The influence of silica pore size and particle size on insulin - a small molecule of protein separation

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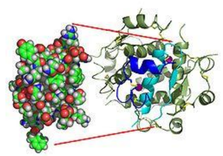
Abstract

Particle size and pore size of silica are very important parameters of a reversed phase column for HPLC analysis. Small particle sizes, less than 3 µm and including sub-2µm, have been widely used for achieving high performance and fast separations. One additional factor that should be considered is the pore size of the silica particle. Normally you use column packings with small pores (60-120Å) for small molecules (less than 5000 Da), use 150-300Å for peptides and low molecular weight proteins and use large pore sizes (1000Å-4000Å) for very high molecular weight proteins and vaccines.

A small protein, such as insulin with a molecular weight of about 5800 Da could potentially be analyzed on columns with small pores or slightly larger pores. To maximize efficiency smaller particle sizes with an optimum pore size should be selected. An insulin regulatory method (USP) with an isocratic mobile phase was used in this study of optimum pore and particle size. The performance and resolution is compared among columns with different pore size including 80Å, 95Å, 120Å, 170Å and 300Å and as well as different particle sizes including 1.8µm, 2.7µm, 3.5µm and 5µm. These choices also include different particle types, including both superficially porous and totally porous particles. The end result of the work shown here are recommendations for achieving the highest efficiency and resolution while still meeting the requirements of the regulatory methods.



Experimental

All the columns explored in this paper were C18 columns, meeting pharmacopeia definitions for octadecyl silane (C18), chemically bonded to porous silica.

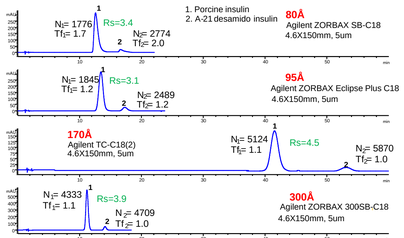
HPLC Conditions

Sample: Porcine insulin (NIFDC China)
Columns: Agilent ZORBAX SB-C18, 4.6 x 150 mm, 5 µm (p/n 883975-902)
ZORBAX Eclipse Plus C18, 4.6 x 150 mm, 5 µm (p/n 959993-902)
ZORBAX 300SB-C18, 4.6 x 150 mm, 5 µm (p/n 883995-902)
ZORBAX SB-C18, 4.6 x 100 mm, 3.5 µm (p/n 861953-902)
ZORBAX Eclipse Plus C18, 4.6 x 100 mm, 3.5 µm (p/n 959961-902)
ZORBAX SB-C18, 4.6 x 100 mm, 1.8 µm (p/n 828975-902)
ZORBAX Eclipse Plus C18, 4.6 x 100 mm, 1.8 µm (p/n 959964-902)
Agilent Poroshell 120 SB-C18, 4.6 x 100 mm, 2.7 µm (p/n 685975-902)
Agilent Poroshell 120 EC-C18, 4.6 x 100 mm, 2.7 µm (p/n 695975-902)
Agilent TC-C18(2), 4.6 x 150 mm, 5 µm (p/n 588935-902)
System: Agilent 1200 SL LC with binary pump, thermostatted column compartment, high performance autosampler and diode array detector
Mobile Phase: 74% A:26% B, where A: 0.2 mol/L sulfate (dissolve 28.4g anhydrous sodium sulfate in 1000 mL of water, pipet 2.7 mL of phosphoric acid into the solution and adjust with ethanolamine to pH 2.3, and mix) B: acetonitrile
Flow Rate: 1.0 mL/min
Injection Vol: 20 µL
Temp: 40 °C
Detector: UV 214 nm

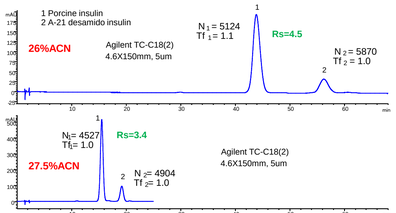
Results and Discussion

Influence of silica pore size, 5µm particles

The method for insulin was first run on four traditional columns with a 5 µm particle size, but with four different pore sizes.

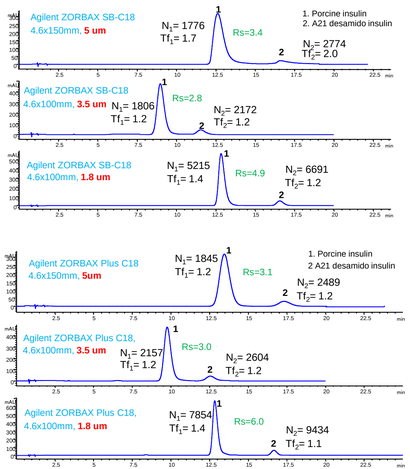


Insulin has the poorest performance both on the SB-C18, 5 µm and the Eclipse Plus C18, 5 µm columns with the smallest pore sizes of 80Å and 95Å. The Agilent ZORBAX 300SB-C18 column has the lowest surface area, but the retention is only slightly less than the small pore size columns. Once the insulin 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 m2/g).



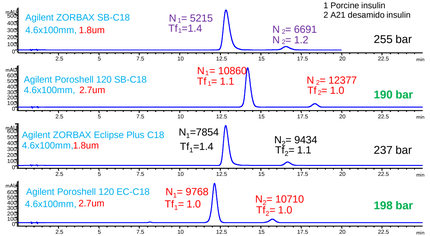
The retention on the Agilent TC-C18(2) column is very long, at over 40 minutes, and not ideal. Therefore the retention is reduced from 44 minutes to 15 minutes by increasing the organic in the mobile phase by only 1.5% while still maintaining good peak shape and efficiency

Influence of silica particle size

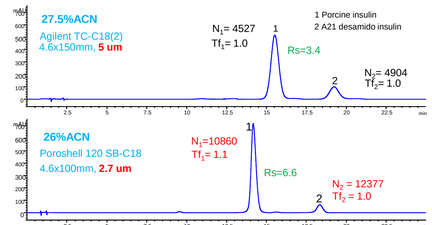


The insulin method was run on 5 µm, 3.5 µm, and 1.8 µm Agilent ZORBAX SB-C18 columns and Agilent ZORBAX Eclipse Plus C18 columns. The data demonstrate improved efficiency using the smaller 1.8 µm particle columns. While these columns are not the ideal pore size columns, efficiency should still be expected to improve with smaller particle sizes, as it does here with insulin.

Comparison between superficially porous and totally porous columns



In these chromatograms the Poroshell 120 SB-C18 2.7um column provides 2X the efficiency of the 80Å SB-C18 1.8µm. This is due to the larger pore size and more rapid diffusion in the 120Å pores. Switching from the ZORBAX Eclipse Plus C18 to 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.



In addition, the Poroshell 120 column provides higher efficiency than the 5um Agilent TC-C18 (2). The chromatogram shown on the TC-C18 (2) column is the one 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.

Conclusions

- Columns with a small a pore size, <100Å did not provide the best results for insulin, because of the restricted access to the bonded phase in the pores of these columns.
- Columns with a larger pore size, >100Å, such as the Poroshell 120, Agilent TC-C18(2), and 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, the Poroshell 120 and TC-C18 (2) columns were suitable and would therefore be a good choice for separations of other small proteins or peptide mapping.
- The smaller particle sizes provided the highest efficiencies. The Poroshell 120 columns had a 2.7µm particle size with a pore size of 120Å and were suitable for the highly efficient analysis of insulin.

Reference

[1] China Pharmacopoeia (2010 edition), insulin, page. 845 – 846.
[2] The United States Pharmacopoeia USP 31 (vol 2) – Insulin, page. 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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