Analysis of Oxidized Insulin Chains using Reversed Phase Agilent ZORBAX RRHD 300SB-C18

Application Note

BioPharma

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Abstract

A new reverse phase media, Agilent ZORBAX RRHD 300SB-C18 1.8 µm, was used for the separation of a typical protein biopharmaceutical, insulin. The value of sub 2-µm particles for protein separations was assessed under denaturing conditions. The advantages of these particles in separating small molecules were also realized in protein separations.

The use of a 1.8 µm column designed for UHPLC systems significantly reduced analysis time, critical for increasing the efficiency of QC for protein primary structure analysis. The separations also demonstrated how this technology achieved resolution of various insulin isoforms.

The eluents routinely used for reverse phase analysis are acidic, containing trifluoroacetic acid or formic acid, which can limit the lifetime of many HPLC columns. Using StableBond technology, it was possible to produce a 300Å pore-size media that was stable under acidic conditions, to provide the robust reproducible separations required for protein QC.

Agilent Technologies

Introduction

Due to the heterogeneity of a protein biopharmaceutical, it is necessary to use a number of chromatographic techniques to fully characterize the API. Methods include size exclusion chromatography for the quantitation of dimers and aggregates and ion exchange for the identification of charge variants. Both of these techniques use aqueous eluents and non-denaturing conditions. As part of the full characterization of a protein it is also necessary to look at the primary amino acid sequence and any post translational modifications to the sequence that may have occurred during the purification or formulation steps of manufacture. To perform this type of analysis, denaturing conditions are required, and so reversed phase HPLC is normally the technique of choice. In this example, we use Agilent ZORBAX Rapid Resolution High Definition (RRHD) columns, which benefit from improved packing processes to achieve stability up to 1200 bar for use with the Agilent 1290 Infinity LC.

Materials and Methods

The conditions in Table 1 were used throughout the investigation, with variations as noted in the relevant chromatograms.

Table 1. Standardized Chromatographic Conditions

Parameter	Item
Column	Agilent ZORBAX 300SB-C18 1.8 µm, 2.1 × 50 mm (p/n 857750-902) (Agilent Technologies, Wilmington DE)
Sample	Insulin, oxidized insulin chain A and chain B from bovine pancreas (Sigma Aldrich, St. Louis, MO.)
Sample concentration	1 mg/mL
Injection volume	3 µL
Flow rate	1.0 mL/min
Pressure	~ 650 bar
Mobile phase A	0.1% TFA in distilled water
Mobile phase B	80% ACN + 0.01% TFA in distilled water
Detector	UV, 280 nm
System	Agilent 1290 Infinity HPLC

Results

Speed

The system separated the test mixture very quickly, distinguishing insulin, a small molecule, from its impurities in less than five minutes (Figure 1). Using multiple gradients achieves the same fast analysis time (Figure 2). Rapid equilibration is evident even with the screening gradient, which starts from a highly aqueous eluent, demonstrating that the column is suitable for use with a wide range of organic content.

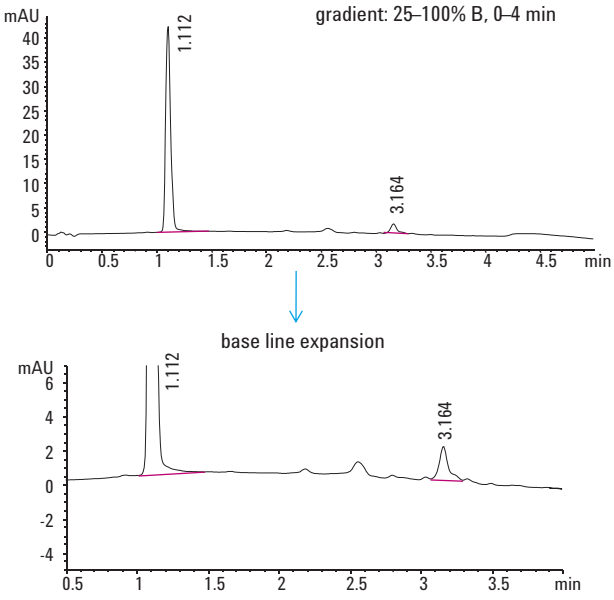

Figure 1. Fast resolution of insulin and some impurities on an Agilent ZORBAX RRHD 300SB-C18 1.8 µm column.

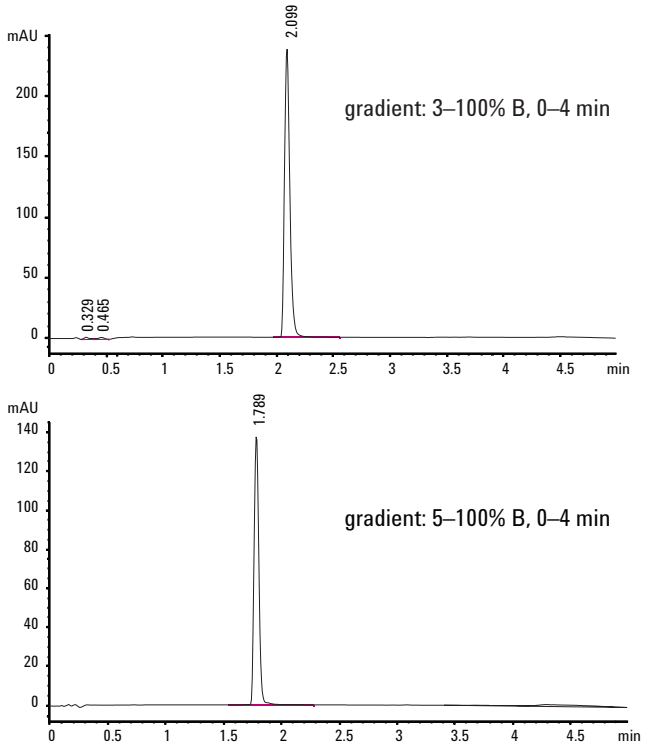

Figure 2. Multiple gradient systems can be selected to separate insulin in less than 5 minutes using the Agilent ZORBAX RRHD 300SB-C18 1.8 µm column, including eluents with high water content.

Flow rate can also be manipulated to provide a fast separation (Figure 3). Peak asymmetry and efficiency remain unchanged (Table 2), a feature of sub 2 µm particles that facilitates rapid separations.

Table 2. Effect of Flow Rate on Retention Time, Asymmetry, and Efficiency in the Analysis of Insulin

Flow rate (mL)	Pressure (bar)	Retention time (min)	Asymmetry	Plate count
0.3	230–150	2.39	0.80	8815
0.5	350–250	2.04	0.82	8390
1.0	680–520	1.78	0.88	8034
1.5	890–670	1.72	0.88	8060

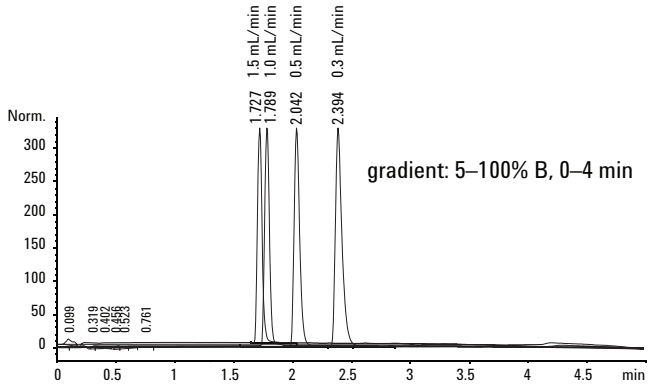

Figure 3. Different flow rates can be selected to separate insulin on the Agilent ZORBAX RRHD 300SB-C18 1.8 µm column.

Reproducibility

Two hundred consecutive injections were done to examine the column's reproducibility. The results show that the integrity of peak shape, asymmetry, retention time and efficiency remained the same after 200 injections of insulin without cleaning the column (Table 3 and Figure 4).

Table 3. Two Hundred Injections of Insulin Demonstrates the Reproducibility of Agilent ZORBAX RRHD 300SB-C18 1.8 µm

Run no	Pressure (bar)	Retention time (min)	Asymmetry	Plate count
1	680–520	1.789	0.86	9758
50	680–520	1.790	0.91	9752
100	680–520	1.788	0.88	9758
200	680–520	1.789	0.87	9741

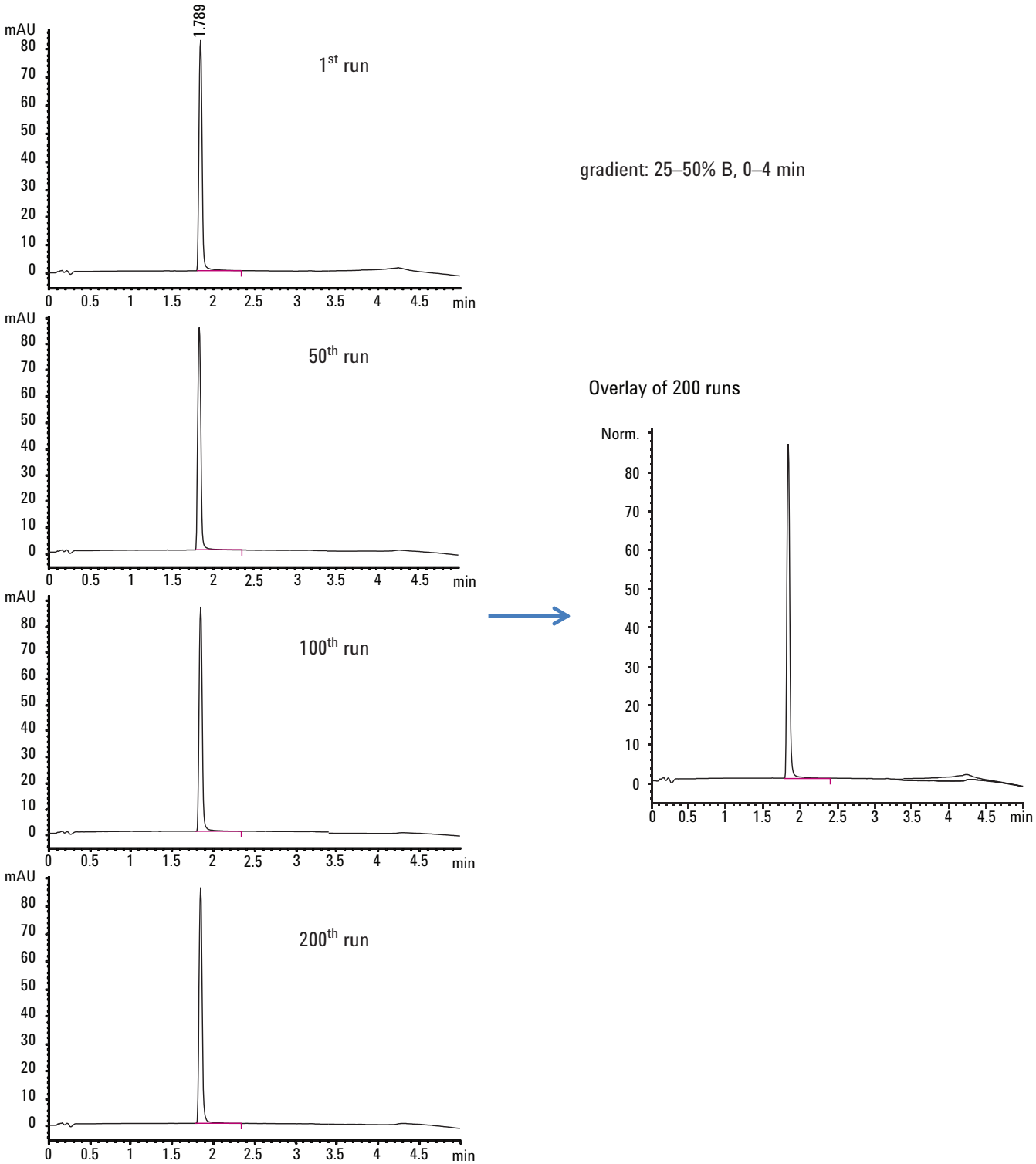

Figure 4. Two hundred injections reveal the reproducibility of the Agilent ZORBAX RRHD 300SB-C18 1.8 µm column.

Separation of Heat-Degraded Insulin

A forced degradation study can be performed by heating APIs, and HPLC used to monitor the degradation products. Heat treating insulin produces degradation products that can be quickly resolved by the column from the monomer insulin (Figure 5).

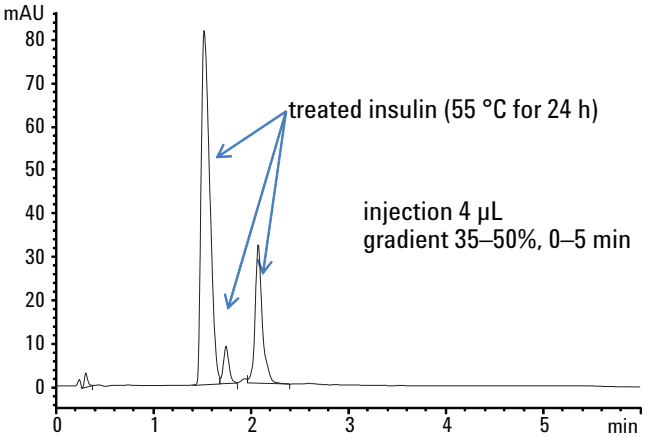

Figure 5. Heat-treated insulin quickly resolved on an Agilent ZORBAX RRHD 300SB-C18 1.8 µm column.

Separation of Insulin Isoforms

Figures 6 and 7 showed that the column can also separate isoforms of insulin; in this case, oxidized insulin chain A (Figure 6) and the mixture of insulin and oxidized insulin chain A (Figure 7). Once again, different gradient systems can be selected with very similar results.

For fast analysis of insulin, high gradient systems are usually used. However, these conditions force oxidized insulin chain A to be eluted rather quickly. To analyze oxidized insulin chain A from its subspecies, a shallow gradient system is required to ensure that the molecule can retain longer on the column.

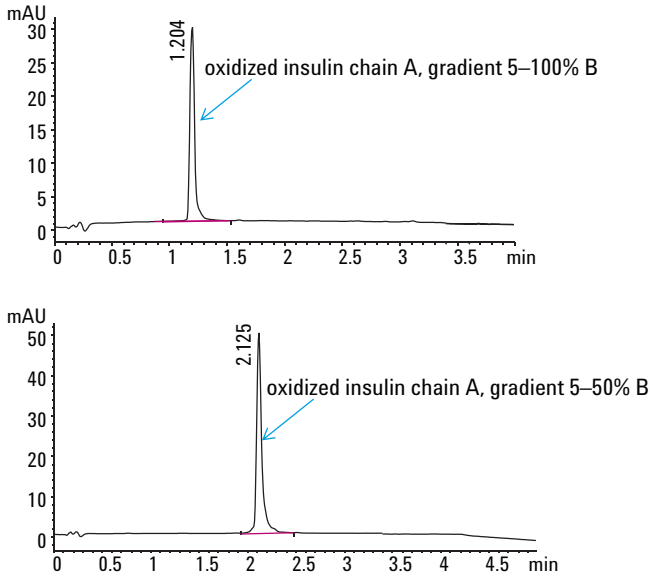

Figure 6. Analysis of oxidized insulin chain A with different gradients.

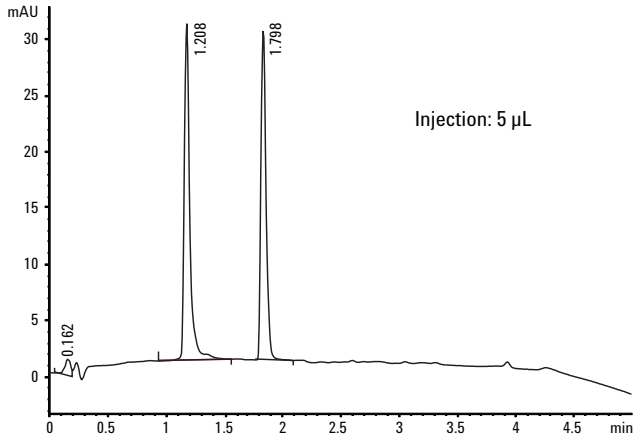

Figure 7. Analysis of insulin and oxidized insulin chain A on Agilent ZORBAX RRHD 300SB-C18 1.8 µm.

Similarly, Figures 8 and 9 demonstrate that the column separates oxidized insulin chain B (Figure 8). Again, different gradient systems can be selected with very similar results. The column also discriminates degradation products of oxidized insulin chain B (Figure 9).

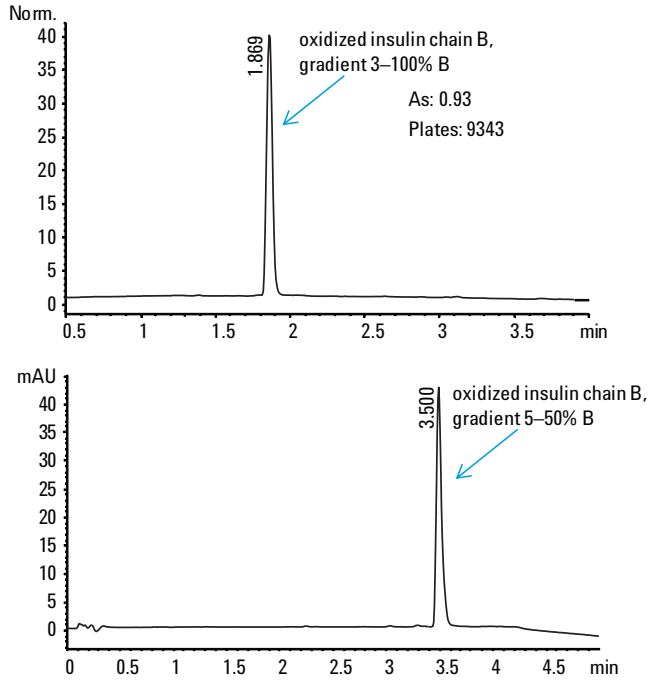

Figure 8. Optimized conditions for fast analysis of oxidized insulin chain B on the Agilent ZORBAX RRHD 300SB-C18 1.8 µm column.

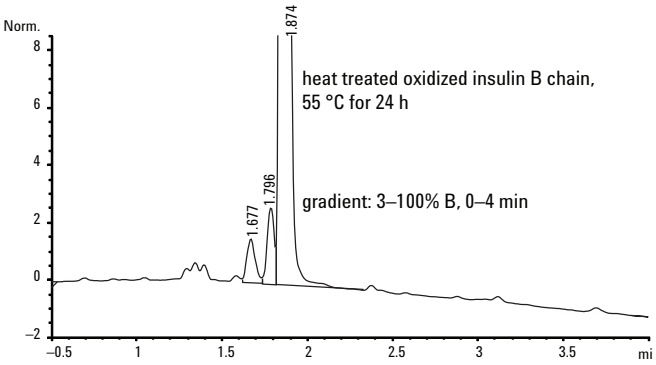

Figure 9. Oxidized insulin chain B and some degradation and impurities are well resolved on Agilent ZORBAX RRHD 300SB-C18 1.8 µm.

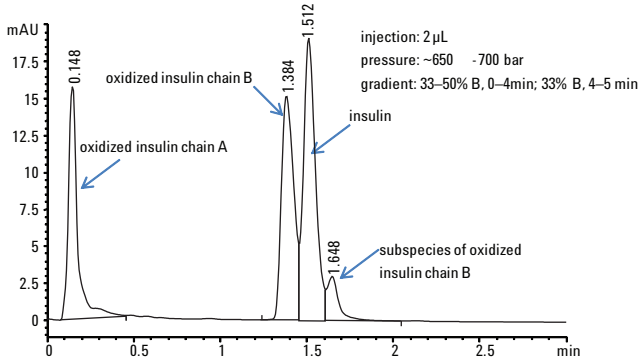

Figure 10. Insulin and oxidized insulin A and B chains are resolved quickly but insulin and oxidized chain B often co-elute.

Conclusions

Analyzing small molecule, protein biotherapeutic insulin, together with its isoforms and breakdown products, is fast and simple with the Agilent ZORBAX RRHD 300SB-C18 1.8 µm column. The column's rapid resolution high definition technology permits high pressure UHPLC, while the StableBond 300Å pore-sized particles are robust when analysis requires acidic conditions. Reproducibility is excellent, with good resolution, asymmetry and efficiency. The column is well suited to the needs of QC when assessing the structure of primary proteins.

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