LABCORP BIOPHARMACEUTICAL CMC SERVICES

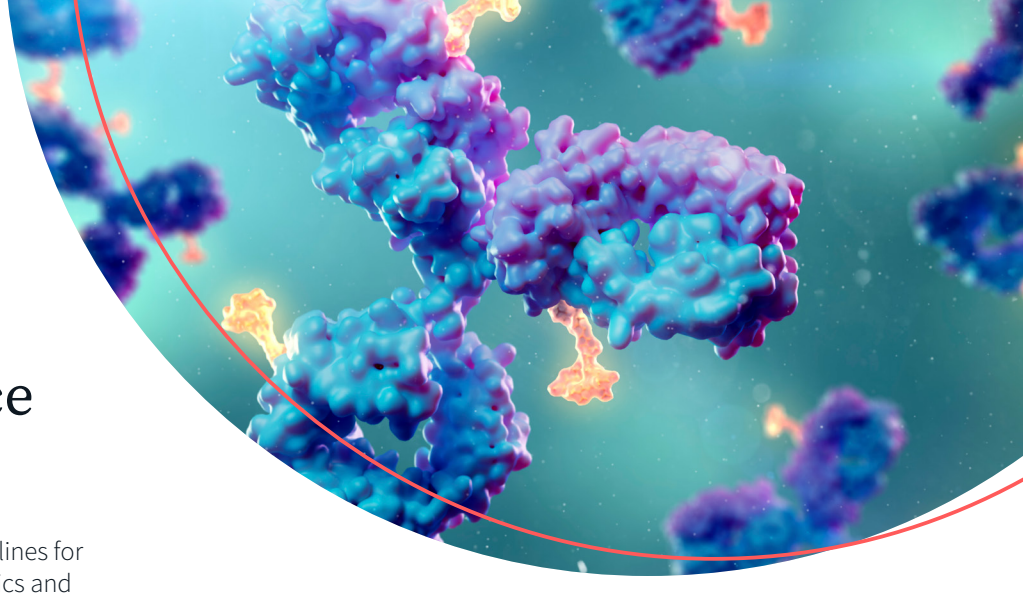
Antibody drug-conjugates (ADCs): Analytical considerations for QC release, stability testing, characterization and comparability

labcorp

Introduction

An antibody-drug conjugate (ADC) represents a biopharmaceutical molecule comprising the monoclonal antibody (mAb) delivery component, which is conjugated traditionally to a highly potent cytotoxin via a chemical linker though other conjugated moieties are being developed.

Regardless of the conjugate, the analytical approach is similar. All three components, mAb, cytotoxin and chemical linker, are crucial to the efficacy of the ADC, as well as the drug antibody ratio (DAR). Additionally, they must be addressed when it comes to the selection of analytics for release testing, stability testing, as well as characterization and comparability analysis. Adding to the complexity of analyzing ADC product is the analytical requirement for mAb intermediate, cytotoxic drug intermediate, drug substance and drug product, with the production of these materials often occurring at different manufacturing sites.



Regulatory guidance and ADC analysis

Although there are no specific regulatory guidelines for ADCs, the guidance documents for both biologics and small molecules apply. These are:

- ICH Q1A (R2)
- ICH Q3C
- ICH Q6B
- ICH Q6A
- ICH Q5C

Hence, for mAb and drug components, the QC release testing and the analytical methods need to evaluate the following critical quality attributes:

- Appearance and description
- Quantity
- Identity
- Purity/impurities
- Potency
- General tests

Due to the complexity of the mAb structure, compared to the small linker/cytotoxin drug, most of the analytical techniques focus on the protein component of the ADC. For the most part, standard techniques for mAb analysis can be used, such as those outlined in the following:

- Appearance
- pH
- Peptide mapping
- Aggregates (SEC HPLC)
- Charge variants (IEX HPLC or cIEF)
- Molecular size (SDS-PAGE red and NR)
- Concentration (A280)
- Ligand binding (ELISA)
- Glycan analysis
- Residual DNA (qPCR)
- Residual HCP (ELISA)
- Residual Protein A (ELISA)
- Particulates (subvisible particles)
- Endotoxin
- Bioburden

For mAbs, many of these methods are either platform assays or compendial, which simplifies the compilation of analytical techniques needed for a release specification. Additionally, a subset of these assays would be stability-indicating and would be applicable for inclusion in stability studies, whether real-time, accelerated or forced degradation studies, such as in a developability risk assessment.

In line with ICH Q6B expectations, in addition to any QC release analytics, a range of techniques need to be applied for protein characterization. It is expected that orthogonal assays be used to provide a wide-ranging, comprehensive understanding of the ADC molecule. ICH Q6B specifically states the need for biophysical techniques in evaluating the higher order structure of the biologic. This is particularly pertinent to ADCs as the potential of the hydrophobic linker/toxin to perturb the secondary/tertiary structure of the mAb. Such techniques could include intrinsic and extrinsic fluorescence, circular dichroism and differential scanning calorimetry. Such structural perturbations also have the potential to increase protein aggregation, and an orthogonal test to evaluate this, such as size exclusion chromatograph (SEC), dynamic light scattering or analytical ultracentrifugation, can be applied.

As an ADC progresses through its life cycle of development, it is expected that comparability studies will be performed to ensure continuity of critical quality attributes associated with manufacturing modifications, as outlined in ICH Q5E. Such comparability studies will involve a suitable selected range of the assays outlined in this document.

ADC-specific analytical techniques

Physicochemical analysis

DAR and drug load distribution

It should also be recognized that ADCs represent a unique set of biopharmaceuticals; consequently, additional specific methods need to be applied. A critical quality attribute of an ADC is the DAR. There are several approaches to determining the number of toxin molecules conjugated to the mAb. The simplest is UV/vis spectroscopy; this method is dependent on the linker/toxin having a different absorbance maximum wavelength from that of the protein. By determining the A280 and the linker/toxin Amax values, it is possible to determine the relative concentrations using the relevant extinction coefficients.

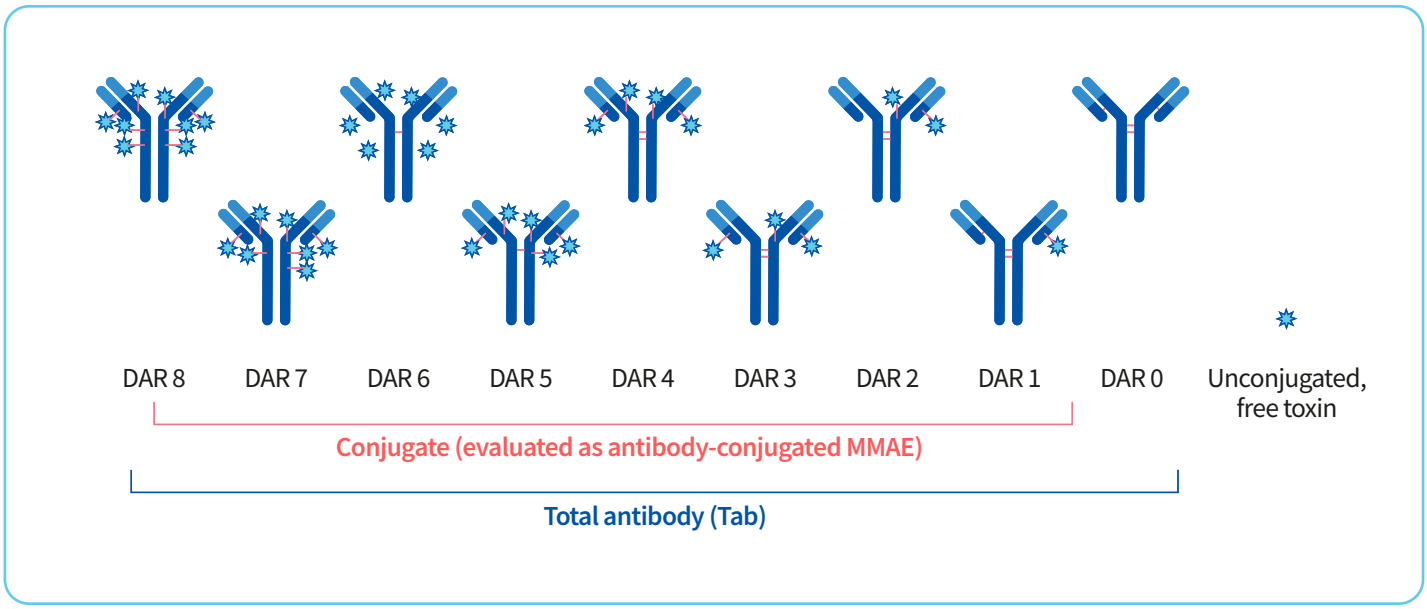


Figure 1: DAR of an ADC

Liquid chromatography (LC) methods can also be used in DAR determination, as the linker/toxin moieties are generally hydrophobic, and progressive conjugation increases the hydrophobic nature of the naked mAb. Reverse-phase HPLC using a PLRP-S column can fractionate the reduced ADC into light chain and heavy chain components; the more hydrophilic unconjugated heavy chains and light chains elute earlier than those conjugated with linker/toxin. Hydrophobic interaction chromatography (HIC) HPLC works on a similar principle of fractionating by hydrophilicity/hydrophobicity. With this technique, the unreduced ADC is fractionated and the retention time relates to the number of linker/toxins conjugated to the mAb. For both of these LC methods, DAR values can be deduced using peak areas quantitation and knowing the respective loading of linker/toxin for each peak.

An advantage of these HPLC methods over that of the UV/vis spectroscopy is that both provide additional information on drug load distribution Drug load distribution can be considered another critical quality attribute, as it is not sufficient to know the average DAR for any ADC; it's also essential to know the distribution of drug-linked species within the ADC, as wel as to what extent, if any, there is unconjugated mAb.

Another more data-rich approach is to use LC/MS to fractionate the various ADC DAR components and use these data to derive an overall DAR value. As with the HPLC approach, this method relies on fractionation of the ADC components and then determination of intact mass using either matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) MS. The subsequent addition of the known molecular weight of the linker/toxin enables the identification and quantification of the various ADC variants.

Sites of conjugation

Depending on the chemistry and site of conjugation—for example lysine, cysteine or non-natural amino acid linked—the resultant ADCs can be either stochastic or have site-directed linkages. Understanding the location of the linker/conjugation is an important characterization component. While this is simpler for site directed conjugations, with the stochastic conjugations the heterogeneous mixture of ADC species makes analysis difficult. Achieving this requires LC/MS/MS using the proteolytic hydrolysis/peptide mapping approach and looking specifically for linker/toxin conjugated peptides.

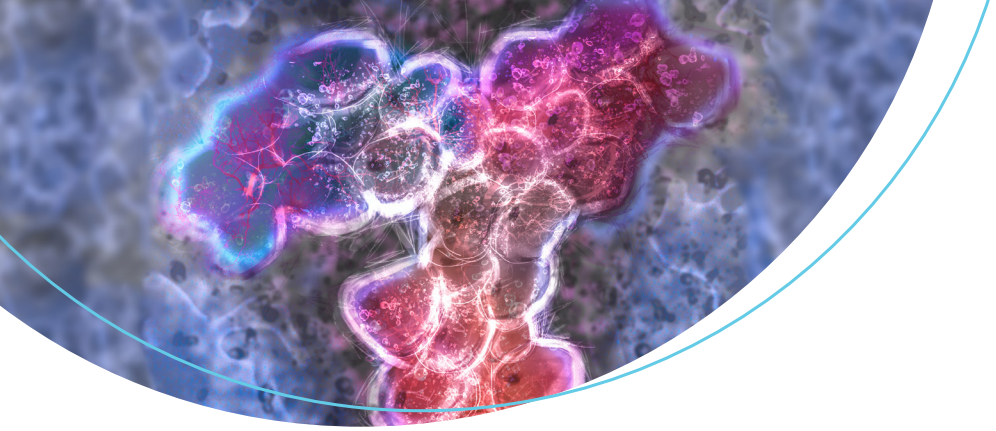
Once the various peptides, including those linked to the cytotoxin, have been identified by MS, the various conjugation sites can be routinely monitored using the LC peptide map. Accordingly, the LC peptide map represents an essential component of an ADC stability study.

Impurities: Free cytotoxin or other conjugate

As discussed in ICH Q6A and B, analysis of impurity levels is an essential part of any QC release package. Generally, impurities that exceed 0.1% need to be characterized and monitored. Standard process and product-related impurity analyses are applied to any biologic or small molecule release specification and hence would be included in an ADC release specification. As it specifically applies to ADCs, the levels of free cytotoxin are a critical quality attribute that need to be determined, particularly as this relates to the toxicity of the product and hence patient safety. Several approaches are possible to determine free toxin, but the most common is HPLC, with the precise method depending on the drug in question. An alternative approach is to use LC/MS, quantifying the toxin-related ions following LC fractionation. The free cytotoxin analysis can involve using a quantitative test to determine the precise levels or alternatively a limit test to ensure the product has suitably low levels of cytotoxin.

Impurities: Residual solvent

The majority of biologic manufacture is aqueous based and involves no organic solvents; in contrast, the production of ADCs uses such solvents to solubilize the linker/toxin components prior to conjugation. As a result, ICH Q3C guidelines apply and residual solvent levels need to be monitored with ADCs. Depending on the solvent used in manufacture, suitable methods using gas chromatography, LC or LC/MS can be applied. As with the free cytotoxin analysis, the assay can be designed to be either a quantitative or a limit test.



Binding and biological activity

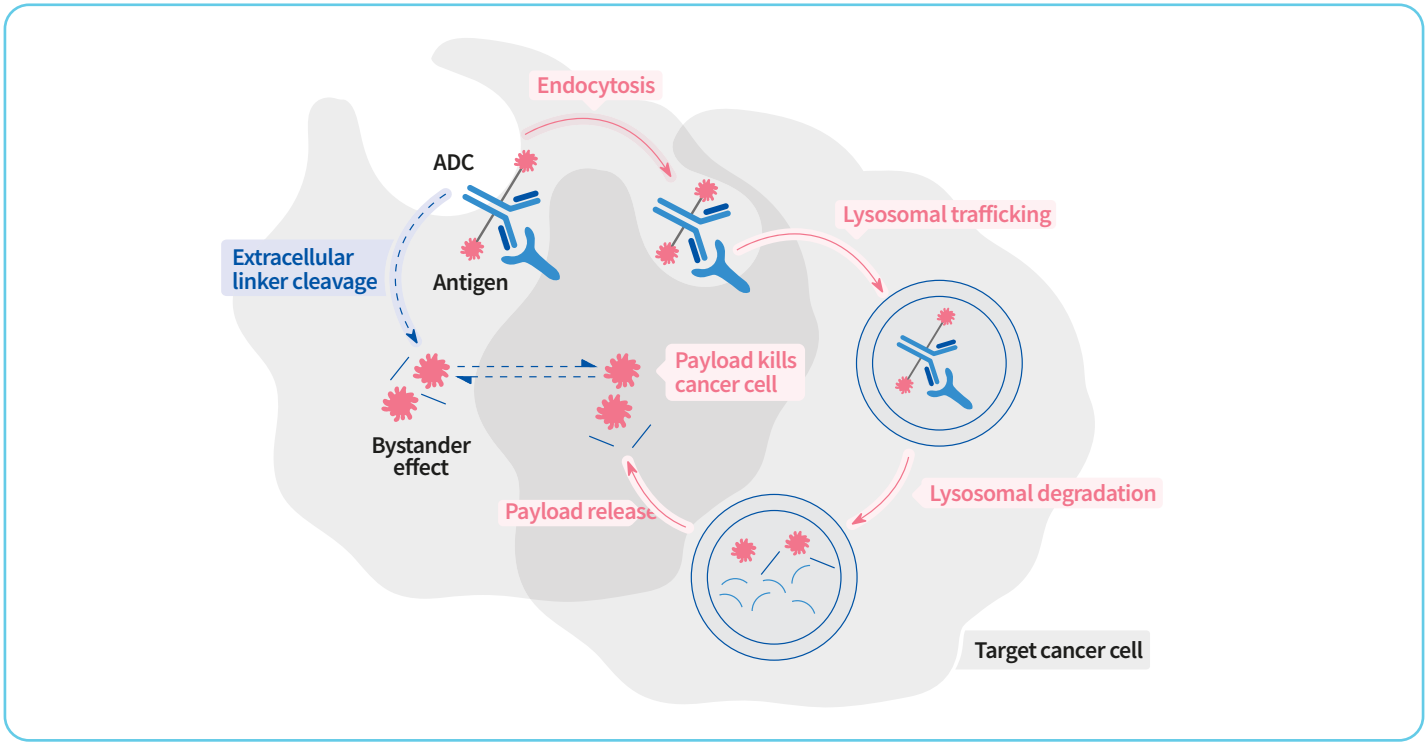


Figure 2: Principal mechanism of action (MOA) for an ADC

Antigen binding

With ADCs, the function of the mAb is to specifically deliver the cytotoxic agent to the target cell. As a delivery device, ligand binding is a critical quality attribute and needs to be determined to demonstrate that the target binding affinity is unaltered compared to the naked mAb. Such ligand binding assays can use standard ELISA formats or extend to the more sophisticated optical interferometry or surface plasmon resonance methods. With these more sophisticated ligand binding techniques, both affinity and kinetic parameters of ADC binding to the target antigen can be determined.

Cell-based cytotoxicity assay

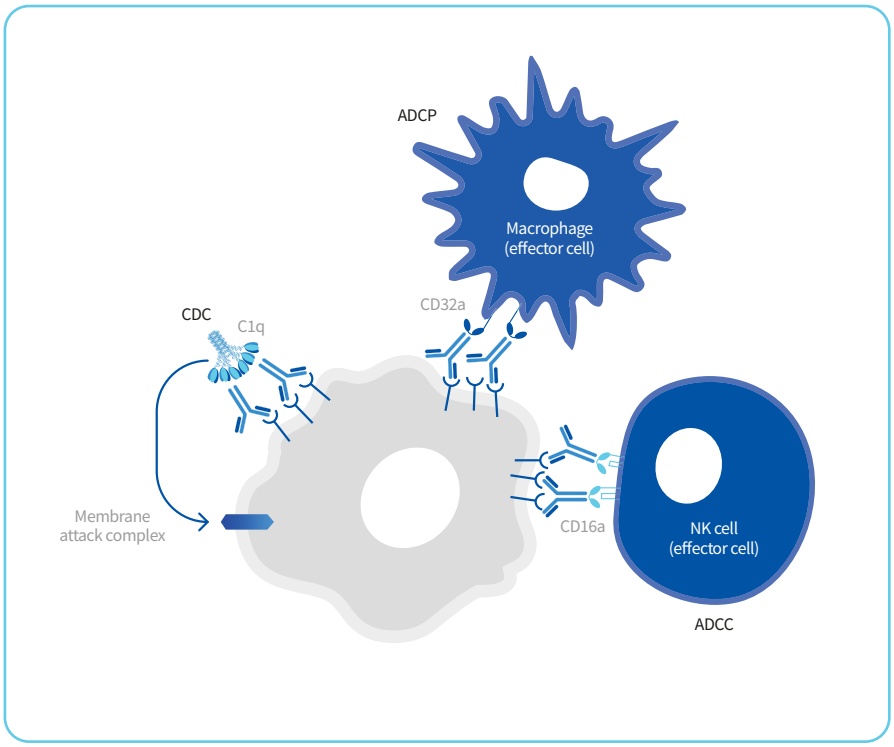
As the ultimate aim of an ADC is to induce cell death in target tumor cells, a method to determine drug efficacy is essential. For CMC purposes, such as QC release and stability testing, a specific cell-based cytotoxicity assay needs to be developed for an ADC. ADC-specific cytotoxicity assays require a cell line that expresses the target antigen and a measurement of cell death, such as LDH release. The advantage of a cell-based assay is that full functionality requires binding to the cell surface antigen, internalization of the ADC and subsequent degradation of the mAb or cleavage of the linker to release the cytotoxin. It is the released cytotoxin that induces cell death. As these cell-based cytotoxicity assays closely resemble the mode of action of the ADC, they represent ideal biological activity assays for QC release and stability testing.

CDR-associated function

The complementarity determining region (CDR) of the mAb is the variable region responsible for the specific binding to the antigen. By the very act of binding to the target antigen, the CDR may induce or indeed inhibit a biological response. Binding to the antigen protein could inhibit its functionality either due to steric hindrance or inactivation of critical components of the protein structure. Alternatively, mAb binding could cross-link proteins and induce activation. For example, trastuzumab ADCs, in addition to inducing cell death through the conjugated toxin, can activate an antiproliferative effect as a result of binding to the HER2 EGF receptor. Where the CDR, by virtue of binding to a ligand induces a biological response, the expectation is that this will be investigated as part of the characterization package. As the CDR-associated function will be highly dependent on the antigen, the resultant cell-based assay will be reliant on the biological response being determined using a cell-based assay.

Fc effector function

In addition to binding to their specific antigen, mAbs are able to induce antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cell-mediated cytotoxicity (CDC) immune responses depending on their ability to bind to specific Fcγ receptors, which is predicated by the IgG subtype. The level of ADCC or CDC effector function is high for human IgG1 and IgG3, low for IgG2, and low or absent for IgG4. ADCs can be engineered to either have or be devoid of Fc effector function, depending on the IgG subtype selected. Nevertheless, it is necessary to demonstrate the presence or absence of ADCC and CDC activity.



To analyze for ADCC or CDC activity, the assay needs a specific target cell that expresses the surface antigen specific for the ADC. With ADCC, cell death is mediated by NK or T-cells, using either a specific cell line or alternatively PBMCs. Similarly, with ADCP phagocytosis is determined using a monocyte, macrophage or dendritic cell line. As with the cytotoxicity assay, cell death can be quantified using LDH release. Alternatively, specific ADCC and ADCP reporter bioassays are available using a fluorescence endpoint. While for ADCC and ADCP the reporter bioassay is the simplest approach, for CDC the assay needs to incorporate an appropriate target cell and a source of complement, which can be either plasma or recombinant protein. CDC activity is determined by cell death.

Unless Fc effector function is central to the function of the ADC, ADCC and CDC analysis can be performed as a characterization assay.

Figure 3: Effector function of an mAb

Additional characterization can be achieved by determining ADC binding, or lack thereof, to the various Fc receptors. Such ligand binding assays can be performed using optical interferometry or surface plasmon resonance to determine the affinity and kinetic binding to the range of Fcγ receptors.

Bystander effect

A third type of cell-based bioassay that can be applied to ADC characterization is the bystander assay. The bystander effect is an ADC's ability to induce cytotoxicity in antigen-positive, as well as in closely associated antigen-negative, cells. The effect relies upon cellular uptake and metabolism of the ADC by the antigen-positive cells with subsequent release of the free toxin into the tumor microenvironment. In a cell-based bystander bioassay, antigen-positive and -negative cells are co-cultured with ADC. Using specific markers for cell death and the antigen-negative cells, fluorescent-activated cell sorting (FACS) can be used to determine the extent of ADC-induced cytotoxicity in the bystander cell population.

Summary

An ADC represents a complex biopharmaceutical molecule comprising an mAb and a highly potent cytotoxin or other conjugate, both linked via a chemical linker (i.e., a drug that comprises both biologic and small molecule). This complexity introduces significant issues for the analytical scientist.

In addition to the standard analytical techniques for proteins and small molecule drugs, ADCs require a range of specific methods to gain a comprehensive understanding of the dual nature of the ADC molecule. Additionally, it is necessary to fully characterize the mode of action of the ADC, as the molecule can have more subtle biological effects other than the guided delivery of the cytotoxin to the antigen-expressing tumor cells. Even more complexity comes from the fact that analysis must be performed on mAb intermediate, cytotoxic drug intermediate, drug substance and drug product, which are more than likely to be manufactured at different GMP sites.

One potential risk mitigation strategy around ADC analysis is to centralize the analytical testing. This approach can reduce the number of analytical methods that need to be developed/established, as some of the methods will apply to mAb intermediate, drug substance and drug product. Moreover, this approach creates data consistency, reducing the potential for different data outputs from two or more laboratories. Of course, it also reduces the analytical burden when it comes to qualification or validation of the assays for GMP compliance. A further risk mitigation strategy is to identify an analytical research laboratory experienced in handling the complexity that is the ADC biopharmaceutical.

Visit us at **labcorp.com**

labcorp