The Influence of Silica Pore Size and Particle Size on Insulin – A Small Protein Molecule Separation

Application Note

Biopharm

Authors

Rongjie Fu
Agilent Technologies (Shanghai) Co., Ltd.
412 Ying Lun Road
Shanghai 200131
China

Maureen Joseph
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808
USA

Abstract

Insulin, a small protein molecule, was separated on columns with different pore size and particle sizes. The efficiency and resolution was compared among columns with different pore sizes including 80Å, 95Å, 120Å, 170Å, and 300Å and different particle sizes including 1.8 µm, 2.7 µm, 3.5 µm, and 5 µm.

The comparison shows that a larger pore size gives higher efficiency for insulin analysis. A pore size larger than 100Å is enough for the efficient separation of insulin and a macro pore size of 300Å is not necessary for such a moderate molecular weight molecule. A small particle size gives higher efficiency for insulin analysis. This is demonstrated by changing the particle size from 5 µm to 3.5 µm then to 1.8 µm.

The Agilent Poroshell 120 columns combination of pore size (120Å) and superficialy porus particle size (2.7 µm), makes it the best choice for insulin analysis.

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Introduction

Particle size and pore size of silica are very important parameters of a reversed phase column when chosen for HPLC analysis. Small particle size columns, those with particles less than 3 µm including sub-2 µm particles, have been widely used for achieving high performance and fast separations. Additionally, the pore size of silica particles should be considered when a column is selected. For molecules with molecular weights less than 5000 Da, a column with a small pores size (60-120Å) is typically selected, a pore size of 200-300Å is typically used for low molecular weight proteins (5 kDA – 50 kDa), and larger pore sizes (1000Å-4000Å) are used for very high molecular weight proteins and vaccines.

A small protein, such as insulin with a molecular weight of about 5800 Da, could be analyzed on columns with small pores, but larger pore size should be evaluated. The proper pore size choice maximizes efficiency. Too small a pore size and the molecule experiences restricted diffusion in and out of the pores. With an appropriate pore size, higher efficiency is seen. To maximize efficiency, smaller particle sizes with an optimum pore size should be selected.

A regulatory method (USP) for insulin with an isocratic mobile phase was used in this study of optimum pore and particle size. The performance, including efficiency and resolution, is compared among columns with different pore sizes including 80Å, 95Å, 120Å, 170Å, and 300Å and different particle sizes including 1.8 µm, 2.7 µm, 3.5 µm, and 5 µm. These choices also include different particle types, including both superficially porous and totally porous particles. The end result of this application note is recommendations for achieving the highest efficiency and resolution while still meeting the requirements of the regulatory methods of the China Pharmacopeia [1] and USP [2].

Materials and Methods

HPLC conditions

Columns	All columns were C18 columns, meeting pharmacopeia definitions for 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	74% A:26% B, where 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 into the solution, and adjust with ethanolamine to a pH of 2.3, and mix) B: acetonitrile

Materials

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 ZORBAX 300SB-C18, 4.6 × 150 mm, 5 µm (p/n 883995-902)
Agilent TC-C18(2), 4.6 × 150 mm, 5 µm (p/n 588935-902)
Agilent ZORBAX SB-C18, 4.6 × 100 mm, 3.5 µm (p/n 861953-902)
Agilent ZORBAX Eclipse Plus C18, 4.6 × 100 mm, 3.5 µm (p/n 959961-902)
Agilent ZORBAX SB-C18, 4.6 × 100 mm, 1.8 µm (p/n 828975-902)
Agilent ZORBAX Eclipse Plus C18, 4.6 × 100 mm, 1.8 µm (p/n 959964-902)
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 695975-902)

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

Influence of silica pore size on efficiency and resolution

To find out how the pore size of silica influences the efficiency, resolution, and tailing factor of the insulin separation, the same method for insulin was run on four traditional columns with a 5 µm particle size (Figure 1), but with four different pore sizes. Insulin has the poorest performance both on the SB-C18, 5 µm and the Eclipse Plus C18, 5 µm columns with the smallest pore sizes of 80Å and 95Å. A slight increase of the pore size from 80Å to 95Å provided a minimal increase in the efficiency of insulin.

The Agilent TC-C18(2) and Agilent ZORBAX 300SB-C18 columns both have a larger pore size and gave higher efficiency for insulin. Columns with a 300Å are often used for a molecular weight of more than 5000 Da. As seen in Figure 1, the efficiency of insulin more than doubled when comparing the results from the columns with <100Å pore sizes to a column with a 300Å pore size. The peak shape also improved dramatically between the smallest pore size column, (Agilent ZORBAX SB-C18) and the largest pore size column, (300SB-C18). These two columns differ only in pore size, the type of bonding and particle size of the columns are the same. This provides the most exact comparison and shows clearly that a pore size larger than 80Å is needed for the most efficient separation of insulin.

The fourth column compared, the Agilent TC-C18(2) has a 170Å pore size that is intermediate between the 80Å, 95Å, and 300Å pore sizes. The efficiency on this column was also more than double the efficiency of the smaller pore size columns, indicating that a 300Å pore size is not necessary, but a pore size >100Å is needed for insulin molecule at 5800 Da to fully access the pores for an efficient separation.

When we compare the four 5 µm columns in more detail, we see some differences in retention. These four columns have different surface area and types of bonding. The 300SB-C18 column has the lowest surface area at about 45 m^2/g, followed by the Agilent ZORBAX Eclipse Plus C18 at 160 m^2/g, then SB-C18 at 180 m^2/g, and finally the TC-C18(2) at 290 m^2/g. The retention is most similar on the two columns with the smallest pore sizes, the Eclipse Plus C18 and the SB-C18, however these are not the lowest surface area. Without being able to fully access the pores and the bonded phase, the retention is limited. The C18 bonding on the Eclipse Plus C18 and the SB-C18 is very different. The SB-C18 has a low carbon load and no endcapping. The Eclipse Plus C18 has a higher carbon load, with denser bonding and thorough endcapping. While this change in the bonding helps improve peak shape it does not change retention because the insulin is not interacting much with the bonded phase in the pores.

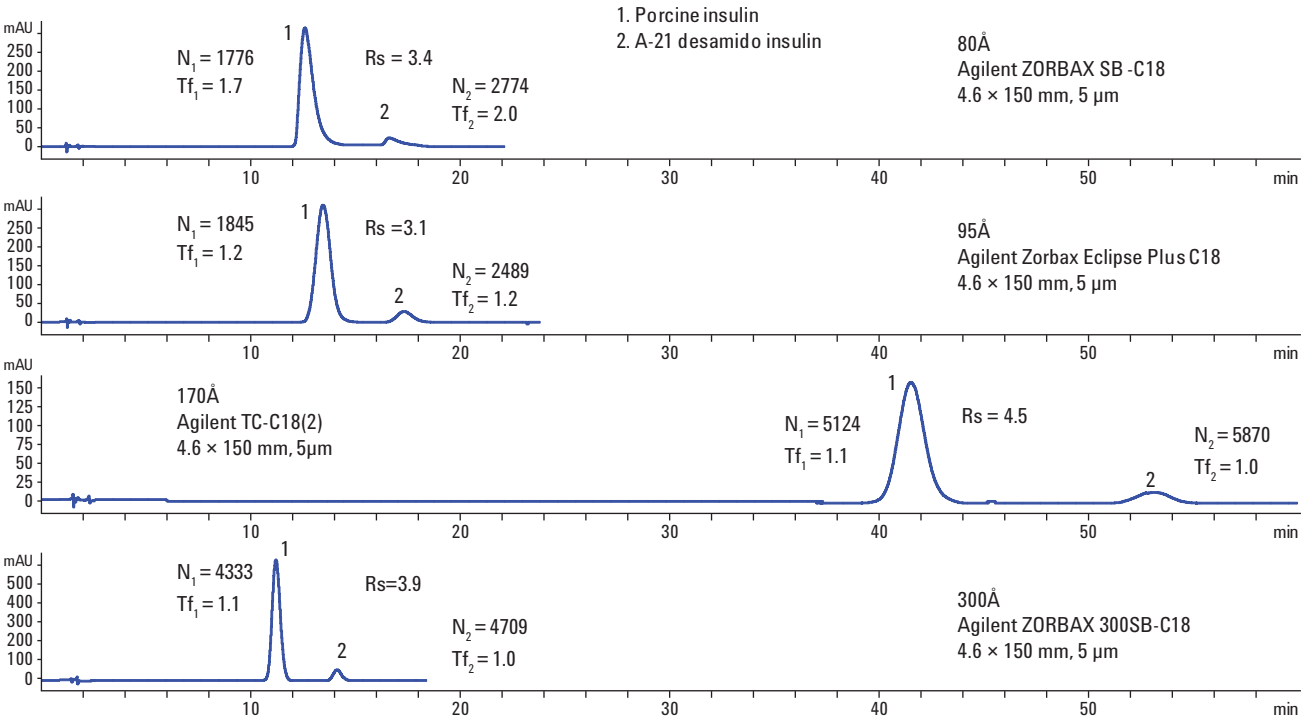

Figure 1. Chromatograms on 4.6 × 150 mm, 5 µm columns with different pore size.

The Agilent ZORBAX 300SB-C18 column has the lowest surface area, but the retention is only slightly less than the small pore size columns. Once the insulin can access the pores and interact with all of the bonded phase in the pores it is better retained. This more efficient access is also seen in the improved peak shape, even on a non-endcapped bonded phase. The Agilent TC-C18(2) column has the highest surface area and the strongest retention, most likely due to accessing all the pores and retention increasing with this higher surface area of silica particles (290 m^2/g).

The retention of the Agilent TC-C18(2) column is very long, at over 40 minutes, and not ideal. Therefore the method on the TC-C18(2) column could be modified with a higher organic component in the mobile phase to substantially reduce the retention time, while still maintaining good peak shape and efficiency (Figure 2). The retention is reduced from 44 minutes to 15 minutes by increasing the organic in the mobile phase by only 1.5%. When organic content in the mobile phase is changed, large molecules (such as proteins) show a much greater change in retention than small molecules, so it is easy to adjust the retention time substantially. The resolution is slightly reduced, but similar to the other 5 µm columns once the retention matches the other columns.

The result of comparing these four 5 µm columns is that the 170Å pore size is large enough to provide access to the pores and results in an efficient separation. Pore sizes >100Å are too small, and for a mid molecular weight compound a pore size of 300Å is not necessary.

Table 1. Theoretical Plates per M on 5 µm Columns with Different Pore Sizes

N/m	80Å	95Å	170Å	300Å
Peak 1	11840	12300	34160	28887
Peak 2	18493	16593	39133	31393

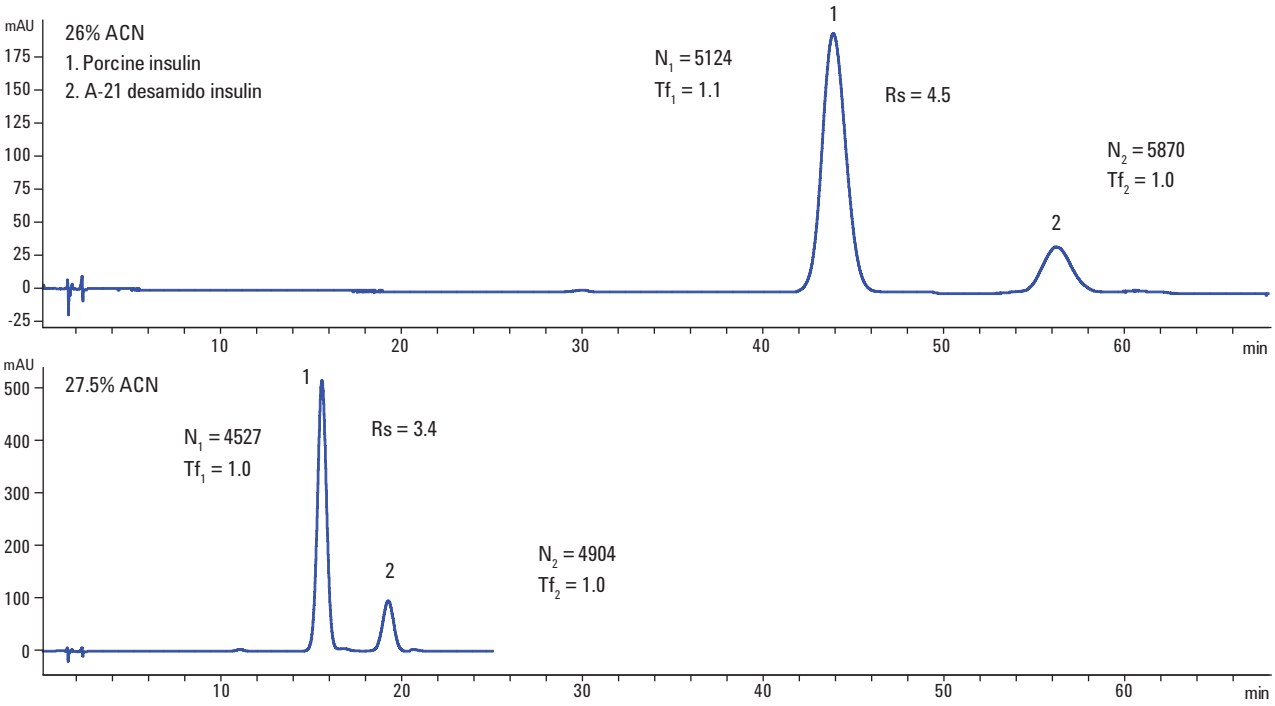

Figure 2. Organic phase modification on an Agilent TC-C18(2), 4.6 ×150 mm, 5 µm column.

Influence of silica particle size

For method development, the standard particle size for HPLC columns was 5 µm until the mid-1990s, when 3.5 µm increased in popularity. More recently, as higher speed and higher resolution is required, chromatographers often select packings with particle sizes less than 3 µm or 2 µm, such as 1.8 µm particles.

The insulin method was run on 5 µm, 3.5 µm, and 1.8 µm Agilent ZORBAX SB-C18 columns and Agilent ZORBAX Eclipse Plus C18 columns. To make sure the data are comparable with different length of columns, the theoretical plate counts were all divided by the length of column to represent the efficiency in plates/meter (N/m). The data in Tables 2 and 3 shows improved efficiency using the smaller 1.8 µm particle columns. While these columns are not the ideal pore size columns, efficiency should still be expected to improve with smaller particle sizes, as it does here with insulin.

Table 2. Theoretical Plates per M on an Agilent ZORBAX SB-C18 with Different Particle Sizes

N/m	SB-C18 5 µm	SB-C18 3.5 µm	SB-C18 1.8 µm
Peak 1	11840	18060	52150
Peak 2	18493	21720	66910

Table 3. Theoretical Plates per M on an Agilent ZORBAX Eclipse Plus C18 Columns with Three Different Particle Sizes

N/m	Plus 5 µm	Plus 3.5 µm	Plus 1.8 µm
Peak 1	12300	21570	78540
Peak 2	14580	26040	94340

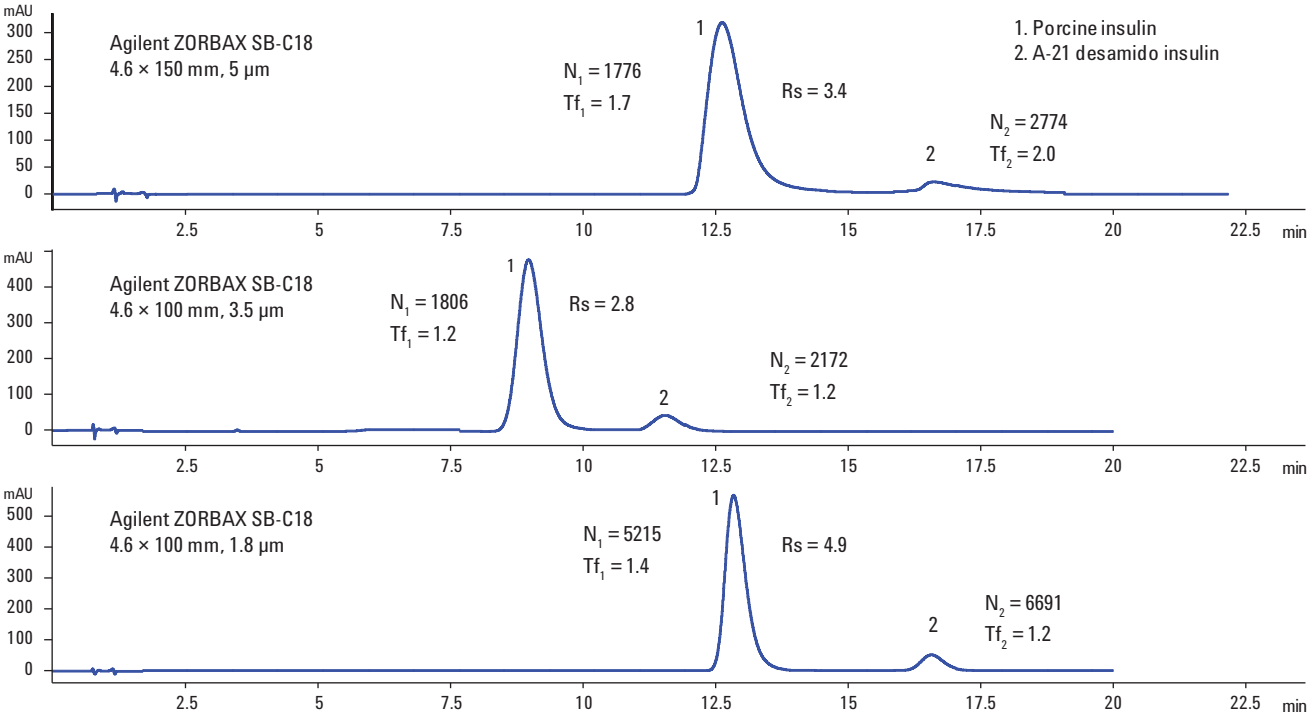

Figure 3. Chromatograms on Agilent ZORBAX SB-C18 columns with different particle sizes.

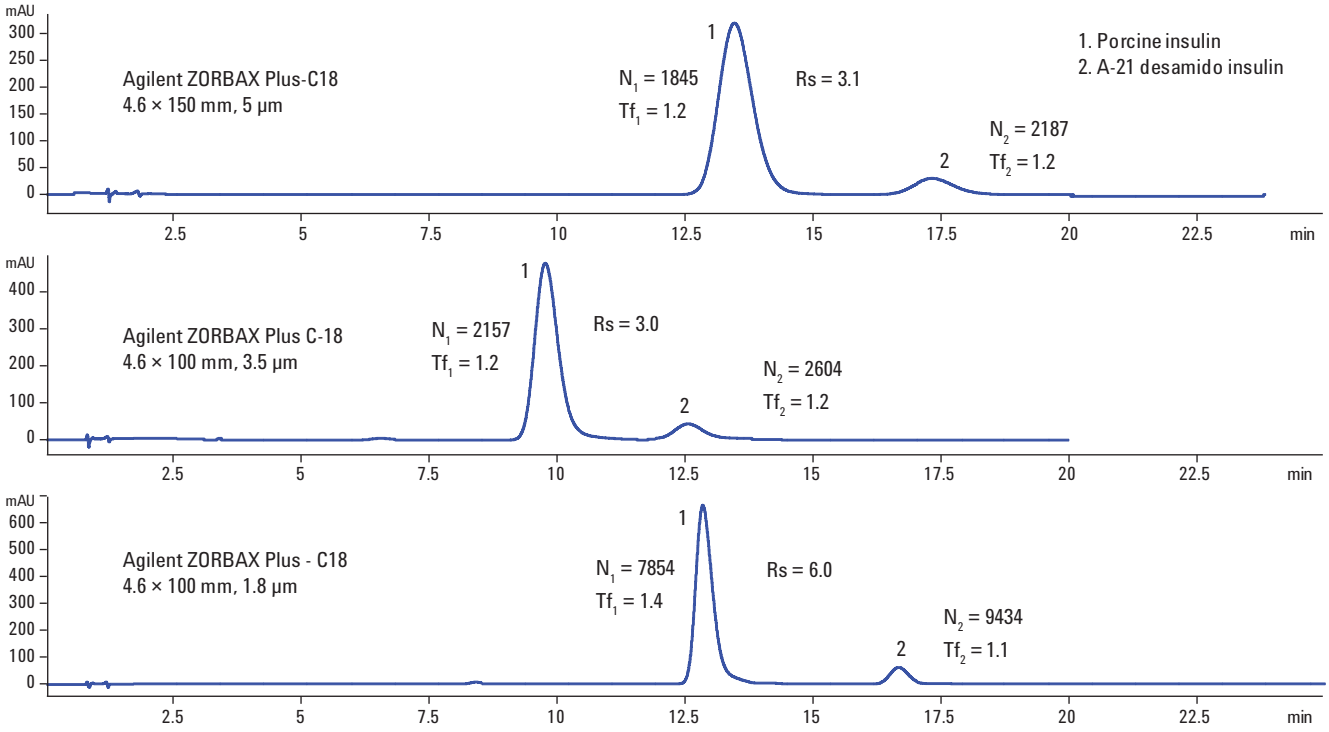

Figure 4. Chromatograms on Agilent ZORBAX Eclipse Plus C18 columns with three different particle sizes.

Comparison between superficially porous Agilent Poroshell 120 2.7 µm and totally porous sub-2 micron columns

The columns compared thus far have all been totally porous columns, most available in a range of pore and particle sizes. The Agilent Poroshell 120, 2.7 µm superficially porous particle column, has a 120Å pore size and a sub 3 µm particle size. The 120Å pore size is >100Å but smaller than the 170Å pore size previously compared. In addition, the 2.7 µm particle size is known to provide similar performance to the sub-2 micron particle columns for small molecules (less than 2000 Da). For molecules around 5000 molecular weight, like insulin, a comparison is needed.

Figure 5 shows the direct comparison of two Poroshell 120 columns with different bonded phases to the closest matching 1.8 µm particle size columns, the Agilent ZORBAX SB-C18 and Agilent ZORBAX Eclipse Plus C18. In these chromatograms, the Poroshell 120 SB-C18 120Å column provides double the efficiency of the SB-C18 80Å. This is due to the larger pore size and more rapid diffusion in the 120Å pores. Switching from the Agilent ZORBAX Eclipse Plus C18 to Agilent Poroshell 120 EC-C18 also provided an increase in efficiency with the change from 95Å to 120Å pore size. In addition, the peak shape of insulin on both Poroshell 120 columns was improved with greater access to the pores (Figure 5). The data in Table 4 provides the theoretical plates per meter (N/m) of the 2.7 µm and 1.8 µm particle sized and shows the increase when using a Poroshell 120 column.

The pressure of the 1.8 µm columns is around 250 bar. The pressure of the Poroshell 120 columns is below 200 bar. Both kinds of column can be used on standard instruments, but Porshell 120 columns are more suitable for standard instruments because they have about 30% lower pressure than the 1.8 µm columns.

Figure 6 shows the Agilent Poroshell 120 SB-C18 column provides higher efficiency than the 5 µm Agilent TC-C18(2). The chromatogram shown for the TC-C18(2) column was run with 27.5% organic in the mobile phase so that the retention times could be more closely matched. The improvement in the efficiency when comparing these two columns is due to the benefit of the smaller particle size with a larger pore size.

Table 4. Theoretical Plates per M on an Agilent Poroshell 120 and Sub-2 µm Columns

N/m	SB-C18, 1.8 µm	Poroshell 120, SB-C18, 2.7 µm	Plus C18, 1.8 µm	Poroshell 120, EC-C18, 2.7 µm
Peak 1	52150	108600	78540	97680
Peak 2	66910	123770	94340	107100

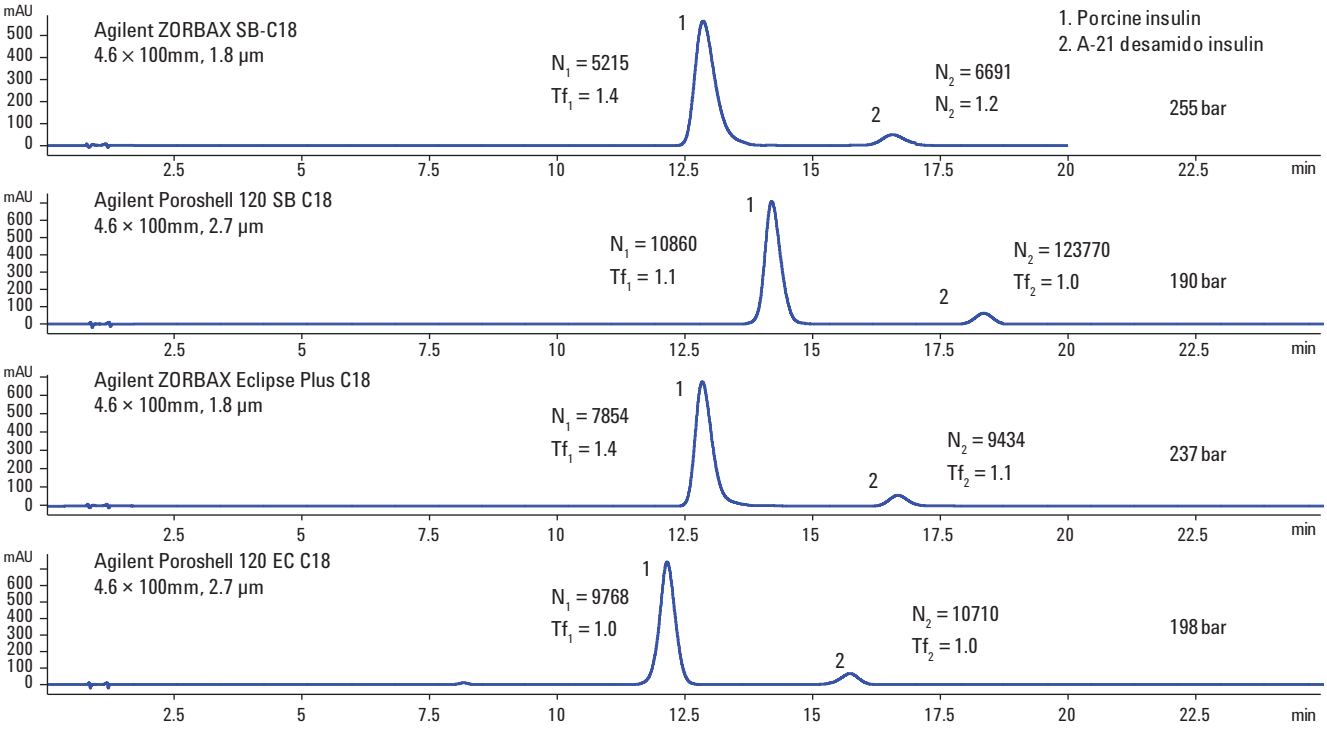

Figure 5. Chromatograms for comparison between Agilent Poroshell 120 and sub-2 micron columns.

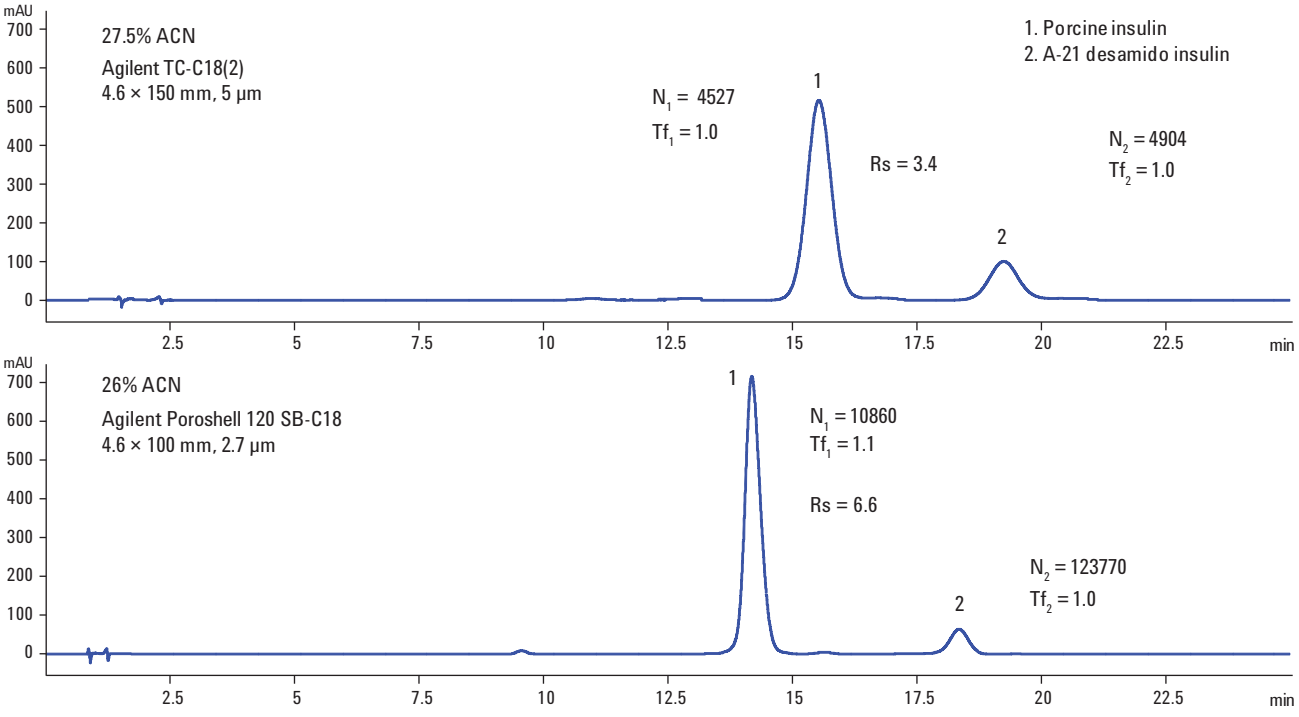

Figure 6. Chromatograms for comparison between Agilent Poroshell 120 SB-C18 and Agilent TC-C18(2) columns.

The remaining obvious column comparison would be between the sub-2 µm particles with 300 Å pore size in the 300SB-C18 material and the 80Å SB-C18. This is something that will be done in the future with the mobile phase called for in this method, but under different conditions, insulin has been shown to have good performance on this column [3].

Conclusion

The comparisons shown here were done to determine what type of C18 columns could effectively be used for the analysis of insulin according to pharmacopeia methods. Columns with too small a pore size, <100Å did not provide the best results. The efficiency on these columns was low and the tailing factors were a little high due to the restricted access to the bonded phase in the pores of these columns. Columns with a larger pore size, >100Å, such as the Agilent Poroshell 120, Agilent TC-C18(2), and Agilent ZORBAX 300SB-C18 provided much higher efficiency and lower tailing factors. A pore size as large as 300Å was not needed for an efficient separation of insulin. The intermediate pore size columns, Poroshell 120 and TC-C18(2), were suitable and would be a good choice for separations of other small proteins or peptide mapping.

Particle size was evaluated to compare efficiency and resolution. Smaller particle sizes provided the highest efficiencies. The Agilent Poroshell 120 columns delivered the most efficiency. These columns had a 2.7 µm particle size with a pore size of 120Å and are suitable for the highly efficient analysis of insulin.

References

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2. The United States Pharmacopoeia USP 31 (vol 2) Insulin, 2403–2404.
3. Phu T Duong, Analysis of Oxidized Insulin chains using Reversed Phase Agilent ZORBAX RRHD 300 SB-C18, Agilent application note, 5990-7988EN.

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