Scale-Up of Protein Separations from Analytical to Semipreparative HPLC

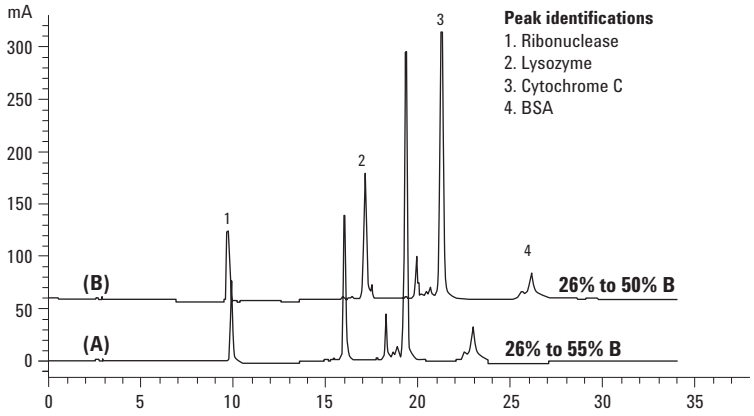
Application

Biochemistry

William Long

Introduction

Method development for preparative HPLC is the same as for analytical HPLC–adjusting conditions for optimal chromatographic performance. That is why it is convenient and cost-effective to modify the analytical column method by scaling up the analytical conditions to achieve the preparative separation needed for collection/isolation of the compound. Data presented here demonstrate how easy it is to develop a gradient separation for four proteins on a 4.6 × 250 mm, ZORBAX 300SB-C8, 5 µm, analytical column and then optimize for sample load on a 9.4-mm id column of identical column length and packing material.



Conditions

Column	**ZORBAX 300SB-C8** 4.6 × 250 mm, 5 µm
Agilent part number	880995-906
Injection volume	5 µm
Flow rate	1.0 mL/min
Detector	UV (280 nm)
Temperature	40 °C

Figure 1. Optimizing gradient conditions for protein separation on a ZORBAX 300SB-C8, 4.6 × 250 mm, 5-µm column.

Highlights

- Use of ZORBAX 300SB analytical columns provide quick and easy scale-up of protein separations from analytical to preparative HPLC.
- Use of ZORBAX 300SB analytical columns optimize separations and reduce costs.
- ZORBAX scalable analytical and semipreparative columns minimize time spent on method development.

Mobile phase

A = 0.2% TFA in water

B = 0.15% TFA in acetonitrile

Gradient timetable

Solvent change	Time (min)
(A) = 26%–55% B	30
(B) = 26%–50% B	30

Method Development

Using an analytical column to develop a preparative method not only streamlines the process of preparative method development, but minimizes the amount of solvent and sample required for method optimization. For this protein scale-up exercise, a wide-pore packing, ZORBAX 300SB-C8 is selected, which maximizes diffusion of large molecules into the pore structure of the packing, thereby maximizing protein peak separation efficiency and sample loading. The ZORBAX 300SB-C8 analytical and semipreparative columns chosen have inner diameters of 4.6 and 9.4-mm id respectively and have identical column lengths, making gradient scale-up, in particular, straightforward.

Using a trifluoroacetic acid/acetonitrile (TFA/ACN) gradient, four proteins are resolved on the selected analytical column, as shown in Figure 1 (A). Gradient conditions were optimized to give a good overall separation. Since our goal was to scale up to a preparative column and we wanted maximum resolution between the individual proteins, the actual gradient used was slightly shallower (Figure 1 (B)) with a slight increase in analysis time. In general, gradient conditions accommodate larger injection volume—up to 100 µL, equivalent to a loading of 1 mg/protein. This separation was performed under these finalized conditions without significant loss in resolution (results not shown here but consult reference [1]). Now we are ready for preparative scale-up.

Preparative Scale-Up

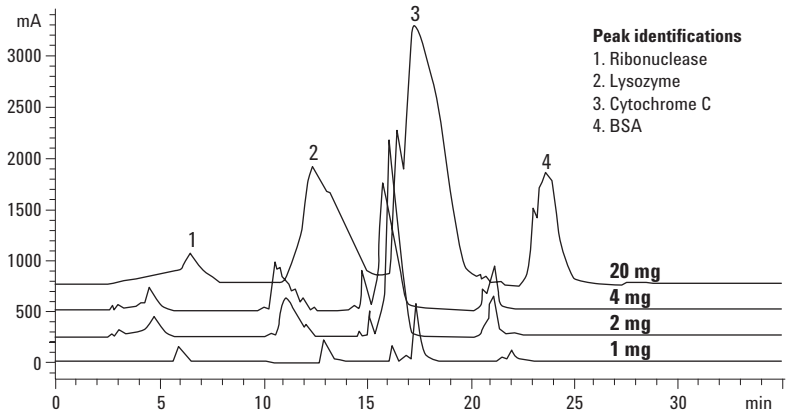
As stated previously, since Agilent ZORBAX analytical, semiprep and PrepHT preparative columns are packed with identical packings and with identical column lengths, scale-up can simply involve the change of columns and flow rate. Flow velocity must be constant to maintain the same time as for the analytical separation. The flow rate used in preparative separations is higher at the same linear velocity because the column diameter, and thus the volume, is larger.

Flow rate scale-up

$$F_{preparative} = [d_{preparative}/d_{analytical}]^2 \times F_{analytical}$$

d = Diameter of the column
F = Flow rate

Using the scale-up calculation above, Figure 2 shows the final separation achieved using the ZORBAX 300SB-C8 semipreparative (9.4 × 250 mm, 5 µm) column under various loading conditions. Because the lengths of the analytical semipreparative columns used here are the same, we are able to ratio the column diameters to calculate the semipreparative flow rate of 4.18 mL/minute, while leaving all other gradient conditions unchanged. Good resolution is maintained as the concentration load is increased from 1 to 4 mg/protein. There is a slight decrease in retention time as the sample load increases as well as some peak fronting as the column approaches an overloaded condition. Note that for the largest mass injection (20-mg/protein), a 0.5-mL loop was required and this extra volume caused a longer shift in retention. A total of 80 mg of total protein was successfully loaded and separated on this semipreparative column.



Mobile phase

A = 0.2% TFA in water

B = 0.15% TFA in acetonitrile

Gradient timetable

Solvent change	Time (min)
26%–50% B	30

Conditions

Column	**ZORBAX 300SB-C8** 9.4 × 250 mm, 5 µm
Agilent part number	880995-206
Injection volume	Varies (100 to 500 µL)
Flow rate	4.18 mL/min
Detector	UV (280 nm)
Temperature	40 °C

Figure 2. Scaling-up a gradient separation for a mixture of proteins.

Conclusion

This work demonstrates that with scalable columns, such as ZORBAX analytical, semipreparative and PrepHT columns, carrying out method development work on less expensive analytical columns can save time and money, especially for solvent costs. The process of optimizing the separation on small matched columns, adjusting mobile phase conditions to achieve the necessary separation factor and plate count, is successful because the column surface chemistry is identical, minimizing problems when implementing the methods on larger scale preparative columns.

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.

Reference

1. W. Long, (2003), *Separation Times*, **16** (2), 15–16, Agilent Technologies publication 5988-9110EN www.agilent.com/chem

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The author, William Long, is a technical support chemist based at Agilent Technologies, Wilmington, Delaware.

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