Separation and Analysis of Peptides by HPLC Using the Evaporative Light-Scattering Detector

Introduction

Recent developments in peptide chemistry have demonstrated that both naturally occurring or synthetic analogues are useful as therapeutic agents and extremely powerful tools for biomedical research [1,2]. The intensification of research is credited to instrument automation and advanced technologies for synthesis, purification, and characterization. These have resulted in the isolation of peptides in large enough quantities to effectively conduct investigations of structure activity relationships, in-vitro and in-vivo (animal) drug screening which ultimately results in the selection of leading compounds for clinical trials/research [3].

Preparation of peptides in large quantities (multigram) requires Good Manufacturer Practice (GMP) conditions which includes compound purity, to equate biological function to a specific peptide. Because peptides are synthesized from amino acids, amino acid derivatives and reagents, it is imperative to follow stringent analytical procedures [2]. HPLC-RP (reversed phase) has emerged as the chromatography of choice for peptide and amino acid analysis. It has been utilized to assess purity, quantity, and to monitor synthesis by-products. Optimization strategies for the separation of peptides from complex matrices have been recently demonstrated [3]. The intent was to achieve a rapid separation based on a hybrid between experimental design used for mobile phase optimization and gradient predictions with sufficient resolution [4]. Furthermore, HPLC-RP was utilized to study the effect of the secondary structure of peptides through the use of bandwidth [5].

Many detection techniques in HPLC have been proposed for the determination of peptides, including radioimmuno assays ultraviolet absorption, electrochemical and fluorescence measurements [7,8]. Fluorescence detection utilizing fluorescamine, o-phenalddehyde, dansyl chloride, and other derivatives reagents have been successfully utilized for low level measurements (10 picomole to 100 femtomole) [8]. Although fluorescence tagging is advantageous for low level determinations, it is time consuming and difficult to quantitatively tag secondary amine groups.

The objective of the present study was to evaluate the evaporative light-scattering detector (ELSD) for the measurements of several peptides without derivatization.

Reagents

Trifluoroacetic acid (TFA), and peptide standards (glycine-tyrosine, G-Y; glycine-leucine-tyrosine, G-L-Y; and lysine-aspartate, K-D) [9] were purchased from Sigma (St. Louis, MO, USA). HPLC-grade solvents were purchased from Alltech Associates (Deerfield, IL, USA). High-purity water was produced with a Milli-Q purification system (Millipore, Bedford, MA, USA).

Apparatus and HPLC Conditions

The system consisted of a solvent delivery system (Hitachi model L-6200A), a Rheodyne fixed 5µL loop (model 7161), an ELSD MK III, and a 250 x 4.6mm ID, 5µm Alltima™ C18 column, were purchased from Alltech Associates. The elution solvent consisted of water-acetonitrile with 0.05% TFA at a flowrate of 0.6mL/min. Sample preparation consisted of individual stock solutions of individual peptides in 0.01 N HCl. Dilute mixtures were prepared from stock to reflect an on column concentration range of 0.05 - 5.0µg. Routinely a 5µL sample was injected. Curve fits were performed using a least squares regression fit to a 3rd order polynomial.

Results and Discussion

Figure 1 is a sample representation of a 5µL injection of a 0.1mg/mL mix. A standard mix in a concentration range of 50 to 5000ng was utilized to evaluate the detector response, and chromatography reproducibility. In Table 1, the results of a series of mixes show the mean and standard deviation (SD) of the detector response, peak width and retention time. The coefficient of variation (CV) was < 3.0 for most data, except for K-D (see Table 1, conc. 0.05 and 0.25µg). The data in Table 1 was treated to a 3rd order polynomial (Figure 2). The results showed a correlation coefficient (r) of 0.998, 0.999 and 0.998 for G-Y, G-L-Y and K-D respectively.

Conclusion

Highly automated instruments and novel approaches to the production of large synthetic peptides/proteins [10] for therapeutics and biomedical research applications has led to a greater need to identify, measure, and determine peak purity of peptides of interest. HPLC-RP has proven to be an effective tool to meet market demands. The evaporative light-scattering detector, described elsewhere [11, 12], coupled with a HPLC-RP system provides a rapid means for peptide evaluation in the low nanogram levels without pre or post-column derivatization.
ELSD response to peptides. A 5µL sample of each standard was chromatographed onto a C18 column. The detector response is equal to the area under the curve for each peak. Data fitted with 3rd order polynomial.

Table 1

Detector Response Data

<table>
<thead>
<tr>
<th>Conc. (1) in µg</th>
<th>G-Y Area (2)</th>
<th>1 SD Peak G-L-Y Area (2)</th>
<th>1 SD Width (3)</th>
<th>Peak K-D Area (2)</th>
<th>1 SD Width (3)</th>
<th>K-D 1 SD Width (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>80.5</td>
<td>2.1</td>
<td>0.073</td>
<td>28.5</td>
<td>0.71</td>
<td>0.130</td>
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<tr>
<td>0.25</td>
<td>486.0</td>
<td>7.1</td>
<td>0.056</td>
<td>216.0</td>
<td>1.41</td>
<td>0.130</td>
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<tr>
<td>0.50</td>
<td>1078.0</td>
<td>5.7</td>
<td>0.060</td>
<td>686.0</td>
<td>4.20</td>
<td>0.110</td>
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<td>1.00</td>
<td>2329.0</td>
<td>37.5</td>
<td>0.056</td>
<td>1669.0</td>
<td>19.10</td>
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<td>2.00</td>
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<td>10.6</td>
<td>0.060</td>
<td>4537.0</td>
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<td>2.50</td>
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<td>59.4</td>
<td>0.060</td>
<td>6233.0</td>
<td>18.40</td>
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<td>3.00</td>
<td>8721.0</td>
<td>2.8</td>
<td>0.060</td>
<td>7814.0</td>
<td>21.20</td>
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<tr>
<td>3.50</td>
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<td>0.063</td>
<td>9405.0</td>
<td>93.30</td>
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<tr>
<td>4.00</td>
<td>10920.0</td>
<td>168.0</td>
<td>0.063</td>
<td>10643.0</td>
<td>161.00</td>
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<td>223.0</td>
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<td>Rt. (4)</td>
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<td>3.90</td>
<td>0.01</td>
<td>4.87</td>
<td>0.01</td>
</tr>
</tbody>
</table>

1. Conc. mix reflects micrograms on column (5µL injection).
2. Mean detector response of triplicate injections.
3. Peak width expressed in minutes.
4. Retention time in minutes +/- S.D.

References