Use of Temperature to Increase Resolution in the Ultrafast HPLC Separation of Proteins with ZORBAX Poroshell 300SB-C8 HPLC Columns

Application

Pharmaceutical

Robert D. Ricker, Jerry J. Lewis, Sr., and Erin Arenz

Introduction

For separations containing ionizable compounds, temperature is a simple tool that can effectively increase separation selectivity and, therefore, resolution. For peptides and proteins, noted peak shifts with increased or decreased temperature may be more significant than those observed with more time consuming mobile phase modifications. Since ZORBAX Poroshell 300SB columns are stable at temperatures up to 90 °C at low pH, these columns not only provide ultrafast, high resolution separations, they also offer the opportunity to use temperature to assist in optimizing resolution of these bioseparations.

Results

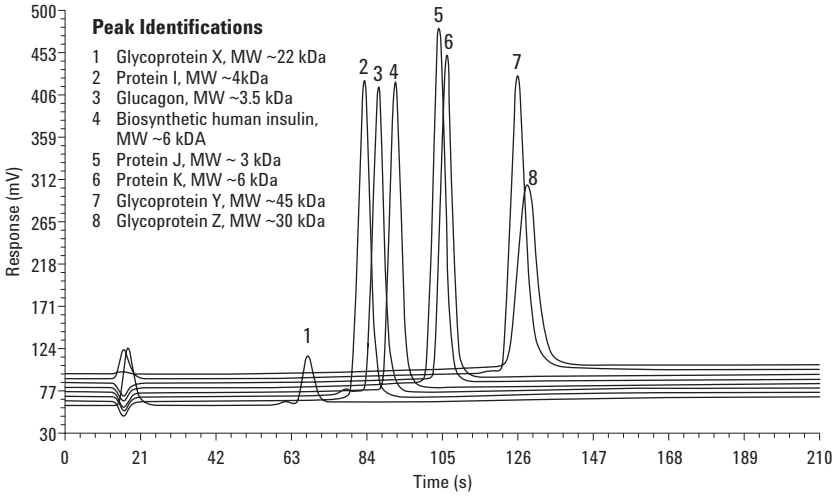
Figures 1 through 3 show overlayed chromatograms for eight proteins, varying in molecular weight from 3 to 45 kDa, and including insulin, glucagon, and glycosylated proteins. Separation of this diverse group of proteins is an obvious challenge. Each analyte, eluted from a ZORBAX Poroshell 300SB-C8, 2.1 × 75 mm, 5-µm column, with an impressive retention time (RT) of less than 2 min. The gradient used in each chromatographic set is linear, from 20%–70% B in 3 min; the flow rate is 1.0 mL/min, while column temperature is changed from 40 to 60 °C and then to 75 °C.

Comparing the overlayed sets of chromatograms in Figures 1 and 2, the resolution between peaks 4, 5, and 6 increased substantially, while little movement was noted between peak pairs 2/3 and 7/8 when elevating the column temperature from 40 to 60 °C. Elevating the operating temperature to 75 °C (see Figure 3) results in shifts in elution times for peaks 2, 3, 4, 5, and 6. The usefulness of temperature change in providing resolution is more obvious if one considers individual peak pairs 2 and 4, 3 and 4, and 5 and 6. Each pair shows significant improvement in resolution as temperature is elevated from 40 to 60 °C and then to 75 °C. Resolution between the peak pair 7 and 8 is best at 75 °C, further demonstrating that temperature change can induce considerable shifts in protein retention.

Highlights

- ZORBAX Poroshell 300SB HPLC columns are designed to provide ultrafast high resolution of peptides and proteins with excellent reproducibility – resolving complex peptide and protein samples in minutes.
- The unmatched stability of StableBond (SB) bonding at low pH enables the routine use of Poroshell 300SB columns at temperatures as high as 90 °C.
- The dynamic temperature range of the ZORBAX Poroshell 300SB columns allows the use of temperature as an effective tool to increase resolution, reduce back pressure, and shorten run times – even for the most rapid protein separations.

In addition to its utility of operation at elevated temperatures, design of the particles comprising in Poroshell SB packings facilitates the use of higher flow rates without loss in resolution. This is possible because of Poroshell's superficially porous particles. Large molecules, which diffuse very slowly compared to small molecules, can move very quickly into and out of Poroshell's very thin (0.25 µm) outer porous layer–stopped by the solid silica core. A flow rate of 1.0 mL/min is five times that commonly used with a 2.1-mm id column and permits the use of a short gradient time of 3 min, with resulting rapid sample throughput.



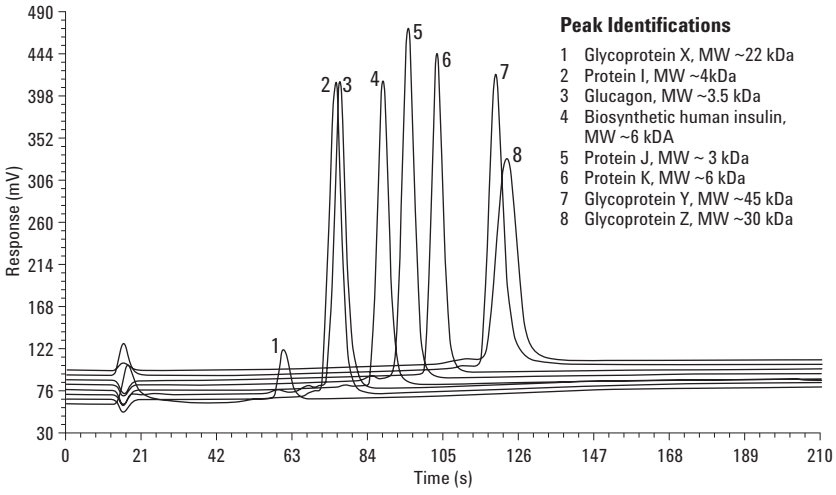
Mobile phase
A = 0.1% TFA in H_2O
B = 0.1% TFA in ACN

Gradient	
Time min	Solvent B
3	20%–70%

Conditions

Column:	**ZORBAX Poroshell 300SB-C8**, 2.1 × 75 mm, 5 µm (p/n 660750-906)
Column temperature:	40 °C
Flow rate:	1.0 mL/min
Detection:	UV (214 nm)

Figure 1. Protein elution pattern on ZORBAX Poroshell 300SB-C8 column at 40 °C.



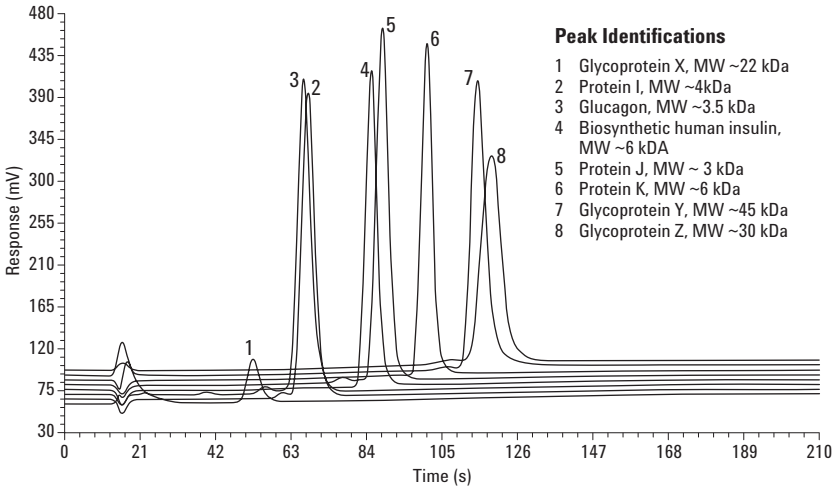
Mobile phase
A = 0.1% TFA in H_2O
B = 0.1% TFA in ACN

Gradient	
Time min	Solvent B
3	20%–70%

Conditions

Column:	**ZORBAX Poroshell 300SB-C8**, 2.1 × 75 mm, 5 µm (p/n 660750-906)
Column temperature:	60 °C
Flow rate:	1.0 mL/min
Detection:	UV (214 nm)

Figure 2. Protein elution pattern on ZORBAX Poroshell 300SB-C8 column at 60 °C.



Mobile phase
A = 0.1% TFA in H_2O
B = 0.1% TFA in ACN

Gradient	
Time min	Solvent B
3	20%–70%

Conditions	
Column:	**ZORBAX Poroshell 300SB-C8**, 2.1 × 75 mm, 5 µm (p/n 660750-906)
Column temperature:	75 °C
Flow rate:	1.0 mL/min
Detection:	UV (214 nm)

Figure 3. Protein elution pattern on ZORBAX Poroshell 300SB-C8 column at 75 °C.

Conclusions

ZORBAX Poroshell 300SB, 2.1 × 75 mm, 5-µm HPLC columns are an excellent choice for the rapid separation of proteins, offering high-resolution separations in minutes and the best option for using elevated temperature at low pH to increase resolution between overlapping protein peak pairs. Investigating the effects of temperature on resolution is a straightforward approach to separation optimization that can be more effective and convenient than changing other separation parameters (for example, the mobile phase composition). In this example, a mixture of very different protein molecules (insulin, glucagon, other proteins and glycoproteins) could be eluted with significantly different selectivity in less than 2 min. The actual turn-around time from injection to injection would be about 3.5 min. The power of Poroshell in method development should not be underestimated, as gradient, temperature, mobile-phase type, and detection settings can be changed, the column re-equilibrated, and the separation reassessed, all very rapidly.

Instrumentation

All work was performed using an Agilent 1100 LC equipped with a solvent degasser, binary pump, auto-sampler, heated column compartment and an ultraviolet detector.

For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem.

The authors, Robert D. Ricker (Applications Specialist) is based at Agilent Technologies, Wilmington, Delaware, Jerry J. Lewis, Sr. Research Scientist and Erin Arenz, Analytical Chemist, Biopharmaceutical R and D are located at Lilly Research Laboratories, Eli Lilly and Company, Lilly Tech Center, Indianapolis, Indiana 46221.

Printed in the USA
February 24, 2004
5989-0589EN

Agilent Technologies