CONSIDERATIONS IN SELECTING CHROMATOGRAPHIC MEDIA AND OPTIMIZING SEPARATION CONDITIONS FOR THE ISOLATION AND PURIFICATION OF SYNTHETIC PEPTIDES

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INTRODUCTION

Peptides are used in many areas of basic research as well as in biopharmaceutical development. In these applications, it is common and practical to use synthetic peptides rather than isolating natural products. An impure peptide, however, heterogeneous at even a trace level, can make the results of other biological or chemical studies ambiguous. Because the range of contaminating sequences and side products can be large, it is important to use the highest possible resolution to ensure that no contaminant is co-purified. It is, therefore, necessary to optimize chromatography for each peptide. The large range of peptide properties requires efficient method development. Successful isolation can be ensured by basing the separation on the analytical method. The chemistry of the isolation-scale column must match the analytical column. Successful chromatography is influenced by the selection of column, by the mobile phase, and by the operating conditions. The chemistry of the BEH Technology™ particles used in UPLC® separations has proven advantageous for chromatography of peptides. The synthesis and structure of this packing material is shown in Figure 1. Columns are available with both 130Å and with 300Å pores in a range of particle sizes. Good peak shape is observed for a wide range of samples. In these experiments we test the separations of several synthetic peptides covering a wide range of properties including size, hydrophobicity, and isoelectric point. In addition to examining column chemistry, the effects of mobile phase modifier, extremes of pH, and organic solvent are evaluated for peptides with particular properties.

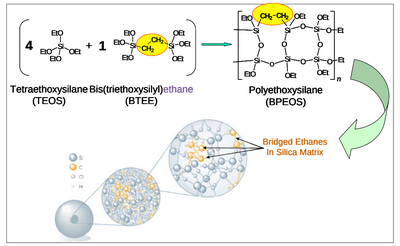

Figure 1. Synthesis of BEH™ Technology Packing Material

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

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

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 2,3,4,5,6,8,12)
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 7,9,10,11)
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

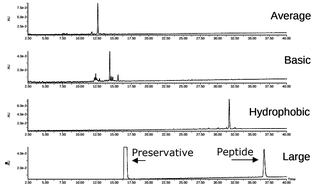

Figure 2. Separation of test peptides in the presence of TFA on XBridge™ BEH130 C_{18} 3.5 µm, 4.6x150mm

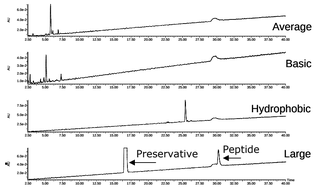

Figure 3. Separation of test peptides in the presence of formic acid on XBridge™ BEH130 C_{18} 3.5 µm, 4.6x150mm

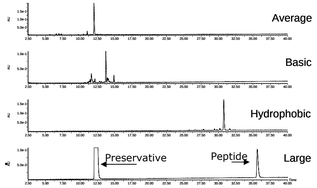

Figure 4. Separation of test peptides in the presence of TFA on XBridge™ BEH300 C_{18} 3.5 µm, 4.6x150mm

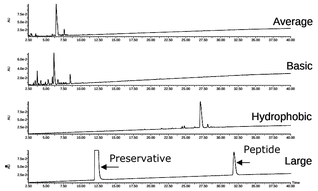

Figure 5. Separation of test peptides in the presence of formic acid on XBridge™ BEH300 C_{18} 3.5 µm, 4.6x150mm

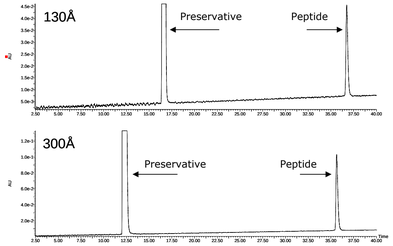

Figure 6. Effect of Pore Size: Large Biopharmaceutical Peptide separated in TFA on either XBridge™ BEH130 C_{18} 3.5 µm, 4.6x150mm or XBridge™ BEH300 C_{18} 3.5 µm, 4.6x150mm

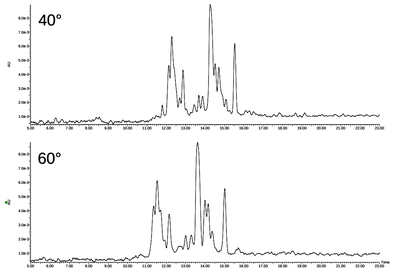

Figure 7. Effect of Temperature: Basic Peptide separated at either 40° or 60° in TFA on XBridge™ BEH130 C_{18} 3.5 µm, 4.6x150mm

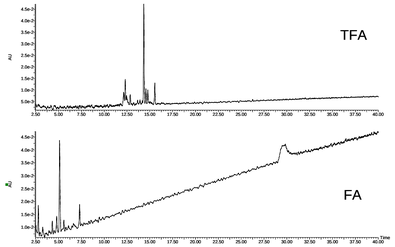

Figure 8. Effect of Mobile Phase Modifier: Basic Peptide in the presence of TFA or formic acid on XBridge™ BEH130 C_{18} 3.5 µm, 4.6x150mm

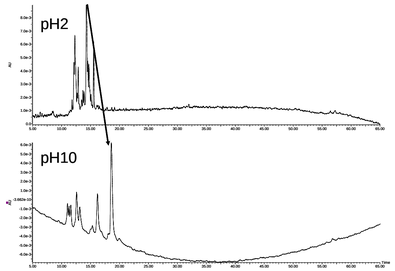

Figure 9. Effect of pH on Separation Selectivity: Basic Peptide in TFA or ammonium bicarbonate on XBridge™ BEH130 C_{18} 3.5 µm, 4.6x150mm

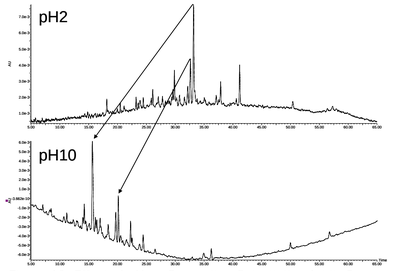

Figure 10. Effect of pH on Separation Selectivity: Acidic Peptide in TFA or ammonium bicarbonate on XBridge™ BEH130 C_{18} 3.5 µm, 4.6x150mm

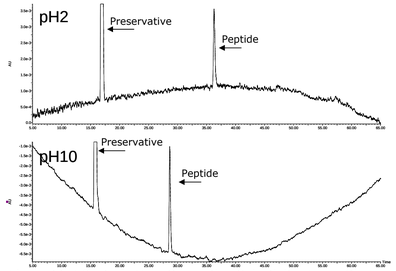

Figure 11. Effect of pH on Separation Selectivity: Large Biopharmaceutical Peptide in TFA or ammonium bicarbonate on XBridge™ BEH130 C_{18} 3.5 µm, 4.6x150mm

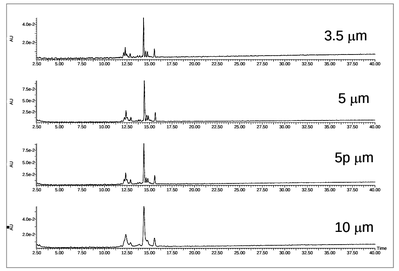

Figure 12. Effect of Particle Size: Basic Peptide separated in TFA or ammonium bicarbonate on XBridge™ BEH130 C_{18} , 4.6x150mm in the four available particle sizes

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.**
- **Temperature has small but useful effects on selectivity.**
- **Peptide Separation Technology columns give consistent selectivity across a range of particle sizes for scaling peptide isolation and purification.**

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