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HEPARINS, LOW-MOLECULAR-MASS

Heparina massae molecularis minoris

DEFINITION

Salts of sulfated glycosaminoglycans 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 *Heparin sodium (0333)* or *Heparin calcium (0332)*, whichever is appropriate, 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.

CHARACTERS

Appearance: white or almost white, hygroscopic powder.

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:

- *field strength*: 75 MHz;
- *temperature*: 40 °C;
- *cell diameter*: 5 mm.

Processing:

- Fourier transformation;
- deuterated methanol reference signal set at 50.0 ppm.

Results: the ^{13}C NMR spectrum obtained is similar to that obtained with 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 with *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 an 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.

The mass-average relative molecular mass is defined by the following expression:

$$\frac{\sum (\mathrm{RI}_i M_i)}{\sum \mathrm{RI}_i}$$

RI_i = mass of substance eluting in the fraction *i*;

M_i = relative molecular mass corresponding to fraction *i*.

Any low-molecular-mass heparin covered by a specific monograph complies with the requirements for identification C prescribed in the corresponding monograph.

Where no specific monograph exists for the low-molecular-mass heparin to be examined, the mass-average relative molecular mass is not greater than 8000 and at least 60 per cent of the total mass has a relative molecular mass lower than 8000. In addition, the molecular mass parameters (mass-average molecular

mass and mass percentages of chains comprised between specified values) correspond to those of the manufacturer's reference preparation.

D. It gives reaction (a) of sodium or the reactions of calcium (as appropriate) (*2.3.1*).

TESTS

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.

Nitrogen (*2.5.9*): 1.5 per cent to 2.5 per cent (dried substance).

Calcium (*2.5.11*): 9.5 per cent to 11.5 per cent (dried substance), if prepared from heparin complying with the monograph *Heparin calcium (0332)*. Use 0.200 g.

Sodium: 10.5 per cent to 13.5 per cent (dried substance), if prepared from heparin complying with the monograph *Heparin sodium (0333)*.

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

Test solution. Dissolve 50 mg in *0.1 M hydrochloric acid* containing 1.27 mg of *caesium chloride R* per millilitre and dilute to 100.0 mL with the same solvent.

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

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).

Molar ratio of sulfate ions to carboxylate ions (*2.2.38*): minimum 1.8.

The sample of heparin used in this titration must be free from ionisable impurities, particularly salts.

Weigh 0.100 g of the substance to be examined taking the necessary measures to avoid the problems linked to hygroscopicity.

Take up into about 20 mL of double-glass-distilled *water R*. Cool to 4 °C and apply 2.0 mL of this solution to a pre-cooled column (approximately 10 × 1 cm), packed with a suitable *cation-exchange resin R*. Wash through with double-glass-distilled *water R* into the titration vessel up to a final volume of about 10-15 mL (*the titration vessel must be just large enough to hold the electrodes from the conductivity meter, a small stirrer bar and a fine flexible tube from the outlet of a 2 mL burette)*. Stir magnetically. When the conductivity reading is constant, note it and titrate with *0.05 M sodium hydroxide* added in approximately 50 µL portions. Record the burette level and the conductivity meter reading a few seconds after each addition until the end-point is reached.

For each measured figure, calculate the number of milliequivalents of sodium hydroxide added from the volume and the known concentration of the sodium hydroxide solution. Plot on a graph the figures for conductivity (as *y*-axis) against the figures of milliequivalent of sodium hydroxide (as *x*-axis). The graph will have 3 approximately linear sections: an initial steep downward slope, a middle slight rise and a final steep rise. Estimate the best straight lines through these 3 parts of the graph. At the points where the 1st and 2nd lines intersect, and where the 2nd and 3rd lines intersect, draw perpendiculars to the *x*-axis to estimate the milliequivalents of sodium hydroxide taken up by the sample at those points. The point where the 1st and 2nd lines intersect will give the number of milliequivalents of sodium hydroxide taken up by the sulfate groups, and the point where the 2nd and 3rd lines intersect will give the number of milliequivalents taken up by the sulfate and carboxylate groups together. The difference between the 2 will therefore give the number of milliequivalents taken up by the carboxylate groups.

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 10.0 per cent, determined on 1.000 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 0.67 kPa for 3 h.

Bacterial endotoxins (*2.6.14*): less than 0.01 IU per International Unit of anti-Xa activity, 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 to fulfil the validation criteria.

ASSAY

The anticoagulant activity of low-molecular-mass heparins is determined *in vitro* by 2 assays which determine its ability to accelerate the inhibition of factor Xa (anti-Xa assay) and thrombin, factor IIa (anti-IIa assay), by antithrombin III.

The International Units for anti-Xa and anti-IIa activity are the activities contained in a stated amount of the International Standard for low-molecular-mass heparin.

Heparin low-molecular-mass for assay BRP, calibrated in International Units by comparison with the International Standard using the 2 assays given below, is used as reference preparation.

ANTI-FACTOR Xa ACTIVITY

Reference and test solutions

Prepare 4 independent series of 4 dilutions each, of the substance to be examined and of the reference preparation of low-molecular-mass heparin in *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R*; the concentration range should be within 0.025 IU to 0.2 IU of anti-factor Xa activity per millilitre and the dilutions chosen should give a linear response when results are plotted as absorbance against log concentration.

Procedure

Label 16 tubes for the dilutions of the substance to be examined and 16 tubes for the dilutions of the reference preparation: T_1, T_2, T_3, T_4 for each of the 4 series of dilutions of the substance to be examined and S_1, S_2, S_3, S_4 for each of the 4 series of dilutions of the reference preparation. To each of the 32 tubes add 50 µL of *antithrombin III solution R1* and 50 µL of the appropriate dilution of the substance to be examined, or the reference preparation. After each addition, mix but do not allow bubbles to form. Treating the tubes in 2 subsequent series in the order S_1, S_2, S_3, S_4, T_1, T_2, T_3, T_4, T_1, T_2, T_3, T_4, S_1, S_2, S_3, S_4, allow to equilibrate at 37 °C (water-bath or heating block) for 1 min and add to each tube 100 µL of *bovine factor Xa solution R*. Incubate for exactly 1 min and add 250 µL of *chromogenic substrate R1*. Stop the reaction after exactly 4 min by adding 375 µL of *acetic acid R*. Transfer the mixtures to semi-micro cuvettes and measure the absorbance (*2.2.25*) at 405 nm. Determine the blank amidolytic activity at the beginning and at the end of the procedure in a similar manner, using *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R* instead of the reference and test solutions; the 2 blank values do not differ significantly. Calculate the regression of the absorbance on log concentrations of the solutions of the substance to be examined and of the reference preparation of low-molecular-mass heparins and calculate the potency of the substance to be examined in International Units of anti-factor Xa activity per millilitre using the usual statistical methods for parallel-line assays.

ANTI-FACTOR IIa ACTIVITY

Reference and test solutions

Prepare 4 independent series of 4 dilutions each, of the substance to be examined and of the reference preparation of low molecular-mass heparin in *tris(hydroxymethyl)aminomethane sodium chloride*

buffer solution pH 7.4 R; the concentration range should be within 0.015 IU to 0.075 IU of anti-factor IIa activity per millilitre and the dilutions chosen should give a linear response when results are plotted as absorbance against log concentration.

Procedure

Label 16 tubes for the dilutions of the substance to be examined and 16 tubes for the dilutions of the reference preparation: T_1, T_2, T_3, T_4 for each of the 4 series of dilutions of the substance to be examined and S_1, S_2, S_3, S_4 for each of the 4 series of dilutions of the reference preparation. To each of the 32 tubes add 50 µL of *antithrombin III solution R2* and 50 µL of the appropriate dilution of the substance to be examined or the reference preparation. After each addition, mix but do not allow bubbles to form. Treating the tubes in 2 subsequent series in the order S_1, S_2, S_3, S_4, T_1, T_2, T_3, T_4, T_1, T_2, T_3, T_4, S_1, S_2, S_3, S_4, allow to equilibrate at 37 °C (water-bath or heating block) for 1 min and add to each tube 100 µL of *human thrombin solution R*. Incubate for exactly 1 min and add 250 µL of *chromogenic substrate R2*. Stop the reaction after exactly 4 min by adding 375 µL of *acetic acid R*. Transfer the mixtures to semi-micro cuvettes and measure the absorbance (*2.2.25*) at 405 nm. Determine the blank amidolytic activity at the beginning and at the end of the procedure in a similar manner, using *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R* instead of the reference and test solutions; the 2 blank values do not differ significantly. Calculate the regression of the absorbance on log concentrations of the solutions of the substance to be examined and of the reference preparation of low-molecular-mass heparins, and calculate the potency of the substance to be examined in International Units of anti-factor IIa activity per millilitre using the usual statistical methods for parallel-line assays.

LABELLING

The label states:

- the number of International Units of anti-factor Xa activity per milligram;
- the number of International Units of anti-factor IIa activity per milligram;
- the mass-average molecular mass and the percentage of molecules within defined molecular mass ranges;
- where applicable, that the contents are the sodium salt;
- where applicable, that the contents are the calcium salt.

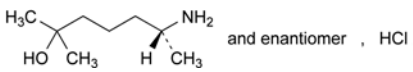
STORAGE

In an airtight tamper-proof container. If the product is sterile and free of bacterial endotoxins, store in a sterile and apyrogenic container.

01/2008:1980
corrected 6.0

HEPTAMINOL HYDROCHLORIDE

Heptaminoli hydrochloridum



$C_8H_{20}ClNO$ M_r 181.7
[543-15-7]

DEFINITION

(6*RS*)-6-Amino-2-methylheptan-2-ol hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water, soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: *B, D.*

Second identification: *A, C, D.*

A. To 1 mL of solution S (see Tests) add 4 mL of *water R* and 2 mL of a 200 g/L solution of *ammonium and cerium nitrate R* in *4 M nitric acid*. An orange-brown colour develops.

B. Infrared absorption spectrophotometry (*2.2.24*).

Comparison: *heptaminol hydrochloride CRS*.

C. Examine the chromatograms obtained in the test for related substances.

Detection: examine in daylight.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (b).

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

Appearance of solution. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution BY_6 (*2.2.2, Method II*).

Acidity or alkalinity. To 10 mL of solution S add 0.1 mL of *methyl red solution R* and 0.3 mL of *0.01 M hydrochloric acid*. The solution is red. Add 0.6 mL of *0.01 M sodium hydroxide*. The solution is yellow.

Related substances. Thin-layer chromatography (*2.2.27*).

Test solution (a). Dissolve 0.50 g of the substance to be examined in *methanol R* and dilute to 5.0 mL with the same solvent.

Test solution (b). Dilute 1.0 mL of test solution (a) to 10 mL with *methanol R*.

Reference solution (a). Dilute 3.0 mL of test solution (a) to 10.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 50.0 mL with *methanol R*.

Reference solution (b). Dissolve 0.10 g of *heptaminol hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

Reference solution (c). Dissolve 10.0 mg of *heptaminol impurity A CRS* in *methanol R* and dilute to 5.0 mL with the same solvent.

Reference solution (d). Dilute 1.0 mL of reference solution (c) to 10.0 mL with *methanol R*.

Reference solution (e). To 2.5 mL of reference solution (c) add 0.5 mL of test solution (b) and dilute to 5 mL with *methanol R*.

Plate: *TLC silica gel G plate R*.

Mobile phase: *concentrated ammonia R*, *dioxan R*, *2-propanol R* (10:50:50 *V/V/V*).

Application: 10 µL; apply test solutions (a) and (b) and reference solutions (a), (b), (d) and (e).

Development: over 2/3 of the plate.

Drying: in air.

Detection: expose the plate to iodine vapour for at least 15 h.

System suitability: the chromatogram obtained with reference solution (e) shows 2 clearly separated principal spots and the chromatogram obtained with reference solution (a) shows a single principal spot.

Limits: in the chromatogram obtained with test solution (a):

- *impurity A*: any spot corresponding to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (d) (0.2 per cent),