⟨209⟩ LOW MOLECULAR WEIGHT HEPARIN MOLECULAR WEIGHT DETERMINATIONS

This chapter provides procedures used to determine molecular weight (MW) distribution and weight-average molecular weight for low molecular weight heparins (LMWH).

INTRODUCTION

Low molecular weight heparins are prepared from Heparin Sodium, USP, by partial depolymerization. LMWHs are polydisperse, i.e., they are made up of polysaccharide chains with a range of molecular weights. The MW distribution is a defining characteristic for each LMWH product. The *USP* contains specific monographs for LMWH products, including limits on molecular weight distribution parameters.

Most techniques for determination of the MW distribution of LMWHs make use of gel permeation chromatography (GPC), sometimes referred to as size exclusion chromatography (SEC). In order to derive molecular weight distribution from a chromatogram, it is necessary to know the relationship between retention time and MW. This chapter describes a GPC method that is calibrated using the Low Molecular Weight Heparin Molecular Weight Calibrant Reference Standard (RS). This preparation is a single polydisperse Standard for which the distribution of molecular weights is described in the form of a Broad Standard Table (see the USP Certificate for USP Low Molecular Weight Heparin Molecular Weight Calibrant RS) that lists the log of the molecular weight (log MW) of a number of reference points and the matching percent fractions by weight of the calibrant above or below these reference points. The Broad Standard Table is used together with a chromatogram of the calibrant to fit a suitable function, in this case a third degree polynomial, to the relationship between log MW and retention time, thus generating a calibration curve for the chromatographic system. With this calibration curve, the analyst calculates the weight-average molecular weight and distribution parameters from the chromatogram of a sample of LMWH.

All the calculations described in this chapter may be performed using a spreadsheet program, but this method is not recommended as it is laborious and open to human error. Proprietary software capable of automating the calibration and molecular weight calculations can be obtained from a number of chromatography systems manufacturers.

PROCEDURE

• **Molecular Weight Measurements of Low Molecular Weight Heparins by Gel Permeation Chromatography**

The following procedure, with any necessary variations, is used where specified in the individual monographs.

Ammonium acetate stock solution: 1 M ammonium acetate in water

Sodium azide solution: 1% (w/v) sodium azide in water

Mobile phase: Transfer 100 mL of *Ammonium acetate stock solution* to a 1-L volumetric flask, add 20 mL of *Sodium azide solution*, and dilute with water to volume. Filter using a nylon membrane of 0.45-µm pore size prior to use.

Calibration solution: Add 2 mL of *Mobile phase* to a vial containing USP Low Molecular Weight Heparin Molecular Weight Calibrant RS. Filter using a nylon membrane of 0.45-µm pore size prior to use.

Sample solution: 5 mg/mL of low molecular weight heparin sample in *Mobile phase*. Filter using a nylon membrane of 0.45-µm pore size.

System suitability solution: 5 mg/mL of USP Dalteparin Sodium RS in *Mobile phase*. Filter using a nylon membrane of 0.45-µm pore size.

Chromatographic system

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

[Note—The temperature of the refractive index detector must be set at the same temperature as that of the *Column temperature*.]

Mode: LC

Detector: Refractive index

Columns

Analytical: 7.8-mm × 30-cm; 5-µm packing L59 in series with a 7.8-mm × 30-cm; 5-µm packing L59[1]

Guard: 6-mm × 4-cm; 7-µm packing L59

Column temperature: 30°

Flow rate: 0.5 mL/min

Column equilibration: 0.5 mL/min for at least 2 h

Injection volume: 20 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: There is baseline resolution between the last peak of the USP Low Molecular Weight Heparin Molecular Weight Calibrant RS and the salt peak, or negative exchange peaks.

Calibration curve: The coefficient of determination of the calibration curve fitted to the Broad Standard Table values must be NLT 0.990, using a third-order polynomial equation.

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 to the nearest 50 Da. The chromatographic system is suitable if the M_w of the USP Dalteparin Sodium RS is within 150 Da of the labeled value as stated in the USP Certificate for USP Dalteparin Sodium RS.

[1] The method was validated using a guard column TSK SWXL 6-mm × 4-mm; 7-µm in series with two analytical columns: TSK G3000 SWXL 7.8-mm × 30-cm; 5-µm in series with a TSK G2000 SWXL 7.8-mm × 30-cm; 5-µm.

Analysis

Samples: Inject 20 µL of the *Calibration solution* (single injection), *System suitability solution* (duplicate injections), and *Sample solution* (duplicate injections), 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 low molecular weight heparin will give a broad peak between about 25 and 45 min, followed by a later eluting narrow salt peak, as illustrated in the USP Certificate for USP Low Molecular Weight Heparin Molecular Weight Calibrant RS.]

Calculations: Calculate the total area under the low molecular weight heparin peak from the *Calibration solution* 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 Low Molecular Weight Heparin 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 Broad Standard Table, and assign the MW in this 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 $\log MW = a + (b \times RT) + [c \times (RT)^2] + [d \times (RT)^3]$.

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:

$$M_w = \Sigma(RI_i \times M_i)/\Sigma RI_i$$

RI_i = detector response at each point

M_i = MW at each point

Round the mean value of M_w to the nearest 50 Da. Using the same GPC software, determine for each of the duplicate *Sample solution* chromatograms the percentage of product with molecular weight as indicated in the product monograph.

Acceptance criteria: As indicated in the product monograph

ADDITIONAL REQUIREMENTS

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Dalteparin Sodium RS

USP Low Molecular Weight Heparin Molecular Weight Calibrant RS