Molecular Weight Determinations of Heparin and Low-Molecular-Weight Heparins Using BioCore SEC-HP Columns

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Applicable field: Heparin analysis

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Abstract

In this article, we describe the method for molecular weight determinations of unfractionated heparin and low molecular weight heparins (LMWHs) using the tailored BioCore SEC-HP columns. The results obtained show better resolution and longer durability than SEC columns widely used in the last decade.

Introduction

Molecular weight (MW) and its distribution are important characteristics of heparin and low molecular weight heparins (LMWHs), a family of anticoagulants with polydisperse, heterogeneous polysaccharide chains.

Current compendial methods for heparin molecular weight determinations involve separation on size-exclusion chromatography (SEC) columns, followed by detection with refractive index detectors (RID). While some SEC columns have been widely used in heparin industry before, they are subject to short life time.

BioCore SEC-HP columns, a series of SEC columns tailored for heparin MW determinations, are used for MW determinations of heparin and LMWHs. The results show good performance which meets the system suitability required by the latest methods specified in pharmacopoeias. Besides, these columns show very long life time, without performance loss even after 100 injections, which can reduce cost per injection for heparin industry.

Experiment

Equipment

HPLC system: Shimadzu LC-2030C

Detector: RID-20A refractive index detector

Columns

Table 1. SEC columns

Cat. #	Description	Usage
B213-050030-07830S-HP	BioCore SEC-300HP Analytical Column, 300 Å, 5 µm, 7.8×300 mm	Unfractionated heparin
B213-050050-07830S-HP	BioCore SEC-500HP Analytical Column, 500 Å, 5 µm, 7.8×300 mm	
B213-050015-07830S-HP	BioCore SEC-150HP Analytical Column, 150 Å, 5 µm, 7.8×300 mm	Low-molecular-weight heparins
B213-050015-04601S-HP	BioCore SEC-150 Guard Column, 5 µm, 4.6×10 mm	

Reagents

Reference standard: Heparin low-molecular-mass for calibration CRS (European Directorate for the Quality of Medicines, Cat. # H019000), Heparin molecular weight calibrant (National Institutes for Food and Drug Control, Cat. # 140819), Heparin reference standard (National Institutes for Food and Drug Control, Cat. # 140818)

Sample: Low-molecular-weight heparin (provided by a customer)

Chromatographic conditions

Table 2. Chromatographic conditions

Parameter	Value
Mobile phase	100 mM ammonium acetate
Elution mode	Isocratic
Flowrate	0.5 mL/min
Injection volume	25 µL (5 µL)
Column temperature	30 ℃ (for system suitability test of UFH) 35 ℃ (for system suitability test of LWMHs and durability test)
Detector temperature	30 ℃ (for system suitability test of UFH) 35 ℃ (for system suitability test of LWMHs and durability test)
Sample concentration	5 mg/mL in mobile phase (for system suitability test of UFH) 10 mg/mL in mobile phase (for system suitability test of LMWHs) 10 mg/mL in water (for durability test)

Procedure

The system suitability test of unfractionated heparin follows the method specified in Chinese Pharmacopoeia[1].

The system suitability test of low-molecular-weight heparins and the durability test follow the method specified in European Pharmacopoeia[2].

Results and discussion

System suitability test on heparin

Inject the heparin molecular weight calibrant solution into the HPLC system, and record the chromatogram, as shown in Figure 1.

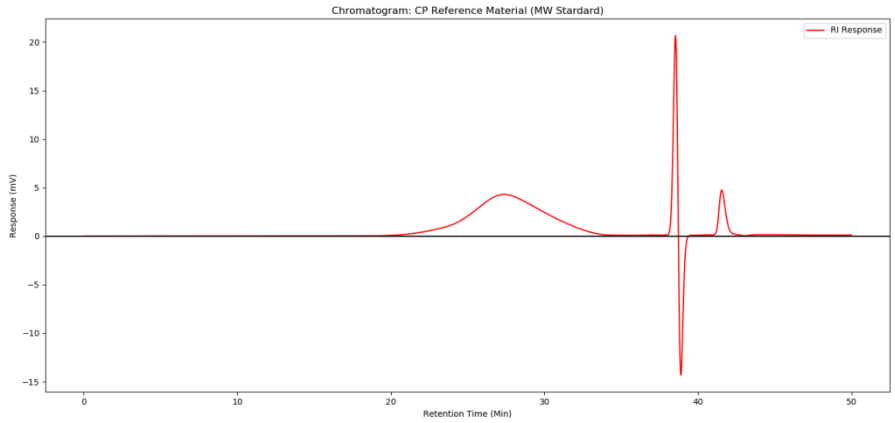

Figure 1. Chromatogram of heparin molecular weight calibrant, analytical columns: BioCore SEC-300HP + SEC-500HP, guard column: none, injection volume: 25 µL

By looking up the broad table of heparin molecular weight calibrant, we can identify each component peak corresponding to a specific molecular weight, as in Table 3.

Table 3. Peaks of heparin molecular weight calibrant

Peak #	RT (min)	Response (mV)
1	21.86667	40000
2	22.53333	36000
3	23.21667	32000
4	24.00000	28000
5	24.45833	26000
6	24.91667	24000
7	25.41667	22000
8	25.95833	20000
9	26.55000	18000
10	27.20000	16000
11	27.93333	14000
12	28.75833	12000
13	29.71667	10000
14	30.84167	8000
15	32.25000	6000

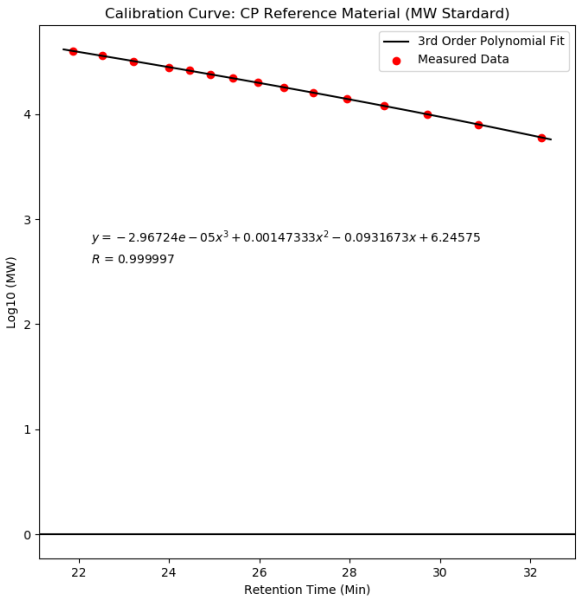

Figure 2. Calibration curve of heparin molecular weight calibrant

The calibration curve relating molecular weight to retention time can be obtained using a 3rd order polynomial fit, as shown in Figure 2.

The calibration formula is $y = -2.96724 \times 10^{-5}x^3 + 0.00147333x^2 - 0.093173x + 6.24575$, with a correlation coefficient $R = 0.999997$.

Inject the heparin reference standard solution into the HPLC system, and record the chromatogram, as shown in Figure 3.

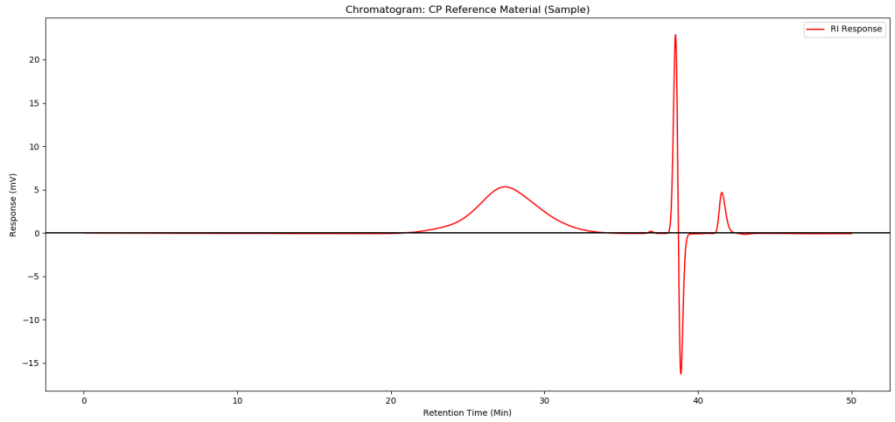

Figure 3. Chromatogram of heparin reference standard, analytical columns: BioCore SEC-300HP + SEC-500HP, guard column: none, injection volume: 25 µL

Table 4. Peaks of heparin reference standard

Peak #	RT (min)	Molecular weight	Response (mV)
1	24.22500	40000	1.177
2	24.46667	36000	1.383
3	24.75833	32000	1.712
4	25.14167	28000	2.225
5	25.39167	26000	2.608
6	25.66667	24000	3.071
7	26.00833	22000	3.663
8	26.39167	20000	4.339
9	26.83333	18000	4.974
10	27.35000	16000	5.312
11	27.93333	14000	5.103
12	28.59167	12000	4.379
13	29.35833	10000	3.333
14	30.26667	8000	2.127
15	31.43333	6000	0.992

The weight-averaged molecular weight ($\bar{M}_w$) can be calculated using the following formula

$$\bar{M}_w = \frac{\sum RI_i \times M_i}{\sum RI_i} = 16{,}611$$

Where RI_i is the RI response of the i[th] peak, and M_i is the corresponding molecular weight calculated using the calibration formula.

The labelled weight-average molecular weight of heparin reference standard is 16,200. The difference between the labelled value and the calculated value is 411, which is within the range required by Chinese Pharmacopoeia, i.e. ± 500.

To conclude, the correlation coefficient and the $\bar{M}_w$ meet the system suitability requirements specified by the method of molecular weight determination in Chinese Pharmacopoeia.

System suitability test on low-molecular-weight heparins

SEC chromatograms of low-molecular-weight heparins, such as enoxaparin sodium, show fine structure of sub-peaks. The resolution between sub-peaks is an indicator of column performance.

Inject the heparin low-molecular-mass for calibration CRS solution into the HPLC system. The chromatogram of calibration CRS solution is shown in Figure 4.

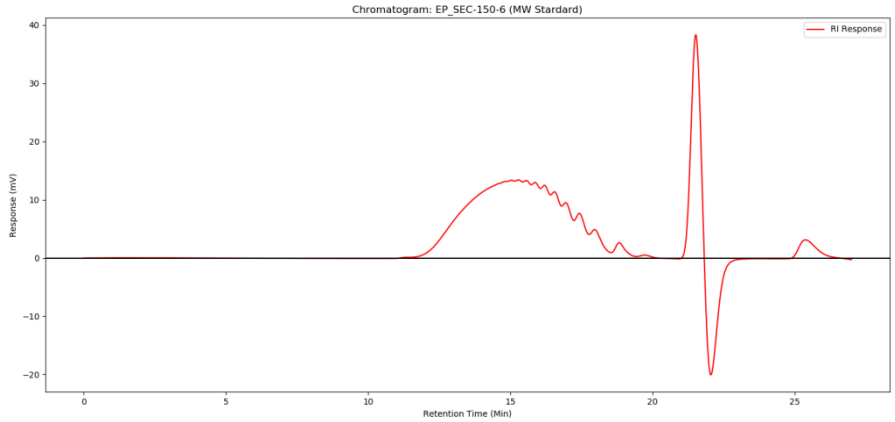

Figure 4. Chromatogram of heparin low-molecular-mass for calibration CRS, analytical columns: BioCore SEC-150HP, guard column: BioCore SEC-150, injection volume: 25 µL

Good resolution between sub-peaks of lower MW components can be observed, as shown in Figure 5. In particular, baseline separation is almost achieved between the two sub-peaks corresponding to MW 1,200 and 600.

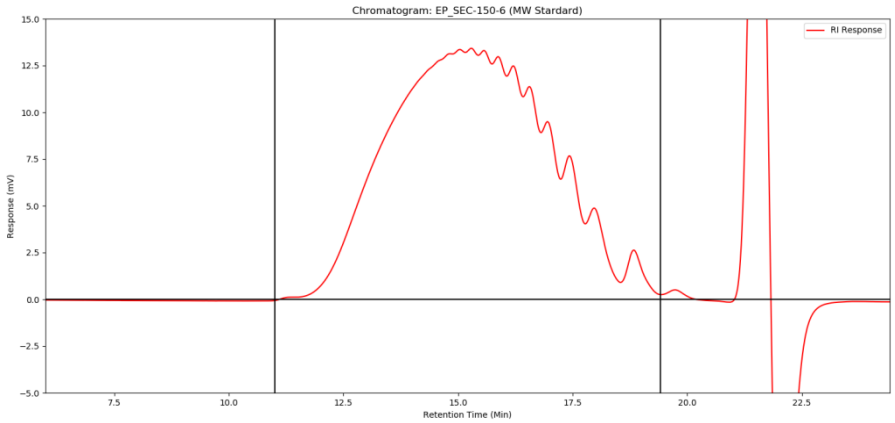

Figure 5. Enlarged clip of Figure 4, showing sub-peaks

By looking up the broad standard table in the leaflet of heparin low-molecular-mass for calibration CRS, we can identify all peaks, as shown in Table 5.

Table 5. Peak identification corresponding to Figure 4

Peak #	RT (Min)	MW
1	12.65000	18000
2	12.87500	15600
3	13.14167	13600
4	13.40833	12000
5	13.93333	9600
6	14.29167	8400
7	14.70000	7200
8	14.93333	6600
9	15.17500	6000
10	15.44167	5400
11	15.71667	4800
12	16.01667	4200
13	16.33333	3600
14	16.66667	3000
15	17.05000	2400
16	17.48333	1800
17	18.06667	1200
18	19.05833	600

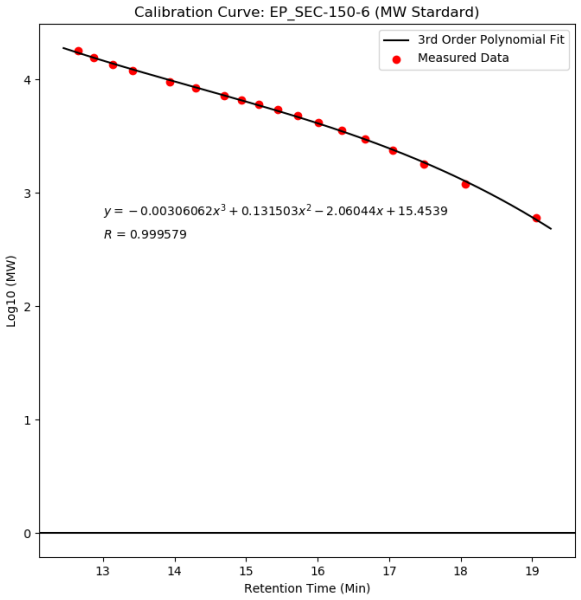

Figure 6. Calibration of heparin low-molecular-mass for calibration CRS

The calibration curve relating molecular weight to retention time can be obtained using a 3rd order polynomial fit, as shown in Figure 6.

The calibration formula is $y = -0.00306062x^3 + 0.131503x^2 - 2.06044x + 15.4539$, with a correlation coefficient $R = 0.999579$.

Column durability

Heparin samples are very complex and cause great challenge for column durability. To test the durability of BioCore SEC-HP columns, we overlay chromatograms after multiple injections of the sample solution.

As we can see in Figure 7, BioCore SEC-HP column brings minimal variation between 30 injections of heparin samples, even without using the guard column.

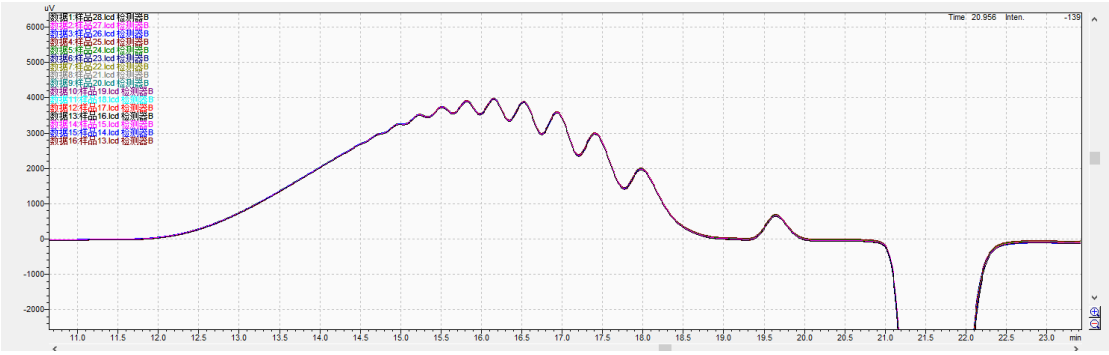

Figure 7. Overlay of first 16 consecutive chromatograms of 30 injections of sample solution, injection volume 5 µL, obtained with BioCore SEC-150HP, no guard column

By incorporating the guard column, chromatograms obtained show almost no variation between 10 injections, even with the injection volume increased from 5 µL to 25 µL, as shown in Figure 8 and Figure 9.

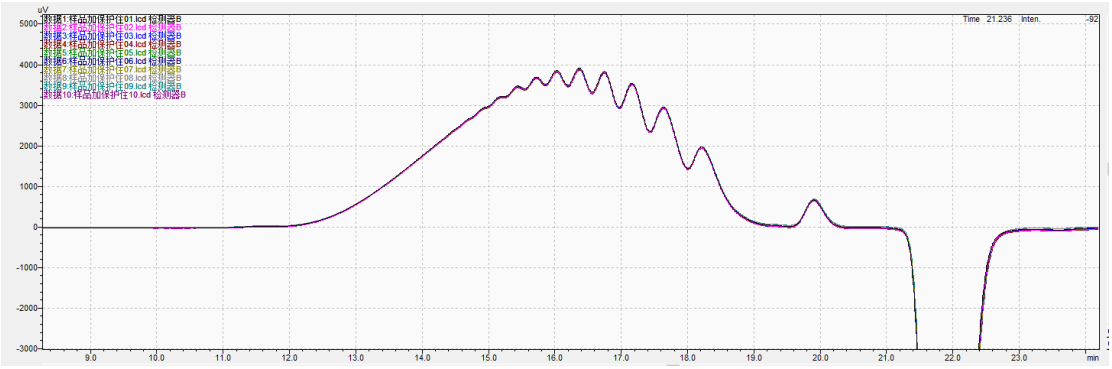

Figure 8. Overlay of consecutive chromatograms of 10 injections of sample solution, injection volume 5 µL, obtained with BioCore SEC-150HP with BioCore SEC-150 guard column

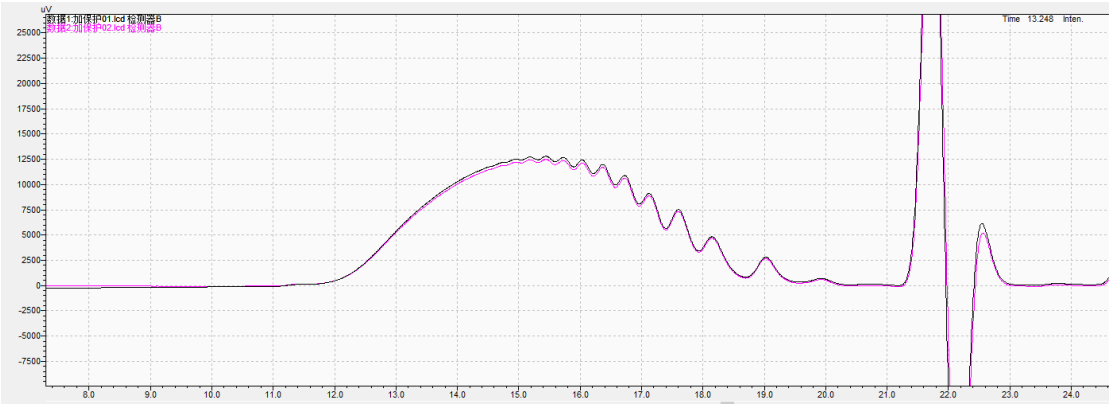

Figure 9. Overlay of consecutive chromatograms of 10 injections of sample solution, injection volume 25 µL, obtained with BioCore SEC-150HP with BioCore SEC-150 guard column

Conclusion

As we can see in the last section, BioCore SEC-HP columns provide better resolution and longer durability for heparin MW determinations.

The superior performance of BioCore SEC-HP columns come in a large part from their monodisperse, low-absorption packing material.

To inspect the difference of packing materials, we take SEM of them.

As shown in Figure 10, the particle size distribution of BioCore SEC-HP is very narrow, with a mean size of 5 µm and CV<5%.

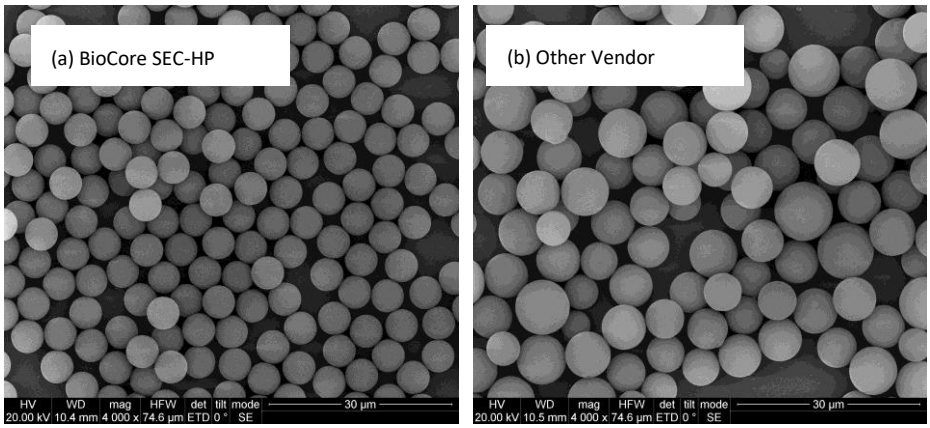

Figure 10. SEMs of particle size distributions of two packing materials

According to feedbacks from local heparin manufacturers, chromatographic profiles differ a lot after 10-20 injections if using competitors' columns. While BioCore SEC-HP columns demonstrate longer life time, with negligible performance lose after 50-60 injections of heparin samples.

The long life time of BioCore SEC-HP columns can be attributed to the superb mechanical strength of their packing material. To confirm that, we pack two columns under 6,000 psi using packing materials from BioCore SEC-HP columns and the other vendor's columns, followed by SEM of the packing materials. As shown in Fig. 9, no particle breakage occurs for BioCore SEC-HP packing material (a), while there is obvious breakage for the other (b).

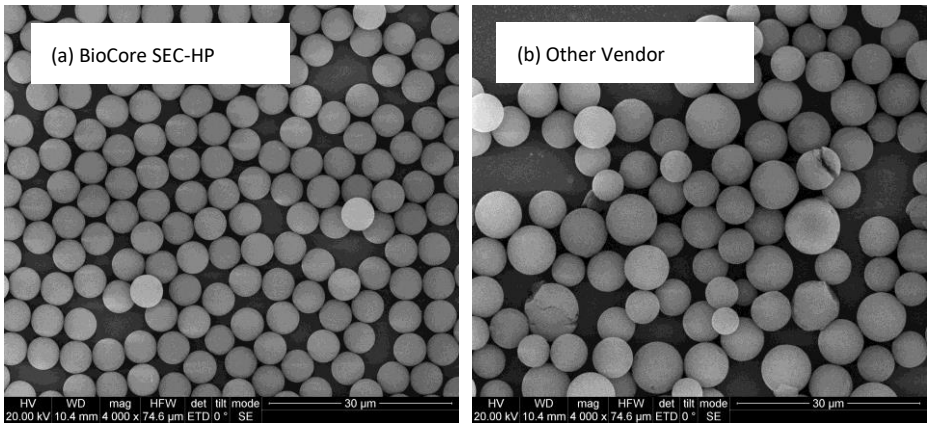

Figure 11. SEMs of packing materials after 6,000 psi packing

A conclusion can be drew that BioCore SEC-HP columns are very stable under high pressure and have great reproducibility in analyzing samples as complex as heparins. This durability performance gives a longer life time and can reduce cost.

Reference

1. Chinese Pharmacopoeia Commission. Chinese Pharmacopoeia (2015 Edition). Beijing: China Medical Science and Technology Press; 2015.6. p. Heparin Sodium; pp.517-518.

2. Council of Europe. European Pharmacopoeia. In: Supplement 9.8. Strasbourg: Council of Europe; 2019. p. Heparins, Low-Molecular-Mass; pp.6923-6924.