UltraPerformance LC® Techniques for Peptide Analysis

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INTRODUCTION

Peptide maps are used for protein characterization, and similar chromatographic techniques are used to analyze synthetic peptides. Analysis of a protein digest is a challenging analytical problem since the sample can include a hundred or more significant components covering a wide range of size and chemical properties. The same kind of reversed-phase chromatography is used to analyze the purity and identity of synthetic peptides where the sample includes several very similar structures. Both applications require the highest possible resolution. UltraPerformance Liquid Chromatography is a new class of separation science. It improves resolution, sensitivity, and speed by using columns packed with very small particles on optimized instruments. We will describe fundamental investigations that show how the physical and chemical properties of the column are responsible for improving resolution. We will give special attention to the analysis of peptides representing different glycoforms of a protein. UPLC® peptide maps are also used for quantitative analysis. Measurements of peak areas were generated over wide range of concentrations, approaching three orders of magnitude without distortion of retention time or peak shape. The utility of this technology in supporting the isolation of synthetic peptides will be described.

METHODS

Chromatographic Conditions

Instrument: Waters AQUITY UPLC® System
Mixer: High Sensitivity Peptide Analysis
Columns: Peptide Separation Technology
ACQUITY UPLC® BEH 130 C18, 1.7 µm 2.1 x 50mm
ACQUITY UPLC® BEH 130 C18, 1.7 µm 2.1 x 150mm
ACQUITY UPLC® BEH 300 C18, 1.7 µm 2.1 x 150mm
Temperature: 40°C
Flow Rate: 200 µL/min (unless otherwise noted)
Mobile Phase A: 0.02% TFA in water
Mobile Phase B: 0.018% TFA in acetonitrile
Example Gradient Table:

Time	%A	%B	Curve
0	100	0	initial
5	50	50	6
5.5	20	80	1
6	100	0	1

UV Detection: 214 nm
Detection Rate: 10 scans/sec
MS Detection: m/z 400—1800scan, 1/sec
Test samples were Waters MassPREP™ Digest Standards enolase or phosphorylase dissolved in 0.5 mL of 95% water and 5% Acetonitrile with 0.1% TFA (unless otherwise noted), resulting in a concentration of 2 pmoles/µL.
Injection Amount: 10 µL in Partial Loop Mode

RESULTS

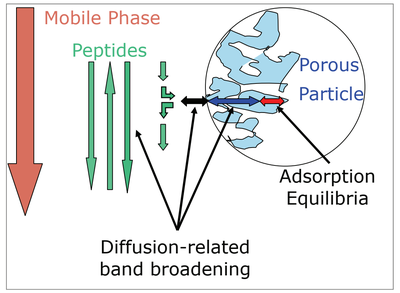

*Figure 1: Chromatography Principles-Mass Transfer and Diffusion:The benefits of UPLC derive from reduced band-broadening that is a consequence of reduced diffusion distances in small particles. In 1.7 µm packing material, efficiency (**N**) is greater with a concomitant increase in pressure.*

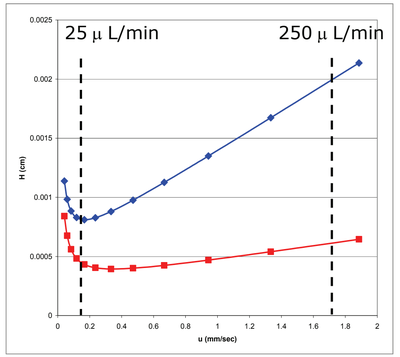

Figure 2 Chromatography Principles-van Deemter Plots for 1500Da Peptide: Diffusion-related band broadening is quantitatively described in the van Deemter equation that relates HETP (H) to linear velocity (u). This relationship is shown graphically for a peptide of 1500 molecular weight on 3.5 µm and on 1.7 µm packing. The minimum in each curve corresponds to the maximum efficiency, and resolving power. The smaller particles have higher resolving power at a higher linear velocity.

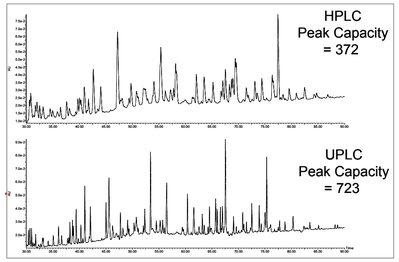

Figure 3. Direct comparison of HPLC and UPLC peptide mapping. Resolution is better with the smaller particle packing.

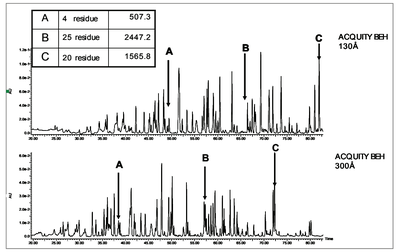

Figure 4. Separation of a tryptic digest of phosphorylase b on ACQUITY UPLC™ BEH130, 1.7mm and on ACQUITY UPLC™ BEH300, 1.7mm Peptide Separation Technology Columns. Larger pore size materials are often preferred for peptide separations. For tryptic peptides, Both columns work well, with the smaller pore size being more retentive.

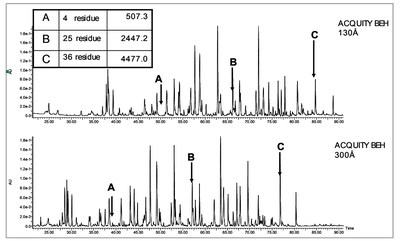

Figure 5. The larger peptides derived with LysC digestion are expected to require larger pore sizes. In this comparison, however, both the 130Å and 300Å give narrow symmetrical peaks for peptides up to 4470Da. Certainly a size will exist where size exclusion effects become prominent.

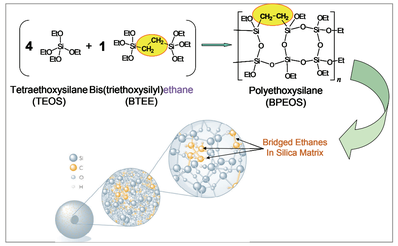

Figure 6. Synthesis of BEH™ Technology Packing Material

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.

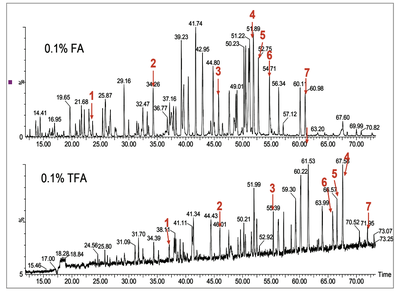

Figure 7. Comparison of the separation of Phosphorylase b peptides with FA and TFA mobile phase modifiers. Formic acid is often preferred as the mobile phase modifier in LC-MS peptide mapping because sensitivity is better than with TFA. That substitution usually is best with specific columns that do not require ion-pairing for peptide separations. With UPLC BEH material, narrow symmetrical peaks are observed with either acid. Retention is greater with TFA and signal intensity is better with formic acid. Tracking of individual shows the changes in selectivity associated with changing the modifier.

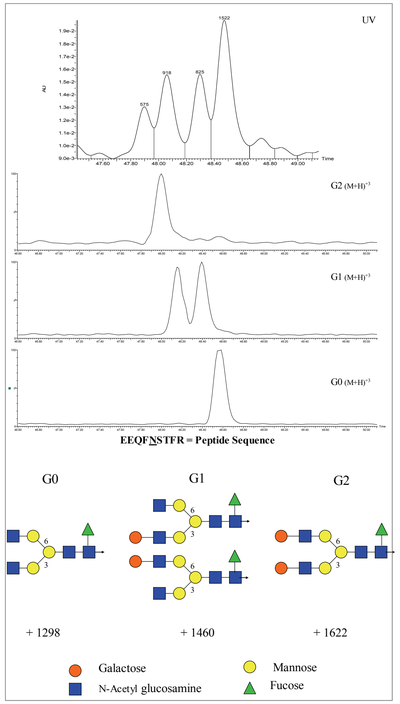

Figure 8. Mouse IgG glycoforms were detected by extracted ion chromatograms of the triply-charged glycopeptides are shown. Four peaks were observed for the three molecular weights indicating that the positional isomers of G1 have been separated by UPLC™.

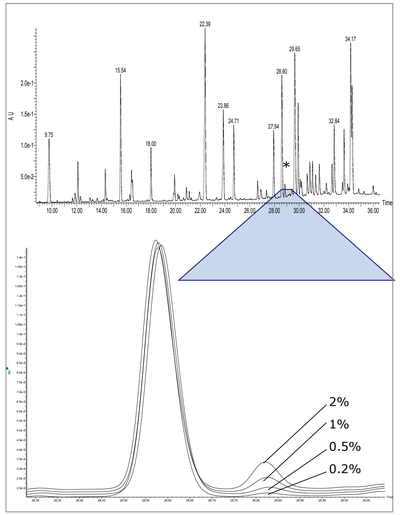

*Figure 9. A peptide map of bovine hemoglobin with a spiked trace contaminant designated *. The peptide mixture was added to the digest from 0.2 to 2% on a molar basis. The linear gradient from 0 – 50% buffer B occurred over 58 minutes. The region at 28 minutes is highlighted. This successful trace analysis requires the high resolution of UPLC for peaks that are only 15 seconds apart, center-to-center. The symmetrical peak shapes at this extreme concentration ratio permits the quantitation of the small peak eluting so closely after the large peak.*

SUMMARY AND CONCLUSIONS

- **UPLC® separations yield higher resolution by reducing diffusion-related band-broadening**
- **Significant improvements in peptide resolution are achievd with UPLC**
- **UPLC packing materials with 130Å and with 300Å pores give usful separations of Tryptic and Lys-C peptides.**
- **The BEH technology™ particles used in UPLC minimize secondary interactions with peptides for improved peak shape.**
- **Symmetrical, well-resolved peaks are observed with IgG glycopeptides.**
- **Mobile phase modifiers can be selected to optimize sensitivity and selectivity.**
- **The combination of column chemistry and instrument characteristics permits quantitative analysis over three orders of magnitude, with UV sensitivity of a few hundred femtomoles.**

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