Reference: PA/PH/Exp. 6/T (12) 38 ANP

NOTE ON THE MONOGRAPH

***Definition**. It is proposed to restrict the scope to heparin material of porcine origin since some of the latest requirements do not apply to materials of other origins. In addition, an enquiry among national authorities has shown that heparin medicinal products currently on the European market are all of porcine origin.*

Further to the replacement of the clotting assay by 2 chromogenic assays for anti-factor IIa activity and anti-factor Xa activity in general chapter 2.7.5. Assay of heparin, it is suggested that potency be measured by the assay of anti-factor IIa activity.

A requirement for the ratio of anti-factor Xa activity to anti-factor IIa activity has furthermore been introduced; a ratio of 1 is typical of unfractionated heparin.

***Production**. A statement was introduced during the last revision, which requires testing for identity of the source species and the absence of material from other likely cross-contaminant species. Further indications have been added that reflect current widely spread practices.*

XXXX:0332

HEPARIN CALCIUM

Heparinum calcicum

DEFINITION

Preparation containing the calcium 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), determined by the assay of anti-factor IIa activity as described under Assay; the ratio of anti-factor Xa activity to anti-factor IIa activity, determined as described under Assay, is 0.9-1.1.

PRODUCTION

The animals from which heparin calcium 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~~ likely contaminant species is verified by appropriate testing during production. The method used to confirm identity of the source species, and the point of application in the process, have been validated and shown to be capable of identifying the presence of heparin of other species at the level of 0.1 per cent (*m/m* heparin). Species verification by methods based on polymerase chain reaction (PCR) amplification of species-specific DNA sequences has been widely shown to be appropriate. If such a method has been chosen, it is also used to test for porcine DNA and to determine that it is present at a consistent level, in line with the manufacturing process used.

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)~~ complies with the requirements described under Assay.

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

Preparation: dissolve 20 mg of the substance to be examined in 0.7 mL of a 20 µg/mL solution of *deuterated sodium trimethylsilylpropionate R* in *deuterium oxide R*.

Comparison: dissolve 20 mg of *heparin calcium for NMR identification CRS* in 0.7 mL of a 20 µg/mL solution of *deuterated sodium trimethylsilylpropionate R* in *deuterium oxide R*.

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 calcium signals must be present: 2.05 ppm, 3.29 ppm (doublet), 4.37 ppm, 5.35 ppm and 5.43 ppm, all within ± 0.03 ppm;

— the ^{1}H-NMR spectrum obtained with the test sample and that obtained with *heparin calcium 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 5.43 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.

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

The following chromatogram is shown for information but will not be published in the European Pharmacopoeia.

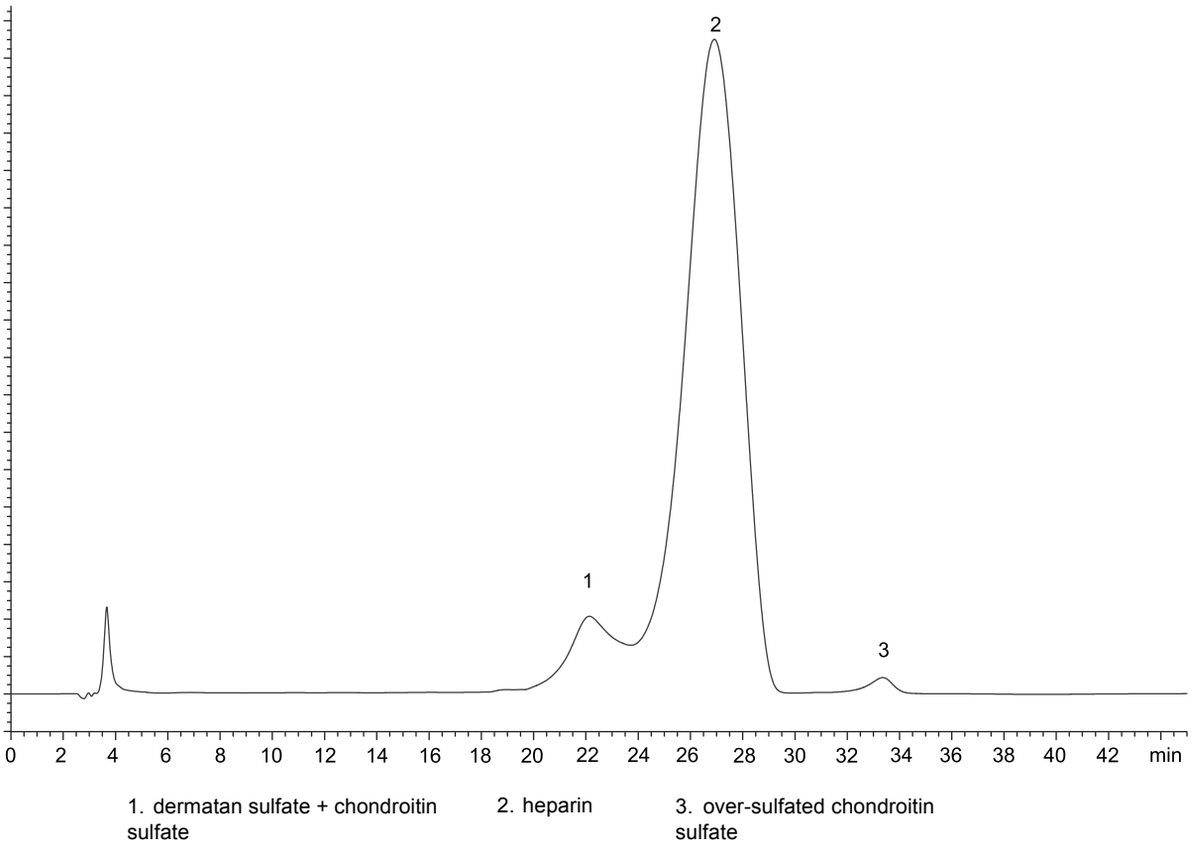


Figure 0332.-1. – *Chromatogram for identification test C of heparin calcium: reference solution (c) (chromatogram obtained after subtraction of the blank)*

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 gives the reactions of calcium (*2.3.1*).

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(1) 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 R1* 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*.

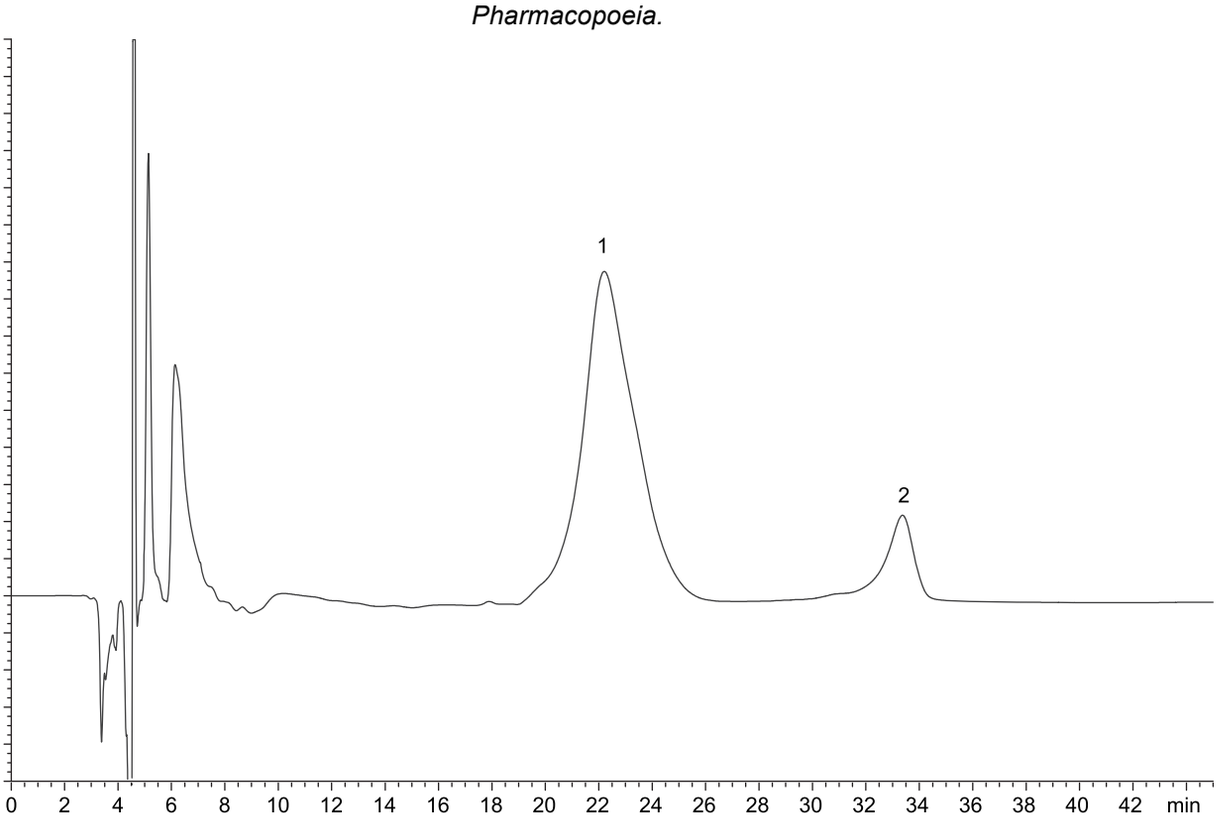
Blank: *water R*.

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. Filter through a membrane filter (nominal pore size 0.45 µm). 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.*

The following chromatogram is shown for information but will not be published in the European Pharmacopoeia.



1. dermatan sulfate + chondroitin sulfate 2. over-sulfated chondroitin sulfate

Figure 0332.-2. – *Chromatogram for the test for related substances of heparin calcium: reference solution (e) (chromatogram obtained after subtraction of the blank)*

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.

(1) Folin-Ciocalteu's phenol reagent from Merck (reference 1.09001.0500) is suitable.

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*[(2)]. 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)[(3)].

Column:

— *size*: *l* = 0.25 m, Ø = 2 mm;

— *stationary phase*: *anion exchange resin R* (9 µm)[(4)];

— *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*[(5)] 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;

(2) Sodium nitrite, analytical reagent grade from Fischer scientific (batch 0886083) is suitable.
(3) AG11-HC from Dionex (reference 052963) is suitable.
(4) AS11-HC from Dionex (reference 052961) is suitable.
(5) Normapur from VWR/Prolabo (reference 27988.232) is suitable.

— *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.

Calcium: 9.5 per cent to 11.5 per cent (dried substance), determined on 0.200 g by complexometric titration (*2.5.11*).

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. The addition of divalent cations may be necessary in order to fulfil the validation criteria.

ASSAY

Carry out the assay of anti-factor IIa activity 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.~~