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UPLC FOR THE ANALYSIS OF SYNTHETIC PEPTIDES AND FOR THE DEVELOPMENT OF ISOLATION STRATEGIES

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INTRODUCTION

Peptides are used in many areas of basic research as well as in biopharmaceutical development.

- A peptide representing a structural feature of a protein may be used to probe specific functional properties.
- An antigenic determinant may be represented by a peptide for developing vaccines or for studying the immune response.
- Peptides may be substrates for enzymes.
- Many peptides play important roles in the physiology of organisms.

In all these applications, it is common and practical to use synthetic peptides rather than isolating natural products. The use of synthetic peptides is preferred because they can be obtained in any required quantity. It is easy to modify the sequence to analyze function, and the identity and purity can be assured. The last point is particularly important since an impure peptide, heterogeneous at even a trace level, can make the results of other biological or chemical studies ambiguous.

Because the range of contaminating deletion sequences and side reaction products can be large, it is very important to use the highest possible chromatographic resolution to ensure that no contaminant coelutes with the main peak. It is, therefore, very useful to apply UltraPerformance LC® (UPLC®) to obtain the best resolution and sensitivity in the minimum runtime.

The enhanced chromatographic resolution associated with UPLC has been demonstrated for peptide mapping (Mazzeo, et al., BioPharm.). The technique improves resolution by a factor of three or more. In addition, the surface chemistry of the BEH Technology™ used in UPLC has proven especially advantageous for peptide mapping. Good retention and peak shape are observed with either TFA or formic acid as a modifier.

These chromatographic characteristics will be useful for synthetic peptides, but there are additional, special requirements. With peptide mapping, the relevant sample components represent a very wide range of chemical and physical properties. Since the map must accommodate all of these components, long, shallow gradients are used. In contrast, synthetic peptides involve a single structure with the contaminants all being closely related structures. It is, therefore, reasonable for the analytical method to be a segmented gradient that is very shallow at the point where the product elutes.

Steep gradients can be used to reduce runtime during regeneration and re-equilibration. UPLC should be especially useful for providing the high resolution and high throughput analyses desired for assessing the purity of synthetic peptides. The analytical conditions used in these experiments could also predict the conditions for purifying the peptide if the chemistry was matched in a larger scale column. These experiments test and demonstrate this process with a 23-residue synthetic peptide.

MATERIALS AND METHODS

Sample preparation

A synthetic peptide was purchased as a custom product. The target sequence was: YPIVSIEDPFAEDDWEAWSHFFK. The expected molecular weight is 2827.3. For analytical experiments, it was dissolved in 0.1% TFA containing 10% acetonitrile to a concentration of 0.2 mg/mL. For isolation, it was dissolved in 0.05% TFA containing 10% acetonitrile to a concentration of 9 mg/mL. For fraction analysis, the collected samples were directly injected.

Analytical chromatography

Instrument: Waters ACQUITY UPLC® System
Waters TUV Detector
Waters ZQ™ 2000 MS Detector
Column: Waters ACQUITY UPLC BEH 130 C_{18}
1.7 µm, 2.1 x 100 mm
Flow Rate: 0.2 mL/min
Column Temp.: 40 °C
Mobile Phase: (A) H_2O with 0.02% TFA
(B) ACN with 0.018% TFA

Gradient Table (Vary Segment Start; 3.0%/col.vol.)		
Time	%A	%B
0	100	0
2	72*	28*
6.6	64*	36*
8.6	10	90
11.6	10	90
12.1	100	0
29.4	100	0

(*28-36;*30-38;*33-41)

Gradient Table (Vary Slope)		
Time	%A	%B
0	100	0
2	72	28
6.6	64	36
8.6	10	90
11.6	10	90
12.1	100	0
29.4	100	0

0.25%/col.vol – 55.3 min
0.75%/col.vol – 18.45 min
1.50%/col.vol – 9.2 min
3.00%/col.vol – 4.6 min

Isolation

Instrument: Waters 600 Multisolvent Delivery System
2767 Sample Manager
2996 PDA with Flow Cell
AutoPurification
Column: Waters Peptide Separation Technology
XBridge™ BEH 130 Prep C_{18}
5 µm, OBD™ 19 x 150 mm
Flow Rate: 17.0 mL/min
Column Temp.: Ambient
Mobile Phase: (A) H_2O
(B) ACN
(C) 1% TFA in Water

Gradient Table			
Time	%A	%B	%C
0	93	5	2
2	70	28	2
15	62	36	2
19	48	50	2
19.1	8	90	2
21	8	90	2
21.1	93	5	2
30	93	5	2

RESULTS

Development of analytical method

In developing a rapid analytical method for synthetic peptides, two parameters must be defined: the range of the shallow gradient segment and the slope of the shallow gradient segment. Estimating acetonitrile concentration required to elute a given peptide, the samples are screened with a fast gradient. The known offset between the time observed at the detector and the gradient table defines the elution range (Jablonski and Wheat; Waters Application Note 720000920EN).

This peptide elutes near 35% acetonitrile. A window of ±4% is often useful. To define more closely the exact conditions, 8% segments starting at different points were compared (Figure 1). When the separation segment begins at a lower concentration, the main peak elutes later, as expected, and there are changes in the patterns of the surrounding contaminants.

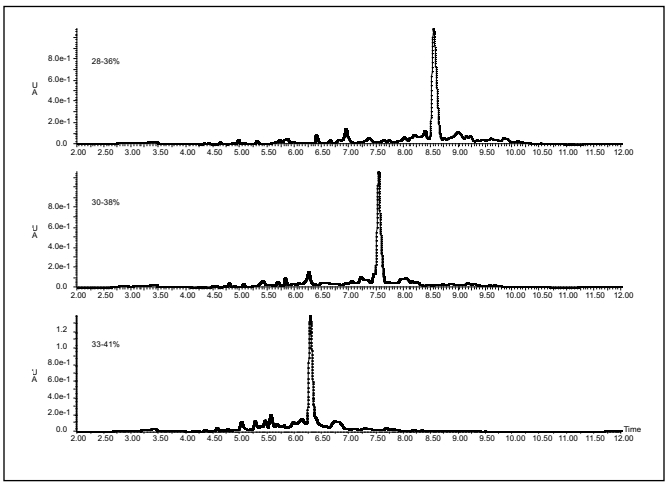

Figure 1. Optimizing the gradient segment for peptide analysis. The synthetic peptide was separated with UPLC at a gradient slope of 3%/column volume over 8% segments beginning at 28, 30, and 33% acetonitrile. Separations were monitored with UV at 214 nm.

To ensure that the product peptide is correctly identified and is well-separated from all the contaminants, the separation was monitored with MS in addition to UV (Figure 2). Significant changes in selectivity can be recognized in this way. Most important, the elution position of one contaminant, m/z 929, varies relative to the desired product peak with starting point of the shallow segment. The most useful analytical gradient runs from 28 to 36% acetonitrile, and that range is used in all subsequent experiments.

The development of the analytical method also requires definition of gradient slope over the narrow segment. It is generally recognized that peptides are best resolved with relatively shallow gradients with a useful range from 0.25%/column volume to 3%/column volume. Better resolution is obtained with shallower gradients, but the run times are longer and elution volumes larger.

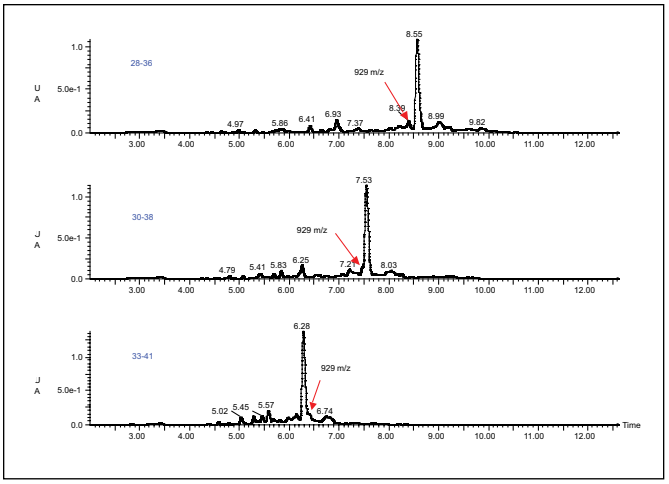

Figure 2. Optimizing the gradient segment for peptide analysis with peak tracking. The same experiments shown in Figure 1 were monitored with ESI/MS detection. Specific selectivity changes are shown with the change in elution position of the m/z 929 contaminant.

Using the segment range determined above, gradient slopes from 0.25% to 3%/column volume were compared (Figure 3). The expected relationship among slope, resolution, and peak volume holds for most components of the mixture. There is, however, a contaminant, m/z 978, that elutes before the target peak at 0.25%/column volume and after at 1.5%/column volume. That contaminant would be unobserved at 0.75%/column volume, directly coeluting with the intended product.

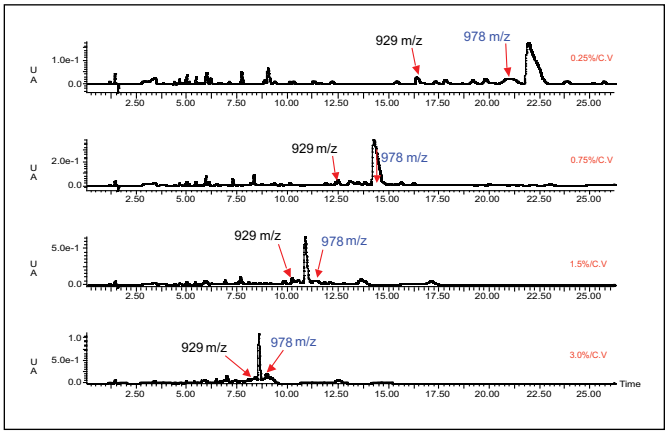

Figure 3. Optimizing the gradient slope for peptide analysis with peak tracking. Various gradient slopes were tested over the range of 28 to 36% acetonitrile. Monitoring the separations with ESI/MS demonstrates the change in selectivity for the m/z 978 contaminant relative to the main product. Note that the separation at 0.75%/column volume is not acceptable while the steeper and shallower gradients give useful resolution.

Isolation of the synthetic peptide

Purification of the synthetic peptide will usually be done on a relatively large column. Successful isolation can be ensured by basing the separation on the conditions developed for the analytical method. This approach requires matching the chemistry of the isolation-scale column to that used for the UPLC analysis.

The column selected here is a Peptide Separation Technology XBridge BEH 130 Prep C_{18} 5 µm OBD™ 19 x 150 mm because it is the same base particle as the UPLC column and is also tested with a peptide separation. The conditions selected for the separation, a gradient from 28 to 36% acetonitrile with a slope of 1.5%/column volume, were selected to best separate the product from the contaminants, particularly the m/z 929 and m/z 978, as well as minimizing the collected volume to facilitate recovery. This separation, on a heavily overloaded column, is shown in Figure 4. Fractions were collected at 15 sec intervals across the main peak.

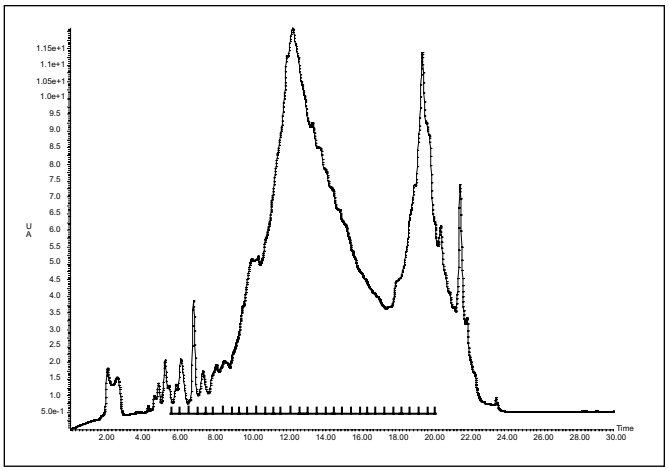

Figure 4. Peptide isolation. The UV trace of an overloaded separation of the synthetic peptide is shown. Because the detector is not a useful guide to collection, time-based fractions were taken at 15 second intervals as shown with the hashmarks.

Analysis of isolated peptide

Each collected fraction was assayed using the analytical method developed above. Results are shown for selected fractions in Figure 5. The examples highlight the early- and late-eluting fractions that contain contaminating material. These observations are consistent with the successful prediction of conditions for larger-scale isolation from the small-scale UPLC peptide analysis. Side fractions were set aside and re-pooled for inclusion in future isolations. The pure fractions were pooled and assayed as shown in Figure 6. The isolated material is greater than 95% pure and contains the intended product, recognized in mass spectrum as the doubly- and triply-charged species. This result confirms the successful isolation.

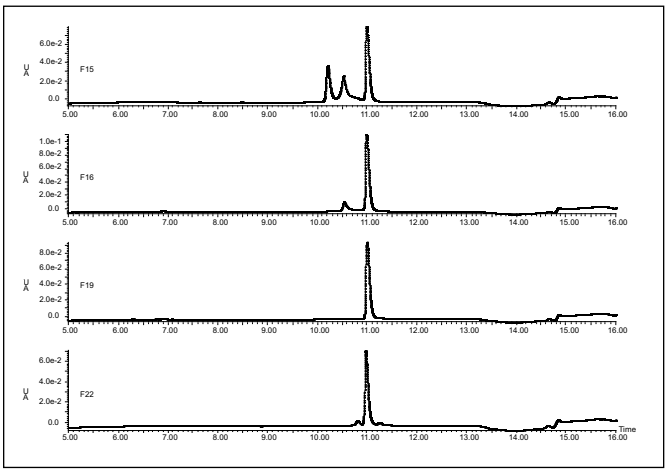

Figure 5: Fraction assay. Fractions across the overloaded isolation peak were assayed with the UPLC separation method. The early- and late-eluting F15, 16, and 22 show the contaminating materials. Fractions 17 to 21 were pooled.

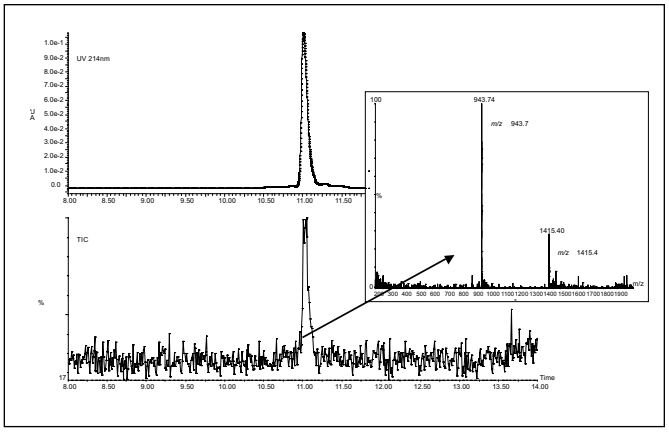

Figure 6: Assay of final product. The pooled fractions were dried and reconstituted for assay with the UPLC Peptide Analysis Application Solution. Both the UV trace at 214 nm and the MS TIC show a single component. The mass spectrum of this peak (inset) shows the doubly- and triply-charged masses for the intended product.

CONCLUSION

The Waters UPLC Peptide Analysis Solution was first described as a tool for protein characterization via peptide mapping. In these experiments, the technique is proven useful for synthetic peptides.

The inherently high resolution of UPLC ensures that contaminants can be quickly detected. Since high resolution is obtained in relatively short run times, it is practical to optimize conditions to eliminate coelution of contaminants. These optimized conditions can be used to predict the best separation conditions for isolation of purified peptide because the same Peptide Separation Technology column chemistry is available in both UPLC and larger formats.

The combination of these techniques should accelerate the production of high purity synthetic peptides for use in a variety of applications.

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