OPTIMIZING SEPARATION CONDITIONS FOR THE ISOLATION AND PURIFICATION OF SYNTHETIC PEPTIDES

Thomas E. Wheat, Jo-Ann Jablonski, Elizabeth Gildea, and Jeffrey R. Mazzeo
Waters Corporation; 34 Maple Street; Milford, MA01757

INTRODUCTION

Peptides are used in basic research as well as in biopharmaceutical development. It is common to use synthetic peptides rather than isolating natural products. Such synthetic products may not be sufficiently homogeneous for use in experiments requiring bioactivity and specificity. Isolation and purification, therefore, become essential steps in a peptide synthesis facility. We have examined the factors that influence purification protocols. This includes selection of a column, operating parameters based on the properties of the peptides and the intended use of the product. A panel of synthetic peptides covering a range of properties including size, hydrophobicity, and isoelectric point is used for these evaluations. These test samples were used to measure the importance of pore size and particle size. Under these controlled conditions, there is little difference between 130Å and 300Å up to 40 residues. More resolution was observed with 5µm particles than with 10µm. The uses of the purified peptides may constrain the mobile phase constituents. The suitability of biocompatible mobile phases, including acetic acid in place of TFA and ethanol instead of acetonitrile affected the selectivity of the separation. In some comparisons, selectivity effects could be related to the peptide sequence while other changes are still open to interpretation. An additional set of experiments has been performed investigating conditions for classes of peptides that have extreme or special properties. We show the impact of elevated temperature as well as the use of high pH and /or alternative solvents for peptides that are very difficult to dissolve in common chromatographic solvents. These experimental observations are combined into a suggested protocol for developing an isolation method.

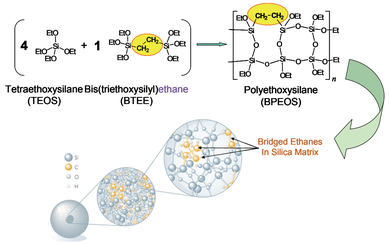

Figure 1. Synthesis of BEH™ Technology Packing Material

Peptide Separation Technology columns are based on the second generation hybrid packing materials that minimize secondary interactions with peptides. The material is stable at both high and low pH for maximum flexibility in developing separations. The synthetic packing material can be prepared with 130Å or 300Å pores in any desired particle size.

METHODS

Instrument

Waters® 2796 AlianceBIO
2996 Photodiode Array Detector
ZQ™ Single Quadrupole Mass Spectrometer
MassLynx™ Software for control and data acquisition
Waters Autopurification System (Figures 9, 10, 11, 12, 13)

Samples

Average Peptide: ISQAVHAAHAEINEAGR;
17 residues;1772.89Da; pI 6.4; HPLC Index 26.5
Basic Peptide: AIKEVKQTIVKHPRY;
15 residues;1809.06Da; pI 10.3; HPLC Index 21.7
Acidic Peptide: DFLEENITALLEEAQ;
15 residues;1733.84Da; pI 3.3; HPLC Index 70.5
Hydrophobic Peptide: WLTGPQLADLYHSLMK;
16 residues;1871.96Da; pI 7.3; HPLC Index 113.3
Biopharmaceutical Peptide: Confidential
~40residues;~4000Da; pI 4.7; HPLC Index 89.6
Thiol Peptide: KLVFFAEDC;
9 Residues; 1070.51Da; pI 4.3 HPLC Index 52.9

Columns

Peptide Separation Technology
XBridge™ BEH130 C_{18} 3.5 µm, 4.6x150mm
XBridge™ BEH130 C_{18} 5 µm, 4.6x150mm
XBridge™ BEH130 C_{18} 5p µm, 4.6x150mm
XBridge™ BEH130 C_{18} 10 µm, 4.6x150mm
XBridge™ BEH300 C_{18} 3.5 µm, 4.6x150mm
XBridge™ BEH300 C_{18} 5 µm, 4.6x150mm
XBridge™ BEH300 C_{18} 5p µm, 4.6x150mm
XBridge™ BEH300 C_{18} 10 µm, 4.6x150mm

CHROMATOGRAPHY
METHOD A (FIGURES 2,3,4,5)
Temperature: 40°C
Flow Rate: 1.5 mL/min
Mobile Phase:
A: 0.1% TFA or 0.1% Formic Acid in water
B: 0.1% TFA or 0.1% Formic Acid in acetonitrile
Gradient:

Time	A%	B%
0.00	95.0	5.0
50.00	50.0	50.0
52.00	10.0	90.0
54.00	10.0	90.0
55.00	95.0	5.0
72.00	95.0	5.0

METHOD B (FIGURES 6,7,8)
Temperature: 40°C or 60°C
Flow Rate: 1.5 mL/min
Mobile Phase:
A: Water
B: Acetonitrile or *iso*-propanol:acetonitrile (80:20)
C:1.0% TFA
D:100 mM NH_4HCO_3, pH10.0
Gradient:

Time	A%	B%	C%*	D%*
0.00	85.0	5.0	10.0	0.0
67.00	25.0	65.0	10.0	0.0
69.00	0.0	90.0	10.0	0.0
71.00	0.0	90.0	10.0	0.0
72.00	85.0	5.0	10.0	0.0
90.00	85.0	5.0	10.0	0.0

*For chromatography at pH 2, use constant 10%C
*For chromatography at pH 10, use constant 10%D

RESULTS

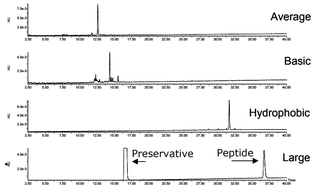

Figure 2. Separation of test peptides in the presence of TFA on XBridge™ BEH130 C_{18} 3.5 µm, 4.6x150mm

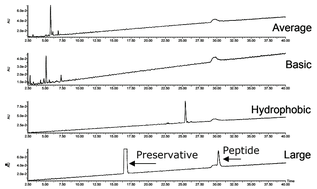

Figure 3. Separation of test peptides in the presence of formic acid on XBridge™ BEH130 C_{18} 3.5 µm, 4.6x150mm

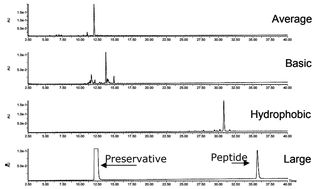

Figure 4. Separation of test peptides in the presence of TFA on XBridge™ BEH300 C_{18} 3.5 µm, 4.6x150mm

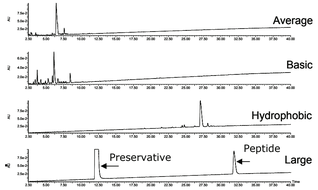

Figure 5. Separation of test peptides in the presence of formic acid on XBridge™ BEH300 C_{18} 3.5 µm, 4.6x150mm

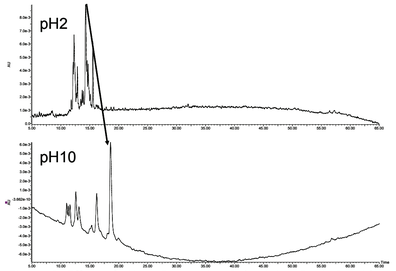

Figure 6. Effect of pH on Separation Selectivity: Basic Peptide in TFA or ammonium bicarbonate on XBridge™ BEH130 C_{18} 3.5 µm, 4.6x150mm

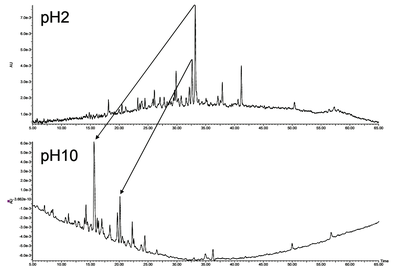

Figure 7. Effect of pH on Separation Selectivity: Acidic Peptide in TFA or ammonium bicarbonate on XBridge™ BEH130 C_{18} 3.5 µm, 4.6x150mm

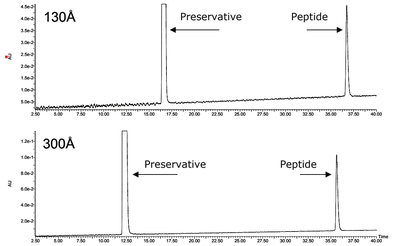

Figure 8. Effect of Pore Size: Large Biopharmaceutical Peptide separated in TFA on either XBridge™ BEH130 C_{18} 3.5 µm, 4.6x150mm or XBridge™ BEH300 C_{18} 3.5 µm, 4.6x150mm

Scaling for Isolation of the Thiol Peptide

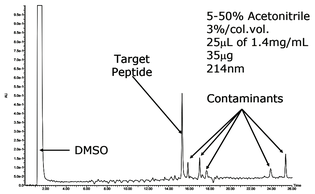

Figure 9. Separation of thiol peptide in the presence of TFA on XBridge™ BEH130 C_{18} 5 µm, 4.6x150mm

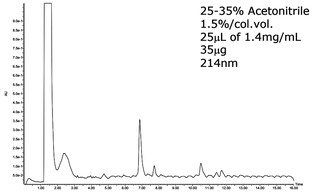

Figure 10. The gradient used in Figure 9 was focused on the elution conditions for the targeted thiol peptide

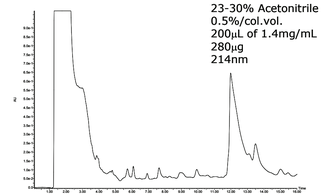

Figure 11. The gradient used in Figure 10 was refined by reducing the slope and by focusing on the target. Loading was increased. Note the tailing of the target peak.

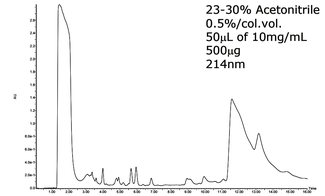

Figure 12. Further increases in loading indicate that peptide solubility defines the capacity of the separation

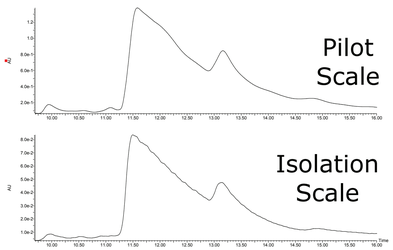

Figure 13. The pilot scale separation developed in Figure 12 was transferred to an XBridge™ BEH130 C_{18} 5 µm, 19x150mm. Load (853 µL; 8.5mg) and conditions (25mL/min; constant gradient) were based on geometric scaling. Separation selectivity is identical at the two scales.

DISCUSSION

A useful protocol for the isolation of synthetic peptides should simplify the number of different separation conditions required by the numerous and varied sequences typically being synthesized. Peptide Separation technology columns are useful for this objective because the surface chemistry minimizes secondary interactions. A single column can, therefore, be used for most peptides with optimization through gradient slope and mobile phase modification. While 300Å pores are often preferred over 130Å, this set of test peptides gives similar selectivity and peak shape on both types of columns with somewhat lower retention with the larger pore sizes. For general use, the use of BEH130 with TFA as modifier will provide a good starting point with the highest retention. If these conditions do not yield adequate purity, a change in modifier provides the largest change in selectivity. Formic acid will usually give lower retention because it gives much less ion pairing than TFA. This mechanism is consistent with the observed larger shift for basic peptides. An alternative change in selectivity is possible by replacing TFA with pH 10 ammonium bicarbonate. With this modifier, acidic peptides will elute earlier and basic peptides later because the side chain ion suppression is inverted at the extremes of pH.

CONCLUSION

- **Peptide Separation Technology columns can be used for synthetic peptides with a wide range of properties.**
- **Either 130Å and 300Å pore size packings can be used for peptides as large as 40 residues.**
- **Formic acid can replace TFA as modifier to change selectivity without deterioration of peak shape.**
- **The differences between formic acid and TFA are consistent with ion pairing with the latter modifier.**
- **Useful separations are possible at either pH 2 or pH 10, and the differences are consistent with estimated isoelectric points. The BEH Technology packing material permits routine use of these pH extremes.**
- **Peptide Separation Technology columns give consistent selectivity across a range of column dimensions for scaling peptide isolation and purification.**

TO DOWNLOAD A COPY OF THIS POSTER, VISIT WWW.WATERS.COM/POSTERS