Towards an in vivo Stability Assay for ADCs and Their Metabolites in Serum by Affinity Capture LC-MS

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One of the challenges of ADCs includes the development of a method to measure ADC and their metabolic products from serum using mass spectrometry. This short report is a suggestion of the direction one might take in order to accomplish such a goal.

1.) Know the Product- ADC
To begin with it is important to know the characteristics of the ADC product prior to in vivo studies. Product should also be used as a control for the developed method (stored under reference standard conditions, in the same formulation buffer that is to be used in the in vivo study). Typical mAb characterization analysis (intact and subunit molecular mass, peptide mapping, glycans, charge heterogeneity, etc) may be desired to characterize the mAb product. These are shown in Table 1, though intact and subunit molecular mass assays, including drug load (drug-antibody ratio), may suffice. Additional information on conjugation site may also need to be elucidated. Product related impurities and degradants differ by ADC type, although those specific to the mAb portion would be similar to unconjugated mAbs. [1]

Table 1. Typical mAb Characterization Assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>Method</th>
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<tbody>
<tr>
<td>Intact Mass +/--PNGaseF</td>
<td>RP Phenyl LC-MS</td>
</tr>
<tr>
<td>Subunit Assay Ides/R +/--PNGaseF</td>
<td>RP C₄ LC-MS</td>
</tr>
<tr>
<td>NR Trypsin Peptide Map</td>
<td>RP C₁₈ UV, LC-MS/MS</td>
</tr>
<tr>
<td>NR Lys-C Peptide Map</td>
<td>RP C₄ LC-MS</td>
</tr>
<tr>
<td>(IgG2 disulfide isomers)</td>
<td></td>
</tr>
<tr>
<td>Released 2-AB Labeled Glycan Mass</td>
<td>HILIC LC-FLD-MS</td>
</tr>
<tr>
<td>Drug Load Distribution/DAR (ADC)</td>
<td>HIC UV; LC-MS; PLRP-S LC-MS</td>
</tr>
</tbody>
</table>

2.) Know Specific Type of ADC- Conjugation Type, Linker, Drug
The product related impurities, degradants, and ADC metabolites are specific features of the ADC, such as the type of conjugation of the drug-linker to the antibody and the chemistry of the linker itself. These properties affect the heterogeneity of the product including drug load (drug-
antibody ratio), micro chemical changes to the linker, how it is metabolized *in vivo*, and how it is degraded.

The *conjugation method* has significant importance on the stability of drug attachment as well as the drug load per antibody. Three common forms of conjugation include:

1. Alkylation of reduced intrachain disulfides (cysteine chemistry): 2-8 drugs/mAb; high heterogeneity
2. Alkylation of engineered cysteines: typically 2 drugs/mAb; low heterogeneity
3. Acylation of lysines: > 8 drugs/mAb; high heterogeneity/non-specific

Conjugation through interchain disulfides is performed by partial reduction followed by addition of the drug (bound to linker). This process lead can lead to multiple mAb isoforms and ~2-8 drug/mAb. An example from the literature of an ADC through reduced intrachain disulfides analyzed by HIC (UV) is shown in **Figure 1**. The HIC fractions from **Figure 1** were collected, reduced and analyzed by RP LC-MS as shown in **Figure 2** and these data were compared to CE-SDS (NR) analysis. These data were combined to obtain isomeric distribution of the HIC purified 2-,4-, and 6-drug/mAb ADCs. [2]

**Figure 1. Intact ADC HIC UV Analysis**
The linker itself can have an effect on the characterization properties of the ADC. Several linker types exist, while the two most common types of linkers are protease-cleavable and non-cleavable linkers. Here they are described conjugated with Auristatin drugs. The first is the protease-cleavable linker which consists of maleimido caproyl-valine-citrulline-p-aminobenzyloxy carbonyl moiety (vcPAB) bound to the drug monomethylauristatin E (MMAE), or vcMMAE, as shown in Figure 3. [3,4] The second type a non-cleavable linker which consists of maleimido caproic acid (mc) (no dipeptide) attached to drug such as monomethylauristatin F (MMAF), or mcMMAF, as shown in Figure 3.
A major drug deconjugation mechanism *in vitro* is **maleimide exchange** between the linker and thiol containing species. It can occur between the maleimide and cysteine, glutathione, or albumin. Maleimide exchange occurs less on the L Chain, most on the H Chain and is observed as a loss of ADC and as shown in Figure 4 as the presence of drug-conjugated albumin.[3]
Another issue with the maleimide linker stability is hydrolysis (opening of the maleimide ring) observed by a mass shift of the ADC by +18 Daltons (i.e. addition of water). [3]

Maleimide exchange is reversible and the reaction can be terminated by hydrolysis of the maleimide ring.

Next, the metabolic products of ADCs differ by ADCs type. ADCs are generally internalized in the target cell through receptor mediated endocytosis. They are delivered through lysosomes where the drug is typically released. The catabolic products of ADCs with vcMMAE and mcMMAF are different. [4] These structures are shown in Figure 5. ADCs with vcMMAE release MMAE as drug as shown in Figure 5, Structure 2 into cells. ADCs composed of mcMMAF are catabolized differently and the drug is release with a cysteine and linker attached so that the “drug” released is actually “cys-mcMMAF” as shown in Figure 5, Structure 4.

Figure 5. Structures of auristatin antibody–drug conjugates and catabolic products

3.) Method to Capture ADCs, Metabolized Products and Degradants in Serum
The loss of drug from the ADC in circulation is a putative safety risk because the drug is highly toxic. As a result, it is of interest to have a method to measure drug loss \textit{in vivo}. In addition to ADC drug-antibody-ratio (DAR), metabolic products, degradants, unconjugated mAb should be measured in an \textit{in vivo} serum or plasma assay. One method to do so would be to use the combination of affinity selection from serum or plasma of the ADC that targets the mAb portion of the ADC followed by LC-MS of the intact species captured. [5] Here, the affinity method target is the antigen so that the beads capture and pull down the mAb plus ADCs (regardless of DAR or linker modifications). The paper by Xu \textit{et al.} describes methodology to biotinylate the target antigen (extracellular domain of receptor). Streptavidin-coated magnetic beads are then coupled to these as shown in \textbf{Figure 6}.

\textbf{Figure 6. Affinity Capture Approach to Characterize ADCs \textit{in vivo}}

A time course assay can be set up. Plasma or serum is incubated with the beads for 2 hrs, washed, deglycosylated, washed and eluted with acetonitrile and formic acid. The intact ADCs and mAbs were analyzed by LC-MS using RP PLRP-S capillary column (Varian/Agilent), which is a RP column made of a highly stable non-bonded hydrophobic stationary phase with 5 µm particles with large pores (4000Å).
This method therefore allows the capture of ADCs from plasma or serum with various DAR including mAb without drug.

An example using this methodology to test stability in matrix is shown below in Figure 7.\cite{3,5} In Figure 7A, ADC was incubated in rat plasma for 0-96 hr and analyzed by LC-MS after affinity selection using the method described above. Loss of drug from the ADC was observed beginning at 6 hr as noted by the signal consistent with ADC DAR1. Major deconjugation of DAR2 was observed by 96 hr.

In the second set of data shown in Figure 7B, ADC was incubated with monkey, rat, and human plasma as well as in buffer. Aliquots were analyzed by the above methodology at 0 and/or 96 hrs. Relative percentage of DAR are listed on the spectra. Minor species (*) were consistent with incomplete deglycosylation (from sample prep). In the three plasma samples, DAR2 degraded to 35-44% of the starting material while DAR0 was observed at 13-17%.

Figure 7A. Deconvoluted Zero-Charge Mass Spectra. Affinity Capture LC-MS Shows Detection of Drug Deconjugation of ADCs in Plasma
In the next experiment, cynos were dosed with ADC at 6, 10, and 20 mg/kg groups. Serum samples were treated and analyzed with the above method and data for 1, 7 and 21 days post-dose. The deconvoluted mass spectra are shown in Figure 8. At 1 day post-dose, some deconjugation of DAR2 was observed as DAR1. By 7 days post-dose, significant deconjugation of DAR2 to DAR1 was reported and the presence of DAR0 was noted. At 21 days post-dose, the major species was DAR1 with increased levels of DAR0 observed.
Figure 8. Drug release observed in a multiple-dose toxicokinetic study of anti-MUC16 ADC in vivo. Deconvoluted Zero-Charge Mass Spectra.

A similar affinity approach to select for the drug may also be used and is shown in Figure 9. However, it does not bind to DAR0. Alternatively, it could be used to assess if the drug was present in plasma (versus target cell). After being treated with the antigen affinity beads, the drug specific beads could be used to capture any drug present in serum or plasma.
Several approaches can be taken begin to develop a specific method measure to measure ADCs, degradants, and metabolites in serum or plasma. This paper includes some ideas on where to start.

References