Roundtable Session 1 – Table 10 – Best Practices in Peptide Map/MS/MS Analysis of Degradation Products and Disulfide Linkage Analysis

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Abstract: Peptide mapping combined with MS/MS analysis is a cornerstone technique for characterizing therapeutic proteins, enabling detailed assessment of primary structure, post-translational modifications, and degradation pathways. This roundtable will focus on best practices for identifying and quantifying degradation products, as well as strategies for accurate disulfide linkage analysis. Key topics include sample preparation considerations, chromatographic separation optimization, MS/MS fragmentation approaches, and data interpretation challenges. Participants will share experiences on method robustness, troubleshooting unexpected species, and leveraging advanced software tools for confident assignment of disulfide bonds and degradation-related modifications. The session aims to foster discussion on harmonizing workflows, improving sensitivity and specificity, and ensuring compliance with regulatory expectations for structural characterization.

Discussion Questions:

1. What are the most common challenges in detecting low-level degradation products during peptide mapping, and how can they be mitigated?

2. Which MS/MS fragmentation strategies (e.g., CID, HCD, ETD) provide the highest confidence for disulfide linkage analysis?

3. How do you differentiate true degradation products from sample preparation artifacts and validate disulfide bond assignments for regulatory compliance?

4. What role do software tools and automated workflows play in improving accuracy, efficiency, and confidence in peptide mapping for stability studies?

5. How do you balance sensitivity and throughput in routine peptide mapping for stability studies?

Notes:

Challenges

- Roundtable participants identified several key challenges, which are discussed in more detail in the sections below
    - Sample preparation
    - Software considerations
    - Resolution of disulfides, especially disulfide knots
    - Potential barriers to adoption of complex MS methods in QC
- Participants also noted the importance of using orthogonal methods to identify low levels of degradation and modifications
    - For example, some isolated acidic and basic variants for more detailed analysis

Fragmentation Method

- Most participants used CID and/or ETD.
    - CID yields good y-ion coverage but often cannot get strong signals from both peptides around the disulfide
    - ETD can be helpful for very complicated disulfides
- Attendees general compared peptide maps with and without reduction to confirm disulfides. It can be challenging to get complete reduction.
    - Reduction efficiency can be impacted by reducing agent – for example, TCEP may yield only partial reduction
    - In some cases, it may be necessary to denature the protein and then reduce
- Participants discussed complexities of disulfide knots, which are generated when there are multiple Cys on a single peptide that participate in disulfide bonding. These are common on IgG2 molecules.
    - For disulfide knots, Isotopically labeled reducing agent can help clarify linkages

Software

- Attendees discussed briefly software packages and capabilities to support disulfide linkage analysis
    - PMI Bios was frequently used
    - BioPharma Finder was also used
    - Participants noted that Chromeleon can be used to identify previously identified disulfides, but not to identify new ones

Sample preparation considerations

- Trypsin and LysC were the most common proteases used. Attendees noted that:
    - Trypsin is less efficient and more pH sensitive
    - Low pH digestion is used with trypsin to reduce sample-prep induced modifications
    - LysC is less pH sensitive and often used in combination with trypsin
    - LysN can also be useful; it yields a strong b ion series, which can provide complementary information
    - Trypsin is generally sufficient for most mAbs, but other proteases may be needed for more complex molecules
- Most participants use alkylation to reduce free thiols and use short digestion times (with higher enzyme: protein ratio (~1:10) and low pH)
- Participants noted that where artifactual deamidation is a concern, O18 water can be used to differentiate native vs artifactual deamidation
- Overall, participants indicated they used control and stressed conditions to identify potential sample prep challenges

Multi-attribute Method (MAM)

- Participants discussed the complexity of analyzing sialylated glycans by MS/ MAM
    - Sialylated glycans are difficult to detect using a standard MAM method
    - Some alternative approaches discussed included:
        - Removing N-linked glycan, then removing sialic acid and focusing on core-O-glycan structure
        - O-glycanase can also be used to remove O-glycans to focus on peptide
        - Proteins can also be digested into multiple subunits, for further resolution of glycosylation sites (for example using IdeS, IdeZ, or other proteases).
- Participants also discussed challenges to adoption of MAM in QC, including:
    - Capability limitations of global QC (and in country testing) labs as well as familiarity of global regulators with MAM can complicate widespread adoption
    - Software and method complexity can be a barrier to adoption in QC

    - Participants noted that even those companies that do implement MAM in QC do some in different ways.
        - Some use different instrumentation in QC vs characterization and some use same system
- Regardless of model, some critical aspects for transitioning to use of Mam in Q include:
    - Building confidence in both QC analysis and MS expertise
    - Automation of sample prep