Size Exclusion Chromatography of Biosimilar and Innovator Insulin

Using the Agilent AdvanceBio SEC column

Application Note

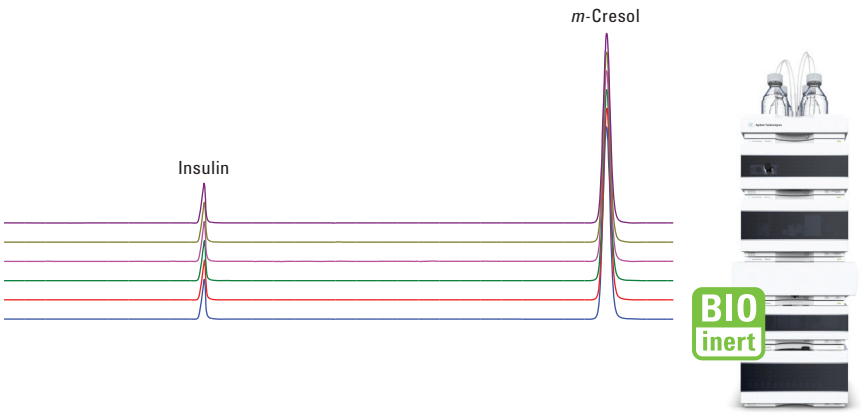
Bio-Pharmaceutical

Authors

M. Sundaram Palaniswamy and
Andrew Coffey
Agilent Technologies, Inc.

Abstract

Insulin is a small polypeptide hormone that controls blood glucose homeostasis. Genetic engineering techniques have enabled biopharma companies to develop diverse, long-acting insulin analogs. There is no pharmacopeia method available for the analysis of insulin analogs. An SEC method identifying innovator and biosimilar insulin analog, following a draft EP method, was developed using an Agilent AdvanceBio SEC 130 Å, 7.8 × 300 mm, 2.7 µm column. The effectiveness of this method, for routine analysis, was confirmed using a system suitability test, and retention time (RT) and area precision studies using innovator insulin as a reference material. This Application Note also presents the application of this column for detecting impurities with molecular masses greater than that of insulin for quantitation studies.



Agilent Technologies

Introduction

Novel insulin analogs are alternatives to human insulin products. Clinical trials have demonstrated equal or superior efficacy outcomes when these analogs are compared with human insulin. Insulin analogs are currently the long-acting basal human insulin on the market. Insulin analog was approved for use by the US Food and Drug Administration (USFDA) in April 2000. Unlike small molecules, biotherapeutics are created using biological processes. Each manufacturer uses an in-house developed process for the production of drug substance and drug product. These production methods can result in impurities derived from the drug substance, such as aggregates and degradation products. Due to the increased demand for antidiabetic drugs, it is a crucial yet challenging task to produce drugs free from impurities, and provide safe medicine free from side effects. In the biopharma industry, LC with UV detection is a versatile tool for lot release and characterization studies[1]. Size exclusion chromatography (SEC) is the method of choice for purity analysis, and for detecting aggregates of drug product. This Application Note describes a SEC-UV approach to determine the molecular similarity between insulin biosimilar and its innovator reference, following system suitability and method precision analysis[2]. These tests ensure that the method can generate results of acceptable accuracy and precision. The criteria selected is based on critical chromatographic parameters and their variation within acceptable limits, which are defined during the method evaluation experiments. An excellent correlation coefficient was observed for the linearity curve of insulin in the range of 10.6 to 3,400 µg/mL, indicating that the method is quantitative. Use of the Agilent AdvanceBio SEC column to monitor and separate impurities with molecular masses greater than the drug product, as determined by forced-stress studies, is also shown.

Materials and Methods

Instruments

A completely biocompatible Agilent 1260 Infinity Bio-inert Quaternary LC System with a maximum pressure of 600 bar was used, consisting of:

- Agilent 1260 Infinity Bio-inert Quaternary LC Pump (G5611A)
- Agilent 1260 Infinity Bio-inert High Performance Autosampler (G5667A)
- Agilent 1200 Infinity Series Thermostat (G1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment containing bio-inert click-in heating elements (G1316C, option 19)
- Agilent 1260 Infinity DAD VL (G1315D with Bio-inert standard 10-mm flow cell)
- Agilent AdvanceBio SEC, 130 Å, 7.8 × 300 mm, 2.7 µm (p/n PL1180-5350)

Software

Agilent ChemStation B.04.03 (or higher)

Size exclusion chromatography parameters

Table 1 shows the chromatographic parameters for size exclusion chromatography using an Agilent 1260 Infinity Bio-inert LC System.

Reagents, samples, and materials

Commercial innovator and biosimilar insulin were purchased from a local pharmacy, and stored according to manufacturer's instruction. Acetic acid and ammonia were purchased from Sigma-Aldrich. All chemicals and solvents used were HPLC grade, and highly purified water from a Milli-Q water purification system (Millipore Elix 10 model, USA) was used.

Procedure

A 10 µL volume of mobile phase was injected as a blank, followed by individual linearity levels in triplicate. Area and retention time (RT) of each level were used to calculate standard deviation (SD) and relative standard deviation (RSD%) values. Limits of detection (LODs) and limits of quantitation (LOQs) were established from the lower linearity level injections. The average area of each linearity level was plotted against the concentration of insulin to determine the calibration curve for the monomers.

Table 1. Chromatographic parameters used for SEC HPLC.

Parameters	Conditions
Mobile phase	200 mL of anhydrous acetic acid, 300 mL of acetonitrile, and 400 mL of water, adjusted to pH 3.0 with concentrated ammonia, and diluted to 1,000.0 mL with water.
TCC temperature	Ambient
Isocratic run	Mobile phase A
Injection volume	10 µL
Flow rate	0.5 mL/min
UV detection	276 nm

Linearity and range

The calibration curve was constructed with nine standard concentrations of innovator insulin in the range 10.6 to 3,400 µg/mL.

LOQ and LOD

The insulin concentration that provides a signal-to-noise ratio (S/N) of > 3 was considered as the LOD, and S/N > 10 was considered as the LOQ.

Preparation of insulin aggregates

Aggregates of insulin were prepared following temperature stress. Briefly, about 3.4 mg/mL of the drug product was incubated at 60 °C for 6 hours in a polypropylene tube. Samples were cooled to room temperature, and immediately analyzed.

System suitability

As per the draft monograph, the following are the system suitability requirements:

- **Symmetry factor**: Maximum 2.0 for the peak due to insulin analog
- **Peak-to-valley ratio**: Minimum 2
- **Total of all impurities with a retention time less than that of insulin analog**: Not more than 0.3 % of the total area of the peaks, disregarding any peak with a RT greater than that of the insulin peak

Results and Discussion

Separation and detection

The biosimilar insulin was compared using the innovator as the reference standard. The optimized SEC HPLC separation of intact biosimilar and innovator insulin on the AdvanceBio SEC 130 Å, 7.8 × 300 mm, 2.7 µm column achieved excellent separation. Homogenous profiles without any indication of aggregation were demonstrated within a total run time of 55 minutes. A peak due to the preservative *m*-cresol was also observed, eluting at approximately 49 minutes (Figure 1).

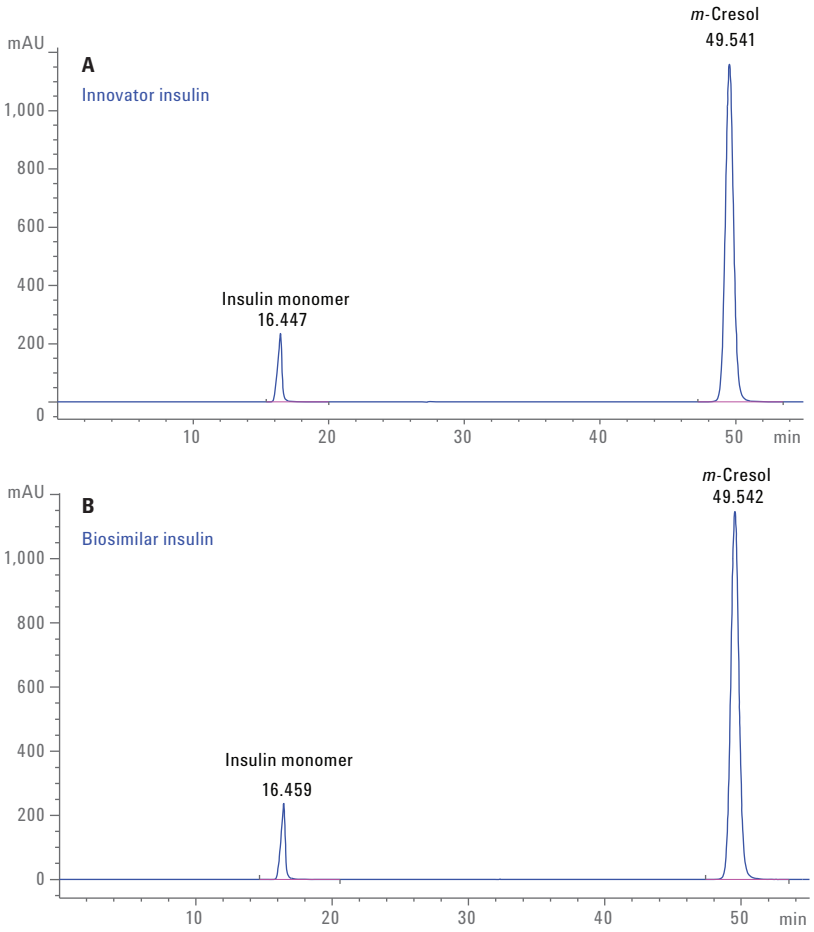


Figure1. SEC HPLC profile of insulin innovator and biosimilar on an Agilent AdvanceBio SEC, 130 Å, 7.8 × 300 mm, 2.7 µm column.

Precision of retention time and area

Figure 2 shows the overlays of six replicates of innovator and biosimilar insulin, demonstrating excellent separation reproducibility. Table 2 lists the average RTs and peak area RSDs for the insulin monomer from six replicates. The RT and peak area RSDs for the insulin monomer were within the acceptable limit of ±3 % and ±5 %, respectively, demonstrating the excellent reproducibility and precision of this method.

System suitability

Table 3 tabulates the acceptance criteria for this system suitability study for insulin analog, and Table 4 presents the summary of the system suitability results.

These results of the system suitability test for insulin innovator and biosimilar demonstrate that the method performed using an Agilent Bio-inert LC and an AdvanceBio SEC column meets the stringent performance requirements for insulin QA/QC analysis.

LOD and LOQ

The LOD and LOQ were tested for insulin innovator, and were found to be 11.3 µg/mL and 28 µg/mL, respectively, indicating that the method is sensitive. Table 5 shows the observed LOD and LOQ values of insulin innovator.

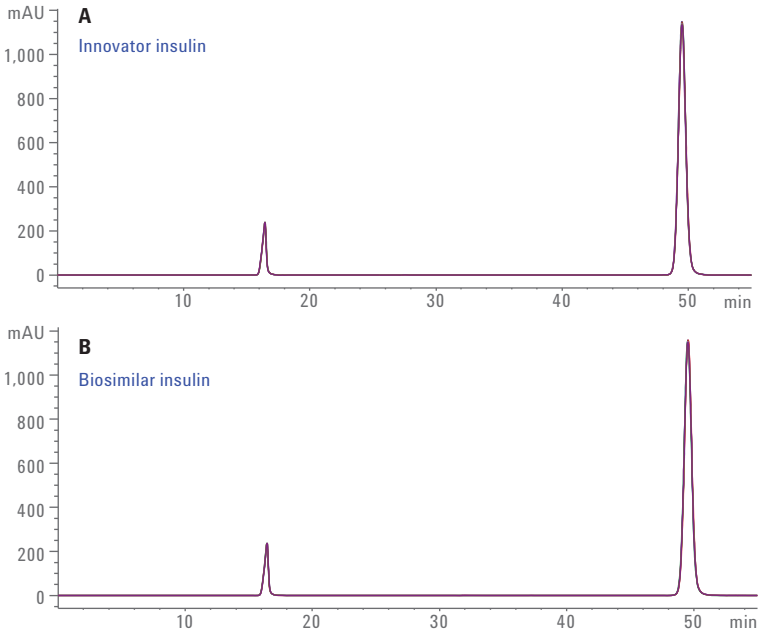


Figure 2. Overlay of six replicates of innovator and biosimilar insulin separated on an Agilent AdvanceBio SEC, 130 Å, 7.8 × 300 mm, 2.7 µm column.

Table 2. RT and peak area precision (n = 6).

	RT		Peak area	
Sample	Mean (min)	RSD	Mean (mAU/min)	RSD
Innovator insulin	16.450	0.057	5,544.91	0.285
Biosimilar insulin	16.460	0.044	5,459.55	0.662

Table 3. Acceptance criteria.

Parameter	Limit
Symmetry factor	Maximum 2.0 for the peak due to insulin analog
Peak-to-valley ratio	Minimum 2
Total of all impurities with an RT less than that of insulin analog	Not more than 0.3 % of the total area of the peaks

Table 4. Summary of system suitability test results.

	Results on an Agilent AdvanceBio SEC, 130 Å, 7.8 × 300 mm, 2.7 µm column			
Sample	Symmetry factor	Peak-to-valley ratio	Total of all impurities with an RT less than that of insulin analog	Passed (Yes/No)
Innovator insulin	1.71	–	0.167	Yes
Biosimilar insulin	1.72	–	0	Yes

Table 5. LOD, LOQ, and S/N results (n = 3) for insulin innovator.

Concentration (µg/mL)	S/N	Average area
10.6 (LOD)	11.9	12.8
31.8 (LOQ)	34.7	37.4

Linearity

Linearity curves for insulin innovator were constructed from the LOD level to the label claim (3.4 mg/mL) in the study, using the area response and concentration of insulin. Figure 3 shows the linearity curve for insulin in the concentration range 10.6 to 3,400 µg. The R^2 value observed was more than 0.99, suggesting excellent dose-dependent correlation between the peak area and the concentration of insulin.

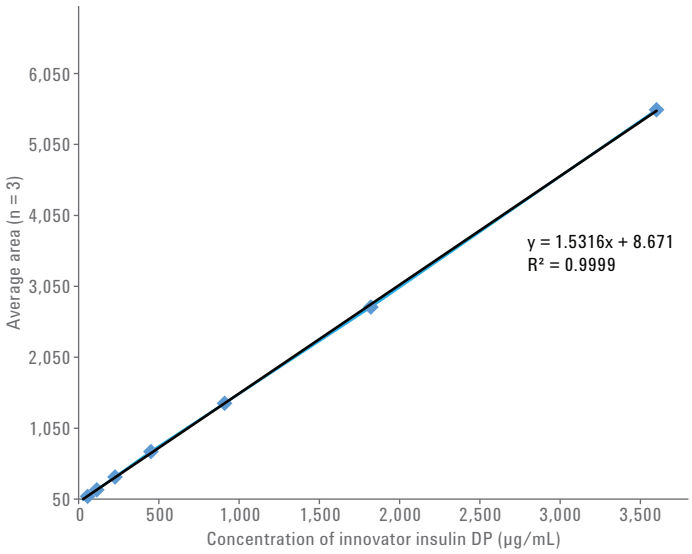


Figure 3. Linearity curve with standard concentrations of insulin ranging from 10.6 to 3,400 µg/mL, showing excellent coefficient value.

Aggregation/degradation analysis and quantification

The impurity profile of biotherapeutics is of increasing importance in drug safety. Although aggregates are present in extremely low concentrations, they may have a big impact on the quality of the product. The AdvanceBio SEC column is designed to have minimum interaction with biomolecules, enabling distinct baseline separation of insulin aggregates. These insulin aggregates elute from the AdvanceBio SEC column at 11.181 and 13.884 minutes, respectively, as shown in Figure 4.

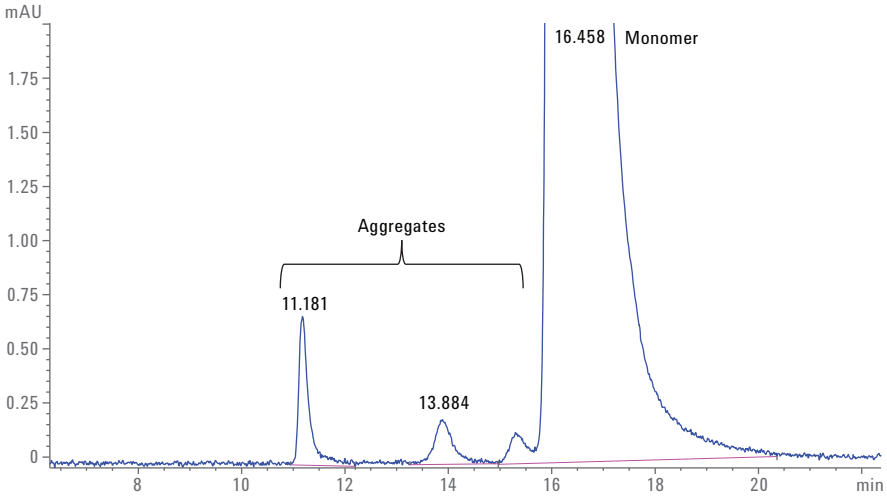


Figure 4. An Agilent AdvanceBio SEC profile of heat stressed insulin showing baseline separation of insulin aggregates.

Economic value and lifetime studies

A laboratory head or group leader may primarily consider cost, particularly when comparing the cost of the AdvanceBio SEC column and other column types. In SEC separation, apart from the cost of the operator and instrument, the most expensive component is the cost of the column itself. If the columns do not last long enough, or there are column-to-column reproducibility issues, multiple columns may need to be screened. Ensuring batch-to-batch reproducibility through control of the entire production process is essential. Figure 5 shows the separation of AdvanceBio 130 Å protein markers on four separate batches of the AdvanceBio SEC 130 Å media, ensuring thorough control of the entire production process.

One of our objectives is to ensure extended column lifetime throughout our customers' development processes. This extended column lifetime provides extra benefits, as the downtime is greatly reduced. Figure 6 shows six overlaid chromatograms of the 250 injections of 3 mg/mL insulin drug substance taken at an interval of 50 runs. Table 6 shows the RT, area, tailing factors, and theoretical plates from the selected runs.

The results clearly demonstrate that there is virtually no change in RT, area, as well as tailing factor over the course of 250 injections. The theoretical plates, a measure of the efficiency of the column, also do not vary significantly.

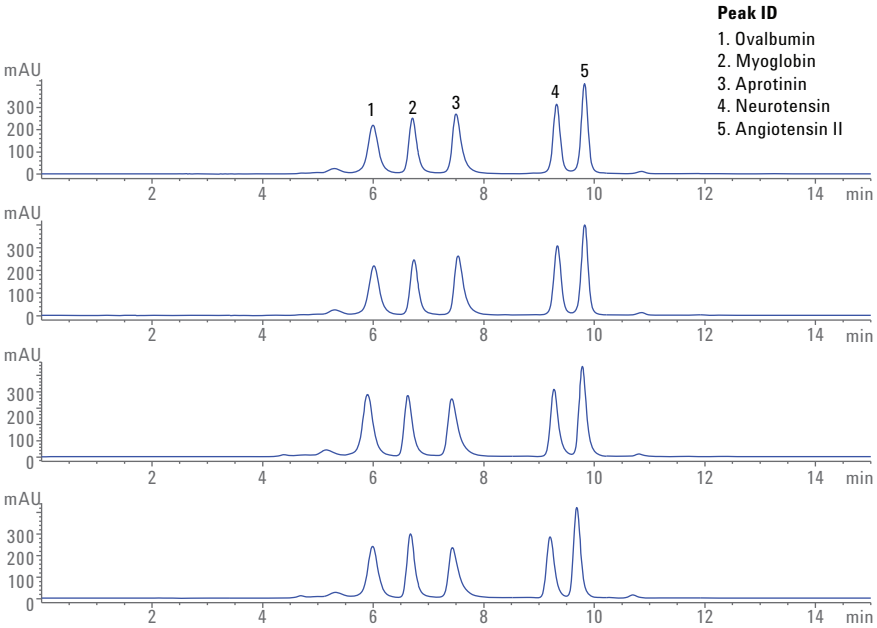


Figure 5. Separation of Agilent AdvanceBio 130 Å protein standards on four separate batches of an Agilent AdvanceBio SEC 130 Å, 7.8 × 300 mm, 2.7 µm media.

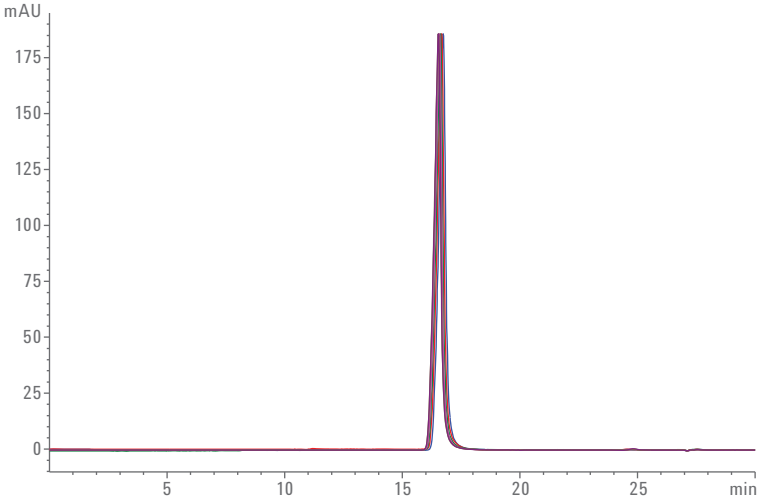


Figure 6. Overlay of six chromatograms for the 250 injection at an interval of 50 runs.

Conclusion

Size exclusion chromatography is the workhorse for detecting and monitoring aggregates and monomers for biopharmaceuticals. This Application Note demonstrates the suitability of an Agilent AdvanceBio SEC 130 Å column as an excellent choice to study insulin analogs. We used the draft pharmacopeia method to develop a simple UV-based approach to define the molecular similarity between biosimilar and innovator insulin drug product using an AdvanceBio SEC 130 Å, 7.8 × 300 mm, 2.7 µm column. RT and area precision of the method were excellent, and met the system suitability requirements. A linear relationship between the peak area and eight standard concentrations of the insulin drug product was observed, with an outstanding coefficient of linearity value. The observed LOD and LOQ was found to be 10.6 and 31.8 µg/mL, respectively, indicating the sensitivity of the method. The AdvanceBio SEC column was able to separate and monitor aggregates analyzed by forced stress study. We have also shown the greater economic benefits of using an AdvanceBio SEC column, some of which are reducing lot-to-lot manufacturing variations, and prolonged column lifetime with reproducible and robust outcomes. This simple and reproducible method, coupled with a bio-inert and corrosion-resistant instrument is considered to be reliable and suitable for routine quality checks of insulin throughout the development process.

Table 6. Observed RT, area, tailing factor, and theoretical plates for 250 injections of insulin drug substance.

Injection no.	RT (min)	Area	Tailing factor	Theoretical plates
1	16.657	3944	0.899	16,001
50	16.671	3966	0.890	15,849
100	16. 681	3968	0.898	15,982
150	16.622	3942	0.893	15,942
200	16.634	3953	0.895	15,919
250	16.634	3963	0.890	15,944

References

1. Kannan V; Narayanaswamy P; Gadamsetty D; Hazra P; Khedkar A; Iyer, H. A tandem mass spectrometric approach to the identification of O-glycosylated glargine glycoforms in active pharmaceutical ingredient expressed in Pichia pastoris. *Rapid Communications in Mass Spectrometry* **2009**, *23(7)*, 1035-42.

2. Pharmeuropa, Vol. 23, No. 2, April **2011**.

www.agilent.com/chem

Published in the USA, May 1, 2016
5991-6872EN

Agilent Technologies