

## Heparin Sodium

### ヘパリンナトリウム

#### *Add the following next to Description:*

**Identification** Dissolve 1 mg each of Heparin Sodium and Heparin Sodium Reference Standard for physicochemical test in 1 mL of water, and use these solutions as the sample solution and standard solution, respectively. Perform the test with 20  $\mu$  L each of the sample solution and standard solution as directed under Liquid Chromatography <2.01> according to the following conditions: the retention times for the major peak from the sample solution and the standard solution are identical.

#### *Operating conditions-*

Detector, column, column temperature, mobile phase A, mobile phase B, flowing of the mobile phase and flow rate: Proceed as directed under the operating conditions in Purity (6).

#### *System suitability-*

System performance: Dissolve 1.0 mg of Heparin Sodium Reference Standard for physicochemical test in 0.60 mL of water. Dissolve 0.10 mg of Over-sulfated Chondroitin Sulfate Reference Standard in 0.20 mL of water. Dissolve 1.0 mg of dermatan sulfate in 2.0 mL of water. To 90  $\mu$  L of the solution of Heparin Sodium Reference Standard add 30  $\mu$  L each of the solutions of over-sulfated chondroitin sulfate and dermatan sulfate, and mix. When the procedure is run with 20  $\mu$  L of the mixture under the above operating conditions, dermatan sulfate, heparin and over-sulfated chondroitin sulfate are eluted in this order with the resolution between the peaks of dermatan sulfate and heparin being not less than 1.0 and that between the peaks of heparin and over-sulfated chondroitin sulfate being not less than 1.5.

#### *Change the Purity (5) to read:*

**Purity (5)** Over-sulfated Chondroitin Sulfate-Dissolve 20 mg of Heparin Sodium in 0.60 mL of a solution of sodium 3-trimethylsilylpropionate- $d_4$  for nuclear magnetic resonance spectroscopy in heavy water for nuclear magnetic resonance spectroscopy (1 in 10000). Determine the spectrum of this solution as directed under Nuclear Magnetic Resonance Spectroscopy <2.21> ( $^1\text{H}$ ) in accordance with the following conditions, using sodium 3-trimethylsilylpropionate- $d_4$  for nuclear magnetic resonance spectroscopy as an internal reference compound: it exhibits no signal corresponding to *N*-acetyl proton of over-sulfated chondroitin sulfate at  $\delta$  2.15 $\pm$ 0.02 ppm, or the signal disappears when determining the spectrum of the sample solution as directed under  $^1\text{H}$  with  $^{13}\text{C}$ -decoupling.

#### *Operation conditions-*

Spectrometer: (1) FT-NMR, Not less than 400 MHz

Temperature: 25°C

Spinning: Off

Number of data points: 32,768

Spectral range: Signal of DHO  $\pm$  6.0 ppm

Flip angle: 90°

Delay time: 20 seconds

Dummy scan: 4

Number of scans: S/N ratio of the signal of *N*-acetyl proton of heparin is not less than 1000

Window function: Exponential function (Line broadening factor = 0.2 Hz)

*System suitability-*

System performance: Dissolve 20 mg of Heparin Sodium Reference Standard for physicochemical test in 0.40 mL of a solution of sodium 3-trimethylsilylpropionate-d<sub>4</sub> for nuclear magnetic resonance spectroscopy in heavy water for nuclear magnetic resonance spectroscopy (1 in 10000). Dissolve 0.10 mg of Over-sulfated Chondroitin Sulfate Reference Standard in 1.0 mL of a solution of sodium 3-trimethylsilylpropionate-d<sub>4</sub> for nuclear magnetic resonance spectroscopy in heavy water for nuclear magnetic resonance spectroscopy (1 in 10000). To the solution of Heparin Sodium Reference Standard for physicochemical test add 0.2 mL of the solution of Over-sulfated Chondroitin Sulfate Reference Standard. When determining the spectrum of this solution under the above conditions, it exhibits the signal of *N*-acetyl proton of heparin and the signal of *N*-acetyl proton of over-sulfated chondroitin sulfate at  $\delta$  2.04 $\pm$ 0.02 ppm and  $\delta$  2.15  $\pm$ 0.02 ppm, respectively.

**Add the following next to Purity (5)**

**Purity (6)** Related substances- Dissolve 2.0 mg of Heparin Sodium in 0.1 mL of water, and perform the test with exactly 20  $\mu$  L of this solution as directed under Liquid Chromatography <2.01> according to the following conditions: it exhibits no peaks after the heparin peak.

*Operating conditions-*

Detector: An ultraviolet absorption photometer (wavelength: 202 nm).

Column: A stainless steel column 2.0 mm in inside diameter and 7.5 cm in length, packed with diethylaminoethyl group bound to synthetic polymer for liquid chromatography (10  $\mu$  m in particle diameter).

Column temperature: A constant temperature of about 35°C.

Mobile phase A: Dissolve 0.4 g of sodium dihydrogenphosphate dihydrate in 1000 mL of water and adjust to a pH of 3.0 with diluted phosphoric acid (1 in 10).

Mobile phase B: Dissolve 0.4 g of sodium dihydrogenphosphate dihydrate and 106.4 g of lithium perchlorate in 1000 mL of water and adjust to a pH of 3.0 with diluted phosphoric acid (1 in 10).

Flowing of the mobile phase: Control the gradient by mixing the mobile phases A and B as directed in the following table.

| Time after injection of | Mobile phase A | Mobile phase B |
|-------------------------|----------------|----------------|
|-------------------------|----------------|----------------|

| sample (min) | (vol%) | (vol%) |
|--------------|--------|--------|
| 0 - 3        | 90     | 10     |
| 3 - 15       | 90→0   | 10→100 |

Flow rate: 0.2 mL per minute.

Time span of measurement: About 2 times as long as the retention time of heparin, beginning after the solvent peak.

*System suitability-*

Test for required detectability: Dissolve 10 mg of Heparin Sodium Reference Standard for physicochemical test in 0.40 mL of water, and use this solution as the Heparin Sodium standard stock solution. Separately, dissolve 0.10 mg of Over-sulfated Chondroitin Sulfate Reference Standard in 0.20 mL of water, and use this solution as the over-sulfated chondroitin sulfate standard solution. To 60  $\mu$  L of the Heparin Sodium standard stock solution add 3  $\mu$  L of the over-sulfated chondroitin sulfate standard solution and 12  $\mu$  L of water, and mix. When the procedure is run with 20  $\mu$  L of the mixture under the above operating conditions, it exhibits a peak for over-sulfated chondroitin sulfate.

System performance: To 120  $\mu$  L of the Heparin Sodium standard stock solution add 30  $\mu$  L of the over-sulfated chondroitin sulfate standard solution, mix and use this solution as the solution for system suitability test. When the procedure is run with 20  $\mu$  L of the solution for system suitability test under the above operating conditions, heparin and over-sulfated chondroitin sulfate are eluted in this order with the resolution between these peaks being not less than 1.5.

System repeatability: When the test is repeated 6 times with 20  $\mu$  L of the solution for system suitability test under the above operating conditions, the relative standard deviation of the peak area of over-sulfated chondroitin sulfate is not more than 2.0%.

**Purity (7)** Galactosamine- Dissolve 2.4 mg of Heparin Sodium in 1.0 mL of water and hydrochloric acid (7 : 5) , and use this solution as the Heparin Sodium stock solution. Dissolve 8.0 mg of D-glucosamine hydrochloride in water and hydrochloric acid (7 : 5) to make exactly 10 mL. Dissolve 8.0 mg of D-galactosamine hydrochloride in water and hydrochloric acid (7 : 5) to make exactly 10 mL. To 99 volumes of the solution of D-glucosamine add 1 volume of the solution of D-galactosamine, and use this solution as the standard stock solution. Transfer 500  $\mu$  L each of the Heparin Sodium stock solution and the standard stock solution to a glass-stoppered test tube, stopper tightly, and heat at 100°C for 6 hours. After cooling to room temperature, evaporate 100  $\mu$  L each of the reaction solutions to dryness. Add 50  $\mu$  L of methanol to each of the residues and evaporate to dryness at room temperature. Dissolve each of the residues in 10  $\mu$  L of water, add 40  $\mu$  L of aminobenzoate derivatization TS, and heat at 80°C for 1 hour. After cooling to room temperature, evaporate the reaction solutions to dryness. Add 200  $\mu$  L each of water and ethyl acetate to each of the residues, shake vigorously, and then centrifuge. After remove the upper

layers, add 200  $\mu$  L of ethyl acetate to each of the lower layers, shake vigorously, and then centrifuge. These lower layers are used as the sample solution and standard solution. Perform the test with 5  $\mu$  L each of the sample solution and standard solution as directed under Liquid Chromatography <2.01> according to the following conditions: the peak area ratio of galactosamine to glucosamine of the sample solution is not larger than that of the standard solution.

*Operating conditions-*

Detector: A fluorescence photometer (excitation wavelength: 305 nm; emission wavelength: 360nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (3  $\mu$  m in particle diameter).

Column temperature: A constant temperature of about 45°C.

Mobile phase: To 100 mL of water and trifluoroacetic acid (1000 : 1) add 100 mL of acetonitrile. Add 140 mL of the solution to 860 mL of water and trifluoroacetic acid (1000 : 1).

Flow rate: 1.0 mL per minute.

Time span of measurement: About 50 minutes after injected.

*System suitability-*

Test for required detectability: Dissolve 8.0 mg of D-mannosamine hydrochloride in 10 mL of water and hydrochloric acid (7 : 5), and use this solution as the mannosamine standard solution. Transfer 500  $\mu$  L of the standard stock solution and the mannosamine standard solution (100 : 1) to a glass-stoppered test tube, stopper tightly, and heat at 100°C for 6 hours. After cooling this solution to room temperature, evaporate 100  $\mu$  L of the reaction solution to dryness. Add 50  $\mu$  L of methanol to the residue and evaporate to dryness at room temperature. Dissolve the residue in 10  $\mu$  L of water, add 40  $\mu$  L of the ethyl aminobenzoate derivatization TS, and heat at 80°C for 1 hour. After cooling this solution to room temperature, evaporate the reaction solution to dryness. Add 200  $\mu$  L each of water and ethyl acetate to the residue, shake vigorously, and then centrifuge. After remove the upper layer, add 200  $\mu$  L of ethyl acetate to the lower layer, shake vigorously, and then centrifuge. The lower layer is used as the solution for system suitability test. When the procedure is run with 5  $\mu$  L of the solution for system suitability test under the above operating conditions, the ratio of the peak area of galactosamine to that of glucosamine is 0.7 – 2.0%.

System performance: When the procedure is run with 5  $\mu$  L of the solution for system suitability test under the above operating conditions, glucosamine, mannosamine and galactosamine are eluted in this order with the resolution each between the peaks of glucosamine and mannosamine and between the peaks of mannosamine and galactosamine being not less than 1.5.

System repeatability: When the test is repeated 6 times with 5  $\mu$  L of the solution for system suitability test under the above operating conditions, the relative standard deviation of the ratio of the peak area of galactosamine

to that of glucosamine is not more than 4.0%.

## Heparin Calcium

### ヘパリンカルシウム

*Change the Identification (2) to Identification (3) and add the following next to Identification (1)*

**Identification (2)** Dissolve 1 mg each of Heparin Calcium and Heparin Sodium Reference Standard for physicochemical test in 1 mL of water, and use these solutions as the sample solution and standard solution, respectively. Perform the test with 20  $\mu$  L each of the sample solution and standard solution as directed under Liquid Chromatography <2.01> according to the following conditions: the retention times for the major peak from the sample solution and the standard solution are identical.

*Operating conditions-*

Detector, column, column temperature, mobile phase A, mobile phase B, flowing of the mobile phase and flow rate: Proceed as directed under the operating conditions in Purity (9).

*System suitability-*

System performance: Dissolve 1.0 mg of Heparin Sodium Reference Standard for physicochemical test in 0.60 mL of water. Dissolve 0.10 mg of Over-sulfated Chondroitin Sulfate Reference Standard in 0.20 mL of water. Dissolve 1.0 mg of dermatan sulfate in 2.0 mL of water. To 90  $\mu$  L of the solution of Heparin Sodium Reference Standard add 30  $\mu$  L each of the solutions of Over-sulfated Chondroitin Sulfate Reference Standard and dermatan sulfate, and mix. When the procedure is run with 20  $\mu$  L of the mixture under the above operating conditions, dermatan sulfate, heparin and over-sulfated chondroitin sulfate are eluted in this order with the resolution between the peaks of dermatan sulfate and heparin being not less than 1.0 and that between the peaks of heparin and over-sulfated chondroitin sulfate being not less than 1.5.

*Change the Purity (8) to read:*

**Purity (8)** Over-sulfated Chondroitin Sulfate-Dissolve 20 mg of Heparin Calcium in 0.60 mL of a solution of sodium 3-trimethylsilylpropionate- $d_4$  for nuclear magnetic resonance spectroscopy in heavy water for nuclear magnetic resonance spectroscopy (1 in 10000). Determine the spectrum of this solution as directed under Nuclear Magnetic Resonance Spectroscopy <2.21> ( $^1\text{H}$ ) in accordance with the following conditions, using sodium 3-trimethylsilylpropionate- $d_4$  for nuclear magnetic resonance spectroscopy as an internal reference compound: it exhibits no signal corresponding to *N*-acetyl proton of over-sulfated chondroitin sulfate at  $\delta$  2.18 $\pm$ 0.05 ppm, or the signal disappears when determining the spectrum of the sample solution as directed under  $^1\text{H}$  with  $^{13}\text{C}$ -decoupling.

*Operating conditions-*

Spectrometer: (1) FT-NMR, Not less than 400 MHz

Temperature: 25°C

Spinning: Off

Number of data points: 32,768

Spectral range: Signal of DHO  $\pm$  6.0 ppm

Flip angle: 90°

Delay time: 20 seconds

Dummy scan: 4

Number of scans: S/N ratio of the signal of *N*-acetyl proton of heparin is not less than 1000

Window function: Exponential function (Line broadening factor = 0.2 Hz)

#### *System suitability-*

System performance: Dissolve 20 mg of Heparin Calcium in 0.40 mL of a solution of sodium 3-trimethylsilylpropionate-d<sub>4</sub> for nuclear magnetic resonance spectroscopy in heavy water for nuclear magnetic resonance spectroscopy (1 in 10000). Dissolve 0.10 mg of Over-sulfated Chondroitin Sulfate Reference Standard in 1.0 mL of a solution of sodium 3-trimethylsilylpropionate-d<sub>4</sub> for nuclear magnetic resonance spectroscopy in heavy water for nuclear magnetic resonance spectroscopy (1 in 10000). To the solution of heparin calcium add 0.20 mL of the solution of Over-sulfated Chondroitin Sulfate Reference Standard. When determining the spectrum of this solution under the above conditions, it exhibits the signal of *N*-acetyl proton of heparin and the signal of *N*-acetyl proton of over-sulfated chondroitin sulfate at  $\delta$  2.04 $\pm$ 0.02 ppm and  $\delta$  2.18 $\pm$ 0.05 ppm, respectively.

#### ***Add the following next to Purity (8)***

**Purity (9)** Related substances- Dissolve 2.0 mg of Heparin Calcium in 0.1 mL of water, and perform the test with exactly 20  $\mu$  L of this solution as directed under Liquid Chromatography <2.01> according to the following conditions: it exhibits no peaks after the heparin peak.

#### *Operation conditions-*

Detector: An ultraviolet absorption photometer (wavelength: 202 nm).

Column: A stainless steel column 2.0 mm in inside diameter and 7.5 cm in length, packed with diethylaminoethyl group bound to synthetic polymer for liquid chromatography (10  $\mu$  m in particle diameter).

Column temperature: A constant temperature of about 35°C.

Mobile phase A: Dissolve 0.4 g of sodium dihydrogenphosphate dihydrate in 1000 mL of water and adjust to a pH of 3.0 with diluted phosphoric acid (1 in 10).

Mobile phase B: Dissolve 0.4 g of sodium dihydrogenphosphate dihydrate and 106.4 g of lithium perchlorate in 1000 mL of water and adjust to a pH of 3.0 with diluted phosphoric acid (1 in 10).

Flowing of the mobile phase: Control the gradient by mixing the mobile phases A and B as directed in the following table.

| Time after injection of sample (min) | Mobile phase A (vol%) | Mobile phase B (vol%) |
|--------------------------------------|-----------------------|-----------------------|
| 0 – 3                                | 90                    | 10                    |
| 3 – 15                               | 90→0                  | 10→100                |

Flow rate: 0.2 mL per minute.

Time span of measurement: About 2 times as long as the retention time of heparin, beginning after the solvent peak.

*System suitability-*

Test for required detectability: Dissolve 10 mg of Heparin Sodium Reference Standard for physicochemical test in 0.40 mL of water, and use this solution as the Heparin Sodium standard stock solution. Separately, dissolve 0.10 mg of Over-sulfated Chondroitin Sulfate Reference Standard in 0.20 mL of water, and use this solution as the over-sulfated chondroitin sulfate standard solution. To 60  $\mu$  L of the Heparin Sodium standard stock solution add 3  $\mu$  L of the over-sulfated chondroitin sulfate standard solution and 12  $\mu$  L of water, and mix. When the procedure is run with 20  $\mu$  L of the mixture under the above operating conditions, it exhibits an over-sulfated chondroitin sulfate peak.

System performance: To 120  $\mu$  L of the Heparin Sodium standard stock solution add 30  $\mu$  L of the over-sulfated chondroitin sulfate standard solution, mix and use this solution as the solution for system suitability test. When the procedure is run with 20  $\mu$  L of the solution for system suitability test under the above operating conditions, heparin and over-sulfated chondroitin sulfate are eluted in this order with the resolution between these peaks being not less than 1.5.

System repeatability: When the test is repeated 6 times with 20  $\mu$  L of the solution for system suitability test under the above operating conditions, the relative standard deviation of the peak area of over-sulfated chondroitin sulfate is not more than 2.0%.

**9.01 Reference Standards**

***Change the following to read:***

Over-sulfated Chondroitin Sulfate Reference Standard: Identification, Purity

***Add the following:***

Heparin Sodium Reference Standard for physicochemical test: Identification, Purity

## 9.41 Reagents, Test Solutions

### *Add the following:*

**Aminobenzoate derivatization TS** Dissolve 280 mg of ethyl aminobenzoate in 600  $\mu$  L of methanol by heating at about 50°C, and add 170  $\mu$  L of acetic acid and 145  $\mu$  L of borane-pyridine complex.

**Lithium perchlorate**  $\text{LiClO}_4$  White, crystals or crystalline powder.

*Content:* not less than 98%. *Assay* - Accurately weigh about 0.2 g of lithium perchlorate, dissolve in 30 mL of water. Transfer the solution to a chromatographic column, prepared by pouring about 25 mL of strongly acidic ion-exchange resin (H type) for column chromatography into a chromatographic tube about 11 mm in inside diameter and about 300 mm in height (after adding 200 mL of 1 mol/L hydrochloride TS and flowing at a flow rate of 3-4 mL per minute, wash the chromatographic column with water until the color of the rinse water changes to yellowish red when adding methyl orange TS to the eluate), and flow at a flow rate of 3-4 mL per minute. Then, wash the column with about 30 mL of water at a flow rate of 3-4 mL per minute 5 times. Combine the rinse water and the eluate, and titrate <2.50> with 0.1 mol/L sodium hydroxide VS (indicator: 3 drops of bromothymol blue TS). Perform a blank determination, and make any necessary correction.

Each mL of 0.1 mol/L sodium hydroxide VS = 10.64 mg  $\text{LiClO}_4$

**D-Galactosamine hydrochloride**  $\text{C}_6\text{H}_{13}\text{NO}_5\cdot\text{HCl}$  White, powder. Melting point: about 180°C (decomposition).

*Optical rotation* <2.49>  $[\alpha]_{\text{D}}^{20}$ : +90 - +97° (1 g, water, 100 mL, 100 mm).

**D-Glucosamine hydrochloride**  $\text{C}_6\text{H}_{13}\text{NO}_5\cdot\text{HCl}$  White, crystals or crystalline powder.

*Content:* not less than 98%. *Assay* - Accurately weigh about 0.4 g of D-Glucosamine hydrochloride, dissolve in 50 mL of water, add 5 mL of diluted nitric acid (1 in 3) and titrate <2.50> with 0.1 mol/L silver nitrate VS (potentiometric titration).

Each mL of 0.1 mol/L silver nitrate VS = 21.56 mg  $\text{C}_6\text{H}_{13}\text{NO}_5\cdot\text{HCl}$

**Calcium acetate monohydrate**  $(\text{CH}_3\text{COO})_2\text{Ca}\cdot\text{H}_2\text{O}$  [K8364, Special class]

**Dermatan sulfate** Dermatan sulfate is mucopolysaccharide purified from the skin and small intestines of pigs by alkaline extraction, followed by digestion with protease and fractionation by alcohol. When cellulose acetate membrane electrophoresis of dermatan sulfate is performed and the membrane is stained in a toluidine blue O solution (1 in 200), a single band appears.

Operation conditions of cellulose acetate membrane electrophoresis -

Cellulose acetate membrane: 6 cm in width and 10 cm in length

Mobile phase: Dissolve 52.85 g of calcium acetate monohydrate in water to make 1000 mL.

Run time: 3 hours (1.0 mA/cm)

**Borane-pyridine complex** C<sub>5</sub>H<sub>8</sub>BN

*Content:* not less than 80%. *Assay* - Accurately weigh about 30 mg of borane-pyridine complex, dissolve in 40 mL of 0.05 mol/L iodine solution, add 10 mL of diluted sulfuric acid (1 in 6), and titrate <2.50> with 0.1 mol/L sodium thiosulfate VS (indicator: starch TS). Perform a blank determination, and make any necessary correction.

Each mL of 0.1 mol/L sodium thiosulfate VS = 1.549 mg C<sub>5</sub>H<sub>8</sub>BN

**D-Mannosamine hydrochloride** C<sub>6</sub>H<sub>13</sub>NO<sub>5</sub>·HCl White, powder. Melting point: about 168°C (decomposition).

*Optical rotation* <2.49>  $[\alpha]_{\text{D}}^{20}$ : -4.2 - -3.2° (0.4 g, water, 20 mL, 100 mm).