Application Note

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Converting a ChP Method of Related Compounds Analysis in Human Insulin to Agilent InfinityLab Poroshell 120 Columns

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Abstract

Quality control of manufactured human insulin requires several analytical tests to be performed. One such test is for analysis of related compounds. In the China Pharmacopeia (ChP)[1], the method for related compounds requires a traditional octadecylsilane (C18) LC column of either 4.6 × 150 mm or 4.6 × 250 mm, 5 µm. The traditional 5 µm particle-size columns provide low-efficiency performance for the insulin peak and insufficient resolution from impurities, and the original method applies a long gradient of over 70 minutes. In this application note, alternative columns for this purpose were tested, and the recommended column was determined to be an Agilent InfinityLab Poroshell 120 EC-C18 column, 3.0 × 100 mm, 2.7 µm. The adjusted methods using Agilent InfinityLab Poroshell 120 columns achieved significant improvements in efficiency performance and resolution, while still meeting the requirements of the ChP method. The analysis time was also reduced to 20 minutes.

Introduction

Insulin is a peptide hormone composed of 51 amino acids and has a molecular weight of 5,808 Da. Regulatory methods in the ChP[1] specify a long gradient for the analysis of related compounds in human insulin. In a previous application note for porcine insulin[2], the gradient method was converted to the Agilent InfinityLab Poroshell 120 SB-C18 column, 4.6 × 100 mm, 2.7 µm. InfinityLab Poroshell 120 columns show many improvements compared to the traditional 5 µm column, including better peak shape, efficiency, and resolution, and their performance is four to six times higher than that of traditional 5 µm columns. Some minor impurities were found in the chromatogram using the InfinityLab Poroshell 120 SB-C18 column due to the improved peak shape, increased efficiency, greater sensitivity, and resolution of these superficially porous columns.[2] However, the retention time was still very long, taking 50 minutes for a single sample.

In this application note, the HPLC method for the related compounds in human insulin was first run on an Agilent ZORBAX SB-C18, 4.6 × 250 mm, 5 µm column or an Agilent ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 µm column. The method was then transferred to a column with superficially porous particles, either the InfinityLab Poroshell 120 SB-C18 or the InfinityLab Poroshell 120 EC-C18, both of which deliver similar performance to columns with sub-2 µm particles for fast separations.

Experimental

Materials and methods

The HPLC conditions for analysis of related compounds of human insulin outlined in the ChP method are shown in Table 1.

Mobile phase for related compounds

A: 0.2 mol/L sulfate/acetonitrile (82/18); to get 0.2 mol/L sulfate: dissolve 28.4 g of anhydrous sodium sulfate in 800 mL of water, pipette 2.7 mL of phosphoric acid into the solution, and adjust with ethanolamine to pH 2.3; mix and add water to a volume of 1,000 mL.
B: acetonitrile/water (50/50).

Referring to the gradient in Table 2, the mobile phase composition and the duration of the isocratic elution was adjusted to obtain a retention time of approximately 25 minutes for insulin, with the A21-desamido insulin eluting

Table 1. ChP HPLC conditions for related compounds of human insulin.

Parameter	Value
Columns	Octadecylsilane (C18) chemically bonded to porous silica
Flow Rate	1.0 mL/min
Injection Volume	20 µL
Column Temperature	40 °C
Wavelength	214 nm

Table 2. Original gradient in the ChP method.

Time (min)	%B
0	22
36	22
61	67
67	67

Table 3. HPLC/UHPLC method parameters.

Column	Mobile Phase	Flow Rate (mL/min)	Gradient	Injection Volume (µL)	Multicolumn Thermostat (°C)	Diode Array Detector
Agilent ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 µm (p/n 959990–902)	A) 0.2 mol/L sulfate/acetonitrile (82/18) B) Acetonitrile/water (50/50)	1.0	Time (min) %B 0 28 36 28 61 67 67 67 67.01 28 73 28	20	40	214 nm, 2.5 Hz
Agilent ZORBAX SB-C18, 4.6 × 250 mm, 5 µm (p/n 880975-902)		1.0	Time (min) %B 0 27 36 27 61 67 67 67 67.01 27 73 27	20	40	214 nm, 2.5 Hz
Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm (p/n 695975-302)		0.8	Time (min) %B 0 28 12 28 17 67 18.20 67 18.21 28 20 28	3	40	214 nm, 20 Hz
Agilent InfinityLab Poroshell 120 SB-C18, 3.0 × 100 mm, 2.7 µm (p/n 685975-302)		0.8	Time (min) %B 0 30 12 30 17 67 18.20 67 18.21 30 20 30	3	40	214 nm, 20 Hz

just before the start of the gradient elution phase. A column equilibrium step should be added at the end of gradient. The actual gradients and other parameters are shown in Table 3. Materials used for the experiment are shown in Table 4.

Results and discussion

Traditional columns

The ChP requires a column with C18 chemically bonded to porous silica as the packing material, which is also within USP L1 materials. Traditional 5 µm columns are commonly used for ChP methods, but smaller particle sizes are allowed if the results meet the requirements. Therefore, the method was first run on a ZORBAX SB-C18, 4.6 × 250 mm, 5 µm column and a ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 µm column. The mobile phase composition was modified according to the requirements of the ChP method to obtain a retention time of approximately 25 minutes for human insulin, as shown in Table 3. The chromatograms from the analysis of related compounds are shown in Figure 1.

The system suitability of insulin analysis requires the resolution between insulin and A21-desamido insulin to be no less than 1.8 and the tailing factor for the insulin peak no more than 1.8. As shown in Figure 1, the tailing factor of A21-desamido insulin on the ZORBAX SB-C18 column barely met the requirements in the ChP. But the tailing factor of A21-desamido insulin on ZORBAX Eclipse Plus C18 column did not meet the requirements in the ChP. There were no other related compounds found in this sample due to low sensitivity on 5 µm columns.

Table 4. Materials used for the experiment.

Standard	Human Insulin
Columns	Agilent ZORBAX SB-C18 column, 4.6 × 250 mm, 5 µm (p/n 880975-902) Agilent ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 µm (p/n 959990-902) Agilent InfinityLab Poroshell 120 SB-C18, 3.0 × 100 mm, 2.7 µm (p/n 685975-302) Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm (p/n 695975-302)
System	Agilent 1260 Infinity II LC system including: Agilent 1260 Infinity II quaternary pump (G7111B) Agilent 1260 Infinity II vialsampler (G7129A) Agilent 1260 Infinity II multicolumn thermostat (G7116A) Agilent 1260 Infinity II diode array detector (G4212B)

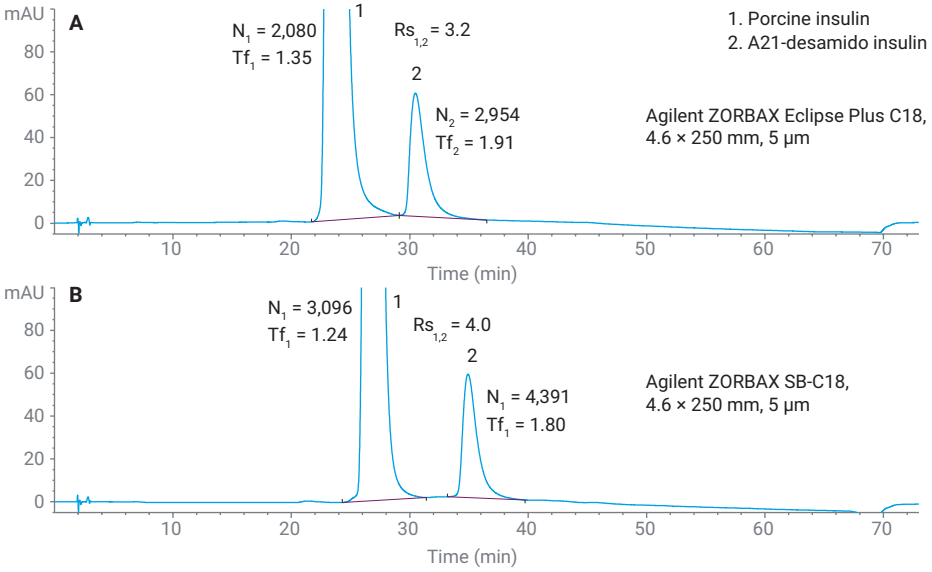


Figure 1. Chromatograms of related compounds analysis on two traditional 5 µm columns: the Agilent ZORBAX Eclipse Plus C18, 4.6 × 250 mm, 5 µm (A) and the Agilent ZORBAX SB-C18, 4.6 × 250 mm, 5 µm (B).

InfinityLab Poroshell 120 columns

To reduce the analysis time and save solvent, the method was transferred to either an InfinityLab Poroshell 120 SB-C18, 3.0 × 100 mm, 2.7 µm column or an InfinityLab Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm column. The mobile phase composition and duration of the isocratic elution was adjusted to achieve the ideal resolution of a related compound with the insulin. The gradient time, followed by the isocratic elution and injection volume, need to be recalculated when scaling the original method to a new one[2]; therefore, the new gradient method for related compounds was run on these columns. The chromatograms are shown in Figure 2.

Both InfinityLab Poroshell 120 columns show many improvements compared to traditional 5 µm columns (Figure 1) including improvements in peak shape, efficiency, and resolution. One important impurity behind insulin was found in the chromatogram using the Poroshell 120 columns due to the improved peak shape, increased efficiency, greater sensitivity, and resolution of the superficially porous columns. The Poroshell 120 EC-C18 column provided better resolution between this impurity and the insulin peak than the Poroshell 120 SB-C18 column. Although both columns meet the minimum resolution requirements of 1.5 or greater according to the ChP, choosing the Poroshell 120 EC-C18 column is recommended to achieve better resolution for this analysis. Some minor impurities may have been present in the separation on the traditional column, but the broader, less-efficient peaks reduced the resolution such that they were not detected on the 5 µm column.

Both Poroshell 120 SB-C18 and EC-C18 columns provide good performance for related compounds analysis. They easily meet the system suitability requirements in the ChP. The dramatic increase in performance is likely due to the smaller 2.7 µm particles of Poroshell 120 and the larger 120 Å pore size of the superficially porous columns. Both columns shorten the analysis time from 73 minutes to only 20 minutes. However, the Poroshell 120 EC-C18 column provides more resolution than the Poroshell 120 SB-C18 column.

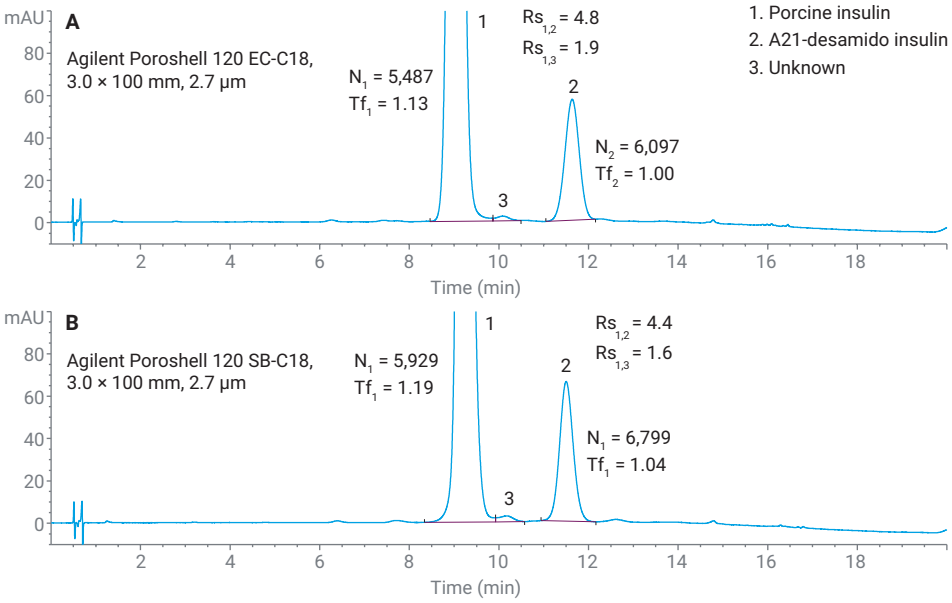
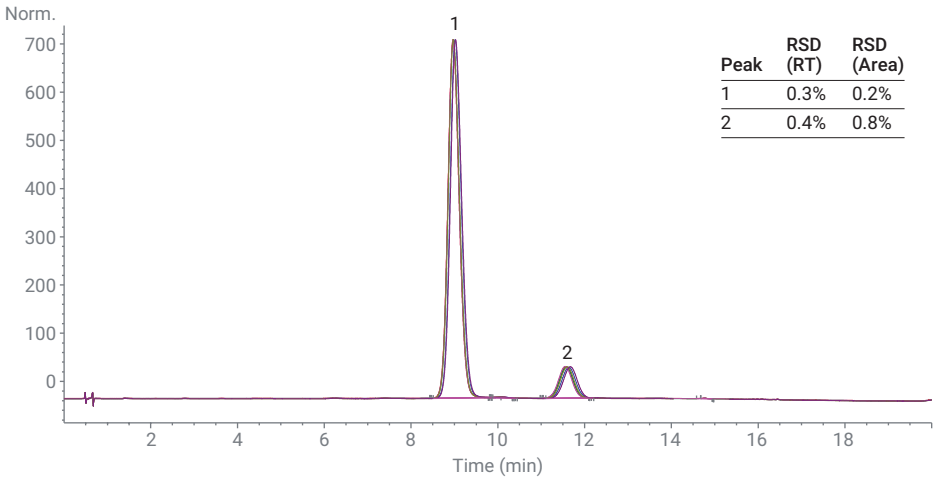


Figure 2. Chromatogram of related compounds analysis on the Agilent InfinityLab Poroshell EC-C18 column, 3.0 × 100 mm, 2.7 µm (A) and the Agilent InfinityLab Poroshell 120 SB-C18 column, 3.0 × 100 mm, 2.7 µm (B).

Reproducibility from injection to injection is important for reliable results. The ChP requirement is a relative standard deviation (RSD) for five replicate injections of not more than 2%. This is a typical requirement for many LC methods. The RSDs of peak area from five replicate injections using the Poroshell 120 EC-C18 column (Figure 3) were 0.2% for porcine insulin and 0.8% for A21-desamido insulin, easily meeting the requirements. The RSDs of retention from five replicate injections using the Poroshell 120 EC-C18 column (Figure 3) were 0.3% for porcine insulin and 0.4% for A21-desamido insulin, which is also excellent for this method.



Peak	RSD (RT)	RSD (Area)
1	0.3%	0.2%
2	0.4%	0.8%

Figure 3. Overlay chromatograms of five injections on an Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm column.

Conclusion

The method for the analysis of insulin was successfully converted from a traditional 5 µm column to two superficially porous Agilent InfinityLab Poroshell 120 columns, with significant improvements in performance. Analysis time was reduced by over 70% from 73 minutes to 20 minutes. This not only greatly increases sample throughput, but also provides significant cost savings in solvent consumption and disposal fees. This benefits human insulin manufacturers and quality control laboratories by running more samples in less time and decreasing the overall operating expenses for this analysis.

Poroshell 120 columns, in particular the Agilent InfinityLab Poroshell 120 EC-C18, 2.7 µm particle column, with a pore size of 120 Å, is suitable for the highly efficient analysis of small proteins such as insulin. Poroshell 120 columns can be used to easily meet the system suitability requirements of the ChP for insulin. This method using a Poroshell 120 column is well suited for quality control testing of manufactured insulin.

References

1. China Pharmacopoeia (2020 edition, III), Human insulin, p. 378, **2020**.
2. Converting a ChP Method of Insulin to Poroshell 120 columns, *Agilent Technologies application note*, publication number 5990-9029EN, **2011**.

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