Converting a CHP Method for Insulin to Agilent Poroshell 120 Columns

Application Note

Biopharm

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Abstract

A regulatory method in the China Pharmacopeia (CHP) for insulin analysis calls for a traditional 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 in either column length. In this application note, the traditional column was replaced with an Agilent Poroshell 120 EC-C18 or Agilent Poroshell 120 SB-C18 column. The adjusted methods using Poroshell 120 columns achieved significant improvements in efficiency performance and resolution while still meeting the requirements of the CHP or United States Pharmacopia (USP) regulatory methods.

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Introduction

Insulin is a peptide hormone composed of 51 amino acids and has a molecular weight of 5808 Da. It is produced in the islets of Langerhans in the pancreas. Its structure varies slightly between species of animals. Insulin "strength" from animals differs from humans because of variations in carbohydrate metabolism control effects. Bovine insulin differs from human in only three amino acid residues, and porcine insulin is close to human insulin. Insulin has been widely used for the treatment of both type 1 and some cases of type 2 diabetes. Regulatory methods in the CHP [1] and USP [2] specify a long isocratic elution for the insulin assay and a long gradient for the analysis of related compounds. Both methods share the same HPLC conditions for the assay but have slightly different gradient methods for the analysis of related compounds.

In this application note, the HPLC methods for the assay and related compounds in the CHP were first run on an Agilent ZORBAX SB-C18, 4.6 × 150 mm, 5 µm or an Agilent ZORBAX Eclipse Plus C18, 4.6 × 150, 5 µm column. The methods were then transferred to a column with superficially porous particles, the Agilent Poroshell 120 column, which delivers similar performance to columns with sub-2 micron particles for fast separations.

Materials and Methods

The CHP HPLC conditions for related compounds and assay of porcine insulin

Columns	Octadecyl silane (C18) chemically bonded to porous silica
Flow rate	1.0 mL/min
Injection volume	20 µL
Column temp	40 °C
Wavelength	214 nm

Mobile Phase for related compounds:

Mobile phase A	0.2 mol/L sulfate (Dissolve 28.4 g anhydrous sodium sulfate in 1000 mL of water, pipet 2.7 mL of phosphoric acid the solution and adjust with ethanolamine to a pH of 2.3, and mix) -acetonitrile (82:18)
Mobile phase B	Acetonitrile:water (50:50)

Referring to the gradient as follows, adjust the mobile phase composition and the duration of the isocratic elution to obtain a retention time of about 25 minutes for insulin, with the A-21 desamido insulin eluting just prior to the start of the gradient elution phase.

Time (min)	%B
0	22
35	22
61	67
67	67

Mobile phase for assay

Mobile phase A	0.2 mol/L sulfate (Dissolve 28.4 g anhydrous sodium sulfate in 1000 mL of water, pipet 2.7 mL of phosphoric acid solution and adjust with ethanolamine to a pH of 2.3, and mix)
Mobile phase B	Acetonitrile (74:26)

Materials used for the note

Sample	Porcine insulin (Provided by NIFDC China)
Columns	Agilent ZORBAX SB-C18, 4.6 × 150 mm, 5 µm (p/n 883975-902)
	Agilent ZORBAX Eclipse Plus C18, 4.6 × 150 mm, 5 µm (p/n 959993-902)
	Agilent Poroshell 120 SB-C18, 4.6 × 100 mm, 2.7 µm (p/n 685975-902)
	Agilent Poroshell 120 EC-C18, 4.6 × 100 mm, 2.7 µm (p/n 695975-302)
System	The Agilent 1200 SL LC system includes a binary pump, a thermostatted column compartment (TCC), a high performance autosampler and a diode array detector (DAD).

Results and Discussion

Traditional columns

The CHP requires a column with octadecyl silane (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 an Agilent ZORBAX SB-C18, 4.6 × 150 mm, 5 µm column. The mobile phase composition was modified according to the requirements of CHP method to obtain a retention time of about 25 minutes for insulin. The chromatogram from the analysis of related compounds is shown in Figure 1 and the assay chromatograms are shown in Figure 2.

The system suitability of insulin analysis requires the resolution between insulin and A-21 desamido insulin not less than 1.8 and the tailing factor for the insulin peak not more than 1.8. As you can see in Figure 1 and Figure 2, both traditional columns meet the suitability requirements in the CHP. The Agilent ZORBAX Eclipse Plus C18 column provides more symmetrical peaks and higher efficiency than the SB-C18 column, which may be due to two differences between the columns. The first difference is the complete endcapping of the Eclipse Plus C18 column versus no endcapping on the SB-C18 column. Endcapped columns typically have better peak shape for basic compounds. The second difference is the larger pore size (95 Å for Eclipse Plus C18, and 80Å for SB-C18). The larger pore size of the Eclipse Plus C18 column improves the efficiency and peak shape with improved diffusion in the larger pore.

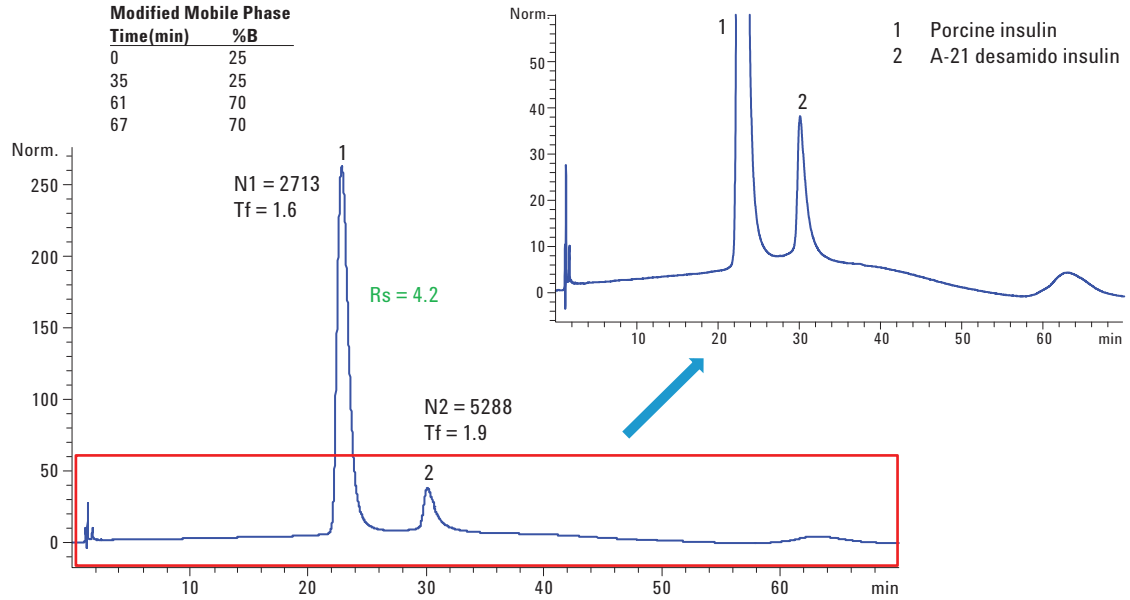

Figure 1. Chromatogram of related compounds analysis on a traditional Agilent ZORBAX SB-C18, 4.6 × 150 mm, 5 µm column.

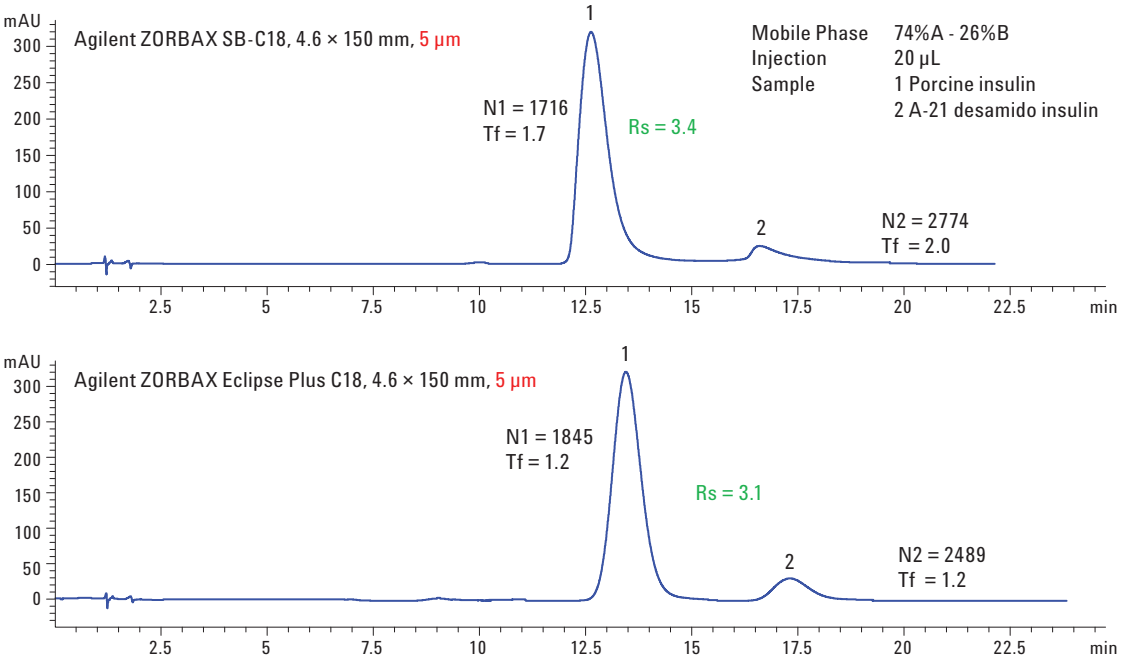

Figure 2. Chromatograms for assay analysis on traditional Agilent ZORBAX SB-C18, 4.6 × 150 mm, 5 µm and Agilent ZORBAX Eclipse Plus C18, 4.6 × 150 mm, 5 µm columns.

Agilent Poroshell 120 Columns

The related compounds analysis method was transferred to an Agilent Poroshell 120 SB-C18, 4.6 × 100 mm, 2.7 µm column. A slight change in the mobile phase composition was made to fit the requirements of the CHP. The gradient time and injection volume need to be recalculated when scaling the original method to a new one.

To maintain the resolution and overall separation the retention index K* in Equation 1 should be kept constant.

Equation 1: $K^* = (t_G F)/(S\ \Delta\Phi\ Vm)$

Where:
t_G is the gradient time
F is the flow rate
S is constant
Vm is volume of column, ($Vm = \Pi\ (d/2)^2(L)(0.6)$, L is the column length, d is the column diameter)
$\Delta\Phi$ is the change in organic percentage across the gradient segment

According to Equation 1, the flow rate and gradient time should be changed with column diameter and length.

To keep almost the same response of the peaks, the injection volume should be changed proportional to the volume of the column (Equation 2).

Equation 2: $(d_1/2)^2 L_1 = (d_2/2)^2 L_2$

Where:
d_1, d_2 are the column diameter for column 1 and column 2 separately
L_1, L_2 are the column length for column 1 and column 2 separately

The new gradient method for related compounds was run on an Agilent Poroshell 120 SB-C18, 4.6 × 100 mm, 2.7 µm column. The chromatogram and data is shown in Figure 3. The assay method was run on a Poroshell 120 SB-C18, 4.6 × 100 mm, 2.7 µm column and a Poroshell 120 EC-C18, 4.6 × 100 mm, 2.7 µm column. The Poroshell 120 columns show many improvements when compared to the traditional SB-C18 column (Figure 1). These improvements include peak shape, efficiency, and resolution. The performance on Poroshell 120 columns is 4-6 times higher than on traditional 5 µm columns. Some minor impurities were found in the chromatogram using the Poroshell 120 columns (Figure 3) due to the improved peak shape, increased efficiency, greater sensitivity, and resolution of the superficially porous columns. While these impurities may have been present in the separation on the traditional column the broader, less efficient peaks reduced the resolution such that they were not detected on the traditional 5 µm column.

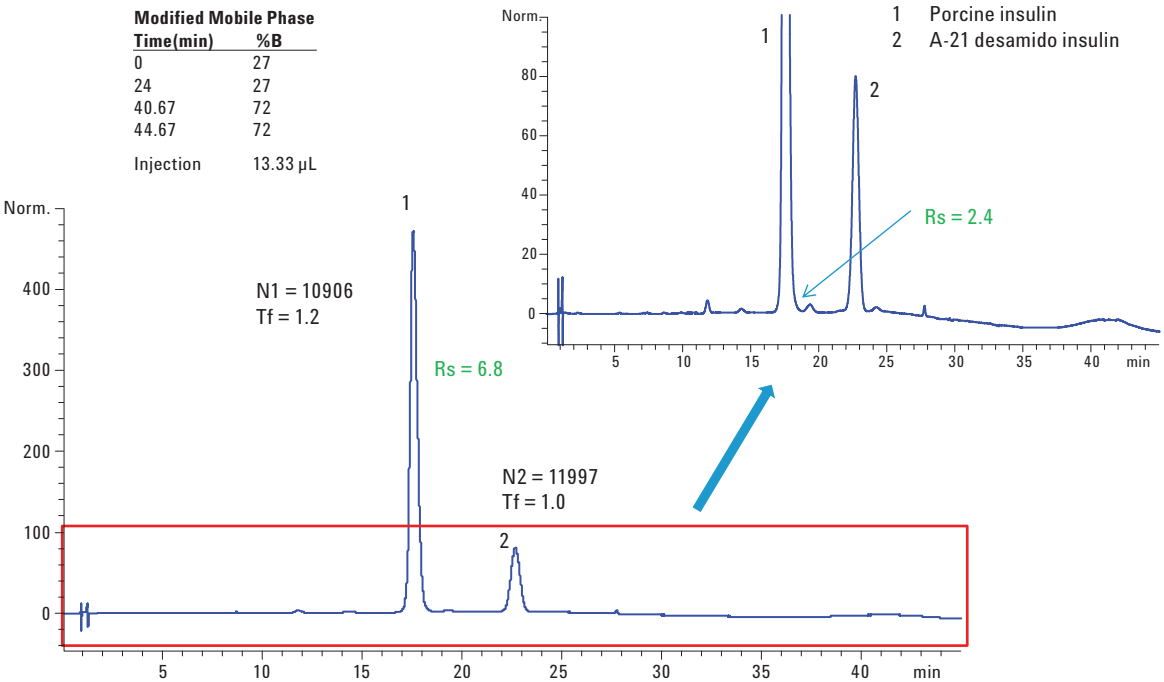

Figure 3. Chromatogram of related compounds analysis on a traditional Agilent Poroshell 120 SB-C18, 4.6 × 100 mm, 2.7 µm column.

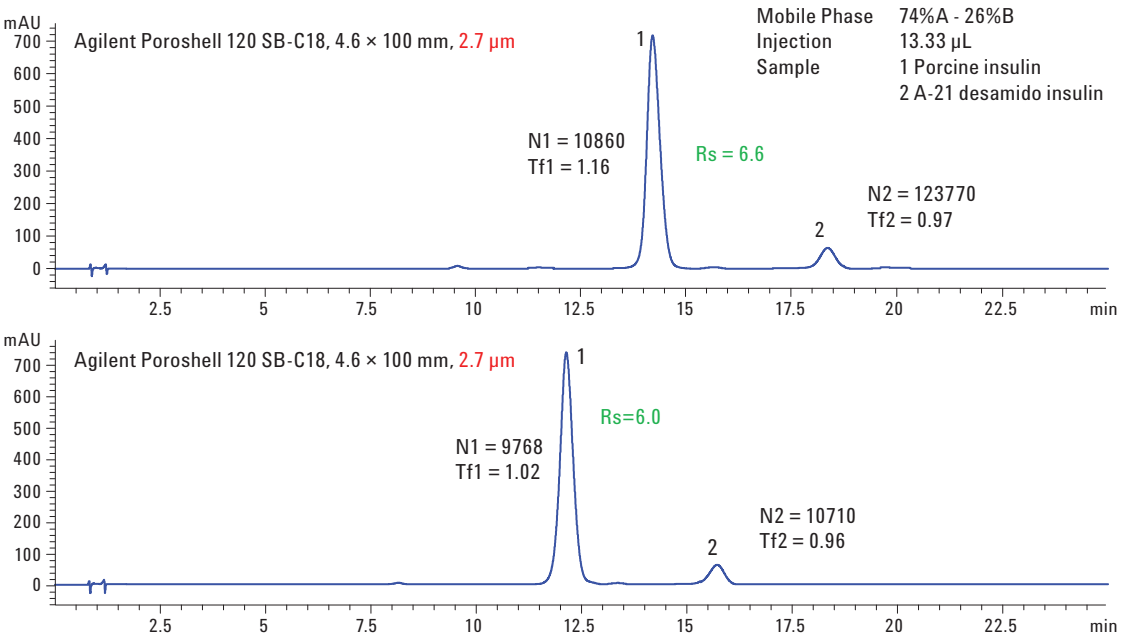

Figure 4. Chromatograms for assay analysis on Agilent Poroshell 120 SB-C18, 4.6 × 100 mm, 2.7 µm and Poroshell EC-C18, 4.6 × 100 mm, 2.7 µm columns.

Agilent Poroshell 120 SB-C18 and EC-C18 columns provide good performance for insulin analysis (Figure 4). They easily meet the system suitability requirements. The dramatic increase in performance is due to the smaller particles (2.7 µm) and the larger pore size (120 Å) of the superficially porous Poroshell 120 columns. For more information on the relationship between pore size, particle size, and molecular weight, consult publication number 5990-9028EN.

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 column (Figure 5) were 0.2% for porcine insulin and 0.4% for A-21 desamido insulin, easily meeting the requirements.

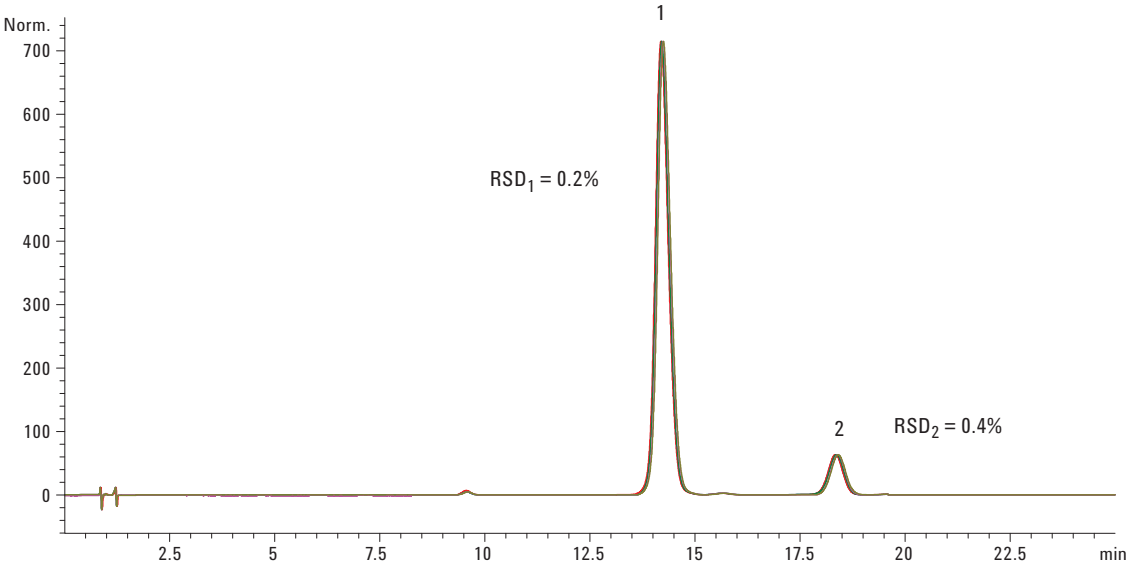

Figure 5. Overlay chromatograms of five injections on an Agilent Poroshell 120 SB-C18, 4.6 × 100 mm, 2.7 µm column.

Conclusion

The method for the analysis of insulin was successfully converted from a traditional 5 µm column to superficially porous Agilent Poroshell 120 columns with significant improvements in performance. A Poroshell column with a particle size of 2.7 µm, and pore size of 120 Å is suitable for the highly efficient analysis of small proteins, such as insulin, and can be used to meet the system suitability requirements of the CHP for insulin. The new method using the Poroshell 120 column is well suited for quality control testing of manufactured insulin.

References

1. China Pharmacopoeia (2010 edition), Insulin, 845–846.
2. The United States Pharmacopoeia USP 31 (vol 2), Insulin, 2403–2404.

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