Finding your way in ADC bioanalytics

Antibody-Drug Conjugates (ADCs) are drugs which combine very potent (i.e. toxic) payloads with a highly selective antibody to guide the payload to its target. Although the approval of the first ADC as a treatment was already in 2000, the number of ADCs currently on the market remains limited: approximately 13 are now used in mainly oncological applications. This is dictated by the complexity of developing this type of molecule for therapeutic purposes. However, due to improvements in the structural design of mainly the linker between antibody and payload, the number of new generation ADCs that is currently being developed is now rapidly growing.

To understand the pharmacokinetics (PK) of ADCs and their metabolism to support drug development programs, it is important to develop accurate and robust bioanalytical methods. This comes with challenges due to the nature of the drug, where several potential PK analytes may need to be quantified, e.g. the conjugated and free antibody, a small molecule toxin, a linker and several combinations of those. Moreover, ADCs are unstable by design, heterogeneous in structure and their individual components have very different concentrations in the circulation, which is further adding to the complexity of their assays.

In this commentary paper we will highlight some of the challenges of typical bioanalytical ADC assays and provide insights into possible approaches for designing a bioanalytical strategy to support ADC drug development. It must be emphasised that each program is unique and that scientific considerations must always be leading in determining what is aimed for, but also what can realistically be achieved.

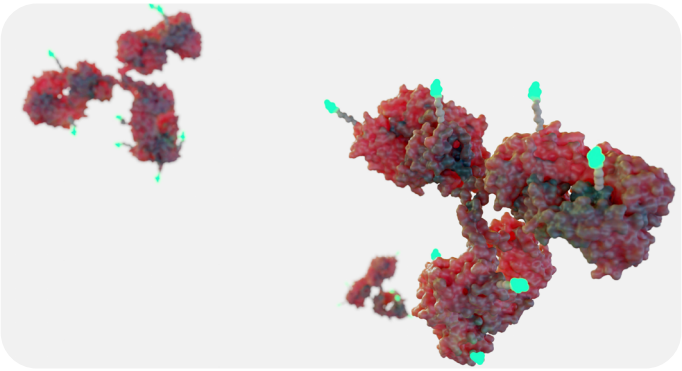
The complexity of ADC bioanalytics

Firstly, it is important to realise that multiple bioanalytical assays are usually needed for ADCs. Typically, a series of PK assays is required, with in its simplest form one for ADC (antibody linked to at least one cytotoxic payload), one for total antibody (including those without payload), and an assay for free payload. This provides information on the degree of payload release. However, the biological situation can easily become more complicated. Many of the linker molecules can be cleaved in several ways under physiological conditions, while some payloads can also undergo metabolism, both after release and while still attached to the antibody. This may lead to several different analytes consisting of free payload or its metabolite connected to various linker fragments. In addition, next to intact ADC containing unmodified payload, it may also be relevant to determine the concentrations of ADC containing metabolised payload. The importance of each determination is dependent on the abundance and therapeutic activity of each of the ADC-related molecules and it is to be decided on a case-by-case basis which assays are required to take informed decisions on the development of the ADC.

Another layer of complexity is added, as an ADC is not a single molecular species, but rather a heterogenous group of molecules, with different numbers of payloads per antibody which is often referred to as the Drug-to-Antibody Ratio (DAR). The DAR of the conjugated antibody may affect its binding capacity in an assay and response in Ligand Binding Assays (LBAs), so different DAR species may not give the same quantification. Finally, the newer generation of ADCs includes molecules of even more structural complexity, such as bispecific ADCs for dual antigen binding and ADCs containing multiple types of payloads.

The type of bioanalytical platform required for the various PK assessments varies. The (small molecule) cytotoxin PK assays often are Liquid Chromatography Mass Spectrometry (LC-MS/MS)-based methods, while the antibody PK is typically measured using LBAs. However, there have been major recent advances that have made LC-MS/MS a reliable complementary technique for protein bioanalysis. Most if not all antibody analyses may also be done using LC-MS/MS-based approaches. Approaches without or with an immunocapture step to selectively extract the molecule(s) of interest from the biological sample can be chosen, the latter usually being referred to as a hybrid LBA – LC-MS/MS assay.

Finally, immunogenicity needs to be assessed by measuring anti-drug antibodies (ADAs), sometimes in a number of different assays, each with their own specificity against the relevant domains of the ADC. For most of these assays critical reagents are required and the assays will only be as good as the quality of the available reagents. While LBAs are typically used for ADA assessment, hybrid LBA – LC-MS/MS approaches have also been recently explored which can provide semi-quantitative concentrations of ADA isotypes and can thus be seen as a useful complementary technique. Finally, ADAs can interfere with the ADC PK assays by competition between the interactions of the critical assay reagents with the drug and the ADAs.



Altogether, it is very important to design assays and develop critical assay reagents that take all of the assay complexities into account. Depending on the questions to be answered on the drug in development, a solid bioanalytical strategy must be designed and updated continuously during the process of assay validation and during sample analysis. Some further considerations are summarized below.

Bioanalytical approaches

Table 1 provides an overview of possible PK bioanalytical assays that may be developed to support development of an ADC and some of their characteristics.

LBA assays

The PK assessment of the antibody part of the ADC is usually performed by LBAs and is separated into at least two PK assays: the ADC (or conjugated antibody) assay and the total antibody assay. The ADC assay uses an assay format in which the payload and antibody part of the ADC are bound with separate capture and detection tools. In this way, the antibody part of the ADC, that is linked to at least one cytotoxic payload, will be detected. The second assay, referred to as the total PK assay, uses an assay format in which both the capture and detection antibodies are directed against the antibody part of the ADC. This allows all antibodies to be detected, including those without payload.

During development of these LBA PK assays for ADCs it is important to take the ADC characteristics such as the DAR and its distribution into account since they can influence the binding process and target recognition in the assay. The ADC drug product and assay reference standard will be available as a heterogeneous mix of antibodies with different numbers of payload molecules per antibody (different DAR). As a result, different assay responses may be obtained for ADC forms with different DARs. Moreover, the ADC will also be heterogeneous by the binding sites of the payload molecules. The presence of payload molecules close to an assay binding epitope may impact the binding properties of an ADC in an LBA and thus the assay response. Since the total antibody assay is dependent on binding of both the capture and detection tools to the antibody part of the ADC, this assay type is likely to be more prone to influences on the LBA responses caused by DAR and its distribution than the conjugated antibody assay. During set-up of these assays, it is important to test the ADC with different DARs to determine the impact on the binding of the capture and detection antibody and assay response. However, the availability of ADC with different DARs, for instance separate fractions of the ADC with DAR = 0, DAR = 2 and DAR = 4 might be limited since its production is not straightforward.

The ADC drug product, which will be used for the preparation of the PK assay calibration curve, will possibly also contain some DAR = 0 antibodies which will not be measured in the conjugated antibody assay. Therefore, it is important to correct for the percentage of DAR = 0 in the reference standard, so information on the percentage of DAR = 0 in the ADC drug product must be available. During validation of the conjugated antibody method, a valuable experiment is a DAR = 0 interference experiment. This experiment provides information on the capability of the conjugated antibody assay to solely measure payload-conjugated ADCs and not the “naked” antibody. For this means, DAR = 0 standard should be spiked at a high concentration to matrix samples; no response of this spike should be visible.

The determination of ADC specific ADAs is generally performed using an LBA in the form of a bridging electrochemiluminescence immunoassay. Due to the multi-facet nature of ADCs, the use of different assays for ADAs against the antibody, payload and linker will provide the complete picture of the treatment-related immunogenic response. A more pragmatic approach would be to develop one ADA assay for ADAs against the complete ADC-linker-payload complex and to perform more specific ADA isotype characterisation for confirmed ADA positive samples. Availability of assay reagents for such assays can be limited, although the “naked” ADC antibody is always available for confirming response against the antibody part of the ADC.

LC-MS/MS assays

Widely used as the first choice for quantitation of small molecules, LC-MS/MS is routinely employed for the determination of free payload in ADC development. Typically, any proteins (including the ADC itself) are removed from the biological sample by protein precipitation or solid-phase extraction and the extract is analysed with reversed-phase LC coupled to a triple quadrupole mass spectrometer against a stable-isotope labeled (SIL) internal standard. The multiplexing capabilities of this technique allow the inclusion of other small molecules into an LC-MS/MS assay, usually without major challenges, such as payload coupled to linker and metabolites of the payload and/or payload-plus linker. Ideally, all these analytes have their own SIL internal standard. One major point of attention for free payload analysis is the fact that they usually occur in the biological matrix of interest in very low concentrations but in the presence of an enormous excess (up to 10,000-fold or more) of the ADC. In vitro release of payload from the ADC, even if very limited, therefore easily leads to a significant overestimation of free payload levels, so this effect should be carefully investigated and minimised.

When assessment of the concentration of conjugated payload is needed, e.g. to obtain information on the average DAR, the LC-MS/MS method for the free payload can be extended with a step to release all payload molecules from the ADC, by forced cleavage of the linker. To distinguish from free payload, the ADC molecules are first isolated from the sample, e.g. by an immunocapture step with an (anti-idiotypic) antibody directed to the ADC or with the pharmacological target. With the same approach, any conjugated metabolised and/or linker-containing forms of the payload can be included in the assay. In preclinical assays, isolation of human ADC can also be done using protein A/G.

Even though the concentration of (total or conjugated) antibody is usually determined with an LBA, LC-MS/MS may also be used for that purpose. When critical LBA reagents are not (yet) available and concentrations are relatively high, the total protein content of the sample, including ADC, can be isolated by precipitation and the protein pellet digested. A unique ADC-specific signature peptide is subsequently quantified by LC-MS/MS as a surrogate for the intact (total) antibody. Ideally, a SIL form of the intact ADC is used as internal standard, but in practice a SIL-signature peptide also does the job. To enhance selectivity and sensitivity, an immunocapture step can be included to extract total ADC-related antibodies from the sample, but an anti-idiotypic antibody (or the pharmacological target) then has to be available. Again, for human antibody-based ADCs in preclinical samples, a more generic extraction with protein A/G or an anti-human Fc antibody can also be performed. Similarly, conjugated antibody concentrations can be determined by first extracting payload-containing antibody from the sample using a payload-directed antibody and subsequently quantifying the ADC-specific signature peptide by LC-MS/MS.

By combining immunocapture with high-resolution mass spectrometry (HRMS), a distribution profile for the DARs of the ADC in a particular sample can be obtained. After dosing, the initial (average) DAR, as present in the pharmaceutical formulation, changes in vivo by the gradual release of payload and this change can be monitored over time. After extracting the ADC molecules with all different DARs from a sample, HRMS can determine the mass of each of the molecular species, either intact or (after reduction and/or enzymatic treatment) at the antibody subunit level. This gives direct insight into the distribution of the number of payloads present on the antibody.

Validation requirements

PK method validations for ADCs are performed according to ICH M10 regulations and do not differ from any other PK method validation study. Some additional validation experiments may be added as driven by scientific considerations. A potential additional experiment for the total antibody assay is specificity to show that the assay detects its target independent of the DAR. This specificity experiment can be performed as an assessment of precision and accuracy (P&A) with the DAR = 0 fraction of the ADC, this reference standard should typically be available. However, additional experiments can also be performed depending on standard availability.

Table 1: Overview of bioanalytical assays for assessing PK of ADCs

Analyte	Technique	Remarks
Free payload (also called warhead, toxin or drug)	LC-MS/MS	– With or without linker attached – With or without metabolite(s) – Often at pg/mL levels – Typically with protein precipitation or solid-phase extraction – Selectivity and stability assessment also in presence of excess ADC
Conjugated payload (total concentration of payload conjugated to antibody)	LC-MS/MS	– Immunocapture with anti-idiotypic antibody or target – Suitable cleavage step to release payload from captured ADC – Quantification of payload with or without linker and/or metabolite(s)
Total antibody	(sandwich) LBA	– Capture with anti-idiotypic antibody or target – Detection with anti-idiotypic or anti-Fc antibody
Total antibody	LC-MS/MS	– Immunocapture with anti-idiotypic antibody or target and enzymatic digestion – Alternatively: direct digestion of the entire sample – Quantification of unique signature peptide
Conjugated antibody (also called ADC)	(sandwich) LBA	– Capture with anti-payload antibody – Detection with anti-idiotypic or anti-Fc antibody
Conjugated antibody	LC-MS/MS	– Immunocapture with anti-payload antibody and enzymatic digestion – Quantification of unique signature peptide
DAR analysis	LC-HRMS	– Immunocapture with anti-idiotypic antibody or target – Intact mass analysis of the extract for ADC with all different DARs by deconvolution of mass spectra

Conclusion

In the widely diverse field of bioanalysis of biotherapeutics, bioanalysis for ADC forms a separate category. This commentary provides a short overview of the different bioanalytical assay types and technologies that can be used for the ADC category. Each assay comes with their own characteristics, requirements and challenges; based on the biology of the ADC and the needs of the drug development project, a suitable analytical approach needs to be determined.

ICON has been working on ADC assays for many years with a team of experienced scientists in LC-MS/MS, LBA PK and ADA assays for ADC drug development.

Please visit ICONplc.com/BioA or ICONplc.com/contact to further discuss your project needs.

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