Introduction

Peptide mapping by RP-HPLC is often used for quality control of therapeutic proteins. Since tryptic digestion generates a complex mixture of peptides typical 25-100, depending on the size of the protein, it is difficult to achieve an ideal separation of all peptides of interest. Separation of Enolase peptides, for example, requires a column peak capacity $P_c$ of 261 to resolve 93% of the peptides. BioSuite PA-B columns with TFA mobile phases provide very high peak capacities. A complete separation (if desirable) requires the use of a longer column and/or shallower gradient. Achieving a complete resolution for peptides in the map requires a gradient time of 18 minutes for the BioSuite PA-B, 150 x 4.6 mm column, for other HPLC conditions see Methods. About 71 peaks are resolved with $R_s > 1$. From total number of Phosphorylase b peptides resolved (column peak capacity $P_c$ measured as 116), the size $s$ of a gradient step is 0.01. Gradient step size also has an impact on the separation selectivity. Further widening the gradient time improves $P_c$ and reduces only marginally. BioSuite PA-B, 50 x 6.6 mm column, for other HPLC conditions see Methods. Use temperature or different type of RP-HPLC columns for fine tuning of separation selectivity. BioSuite PA-B columns with TFA mobile phases provide very high peak capacities. Use longer column, and/or shallower gradient or using different types of C18 columns (Figure 4) to-column with constant $s$ (Figure 5).

Column Selection and Methods

BioSuite™ Peptide Analysis columns were selected for this study because of their great peak capacity for peptide separation. BioSuite C18 PA columns are primarily recommended for LC-MS applications, they maintain high performance with MS compatible mobile phases comprised of aqueous formic acid and acetic acid. BioSuite C18 PA columns perform best with fluorinated acid and TFA mobile phases (LC-MS applications). BioSuite PA columns are packed with silica based C18 bonded-phase. In this study we used 0.1% aqueous FA as mobile phase A and 0.05% TFA at 80% aqueous ACN (mobile phase B). One step was used for separation. $P_c$ correlates with $R_s > 1$. There $s$ is 0.01. $P_c$ from 0 to 100 % ACN. Gradients from 0 to 80 % ACN were used.

More Information

Use longer column, and/or shallower gradient or using different types of C18 columns (Figure 4) to-column with constant $s$ (Figure 5).

Conclusions

• BioSuite™ columns for LC-MS and LC-UV mapping are primarily recommended for LC-MS applications; they maintain high performance with MS compatible mobile phases comprised of aqueous formic acid and acetic acid. BioSuite C18 PA-A columns are primarily recommended for LC-MS optimization is often carried out intuitively by alternating both column length and gradient slope. As a result, the method development for peptide mapping is a lengthy process. A rational approach to RP-HPLC peptide mapping is suggested using a simple statistical model (Shins and Goldings, Anal Chem. 72, 1999, p. 474.) predicting the number of peaks, $p$, resolved on column (observed in chromatogram) with resolution $R_s > 1$.

$$P_c = \frac{1}{\Delta t} + \frac{1}{s}$$

For example, when peak width at 0.1% of peak height in 1 minute and gradient time $t_g$ is 22 minutes, the peak capacity $P_c$ is 269. Peak capacity measured experimentally (Figure 1) for BioSuite C18 3µm PA-A and C18 5µm PA-B using various gradient slopes. Gradient time $t_g$ was calculated according to equation 3. Resolution decreased with gradient length, the gradient time has to be increased in order to keep the gradient slope constant for columns with different lengths. The ac value is 1 for gradients from 0 to 100 % ACN. Gradients from 0 to 80 % ACN were used.

$$S = \frac{\Delta t}{t_g}$$

Results and Discussion

Figure 1: Example of peak capacity measurement. BioSuite PA, 30, 100, and 150 x 4.6 mm columns. $R_s$ values were calculated from the peak area. According to the theory, the $P_c$ increases proportionally to $s^2$. Doubling the column length adds only 40 % to the peak capacity.

Figure 2: Impact of gradient slope on Enolase digestion separation (50 peptides). The number of Enolase peptides resolved with $R_s > 1$ is compared with column peak capacity measured as shown in Figure 1. Note that gradient slope also has an impact on the separation selectivity. Further widening the gradient time improves $P_c$ and reduces only marginally. BioSuite PA-B, 50 x 6.6 mm column, for other HPLC conditions see Methods.

Figure 3: Separation of Phosphorylase b on BioSuite PA-B, 150 x 6.6 mm column. For BioSuite PA-B, 40 °C, $P_c = 116$; 35 °C, $P_c = 70$; and 30 °C, $P_c = 21$. About 71% of the peptides are resolved with $R_s > 1$. From total number of Phosphorylase b peptides resolved (column peak capacity $P_c$ measured as 116), the size $s$ of a gradient step is 0.01. Gradient step size also has an impact on the separation selectivity. Further widening the gradient time improves $P_c$ and reduces only marginally. BioSuite PA-B, 50 x 6.6 mm column, for other HPLC conditions see Methods. Use temperature or different type of RP-HPLC columns for fine tuning of separation selectivity.

Table 1: Summary of $R_s$ measurement/prediction data for Enolase (50 peptide) and Phosphorylase b (100 peptide).

<table>
<thead>
<tr>
<th>Column type</th>
<th>$R_s$ measured</th>
<th>$R_s$ predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>BioSuite PA-B, 40 °C</td>
<td>40</td>
<td>41</td>
</tr>
<tr>
<td>BioSuite PA-B, 35 °C</td>
<td>38</td>
<td>39</td>
</tr>
<tr>
<td>BioSuite PA-B, 30 °C</td>
<td>35</td>
<td>36</td>
</tr>
</tbody>
</table>

Table 2: Selection of peptides for peak capacity measurement.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptides resolved</th>
<th>Peptide peaks completely resolved</th>
<th>Peptide peaks partially resolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enolase</td>
<td>93</td>
<td>93</td>
<td>0</td>
</tr>
<tr>
<td>Phosphorylase b</td>
<td>80</td>
<td>80</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 4: Adjusting the selectivity of peptide separation (A) by altering the separation temperature (B) - by altering the type of C18 stationary phases. Both approaches result in substantial changes in selectivity and can be used for more convenient adjustment of peptide separation rather than changing the mobile phase composition or gradient slope. Columns: 100 x 4.6 mm [A] or 50 x 6.6 mm [B], $s = 0.01$, for other conditions see Methods.

Select column (Table 1) with desirable peak capacity.

Guidelines for RP-HPLC peptide mapping method development.

Optimize the separation temperature. (A) - by altering the separation temperature (B) - by altering the type of C18 stationary phases. Both approaches result in substantial changes in selectivity and can be used for more convenient adjustment of peptide separation rather than changing the mobile phase composition or gradient slope. Columns: 100 x 4.6 mm [A] or 50 x 6.6 mm [B], $s = 0.01$, for other conditions see Methods.

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Impact of column peak capacity on peptide mapping by reversed-phase high-performance liquid chromatography (RP-HPLC)