**Characterization and Stability of Polysorbate 20 in Protein Formulation by 2D-UHPLC-CAD-MS**

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**Abstract**

Polysorbate 20 is a non-ionic surfactant commonly used in the formulation of protein pharmaceuticals to prevent the aggregate formation and protect proteins from denaturation. It is important to understand the composition and stability of polysorbate 20 in protein formulations as polysorbate 20 can gradually degrade in aqueous solution over time and lose their surfactant activity. In this study, the characterization and stability study of polysorbate 20 in the presence of protein formulation sample matrix was first reported using 2D-LC coupled with CAD and MS detection. A mixed mode column that has both anion exchange and reversed phase properties was used in the 1st dimension to separate the proteins in the formulation sample, while polysorbate 20 esters were trapped and then analyzed using an RP-UHPLC column in the 2nd dimension to further separate and characterize the ester subspecies. Another 2D-LC method using a cation exchange column in the 1st dimension and the same RP-UHPLC in the 2nd dimension was developed for the analysis of degradation products of polysorbate 20. Stability samples of a protein drug product were studied using these two 2D-LC-CAD-MS methods to study the change of multiple ester species in polysorbate 20 and their corresponding degradants. This 2D-LC approach allows formulation scientists to characterize polysorbate 20 in real time protein formulation and help understand the potential impact on the stability of the drug products.

**Introduction**

- Polysorbate 20 is a complex mixture of polymeric esters of different polar head groups, different fatty acid tails and different degree of esterification.

![Hydrophilic head group](image1)

- The molecular heterogeneity of polysorbate 20 and the interference from the high concentration of proteins and excipients in the protein formulation make the characterization of polysorbate 20 by conventional one-dimensional LC-MS highly challenging.

**2D-LC set up**

In this heart cutting mode, the eluent that contains the peaks of interest from the 1st D separation is trapped in the loop and then sent to the 2nd D for further analysis by an orthogonal column.

**Results and Discussion**

1. Analysis of PS 20 esters in protein formulation by mixed mode-RP 2D-LC

   ![Image](image2)

   - Low pH mobile phase washes out positively charged protein by electrostatic repulsion, esters in PS20 retain by RP mechanism
   - At PS20 esters elutes in a single peak
   - 1st D: Oasis Max mixed mode column that has both anion exchange and reversed phase properties to separate PS20 esters from protein.
   - 2nd D: Acquity BEH C18 UHPLC column with 1.7 μm particle size provides superior resolution of the esters than the previously reported methods using regular 5 μm columns.

2. Analysis of degradation products of polysorbate 20 in protein formulation by IEC-RP 2D-LC

   - POE sorbitan, POE isosorbide and POE are the by-products in the manufacture of polysorbate 20 and also the degradation products of polysorbate 20.

   ![Image](image3)

   - POE sorbitan mono-laurate
   - POE isosorbide mono-laurate
   - POE mono-laurate
   - POE sorbitan mono-myristate
   - POE isosorbide mono-myristate
   - POE mono-myristate
   - POE sorbitan mono-palmitate
   - POE isosorbide mono-palmitate
   - POE mono-palmitate
   - POE sorbitan di-laurate
   - POE isosorbide di-laurate
   - POE mono-laurate
   - POE sorbitan di-myristate
   - POE isosorbide di-myristate
   - POE mono-myristate
   - POE sorbitan di-palmitate
   - POE isosorbide di-palmitate
   - POE mono-palmitate

**Table 1:** Identification and characterization of major esters in polysorbate 20

<table>
<thead>
<tr>
<th>Peaks</th>
<th>Peak id</th>
<th>Observed [M-H(^+)]</th>
<th>Theoretical [M-H(^+)]</th>
<th>Area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>POE sorbitan mono-laurate (C(_{12}))</td>
<td>1249.76 (n=20)</td>
<td>1249.75 (n=20)</td>
<td>28</td>
</tr>
<tr>
<td>2</td>
<td>POE isosorbide mono-laurate (C(_{12}))</td>
<td>879.54 (n=12)</td>
<td>879.53 (n=12)</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>POE mono-laurate (C(_{12}))</td>
<td>707.47 (n=11)</td>
<td>707.46 (n=11)</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>POE sorbitan mono-myristate (C(_{14}))</td>
<td>1277.79 (n=20)</td>
<td>1277.78 (n=20)</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>POE isosorbide mono-myristate (C(_{14}))</td>
<td>907.58 (n=12)</td>
<td>907.56 (n=12)</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>POE mono-myristate (C(_{14}))</td>
<td>691.49 (n=10)</td>
<td>691.46 (n=10)</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>POE sorbitan mono-palmitate (C(_{16}))</td>
<td>1305.82 (n=20)</td>
<td>1305.81 (n=20)</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>POE isosorbide mono-palmitate (C(_{16}))</td>
<td>1608.04 (n=24)</td>
<td>1608.02 (n=24)</td>
<td>22</td>
</tr>
<tr>
<td>9</td>
<td>POE mono-palmitate (C(_{16}))</td>
<td>1366.06 (n=25)</td>
<td>1366.05 (n=24)</td>
<td>12</td>
</tr>
<tr>
<td>10</td>
<td>POE sorbitan di-laurate (C(_{14}))</td>
<td>1664.10 (n=24)</td>
<td>1664.08 (n=24)</td>
<td>7</td>
</tr>
</tbody>
</table>

**3. Stability of polysorbate 20 in protein drug product**

The protein drug product was stored at 5°C. The stability samples were analyzed by the mixed mode-RP UHPLC 2D-LC to study the composition of ester subspecies in polysorbate 20, and then the same sample was analyzed by the IEC-RP UHPLC 2D-LC to study the change of polyols. Almost all the esters species decreased over time. The degradation was also supported by the increase of polyols.

![Image](image4)

- The change of major polysorbate 20 esters in protein drug product compared to the esters in placebo at different stability time points.

**Conclusion**

- Two 2D-LC methods using different separation mechanisms in each dimension were developed to characterize polysorbate 20 in protein formulation sample matrix.
- Mixed mode-UHPLC 2D-LC method was developed to study the change of polysorbate 20 esters in protein sample matrix.
- Cation exchange-UHPLC 2D-LC method was developed for the analysis of degradation products of PS20.
- These two 2D-LC methods were applied to study the stability of polysorbate 20 in a real protein drug product.
- The decrease of esters in polysorbate 20 and the increase of polyols confirmed the degradation of polysorbate 20 in the protein drug product. Some polysorbate 20 esters exhibit different degradation rates in the protein drug product compared to the placebo.
- The 2D-LC methods we developed provide an important tool to characterize polysorbate 20 in the real protein drug product.

**Acknowledgements**

We thank Jackie Tyler and Yvonne Lentz for providing some of the materials. We also appreciate Colin Medley, Michael Dong and Larry Wigman for helpful suggestions.