Application Note

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Analysis of Covalent High Molecular Weight Insulin

Improvements in speed and resolution with high performance size exclusion chromatography

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Abstract

The analysis of insulin was done with an HPLC size exclusion chromatography (SEC) method using an Agilent AdvanceBio SEC 120 Å pore size, sub-2 µm hydrophilic polymer coated silica packing material. The results of the analysis were compared to traditional methods and competitor columns for performance and efficiency. Resolution of insulin and high molecular weight (HMW) proteins were significantly improved over results from traditional methods with the AdvanceBio column. Chromatographic run times were shorter, and high-throughput insulin sample analysis became a reality.

Introduction

Insulin is a small polypeptide hormone that controls blood glucose homeostasis and is widely used in diabetes treatment. Genetic engineering techniques have enabled biopharma companies to develop diverse, long-acting insulin analogs. It has long been known that, when subjected to acidic conditions and high temperature, insulin monomers form amyloid-like fibrils.[1] For the insulin analogue manufacturer, this is especially problematic, since insulin analogs can have a higher propensity for aggregation than native insulin.[2] One of the critical quality control attributes for injectable insulin is the control of insulin fibrillation, commonly known as high molecular weight (HMW) proteins. The current US (USP) and European (EP) pharmacopoeia monograph methods for HMW aggregates determination are based on HPLC size exclusion chromatography (SEC).[3,4] According to the EP method, the use of a "hydrophilic silica gel for chromatography R (5 to 10 µm) with a pore size of 12 to 12.5 nm, of a grade suitable for the separation of insulin monomer from covalent dimer and polymers" with a length of 30 cm and a minimum internal diameter of 7.5 mm are prescribed. However, the method requires a lengthy 35 minute run time that is cost prohibitive for any laboratory performing high throughput sample analysis. The application presented here describes a SEC method developed using an Agilent AdvanceBio SEC 120 Å pore size, sub-2 µm hydrophilic polymer coated silica packing material. Some of the advantages of this method include faster run times and higher resolving separations of insulin and covalent insulin HMW compared to traditional pharmacopoeia methods.

Experimental

Equipment and materials

All chemicals and reagents were HPLC grade or higher and were obtained from Sigma-Aldrich (now Merck) or VWR Scientific. Water was purified using a Milli-Q A10 (Millipore).

Instrumentation

Agilent 1260 Infinity II Bio-inert LC instrument comprising:

- Agilent 1260 Infinity II Bio-inert Pump (G5654A)
- Agilent 1260 Infinity II Bio-inert Multisampler (G5668A) with sample cooler (option #100)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A) with bio-inert heat exchanger (option #019)
- Agilent 1260 Infinity II Variable Wavelength Detector (G7114A)

Method conditions

HPLC Conditions	
Column	Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 300 mm (p/n PL1580-5250)
Mobile Phase	Arginine (1.0 g/L)/acetic acid/acetonitrile (65/15/20 v/v/v)
Flow Rate	0.30 mL/min
Column Temperature	25 °C
Injection Volume	2 µL
Samples	Human insulin control, Heat-stressed insulin (60 °C for six hours)
Total Run Time	15 minutes

Software

OpenLab 2.2 CDS

Sample preparation

The control human insulin (Sigma, I2643) and heat-stressed insulin samples were prepared as per the Ph. Eur.

Sample was reconstituted and diluted to 4.0 mg/mL in 0.01 N hydrochloric acid solution, then further diluted to 2 mg/mL as the final concentration.

Results and discussion

The focus of this application is a performance evaluation of the AdvanceBio SEC 120 Å, 1.9 µm, 4.6 × 300 mm column under the conditions provided by the USP and EP monographs for the analysis of HMW species in insulin samples. The acidic mobile phase prescribed by both of these pharmacopeial methods is composed of 0.65 g/L L-arginine, 15% acetic acid, and 20% (v/v) acetonitrile. This mobile phase provides an assessment of the levels of covalent HMW present in these preparations while disrupting noncovalent insulin self-association and column interactions. The featured AdvanceBio SEC 1.9 µm columns are designed with hydrophilic polymer coating to minimize undesired secondary effects between analyte and surface particle chemistry. The columns provide superior resolution and accurate HMW protein quantification. Figure 1 shows the chromatogram for system suitability with Ph. Eur. insulin control standard. Monomer and HWM species for the 1.9 µm column have a resolution of 4.03, which far exceeds the resolution of the monograph system suitability requirement of ≥2.0. Peak areas (Table1) for HMW proteins in the insulin control sample are within the <1% suitability requirement. Note the total analysis time required for this method is approximately 15 minutes using AdvanceBio SEC 120 Å, 1.9 µm, 4.6 × 300 mm column compared to reported 35 minutes in Ph. Eur. monograph with traditional hydrophilic silica column using higher particle size.

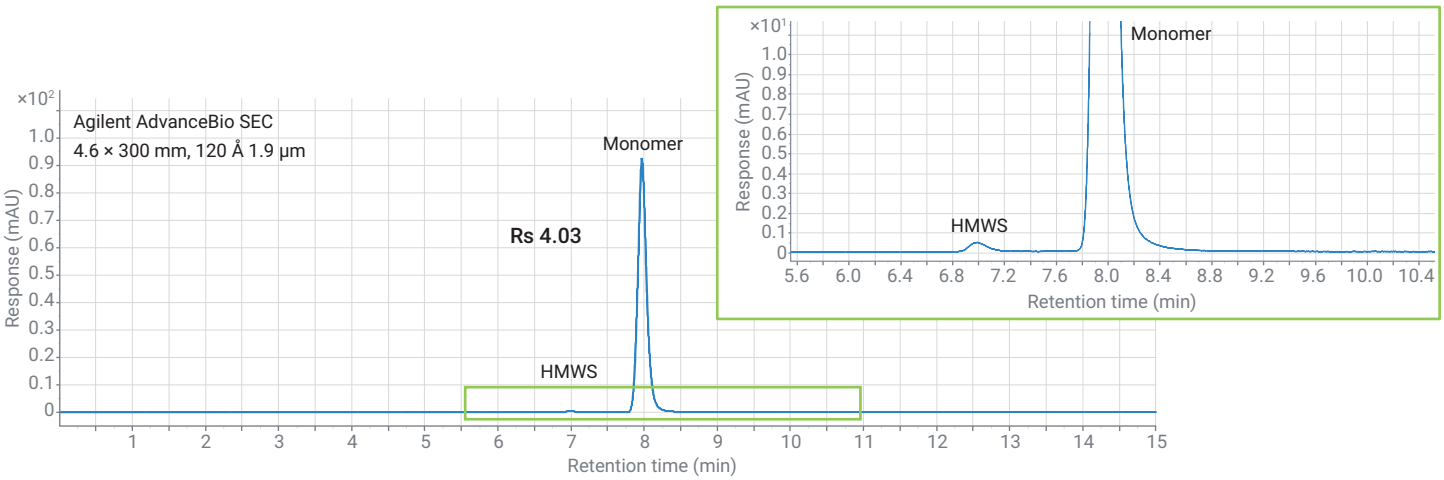

Figure 1. Size-exclusion chromatograms of human insulin control with monomer and HMW species.

Table 1. Results summary for SEC analysis of human insulin control.

Peak ID	RT (min)	%Area	Rs USP	Peak Tailing	Width 50%
Insulin HWM	6.99	0.67		1.48	0.15
Insulin Monomer	7.96	99.33	4.03	1.04	0.12

The insulin SEC HMW determination method in the EP monograph prescribes an SEC particle size of 5 to 10 µm while the USP monograph does not specify a particle size limit. As part of this study, a comparison was performed among AdvanceBio SEC 120 Å 1.9 µm column and other SEC column vendors with sub-2-µm particles and equivalent 300 mm length with 4.6 mm id (Figure 2). The 1.9 µm Agilent column demonstrates a significant increase (>50%) in resolution as compared to the competitor columns. The improved resolution is also apparent in the insulin monomer peak tail, in which lower molecular weight fragment peaks are better resolved with Agilent SEC compared to the vendor 2 SEC column. Significant peak tailing observed in the vendor 2 SEC column might be due to undesired secondary interactions. It is important to note that different elution times of insulin peaks are due to pore size differences between these columns. The percent peak areas of HMW species in the heat stressed insulin sample exceeds 1% for all the columns, indicating that sample would not pass the suitability test limits. However, higher % aggregates were resolved using Agilent SEC 120 Å, 1.9 µm column compared to other vendor columns.

Data are summarized in Table 2. Improvements are seen in efficiency for monomer and covalent dimer peaks in the AdvanceBio SEC 120 Å, 1.9 µm, 4.6 × 300 mm column method, again lending to the increased resolution and reduced run times with the updated method. According to USP (USP37-NF32S1) and EP guidelines, a 50% reduction in particle size and a 25% change in the column inner diameter for isocratic methods are the maximum allowable adjustments. Based on these requirements, featured SEC methods for insulin analysis with sub-2 µm particle size and 4.6 mm id would require further method validation and optimization to incorporate modern particle technology into established methods.

Table 2. Results summary of competitor SEC analysis for stressed insulin sample.

	Agilent AdvanceBio SEC 120 Å 1.9 µm			Vendor 1 SEC 125 Å 1.7 µm			Vendor 2 SEC 150 Å 1.8 µm		
Peak ID	%Area	Peak Tailing	Peak Width 50%	%Area	Peak Tailing	Peak Width 50%	%Area	Peak Tailing	Peak Width 50%
HMWS	1.93			1.57			1.10		
Insulin Monomer	97.66	1.00	0.13	97.85	1.10	0.13	98.80	1.37	0.14
LMWS	0.41			0.58			0.20		

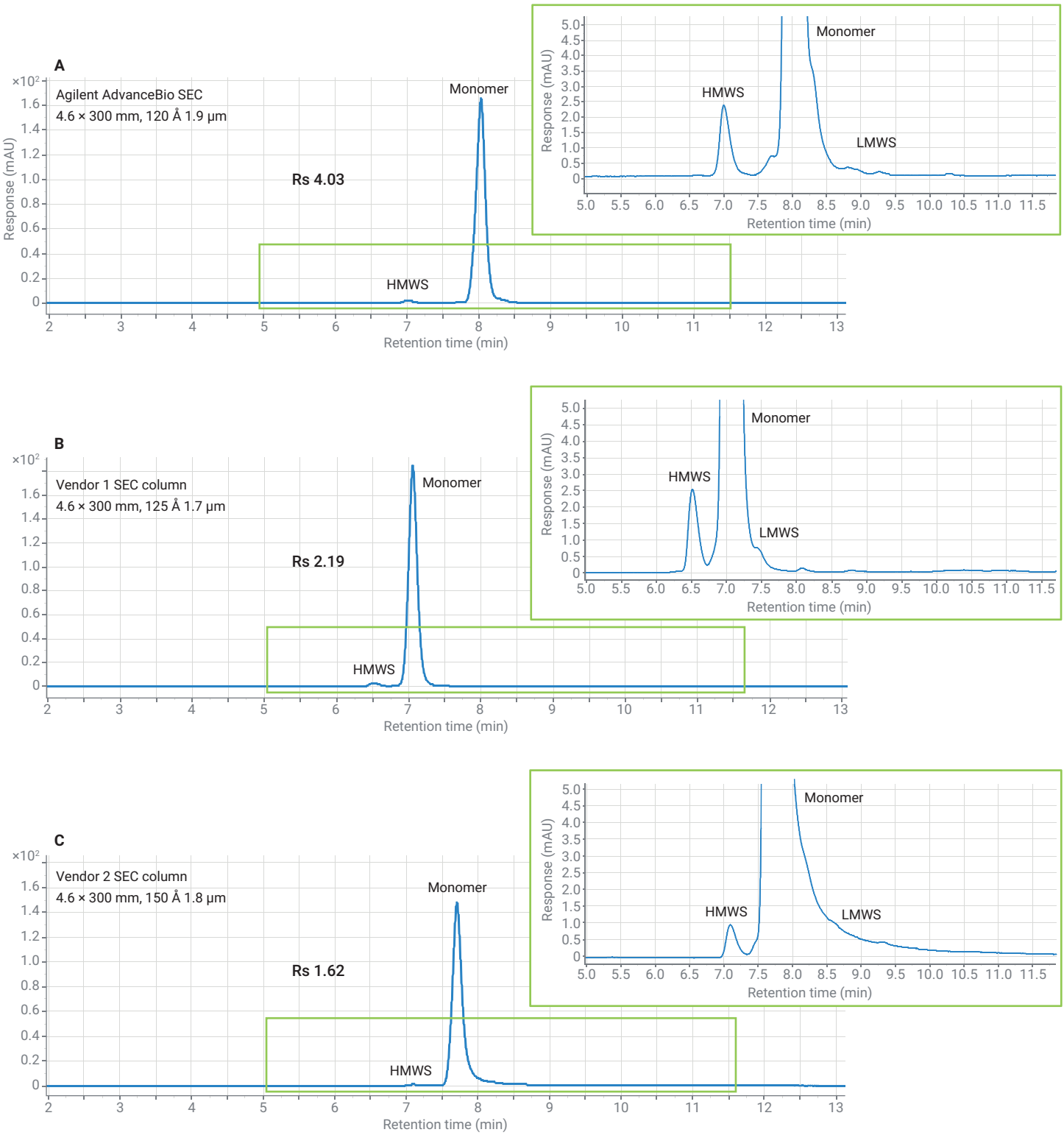

Figure 2. Competitor SEC analysis of stressed insulin with aggregates and low molecular weight fragments.

Conclusion

Size exclusion chromatography is the USP and EP standard method for the analysis of covalent HMW insulin in therapeutic preparations. The chromatographic profiles demonstrating the performance of this method using SEC columns of different competitors have been presented. Based on these results the use of Agilent BioAdvance SEC 120 Å pore size, AdvanceBio SEC 1.9 µm column, and Agilent Infinity II Bio-inert liquid chromatography instrumentation for this SEC-based analysis provides significant improvements in resolution compared to traditional SEC-HPLC methods while reducing analysis time and mobile phase use.

References

1. Yang, Y., *et al.* An Achilles' Heel in an Amyloidogenic Protein and Its Repair: Insulin Fibrillation and Therapeutic Design." *J. Biol. Chem.* **2010**, 10806–10821.
2. Librizzi; Fabio; Rischel. The Kinetic Behavior of Insulin Fibrillation is Determined by Heterogeneous Nucleation Pathways. *Protein Science* **2005**, *12*, 3129–3134.
3. Insulin Monograph, *USP Pharmacopeial Forum*, *31(5)*, 1375.
4. European Pharmacopeia 5.0, *Human Insulin Monograph* **2005**, *838*, 1800–1802.

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