RECENT ADVANCES IN THE APPLICATION OF UPLC TO LC/MS PEPTIDE MAPPING

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INTRODUCTION

The analysis of protein structure is routinely based on enzymatic digestion to more manageable fragments. The separation of these fragments, peptide mapping, has long been a fundamental tool for structural analysis. Reversed phase HPLC has become the preferred tool for peptide mapping. The chromatographic separation is coupled to mass spectral detection for identification of the chromatographic peaks. The analysis of authentic protein samples is, however, more complicated because all samples are mixtures of native and modified or damaged proteins. Low abundance peptides, representing these trace modifications, may co-elute with major components. The analysis benefits therefore from the use of the most highly resolving chromatographic techniques. UPLC® peptide mapping routinely gives higher resolution than is possible with HPLC. UPLC is used in different ways to meet the requirements of specific analyses. First, the inherently high resolution can be used to ensure that all components in a sample are detected. Second, the high resolution can be used to reduce run time while preserving the resolution of a well-established map. Third, a very fast assay for particular diagnostic peptides can be developed. All three strategies will be illustrated with protein digests. The various options for improving selectivity, including gradient slope, temperature, and mobile phase modifier will be tested. Several new UPLC packing materials will also be compared in terms of their effects on selectivity in peptide maps. The systematically optimized chromatography improves the overall sensitivity and dynamic range of MS characterization, thus allowing accurate quantitation of modified proteins.

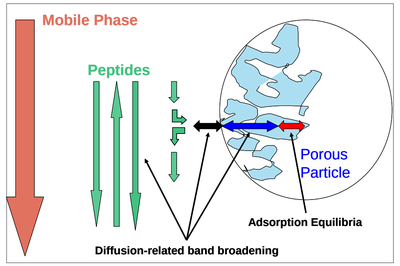

Figure 1. Mechanism of UPLC® Separations

METHODS

Materials
Phosphorylase b MassPREP™ Digestion Standard; Waters
[Glu[1]]-Fibrinopeptide B; Sigma

XBridge™ BEH 300 C_{18} 3.5µm 2.1x250mm Column
ACQUITY UPLC™BEH 300 C_{18} 1.7µm 2.1x150mm Column
ACQUITY UPLC™BEH 300 C_{18} 1.7µm 2.1x50mm Column
ACQUITY UPLC™BEH 130 C_{18} 1.7µm 2.1x100mm Column
ACQUITY UPLC™BEH 300 C_{18} 1.7µm 2.1x100mm Column
ACQUITY UPLC™HSS C_{18} 1.8µm 2.1x100mm Column
ACQUITY UPLC™HSS T3 C_{18} 1.8µm 2.1x100mm Column
ACQUITY UPLC™HSS300 C_{18}SB 1.8µm 2.1x100mm Column

Hydrogen peroxide; Sigma
Formic Acid; Pierce
Trifluoroacetic Acid; Pierce
MilliQ® water
Acetonitrile; Fisher Optima
Methanol; Fisher HPLC Grade

Instruments
Waters® ACQUITY UPLC™System including: ACQUITY Binary Solvent Manager, ACQUITY Sample Manager, ACQUITY Sample Manager, ACQUITY Column Heater, ACQUITY TUV
Waters® Micromass LCT Premier Mass Spectrometer, Waters® Column Heater Module

Samples
Oxidized digest: Add 250µL 0.01% hydrogen peroxide to 1 vial Phosphorylase b MassPREP™ Digestion Standard. Vortex. Incubate at room temperature for 2 hours. Final concentration of digest is 4pmol/µL.

Native digest:
MassPREP™ Phosphorylase b Digestion Standard
MassPREP™ Enolase Digestion Standard
MassPREP™ Hemoglobin Digestion Standard
MassPREP™ Peptide Standards

Add 250 µL water to 1 vialVortex. Final concentration of digest is 4pmol/µL
Lockspray:Glu-fibrinopeptide 1pmole/µL in 0.1% Formic Acid in 50% water, 50% methanol

Chromatography
Mobile phase A1: 0.02% TFA in Water
Mobile phase A2: 0.1% Formic Acid in Water
Mobile phase B1: 0.018% TFA in Acetonitrile
Mobile phase B2: 0.1% Formic Acid in Acetonitrile
Weak needle wash: 0.2%TFA with 5% Acetonitrile
Strong needle wash: 20% Mobile phase A:80% Mobile phase B
Flow rate 0.2mL/min
Injection: 10 µL
Gradient and Temperature as noted in Figure Legends

RESULTS

COMPARING HPLC AND UPLC

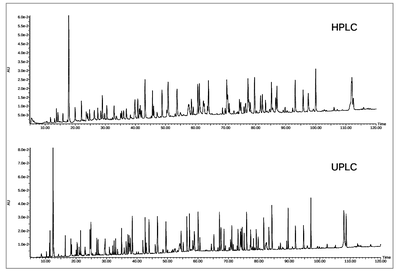

Figure 1. HPLC and UPLC Separations at Constant Gradient Time. Both columns are run from 0-50%B over 180min. The increased number of peaks with UPLC is readily apparent. If the objective is simply more resolution regardless of time, the direct substitution of UPLC will meet the objective. The UPLC column is 150mm long and the HPLC is 250mm. At constant flow rate and time, the UPLC gradient is more shallow.

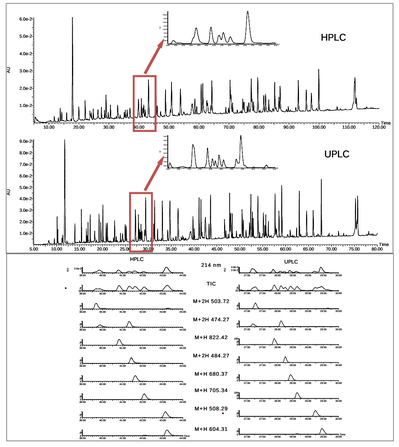

Figure 2. HPLC and UPLC at Constant Gradient Slope. The gradient duration is scaled to column so gradient slope is constant. The UPLC run time is 60% that of the HPLC. Resolution is still improved, but note especially in the inset that selectivity is preserved. Constant selectivity is confirmed with extracted mass chromatograms as shown for the selected region.

DEVELOPING FAST, FOCUSED METHODS

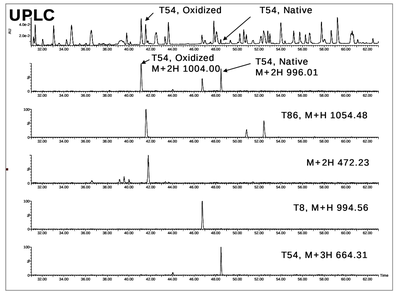

Figure 3. Segment of 120min UPLC gradient showing forms of Peptide T54. The UPLC gradient gives improved resolution of the variant peptide with better sensitivity. For some purposes an even shorter run time may be useful for monitoring particular structural changes.

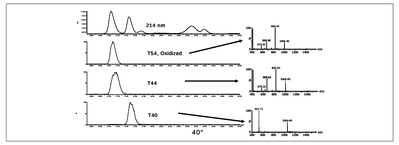

Figure 4. 12.5-16%B in 34min at 40°. Resolution of the desired peptide is inadequate.

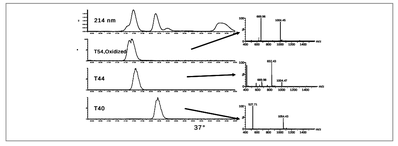

Figure 5. 12.5-16%B in 34min at 37°. Resolution of the desired peptide is improved but still inadequate.

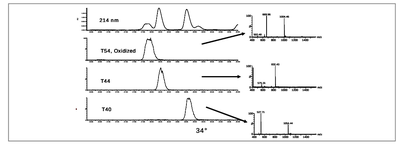

Figure 6. 12.5-16%B in 34min at 34°. Resolution of the desired peptide is better at lower temperature.

OPTIMIZED FOR SPEED

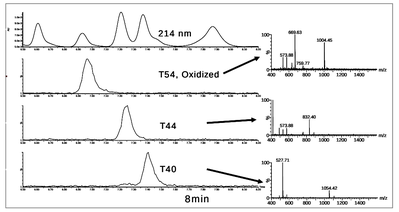

Figure 7. Separation on 2.1x50mm UPLC Column; 12.5-16%B in 7.67min at 34°. Scaling the conditions developed in the above experiments to a 50mm column can reduce run time in proportion to column length. In the resulting separation, the oxidized T54 peptide is well resolved from the adjacent chromatographic peaks. The mass spectra are easily interpreted. The chromatographic peak is sufficiently well-separated for identification and quantitative analysis using UV detection.

ALTERNATIVE COLUMN CHEMISTRIES FOR PEPTIDE UPLC

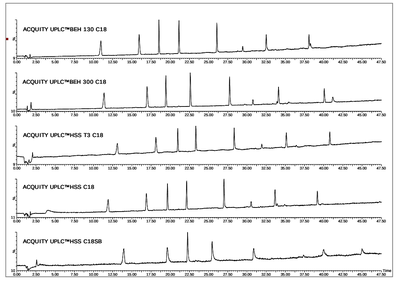

Figure 8. MassPrep Peptide Standards Separated on 5 UPLC columns. The same sample was separated on 5 different UPLC columns. These packings represent two different base particle materials and four different bonding chemistries for the attachment of the C_{18} bonded phase In all examples, elution was effected by a gradient from from 5% to 50% acetonitrile in the presence of constant 0.1% Formic Acid at a flow rate of 0.2mL/min. The columns all give useful separations of this mixture. The selectivity differences among the materials are subtle and will be examined more closely below with complex digests. The HSS C18SB column tends to give tailing peaks for these markers, but note the peptide at 22.5 minutes that is symmetrical. The other four columns show good retention and peak shape with formic acid as a mobile phase modifier. They can be used, therefore, to maximize MS sensitivity while optimizing chromatographic quality.

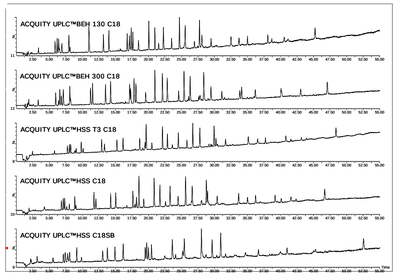

Figure 9. MassPrep Enolase Digestion Standard Separated on 5 UPLC columns.The same sample was separated on 5 different UPLC columns. The columns all give useful separations of this mixture. The selectivity differences among the materials are really quite obvious. No one column gives the best separation across the whole sample. On the other hand every column gives better resolution than others for some specific peptides.The HSS C18SB column does show more peak tailing than the other columns so it is not a likel;y first choice in an optimization experiment.

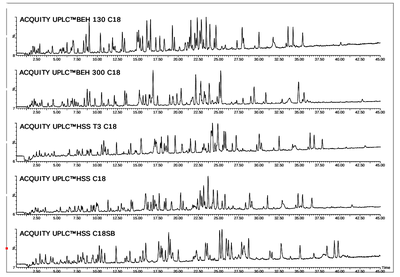

Figure 10. MassPrep Phosphorylase Digestion Standard Separated on 5 UPLC columns. The column assortment gives a set of similar separations, but there are uniquely useful regions associated with each

CONCLUSION

- **UPLC® Peptide Mapping can be used to improve resolution, to reduce runtime without sacrificing resolution, and to develop fast, focused maps for specific structural features.**
- **Developing UPLC maps exploits the usual mechanisms for reversed phase.**
- **New UPLC columns provide alternate selectivities for optimizing Peptide maps.**

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