxyguanosine, 2′-

deoxycytidine, and 5-methyl-2′-deoxycytidine into a 200-mL volumetric flask, add approximately 185 mL of water, and dissolve with sonication and vortexing, if necessary. Dilute with water to volume, and mix. Transfer 2.0 mL of this solution into a 100-mL volumetric flask, dilute with water to volume, and mix.

Adenosine stock solution: Accurately weigh and transfer 25 mg of USP Adenosine RS into a 100-mL volumetric flask, add approximately 85 mL of water, and dissolve with sonication and vortexing, if necessary. Dilute with water to volume, and mix.

Standard solution: Transfer 2.0 mL of the *Adenosine stock solution* into a 200-mL volumetric flask, dilute with water, and mix.

System suitability solution: Transfer 2.0 mL of the *Standard solution* into a 100-mL volumetric flask, dilute with water to volume, and mix.

Reaction buffer: Accurately weigh and transfer 0.41 g of magnesium chloride hexahydrate, 0.24 g of tris (hydroxymethyl)amino methane, and 0.58 g of sodium chloride into a 100-mL volumetric flask, dissolve in 75 mL of water, and mix. Adjust with 1 N hydrochloric acid to a pH of 7.9 ± 0.1. Dilute with water to volume, and mix.

PDE I diluent: Transfer 5.0 mL of glycerol and 5.0 mL of the *Reaction buffer* into a 20-mL flask, and vortex to mix.

PDE I solution: Carefully transfer the contents of one vial of phosphodiesterase I (PDE I) into a 1000-µL eppendorf vial, and reconstitute with 1000 µL of *PDE I diluent*. Store at −20°.

Enzyme digest solution: Add 10 µL of Benzonase[4], 222 Units of alkaline phosphase (AP), and 125 µL of *PDE I solution* to 5.0 mL of *Reaction buffer*. Store at −20°.

Blank: Transfer 100 µL of water and 100 µL of *Enzyme digest solution* into a 250-µL HPLC vial, and mix with a micropipette. Incubate NLT 60 min in the autosampler at 37° before injection.

Sample solution: Accurately weigh and transfer 400 mg of Heparin Sodium into a 20-mL volumetric flask, dilute with water to volume, and mix. Transfer 100 µL of this solution and 100 µL of *Enzyme digest solution* into a 250-µL HPLC vial, and mix. Incubate NLT 60 min in the autosampler at 37° before injection.

Chromatographic system

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

Mode: LC

Detector: UV 260 nm

Column: 4.6-mm × 15-cm; 4-µm packing L1

Column temperature: 20 ± 3°

Autosampler temperature: 37 ± 1°

Flow rate: 1 mL/min

Injection volume: 10 µL

Run time: 25 min

System suitability

Samples: *System suitability solution*, *Standard solution*, and *Nucleoside identification solution*

Suitability requirements

Resolution: The resolution between the 2′-deoxycytidine peak and the uridine peak is NLT 1.3 for the injection of the *Nucleoside identification solution*.

Relative standard deviation: Inject six replicates of the *Standard solution*, and record the chromatograms. The percent relative standard deviation (%RSD) of the areas of the adenosine peak is NMT 10%.

Signal-to-noise ratio: The *S/N* of the adenosine peak in the *System suitability solution* is NLT 10.

Analysis

Samples: *Water*, *Blank*, *System suitability solution*, *Nucleoside identification solution*, *Standard solution*, and *Sample solution*

Record the chromatograms. Calculate the area reject value, Q:

$$Q = (10 \times A_{sss})/(S/N)$$

A_{sss} = peak area of adenosine in the *System suitability solution*

S/N = signal-to-noise ratio of the adenosine peak in the *System suitability solution*

For the *Standard solution*, calculate the concentration of adenosine, in mg/mL:

$$C_s = W_s/DF$$

C_s = concentration of adenosine in the *Standard solution* (mg/mL)

W_s = weight of USP Adenosine RS (mg)

DF = 10,000 (dilution factor)

Calculate the percentage of nucleotidic impurities:

$$\text{Result} = \Sigma[(C_s/A_s) \times A_i \times (MW_{ratio}/RRF_i)] \times (DF/W_{sample}) \times 100$$

C_s = concentration of adenosine in the *Standard solution* (mg/mL)

A_s = average peak area (n = 6) of adenosine in the *Standard solution*

A_i = peak area of each impurity above Q in the *Sample solution*

MW_{ratio} = see Table 4

RRF_i = relative response factor for the corresponding peak (see Table 4)

DF = dilution factor, 40

W_{sample} = sample weight of Heparin Sodium (mg)

Table 4

Name	Relative Retention Time	Relative Response Factor	MW_{ratio}
Cytidine	0.28	0.53	1.2548
2′-Deoxycytidine	0.38	0.56	1.2727
Uridine	0.40	0.75	1.2537
5-Methyl-2′-deoxycytidine	0.66	0.25	1.2569
Guanosine	0.81	0.74	1.2188
2′-Deoxyguanosine	0.89	0.83	1.2319
Thymidine	0.92	0.68	1.2558
Adenosine	1.00	1.00	1.2319
2′-Deoxyadenosine	1.04	1.09	1.2466
Others	—	1.00	1.0000

Acceptance criteria: NMT 0.1% (w/w) is found.▲*USP37*

- **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.

Change to read:

- **PROTEIN IMPURITIES**

▲[NOTE—Treatment for interfering substances is only required for samples previously tested with a protein content greater than 0.1%.]▲*USP37*

Standard stock solution: ~~0.100~~

▲2.0▲*USP37*

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~~

▲0.010 and 0.050▲*USP37*

mg/mL of bovine serum albumin, the concentrations being evenly spaced.

▲**System suitability standard:** Dilute a portion of the *Standard stock solution* with water to obtain a solution containing 0.030 mg/mL of bovine serum albumin.▲*USP37*

Sample solution: ~~5~~

▲30▲*USP37*

mg/mL of Heparin Sodium in water. Prepare in triplicate.

▲**Spiked sample:** Using an appropriate dilution scheme and the *Standard stock*

solution, prepare a *Spiked sample* containing 30 mg/mL Heparin Sodium and 0.030 mg/mL bovine serum albumin in water.▲*USP37*

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*. Using an appropriate dilution scheme and the *Standard stock solution*, prepare a *Spiked sample* solution containing 30 mg/mL Heparin Sodium and 0.030 mg/mL bovine serum albumin in water.

▲Prepare fresh daily.▲*USP37*

Diluted Folin–Ciocalteu's phenol reagent: Dilute Folin–Ciocalteu's phenol reagent ~~2–4~~ ▲1–2▲*USP37* 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~~ ▲10.3 ± 0.3.

Sodium deoxycholate reagent: Prepare a solution of sodium deoxycholate in water having a concentration of 150 mg in 100 mL.

Trichloroacetic acid reagent: Prepare a solution of trichloroacetic acid in water having a concentration of 72 g in 100 mL.▲*USP37*

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*.

▲ Mix.▲*USP37*

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 ▲NLT▲*USP37* 30 min. Determine the absorbance ~~as directed in *Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩*, Method 2*~~ ▲at the wavelength of maximum absorbance at 750 nm with a suitable spectrophotometer, using the solution from the *Blank* to set the instrument to zero and ensuring that all samples and standards absorbances are measured after the same

final incubation time. To remove interfering substances, add 0.1 mL of *Sodium deoxycholate reagent* to 1 mL of a solution of the protein under test. Mix on a vortex mixer, and allow to stand at room temperature for 10 min. Add 0.1 mL of *Trichloroacetic acid reagent*, and mix on a vortex mixer. Centrifuge at a speed that ensures removal of visible particulate matter. [NOTE—NLT 14,100 RCF should be used. Appropriate centrifuge speed should be determined by each laboratory.] The supernatant should be essentially free of visible particulates. A pellet may not be visible. If the interfering substances method is used, dissolve the protein residue in 1 mL of *Lowry reagent C*.▲*USP37*

Calculations ~~*See Biotechnology-Derived Articles—Total Protein Assay* ⟨1057⟩*; Method 2.*~~

▲Using the linear regression method, plot the absorbances of the solutions from the *Standard solutions* versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the absorbance of the *Sample solution*, determine the concentration of protein in the *Sample solution*.

System suitability: The correlation coefficient (*r*) for a linear fit of all standards is NLT 0.99. The percent RSD between triplicate sample results is NMT 10%. If the sample absorbancies are lower than the standard curve, the percent RSD specification is not required. The percent recovery of the *System suitability standard* is 90%–110%. If the interfering substances treatment is performed, the percent recovery of the *Spiked sample* is 85%–115%.▲*USP37*

Acceptance criteria: NMT ~~1.0%~~

▲0.1%▲*USP37*

(w/w) is found.

SPECIFIC TESTS

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

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.

Change to read:

- **USP REFERENCE STANDARDS** 〈11〉

  USP Adenosine 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

  ▲USP Heparin Sodium Molecular Weight Calibrant RS ▲*USP37*

  USP Oversulfated Chondroitin Sulfate RS

---

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

2 ▲ IonPac® AS11-HC 2.0- × 250-mm, Dionex (cat # 052961) High Capacity column is necessary to achieve required resolution.▲*USP37*

3 The method was validated using a guard column TSK SWXL 6-mm × 4-cm, 7-µm in series with two analytical columns: TSK G3000 SWXL 7.8- × 30-cm, 5-µm in series with a TSK G4000 SWXL 7.8- × 30-cm, 8-µm diameter.

4 A suitable ultrapure *Serratia marcescens* nuclease (EC 3.1.30.2) must be ≥99% containing ≥250 units/µL.

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

USP36–NF31 Page 3801

Interim Revision Announcement: *USP32–NF27* No. 5 Page 1085