appropriate procedure for the removal of bacterial endotoxins. The addition of divalent cations may be necessary in order to fulfil the validation criteria.

ASSAY

Carry out the assay of heparin (*2.7.5*). The estimated potency is not less than 90 per cent and not more than 111 per cent of the stated potency. The confidence limits of the estimated potency (P = 0.95) are not less than 80 per cent and not more than 125 per cent of the stated potency.

STORAGE

In an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states:

- the number of International Units per milligram;
- the animal species of origin;
- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

08/2010:0333

HEPARIN SODIUM

Heparinum natricum

DEFINITION

Preparation containing the sodium salt of a sulfated glycosaminoglycan present in mammalian tissues. It is prepared either from the lungs of cattle or from the intestinal mucosae of pigs, cattle or sheep. On complete hydrolysis, it liberates D-glucosamine, D-glucuronic acid, L-iduronic acid, acetic acid and sulfuric acid. It has the property of delaying the clotting of blood.

Potency: minimum 180 IU/mg (dried substance).

PRODUCTION

The animals from which heparin sodium is derived must fulfil the requirements for the health of animals suitable for human consumption. All stages of production and sourcing are subjected to a suitable quality management system. The identity of the source species and the absence of material from the other species is verified by appropriate testing during production.

It is produced by methods of manufacturing designed to minimise or eliminate substances lowering blood pressure.

CHARACTERS

Appearance: white or almost white, hygroscopic powder.

Solubility: freely soluble in water.

IDENTIFICATION

A. It delays the clotting of recalcified citrated sheep plasma (see Assay).

B. Nuclear magnetic resonance spectrometry (*2.2.33*).

Solution A. A solution in *deuterium oxide R* containing 20 µg/mL of *deuterated sodium trimethylsilylpropionate R* and if the signal at 5.22 ppm is smaller than 80 per cent of the signal at 5.44 ppm, 12 µg/mL of *sodium edetate R*.

Preparation: dissolve 20 mg of the substance to be examined in 0.7 mL of solution A.

Comparison: dissolve 20 mg of *heparin sodium for NMR identification CRS* in 0.7 mL of solution A.

If stored, the sodium edetate and deuterated sodium trimethylsilylpropionate solutions must be kept in high-density, natural polyethylene bottles.

Apparatus: spectrometer operating at minimum 300 MHz.

Acquisition of ^{1}H-NMR spectra:

- *number of transients*: minimum 16; it is adjusted until the signal-to-noise ratio is at least 1000:1 for the heparin methyl signal at 2.04 ppm;
- *temperature*: about 25 °C; test sample and reference spectra have to be obtained at the same temperature;
- *acquisition time*: minimum 2 s;
- *repetition time* (acquisition time plus delay): minimum 4 s;
- *spectral width*: 10-12 ppm, centred at around 4.5 ppm;
- *pulse width*: to give a flip angle between 30° and 90°.

Processing:

- *exponential line-broadening window function*: 0.3 Hz;
- Fourier transformation;
- trimethylsilylpropionate reference signal set at 0.00 ppm.

Results:

- the large heparin sodium signals must be present: 2.04 ppm, 3.27 ppm (doublet), 4.34 ppm, 5.22 ppm and 5.42 ppm, all within ± 0.03 ppm;
- the ^{1}H-NMR spectrum obtained with the test sample and that obtained with *heparin sodium for NMR identification CRS* are compared qualitatively after the 2 spectra have been normalised so as to have a similar intensity; dermatan sulfate with a methyl signal at 2.08 ± 0.02 ppm may be observed; no unidentified signals larger than 4 per cent compared to the height of the heparin signal at 5.42 ppm are present in the ranges 0.10-2.00 ppm, 2.10-3.10 ppm and 5.70-8.00 ppm; signals from the solvent or process-related substances may be present and have to be identified to be accepted; variations in the intensity of some signal regions of the spectrum of heparin may occur: the intensity-variable regions are between 3.35 ppm and 4.55 ppm, where the signal pattern is approximately kept but intensity varies.

C. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

Injection: test solution (a) and reference solution (c).

Relative retention with reference to heparin (retention time = about 26 min): dermatan sulfate and chondroitin sulfate = about 0.9; over-sulfated chondroitin sulfate = about 1.3.

System suitability: reference solution (c):

- *peak-to-valley ratio*: minimum 1.3, where H_p = height above the baseline of the peak due to dermatan sulfate + chondroitin sulfate and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to heparin.

Results: the principal peak in the chromatogram obtained with test solution (a) is similar in retention time and shape to the principal peak in the chromatogram obtained with reference solution (c).

D. It complies with the test for sodium (see Tests).

TESTS

Appearance of solution. The solution is clear (*2.2.1*) and not more intensely coloured than intensity 5 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

Dissolve a quantity equivalent to 50 000 IU in *water R* and dilute to 10 mL with the same solvent.

pH (*2.2.3*): 5.5 to 8.0.

Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

Nucleotidic impurities. Dissolve 40 mg in 10 mL of *water R*. The absorbance (*2.2.25*) measured at 260 nm is not greater than 0.15.

Protein: maximum 0.5 per cent (dried substance).

Solution A. Mix 2 volumes of a 10 g/L solution of *sodium hydroxide R* and 2 volumes of a 50 g/L solution of *sodium carbonate R* and dilute to 5 volumes with *water R*.

Solution B. Mix 2 volumes of a 12.5 g/L solution of *copper sulfate R* and 2 volumes of a 29.8 g/L solution of *sodium tartrate R* and dilute to 5 volumes with *water R*.

Solution C. Mix 1 volume of solution B and 50 volumes of solution A.

Solution D. Dilute a phosphomolybdotungstic reagent 2- to 4-fold in *water R*. Suitable dilutions produce solutions of pH 10.25 ± 0.25 after addition of solutions C and D to the test and reference solutions.

Test solution. Dissolve the substance to be examined in *water R* to obtain a concentration of 5 mg/mL.

Reference solutions. Dissolve *bovine albumin R* in *water R* to obtain a concentration of 100 mg/mL. Prepare dilutions of the solution in *water R* as prescribed in general chapter *2.5.33, method 2*.

Blank: *water R*.

Procedure. To 1 mL of each reference solution, of the test solution and of the blank, add 5 mL of solution C. Allow to stand for 10 min. Add 0.5 mL of solution D, mix and allow to stand at room temperature for 30 min. Determine the absorbances (*2.2.25*) of the solutions at 750 nm, using the solution prepared from the blank as compensation liquid.

Calculations. As prescribed in general chapter *2.5.33, method 2*.

Related substances. Liquid chromatography (*2.2.29*).
Reference solutions are stable at room temperature for 24 h.

Test solution (a). Dissolve an accurately weighed quantity of about 50 mg of the substance to be examined in 5.0 mL of *water for chromatography R*. Mix using a vortex mixer until dissolution is complete.

Test solution (b). Dissolve an accurately weighed quantity of about 0.1 g of the substance to be examined in 1.0 mL of *water for chromatography R*. Mix using a vortex mixer until dissolution is complete. Mix 500 µL of the solution and 250 µL of *1 M hydrochloric acid*, then add 50 µL of a 250 mg/mL solution of *sodium nitrite R*. Mix gently and allow to stand at room temperature for 40 min before adding 200 µL of *1 M sodium hydroxide* to stop the reaction.

Reference solution (a). Dissolve 250 mg of *heparin for physico-chemical analysis CRS* in *water for chromatography R* and dilute to 2.0 mL with the same solvent. Mix using a vortex mixer until dissolution is complete.

Reference solution (b). Add 1200 µL of reference solution (a) to 300 µL of *dermatan sulfate and over-sulfated chondroitin sulfate CRS*. Mix using a vortex mixer to homogenise.

Reference solution (c). Add 100 µL of reference solution (b) to 900 µL of *water for chromatography R*. Mix using a vortex mixer to homogenise.

Reference solution (d). Add 400 µL of reference solution (a) to 100 µL of *water for chromatography R* and mix using a vortex mixer. Add 250 µL of *1 M hydrochloric acid*, then add 50 µL of a 250 mg/mL solution of *sodium nitrite R*. Mix gently and allow to stand at room temperature for 40 min before adding 200 µL of *1 M sodium hydroxide* to stop the reaction.

Reference solution (e). To 500 µL of reference solution (b), add 250 µL of *1 M hydrochloric acid*, then add 50 µL of a 250 mg/mL solution of *sodium nitrite R*. Mix gently and allow to stand at room temperature for 40 min before adding 200 µL of *1 M sodium hydroxide* to stop the reaction.

Precolumn:
- *size*: *l* = 0.05 m, Ø = 2 mm;
- *stationary phase*: *anion exchange resin R* (13 µm).

Column:
- *size*: *l* = 0.25 m, Ø = 2 mm;
- *stationary phase*: *anion exchange resin R* (9 µm);
- *temperature*: 40 °C.

Mobile phase:
- *mobile phase A*: dissolve 0.40 g of *sodium dihydrogen phosphate R* in 1 L of *water for chromatography R* and adjust to pH 3.0 with *dilute phosphoric acid R*;
- *mobile phase B*: dissolve 0.40 g of *sodium dihydrogen phosphate R* in 1 L of *water for chromatography R*, add 140 g of *sodium perchlorate R* and adjust to pH 3.0 with *dilute phosphoric acid R*; filter and degas;

Time (min)	Mobile phase A (per cent *V/V*)	Mobile phase B (per cent *V/V*)
0 - 10	75	25
10 - 35	75 → 0	25 → 100
35 - 40	0	100

Flow rate: 0.22 mL/min.

Detection: spectrophotometer at 202 nm.

Equilibration: at least 15 min.

Injection: 20 µL of test solution (b) and reference solutions (d) and (e).

Relative retention with reference to heparin (retention time = about 26 min): dermatan sulfate and chondroitin sulfate = about 0.9; over-sulfated chondroitin sulfate = about 1.3.

System suitability:
- the chromatogram obtained with reference solution (d) shows no peak at the retention time of heparin;
- *resolution*: minimum 3.0 between the peaks due to dermatan sulfate + chondroitin sulfate and over-sulfated chondroitin sulfate in the chromatogram obtained with reference solution (e).

Limits:
- *sum of dermatan sulfate and chondroitin sulfate*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (e) (2.0 per cent);
- *any other impurity*: no peaks other than the peak due to dermatan sulfate + chondroitin sulfate are detected.

Nitrogen (*2.5.9*): 1.5 per cent to 2.5 per cent (dried substance), determined on 0.100 g.

Sodium: 9.5 per cent to 12.5 per cent (dried substance).

Atomic absorption spectrometry (*2.2.23, Method I*).

Test solution. Dissolve 50 mg of the substance to be examined in a 1.27 mg/mL solution of *caesium chloride R* in *0.1 M hydrochloric acid* and dilute to 100.0 mL with the same solvent.

Reference solutions. Prepare reference solutions containing 25 ppm, 50 ppm and 75 ppm of Na, using *sodium standard solution (200 ppm Na) R* diluted with a 1.27 mg/mL solution of *caesium chloride R* in *0.1 M hydrochloric acid*.

Source: sodium hollow-cathode lamp.

Wavelength: 330.3 nm.

Atomisation device: flame of suitable composition (for example 11 L of air and 2 L of acetylene per minute).

Heavy metals (*2.4.8*): maximum 30 ppm.

1.0 g complies with test F. Prepare the reference solution using 3.0 mL of *lead standard solution (10 ppm Pb) R*.

Loss on drying (*2.2.32*): maximum 8.0 per cent, determined on 1.000 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 670 Pa for 3 h.

Bacterial endotoxins (*2.6.14*): less than 0.01 IU per International Unit of heparin, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Carry out the assay of heparin (*2.7.5*). The estimated potency is not less than 90 per cent and not more than 111 per cent of the stated potency. The confidence limits of the estimated potency (P = 0.95) are not less than 80 per cent and not more than 125 per cent of the stated potency.

STORAGE

In an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states:

- the number of International Units per milligram;
- the animal species of origin;
- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

01/2008:0828

HEPARINS, LOW-MOLECULAR-MASS

Heparina massae molecularis minoris

DEFINITION

Salts of sulfated glucosaminoglycans having a mass-average relative molecular mass less than 8000 and for which at least 60 per cent of the total mass has a relative molecular mass less than 8000. Low-molecular-mass heparins display different chemical structures at the reducing, or the non-reducing end of the polysaccharide chains.

The potency is not less than 70 IU of anti-factor Xa activity per milligram, calculated with reference to the dried substance. The ratio of anti-factor Xa activity to anti-factor IIa activity, determined as described under Assay, is not less than 1.5.

PRODUCTION

Low-molecular-mass heparins are obtained by fractionation or depolymerisation of heparin of natural origin that complies with the monograph on *Heparin sodium (0333)* or *Heparin calcium (0332)*, whichever is appropriate, for parenteral administration, unless otherwise justified and authorised. For each type of low-molecular-mass heparin the batch-to-batch consistency is ensured by demonstrating, for example, that the mass-average relative molecular mass and the mass percentage within defined relative molecular-mass ranges lower than 8000 are not less than 75 per cent and not more than 125 per cent of the mean value stated as type specification. The same limits apply also to the ratio of anti-factor Xa activity to anti-factor IIa activity.

Nucleotide and protein impurities of the source material. Dissolve 40 mg of the source material before fractionation in 10 mL of *water R*. The absorbance (*2.2.25*) measured at 260 nm and 280 nm is not greater than 0.20 and 0.15, respectively.

CHARACTERS

Appearance: white or almost white powder, hygroscopic.

Solubility: freely soluble in water.

IDENTIFICATION

A. Nuclear magnetic resonance spectrometry (*2.2.33*).

Preparation: dissolve 0.200 g of the substance to be examined in a mixture of 0.2 mL of *deuterium oxide R* and 0.8 mL of *water R*.

Comparison: dissolve 0.200 g of the appropriate specific low-molecular-mass heparin reference standard in a mixture of 0.2 mL of *deuterium oxide R* and 0.8 mL of *water R*.

Operating conditions: use a pulsed (Fourier transform) spectrometer operating at 75 MHz for ^{13}C. Record the spectra at 40 °C, using cells 5 mm in diameter. Use *deuterated methanol R* as internal reference at δ = 50.0 ppm.

Results: the spectrum obtained is similar to the appropriate specific low-molecular-mass heparin reference standard.

B. The ratio of anti-factor Xa activity to anti-factor IIa activity, determined as described under Assay, is not less than 1.5.

C. Size-exclusion chromatography (*2.2.30*).

Test solution. Dissolve 20 mg of the substance to be examined in 2 mL of the mobile phase.

Reference solution. Dissolve 20 mg of *heparin low-molecular-mass for calibration CRS* in 2 mL of the mobile phase.

Column:

- *size*: l = 0.30 m, Ø = 7.5 mm;
- *stationary phase*: appropriate porous silica beads (5 µm) with a fractionation range for proteins of approximately 15 000 to 100 000;
- *number of theoretical plates*: minimum of 20 000 per metre.

Mobile phase: 28.4 g/L solution of *anhydrous sodium sulfate R* adjusted to pH 5.0 using *dilute sulfuric acid R*.

Flow rate: 0.5 mL/min.

Detection: differential refractometer.

Injection: 25 µL.

Calibration. For detection, use a differential refractometer (RI) detector connected in series to a ultraviolet spectrophotometer (UV) set at 234 nm such that the UV monitor is connected to the column outlet, and the RI detector to the UV-monitor outlet.

It is necessary to measure the time lapse between the 2 detectors accurately, so that their chromatograms can be aligned correctly. The retention times used in the calibration must be those from the RI detector.

The normalisation factor used to calculate the relative molecular mass from the RI/UV ratio is obtained as follows: calculate the total area under the UV_{234} (ΣUV_{234}) and the RI (ΣRI) curves by numerical integration over the range of interest (i.e. excluding salt and solvent peaks at the end of the chromatogram). Calculate the ratio r using the following expression:

$$\frac{\sum \mathrm{RI}}{\sum \mathrm{UV}_{234}}$$

Calculate the factor f using the following expression:

$$\frac{M_{na}}{r}$$

M_{na} = assigned number-average relative molecular mass of the *Heparin low-molecular-mass for calibration CRS* found in the leaflet supplied with the CRS.

Provided the UV_{234} and the RI responses are aligned, the relative molecular mass M at any point is calculated using the following expression:

$$f\frac{\mathrm{RI}}{\mathrm{UV}_{234}}$$

The resulting table of retention times and relative molecular masses may be used to derive a calibration for the chromatographic system by fitting a suitable mathematical relationship to the data. A polynomial of the 3rd degree is recommended. *It must be stressed that the extrapolation of this fitted calibration curve to higher molecular masses is not valid.*

Inject 25 µL of the test solution and record the chromatogram for a period of time, ensuring complete elution of sample and solvent peaks.