ApplicationNOTE

Waters

REDUCTION OF RUN TIME IN PEPTIDE MAPPING

Introduction-

A successful peptide map of a protein pharmaceutical is the result of manipulation and optimization of many chromatographic conditions. These factors include: flow rate, temperature, gradient slope and duration, pH, mobile phase and column. An important column characteristic that affects the separation of peptides is particle size. Decreased particle size enables separations with higher resolution and peak capacity. Currently, many peptide maps of protein pharmaceuticals are separated on columns containing 5µm particles that are 250 mm in length. These columns have similar peak capacity as columns packed with 3µm particles and 150 mm length. Therefore, using a column with similar peak capacity that is shorter in length enables reduced run time while maintaining resolution.

Protein Digestion Sample Preparation-

The Waters MassPREP™ Enolase Digestion Standard (Part Number 186002325) was used in this analysis. One vial of the digestion standard was diluted in 100µL of mobile phase A to obtain the appropriate working standard. The resulting protein digest contains no undigested enolase, trypsin or other hydrophilic components.

Chromatography-

HPLC System:	Waters Alliance® 2695 Separations Module
Column A:	BioSuite™ PA-A Column, 5µm 4.6 x250 mm**
Column B:	BioSuite™ PA-A Column, 3µm 4.6 x150 mm
Flow Rate:	1.0 mL/minute
Mobile phase A:	0.02% TFA in water
Mobile phase B:	0.018% TFA in acetonitrile
Gradient A:	0-48 % B in 100 min (linear)
Gradient B:	0-48% B in 60 min (linear)
Injection Volume:	15µL
Injected Mass:	750 pmol
Column Temp.:	40 °C
Detection:	Waters 996 Photodiode Array Detector; 214 nm
****Prototype column**	

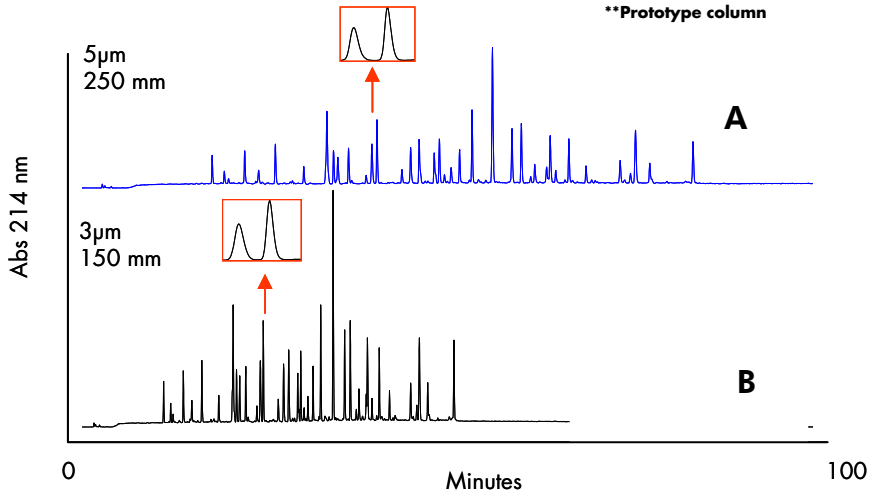

Figure 1. Separation of enolase tryptic digest on a 5µm 4.6 x 250 mm column (A) and a 3µm 4.6 x 150 mm column (B) Resolution of the critical pair is shown in the inset.

Results and Discussion-

Run time, resolution of critical pairs, and peak capacity were examined in this study. *Figure 1* shows the reduction in run time from 116 minutes using the 5µm, 250 mm column to 70 minutes using the 3µm, 150 mm column. This represents a 40 percent reduction in the run time of the peptide map. The data further shows the maintenance of good resolution when using the shorter, 150 mm, column. This is shown by the baseline resolution between a critical pair of peptides *(Figure 1, inset)*. Finally, the peak capacity of each column was calculated. The peak capacity (P_c) of a column is defined as the number of peaks a column can separate within a given gradient time and was calculated by using the equation:

$$\mathbf{P_c = 1 + (t_g / W_{0.5h})}$$

Where t_g represents the time of the gradient in minutes and $W_{0.5h}$ represents the width of the peak at half height in minutes. Five peaks corresponding to five different peptides were selected from each column (Figure 2). Peak widths were then calculated, averaged and the peak capacity of each column was calculated (Table 1) The results of this calculation show equal peak capacities for both columns; 583 for the 3µm, 150mm column and 609 for the 5µm, 250 mm.

Peak Number	3µm 150 mm $W_{0.5h}$	5µm 250 mm $W_{0.5h}$
1	0.092	0.131
2	0.112	0.185
3	0.105	0.173
4	0.095	0.155
5	0.112	0.179
Sum	0.516	0.823
Average	0.103	0.165
Peak Capacity	583	609

Table 1. Peak capacity data

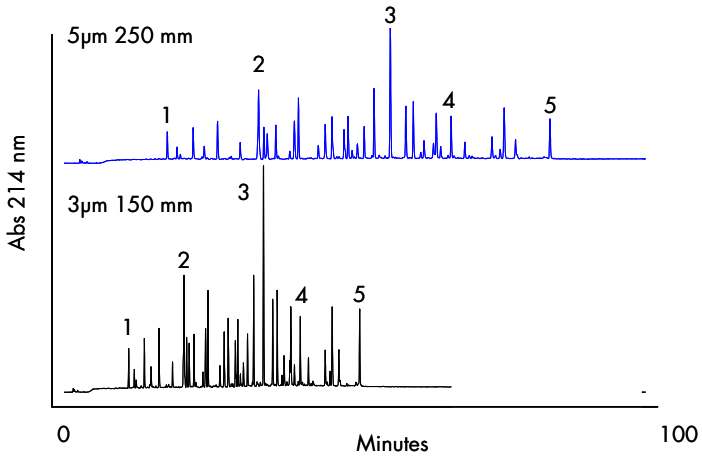

Figure 2. Selected peaks used for peak capacity calculations

Conclusion–

The results of this study show that the use of shorter columns that contain smaller particles results in the following: 1) A reduction in run time of peptide maps, a reduction of 40 percent was obtained in this study. 2) No reduction in peak capacity, the same peak capacity was obtained in this study using a shorter column packed with small particles (150 mm, 3µm) as with the longer column packed with larger particles (250 mm, 5µm). 3) Maintenance of baseline resolution between critical pairs of peptides in a separation. Therefore, one can significantly reduce the analysis time of peptide mapping without losing resolution of critical pairs and peak capacity by using the Waters BioSuite™ $C_{18,}$ 3µm,150 mm, PA-A column.

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