Roundtable Session 1 - Table 8 - Best Practices for Elucidating Antibody Drug Conjugates Molecules by MS

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Abstract:

Antibody Drug Conjugates have become a key modality within biopharma in recent years. With the recent success of some ADCs in the oncology space, they've re-emerged with improved drug-like properties, higher DARs and greater complexity. All of this translates into greater challenges for analytical scientists. Best Practices for Elucidating Antibody Drug Conjugate Molecules by MS will focus on the key strategies needed to characterize antibody drug conjugates and monitor their heterogeneity and stability.

Discussion Questions:

- Native Intact MS for DAR and drug load distribution (DLD)`
- Reduced/Subunit LC-MS for chain occupancy/heterogeneity and DLD
- LC-MS-MS for site localization of conjugation
- Linker-Payload characterization (maleimide hydrolysis, linker cleavage, payload degradation, etc.)

Notes:

1. Overview and analytical strategy
    - ADC analytical characterization requires orthogonal MS strategies (intact, middle-down, and top-down) to capture heterogeneous proteoforms, conjugation site occupancy, and biotransformations. Establishing a coherent strategy across release, stability, and characterization assays is essential given the diversity of conjugation chemistries and payload/linker designs.
    - Biotransformation of linkers and payloads in biological matrices remains a major analytical challenge. Analytical workflows should be designed to detect and quantify both intact conjugates and relevant transformation products.
    - John Valliere Douglas' publication is cited as an early, foundational paper demonstrating SEC-MS for DAR analysis. It remains a frequently referenced starting point for SEC-MS method development. Buffer composition and concentration must be optimized for instrument compatibility and adduct suppression.

2. Cysteine conjugation and DAR heterogeneity

- Cysteine conjugation typically yields broad DAR distributions, particularly at high loading such as DAR 8. Heterogeneity arises from multiple accessible cysteines and variable occupancy.
- Maleimide chemistry is widely used due to its convenient Michael addition kinetics and inexpensive reagents. However, maleimide conjugation is susceptible to:
    - Retro-Michael reactions
    - Maleimide ring opening/hydrolysis which produces succinimide ring-opened species with altered charge and mass.
- Alternative approaches:
    - Engineered cysteines reduce heterogeneity by controlling conjugation stoichiometry and site occupancy.
    - Non-natural amino acid (nnAA) incorporation (via engineered codons and alternate expression hosts such as E. coli) enables site-specific chemistries orthogonal to native residues. Trade-offs include potential impacts on product quality (e.g., altered glycosylation if switching expression host), lower expression titers, and regulatory considerations.

3. Charge variants

- Maleimide ring opening (and other modifications) generates acidic species, complicating charge profiling. icIEF can detect these charge variants, but distinguishing species attributable to payload chemistry versus other modifications (deamidation, oxidation) may be challenging.
- Charge profiling is informative for stability studies, but its value as a release assay should be justified when charge changes are reversible (e.g., pH-dependent conformational/ionic interactions) or attributable to reversible payload-associated shifts.

4. Regulatory considerations and demonstrating DAR distribution

- Regulators expect a comprehensive description of DAR distribution. Intact mass analysis (native or denaturing MS) is commonly used in characterization dossiers to support quantitation of DAR species.
- For lot release, HIC remains a common orthogonal method to quantify DAR distribution (release assay), as it separates species based on hydrophobicity imparted by payloads.
- Other chromatographic options and comparisons:
    - SEC-MS and RPLC can provide complementary DAR and aggregation information. Comparative studies indicate comparable profiles between HIC, SEC, and RPLC under optimized conditions.

    - Online buffer exchange coupled to MS has been implemented as an alternative to SEC-MS for rapid intact mass/DAR characterization, reducing sample handling and column interactions.

5. Tools for payload identification and data processing

- Payload mass can be determined from known chemistry and chemical drawing tools. Observed mass shifts in intact/middle-down spectra are matched to theoretical payload masses.
- Diagnostic fragment ions from payload cleavage or linker-specific fragments accelerate identification of true positives. Software platforms like Genedata can accept user-defined payload masses and diagnostic fragments to automate identification and DAR calculation.
- Best practice: populate analysis software with expected conjugation masses and potential modification masses (e.g., ring-opened maleimide +18 Da, common adducts) to increase automated detection sensitivity and specificity.

6. Peptide-level mapping and intact conjugate analysis

- For peptide mapping of conjugated antibodies, do not attempt to remove the payload prior to digestion and LC-MS/MS. The conjugated peptides should be characterized directly: compare peak areas and MS responses of conjugated versus unconjugated peptides to estimate site occupancy and local DAR measures.
- Conjugation alters peptide physicochemical behavior (retention time, ionization efficiency, fragmentation), so interpretation requires appropriate controls and careful method qualification.

7. Lysine conjugation and stoichiometry control

- Lysine conjugation yields increased heterogeneity due to the abundance of surface-exposed Lys residues. A deliberate conjugation strategy (stoichiometry control, buffer pH, reagent equivalents, and protection strategies to exclude CDR Lys) is required to favor reproducible product profiles.
- Controls: monitor site occupancy and ensure no critical Lys residues (e.g., in CDRs) are modified. Oligo-conjugation at Lys residues is an area of active industrial development.

8. Emerging modalities and complexity

- Bispecific antibodies with single payloads introduced via engineered cysteines, and oligomer- or peptide-conjugated mAbs, introduce additional analytical complexity for DAR/site-of-attachment determination and stability profiling.
- Dual-payload ADCs (two different small-molecule drugs attached to a single antibody) are rare but present substantial analytical and regulatory challenges in separation, quantitation, and bioanalytical readouts.

9. Payload physicochemistry and stability implications
    - Trend: payload/linker designs are moving toward increased hydrophilicity to mitigate aggregation/hydrophobicity issues associated with higher drug loads. However, increased ionic functionality can change charge profiles.
    - Many payload-associated charge changes under physiological or assay conditions are reversible. Such reversible behavior should be characterized and described in regulatory submissions, providing mechanistic justification and supportive stability data that demonstrate whether observed charge shifts are process- or storage-relevant.
    - Lyophilized ADC presentations reduce PTMs induced by solution-phase degradation risk during storage. Though, solution stability post-reconstitution must be characterized.
10. Immunogenicity considerations for non-natural amino acids and payloads
    - Concern exists that nnAAs could generate neo-epitopes. Empirical observations suggest low immunogenicity risk from isolated nnAAs due to typically low titers when nnAAs are present. However, payloads themselves may present higher immunogenic potential, and the overall presentation (epitope context, payload steric effects) will influence immune recognition.
    - Immunogenicity risk assessment should combine in silico risk predictions, in vitro immunogenicity assays, and clinical monitoring strategies. Provide justification in regulatory dossiers addressing potential nnAA-related risk and payload-driven immunogenicity.