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WHITE PAPER

Updated Recommendations for the Bioanalysis of Antibody–Drug Conjugates (ADC) from the ADC working group of the AAPS Bioanalytical Community

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Abstract

Antibody–drug conjugates (ADCs) represent a rapidly evolving therapeutic modality, combining the selective targeting of monoclonal antibodies with highly potent small molecule payloads. Their inherent structural complexity demands a sophisticated and multi-faceted bioanalytical approach spanning preclinical discovery through late-stage clinical development. This white paper, developed by the ADC Working Group of the AAPS bioanalytical community, comprising over 100 members from industry, contract research organizations (CROs), and regulatory agencies, provides updated recommendations for ADC bioanalysis. Building upon the foundational 2013 AAPS position paper and recent publications governing regulatory frameworks on PK considerations, this work addresses advances in bioanalytical quantitation strategies for total antibody (tAb), conjugated ADC, free (unconjugated) payload, and drug-to antibody ratio (DAR); novel immunogenicity assessment considerations; soluble target interference; critical reagent lifecycle management; payload-specific stability requirements; cross-validation strategies; and regulatory considerations. The recommendations presented herein reflect over a decade of scientific progress and are designed to serve as a comprehensive, empirically validated, and industry aligned bioanalytical framework for contemporary ADC drug development.

Keywords antibody–drug conjugate · bioanalysis · crossvalidation · drug-to-antibody ratio · free payload · immunogenicity · LC–MS/MS · ligand binding assay · pharmacokinetics · regulatory guidance

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Introduction

Antibody–drug conjugates (ADC) are a unique chemical modality that are a valuable and increasingly important therapeutic in the biopharmaceutical industry particularly in oncology and other new therapeutic areas such as infectious diseases, immunology, and neuroscience. Their uniqueness comes from combining the selective targeting ability of antibodies with potent payloads. This distinct combination aids in enhanced targeted therapy and increased therapeutic index while addressing drug resistance against small molecule therapies (1). The fundamental structure of an ADC comprises three key components: the antibody, the linker, and the payload/warhead. The antibody, frequently built on IgG backbone, is responsible for targeting specific antigens expressed on cells to deliver the drug directly to its site of action. The linker, which can be either cleavable or non-cleavable, connects the payload to the antibody and plays a critical role in the stability and release of the therapeutic agent within the target cells. The payload, typically a highly potent small molecule drug, is designed to interact with specific biological targets upon release, thus enhancing the overall therapeutic index of the ADC compared to payload alone, and reducing off-target toxicity. Although non-small molecule payloads have gained popularity in recent years, this white paper will focus exclusively on small molecule payloads due to space limitations.

The bioanalysis of ADCs can be challenging due to their inherent structural and functional complexity. To suitably characterize an ADC and what could potentially be a variety of molecular species, a distinctive panel of bioanalytical assays are utilized both in preclinical and clinical stages of drug development. In this white paper, we will use the following terminology, which is aligned with regulatory agencies' guidance to the industry. ADC refers to antibodies or fragments with at least one payload attached by a chemical linker. There are two types of ADC assay, conjugated payload assay and conjugated antibody assay. A conjugated payload assay measures the number of payloads on the ADC, while a conjugated antibody assay measures the number of antibodies with attached payload, regardless of the number. Total antibody (tAb) includes antibodies that are either conjugated or not conjugated to a payload. Unconjugated payload (free payload) refers to the small molecule drug that is not attached to an antibody.

Since the publication of a position paper by Gorovits et al. in 2013 (2) on the bioanalysis of antibody–drug conjugates, both the scientific understanding of ADC and the technologies supporting their bioanalysis have advanced significantly (3, 4). The 2013 AAPS Position Paper established the foundational framework for ADCs bioanalysis by identifying key analytes, characterizing DAR heterogeneity challenges, and underscoring the critical absence of ADC-specific regulatory guidance at that time. Our current publication reflects over a decade of scientific and regulatory advancement, most notably the maturation of liquid chromatography tandem mass spectrometry (LC–MS/MS) as an analytical platform for protein therapeutics bioanalysis, the introduction of formalized phase-appropriate bioanalytical strategies, and the establishment of governing regulatory frameworks including regulatory guidance (5, 6). Furthermore, the white paper addresses several domains not contemplated in the original position paper — among them soluble target interference, inter-laboratory cross-validation strategies, critical reagent lifecycle management, and payload-specific stability considerations for modern cytotoxic agents such as DXd. The current publication represents a logical evolution from the 2013 position paper, translating foundational principles into a comprehensive, empirically validated, and regulatory-aligned bioanalytical playbook for contemporary ADC drug development. This white paper presents current perspectives and methodologies, offering a comprehensive overview of bioanalytical strategies employed in the study of ADC. It covers the quantification of tAb, ADC, and free payload, while also emphasizing the importance of drug-to-antibody ratio (DAR) determination, immunogenicity considerations, and the impact of soluble target.

Following release of the guidance documents on Clinical Pharmacology Considerations for Antibody–Drug Conjugates (5, 6), the AAPS ADC Working Group—comprising more than 100 members from industry, contract research organizations, and regulatory agencies—conducted surveys and engaged in discussions to align on best practices. These activities focused on phase-appropriate testing strategies, cross-validation criteria, stability considerations, and the measurement of target-unbound *versus* target-bound ADCs.

Both guidance documents are aligned on bioanalytical expectations for ADCs, i.e., fully validated bioanalytical methods following the U.S. Food and Drug Administration (FDA)'s M10 Bioanalytical Method Validation and Study Sample Analysis (7) (abbreviated as "M10" hereafter) guidance for core analytes including the ADC, total antibody, free payload, and pharmacologically active metabolites. Notably, however, China's National Medical Products Administration (NMPA)/Center for Drug Evaluation (CDE) guidance extends beyond the FDA's recommendations by explicitly requiring characterization of conjugated payload and DAR evolution over time (6). This article aims to summarize the consensus on these topics among bioanalytical scientists.

Measurement of ADC and its Constituent Parts

The choice of technology platform, depicted in Fig. 1, depends on many factors, including reagent availability, linker type (cleavable *versus* non-cleavable), throughput, and sensitivity. Ligand binding assays (LBAs), including Electrochemiluminescence Immunoassay (ECLIA) and Enzyme-Linked Immunosorbent Assay (ELISA), are high throughput and sensitive but require specialized reagents for both capture and detection. Hybrid immunocapture LC–MS/MS methods have less onerous requirements for capture reagent and offer additional advantages in terms of selectivity and specificity, at the expense of throughput (4, 8–12). LC–MS/MS is increasingly being adopted and applied in the quantitation of ADC drugs (2, 13–16).

The bioanalytical strategies for ADCs should address the dynamic nature of these conjugates *in vivo*, where the DAR can change over time. An understanding of the stability and clearance of ADCs, as well as their potential to induce immune responses, is crucial for appropriate method selection for quantification of tAb and ADC (4).

Measurement of Total Antibody (tAb)

The tAb assay measures the concentration of all circulating DAR forms of a given ADC including fully deconjugated (DAR = 0). Both hybrid LC–MS/MS and LBA methods are feasible for the measurement of tAb.

For LBAs, a target, anti-idiotypic(ID) or anti-Fc antibody can be used for capture, with its complementary detection antibody used for detection. Although the reagents do not bind directly to the payload, the payload can still cause steric hindrance. Capture and detection reagents should be evaluated based on stage of development, specificity, selectivity, and availability of the reagent. Regardless of reagent of choice, it is important to evaluate the impact of both unconjugated antibodies and conjugated antibodies during assay development (4).

For pre-clinical quantification of tAb by LC–MS/MS, trypsin digestion and measurement of a surrogate peptide is typical. Depending on the specificity required and the molecular entity in the ADC that is being measured, different critical reagents can be used. ADC drugs can be captured by protein A and protein G (17, 18), anti-human Ig, target antigen (17, 19), or anti-ID antibody (17), followed by denaturation, reduction, and sometimes alkylation. Proteolysis with trypsin or other sequence-specific proteases is commonly performed to release a unique signature peptide as the surrogate analyte for tAb (19). Generic tryptic peptides have been identified that can be used across preclinical species including Fc region peptides of IgG such as VVSVLTVLHQDWLNGK (20), GPSVFPLAPSSK (21), and TTPPVLDSDGSFFLYSK (22). Peptides from variable region provide additional specificity and ruggedness for quantitation.

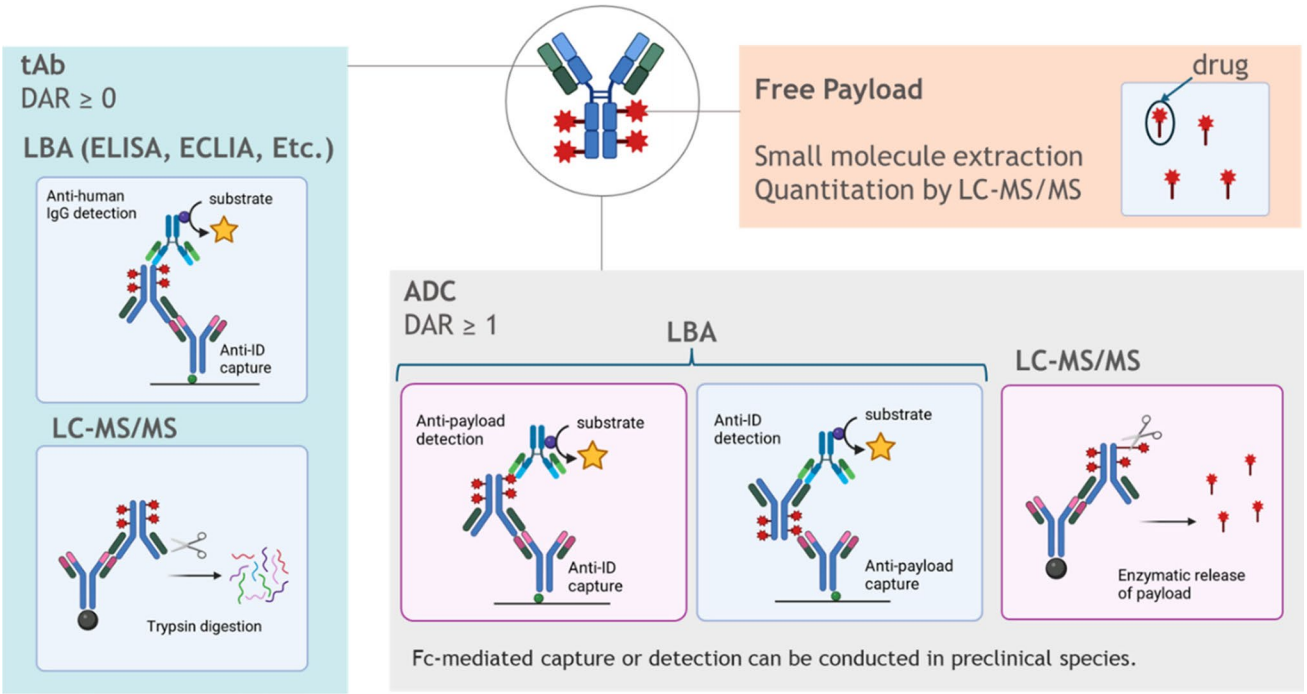

Fig. 1 Analytical methods to measure ADC constituents: payload capture or detection formats may exhibit increased sensitivity to DAR, influenced by reagent quality and the DAR distribution of the ADC

Ideally, the signature peptides should not miss cleavages or post-translational modifications. If possible, two peptides should be selected, one for quantitation and another for confirmation. The use of a stable label generic internal standard, including isotope labeled peptide, or flank peptide (peptide including additional amino acids on either side of the surrogate peptide) have been demonstrated to improve accuracy and precision in various literature. The use of generic mAb, such as Silumab, was shown to correct for digestion efficiency and ionization matrix effects (23) in preclinical workflows. The determination of total antibody in clinical assays commonly employs hybrid LC–MS to improve specificity. First the total antibody is affinity enriched using the target protein or an anti-ID antibody. The enriched sample is proteolyzed to release a highly specific surrogate peptide for quantification.

Measurement of ADC

PK methods to measure ADC refer to the quantification of an antibody or antibody fragment conjugated to at least one payload molecule via a chemical linker, resulting in a DAR of at least one. The DAR sensitivity of the assay for measuring ADC will depend on the assay format and assay reagents being used.

A 'conjugated payload' assay, often use anti-Fc, anti-idiotype, or target-mediated capture to enrich all antibody species, while payload-dependent detection is used to generate signal. These assays In LBAs, anti-payload detection can produce a DAR-sensitive readout because signal intensity may increase with payload coverage; however, the extent of DAR sensitivity may also be influenced by steric hindrance, epitope accessibility, and avidity effects. In hybrid LC–MS/MS, DAR-sensitive measurement is typically achieved by capture of antibody species followed by enzymatic or chemical release of payload, which is then quantified as a surrogate for conjugated antibody.

'Conjugated antibody' assays, which typically employs an anti-payload antibody as the capture reagent and an anti-Fc or anti-idiotype antibody for detection via ligand-binding assay (LBA), may be suitable depending on the study design. Importantly, this assay format is not DAR-sensitive, as the measured concentrations will not reflect changes in DAR occurring during the *in vivo* phase of the study. This limitation arises because the capture antibody does not discriminate based on the number of payload molecules conjugated to the ADC.

The industry is increasingly adopting LC–MS/MS quantitation of ADC due to its enhanced specificity and DAR sensitivity, and the advantage of eliminating the need for an anti-payload reagent. Additionally, LC-MS/MS–based combination assays enabling simultaneous quantification of ADC and total antibody can improve efficiency and turnaround time, while introducing additional assay complexity. Under this process, for ADCs with cleavable linkers, samples may be split after capture, with one portion processed for tAb measurement and the other subjected to enzymatic or chemical payload release to measure ADC. Reported approaches include papain digestion (18, 24, 25), cathepsin B cleavage (2, 26, 27), or chemical release of payload as the surrogate analyte for ADC. Alternatively, a 2-in-1 approach may be used where the first step is used to specifically release payload, followed by a digestion with a sequence specific enzyme, like trypsin, to generate a surrogate peptide for total antibody.

LBA and LC–MS/MS are both appropriate assay formats for ADC analysis. DAR-related quantitation bias may occur when detection (4, 28) or capture reagents are used, highlighting the importance of evaluating assay performance across materials with different DAR distributions during method development. Generally, standards or reference materials with known DAR values may be assessed to understand the impact of DAR on assay precision, accuracy, and interpretability (4).

Measurement of Free Payload

ADCs with stable linkers are designed to release minimal free payload into the systemic circulation, and free payload concentrations are generally low. Guidance (5) recommends measurement of the systemic exposure of the free payload in first-in-human studies. Decision in later development to exclude should take into consideration safety data (e.g., nonclinical data regarding the MOA of the ADC). For example, if the unconjugated payload is undetectable with a sufficiently sensitive assay, measuring the unconjugated payload may not be necessary (5).

LC–MS/MS has been the primary analytical method for quantifying small molecule free payload due to its exceptional sensitivity and specificity. The isolation of free payload from, typically, plasma, is achieved through established small molecule LC–MS/MS workflows, commonly employing sample preparation techniques such as protein precipitation (PP), liquid–liquid extraction (LLE), and solid-phase extraction (SPE). It is essential to ensure that conjugated payload is not inadvertently cleaved from the antibody during these procedures, as this could cause bias in quantitation. These methodologies effectively separate free payloads from plasma proteins and other possible interferences. Payload bioanalysis also presents unique challenges as ADC reference standards often contain the payload molecule and payload-related impurities in the range from sub-ng/mL to low ng/mL, making it more difficult to assess the impact of the ADC on the quantification of the payload. Specific strategies for mitigating the impact of ADC impurities and degradants are described in the Stability Considerations section.

Measurement of DAR Distribution

Currently, regulatory agencies do not require individual DAR data from bioanalytical methods. Measuring individual DAR is useful in decision making and question-driven scenarios. Individual-DAR analysis may be treated as a risk-based tool (cross-species translation issues), most valuable for early de-risking (unstable linker/rapid deconjugation suspected), explaining safety signals, and demonstrating comparability.

As DAR distribution of the ADC is a critical attribute, it may be monitored during preclinical and clinical study sample analysis. Characterization of linker stability and DAR is recommended to be conducted early during ADC development by following the average DAR. ADC data, in combination with tAb measurements, allow for the calculation of the average DAR, as shown in Fig. 2a. Monitoring the average DAR over time following dosing in preclinical species is indicative of the linker stability and can be useful in rank ordering compounds in the discovery phase. During drug development, DAR trending plots are useful to characterize the linker stability of an ADC. These graphical representations are used to track the average DAR following *in vivo* dosing during both preclinical and clinical studies. Once the DAR profile is thoroughly characterized during early clinical studies (e.g., first-in-human studies) to understand linker stability, it may be justified to discontinue further DAR analysis in some circumstances. This may include low systemic exposure of the payload or tAb concentrations that are highly correlated with that of the ADC.

Although average DAR is the generally accepted approach, there are instances where measuring individual DAR species can provide a more granular view of ADC heterogeneity (Fig. 2b). Intact and middle-down high-resolution mass spectrometry (HRMS) approaches can detect shifts in specific DAR species (DAR0, DAR2, DAR4, etc.), including DAR0, which is undetectable via conjugated payload assays. Pool-and-analyze HRMS strategies allow quantitation of individual DAR species (29–31). In recent years, the intact DAR measurement has also been performed by native MS, SEC-native MS, native reversed-phase LC–MS (nRPLC-MS), and hybrid HIC/MS (32, 33).

Immunogenicity Consideration

For ADCs, like all protein therapeutics, an evaluation of the immune responses is important to understand the impact on PK, safety, and efficacy over time (34). The bridging LBA is the most used format for detection of anti-drug antibody

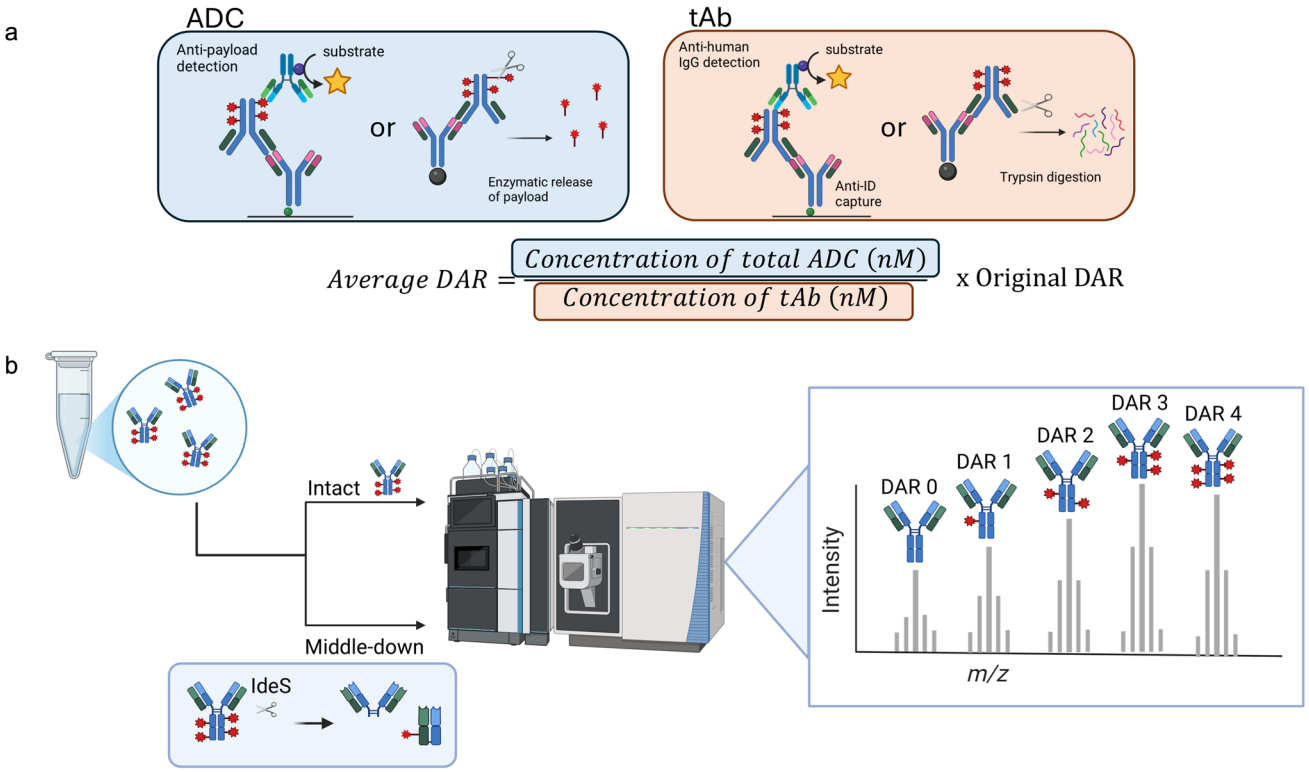

Fig. 2 Methods for DAR distribution measurements by **a**) calculation of average DAR and **b**) measurement of individual DAR using High-Resolution Mass Spectrometry

(ADA) against ADCs. Current regulatory guidelines suggest multi-tiered ADA bioanalytical strategy. The main assumption is that all assays (screen, confirmatory and titer) are orthogonal where the confirmation results empirically remove false positives from the screen assay, and the titer results establish the magnitude of the response. The suitability of the reduced-tier approach is determined by a data driven assessment of assay characteristics including assay range, sensitivity, specificity, and drug tolerance (35). Sponsors are encouraged to consult with regulatory agencies prior to conducting pivotal studies on full implementation of reduced-tier approach, as needed.

ADC is a multi-domain modality, and further ADA characterization for domain specificity, and neutralizing antibody should be based on immunogenicity risk assessment for clinical studies. The product-related risk is based on Ab, linker, payload, or combined epitopes; clinical studies have not demonstrated increased immunogenicity to ADC compared to conventional therapeutic proteins (36–38). As for the patient-related risk, the ADCs approved to date are all in the oncology therapeutic area; patients' prior lines of chemotherapy may affect their immune status at baseline, making them less susceptible to developing ADA (39).

ADC Soluble Target Consideration

ADC and soluble target interaction lead to multiple states in equilibrium, and differentiation of free target, total target, and bound target (target: ADC complex) is desirable. FDA (5) suggests that if ADC target is shed or secreted into the systemic circulation to a significant extent, bioanalytical assays could be recommended to distinguish the target-unbound ADC (i.e., free) from the target-bound ADC. In this case, soluble target could interfere with the required amount of ADC reaching the intended site of action, and impact clinical response. Therefore, developing a free target assay could provide key data that may inform the efficacious dose. LBAs are often the preferred platform for measuring soluble targets, and there are several points to consider when developing a reliable free target assay or the alternative, a total target assay. These include: (1) target biology which comprises low abundance target levels and high assay sensitivity requirement; (2) selection of capture and detection reagents and their impact on target and ADC equilibrium; and (3) associated technical challenges including optimizing assay conditions that minimally perturb target: ADC complexes formed during circulation.

For free target assays, target capture Ab with a similar or a relatively lower avidity compared to that of the ADC therapeutic should be selected. Also, potentially using the ADC therapeutic itself as a capture reagent could be considered to minimize disruption of existing sample equilibrium, and risk of overestimation of free target concentration. Controlled assay conditions, including reduced sample dilution, and shorter incubation time, can be implemented to minimize potential issues. In most cases, the high molar ratio of ADC to free target can prohibit accurate measurement, and thus, in the case when the ADC is at a significantly higher concentration than the target, there may be very little free target in circulation and attempts at quantitation of free target may not result in any biologically relevant or useful data.

For total target assays, the dynamic range is critical since total target data provides information on the effect of ADC on target accumulation. ADCs generally have a longer half-life than the free target, and consequently the ADC-target complex formed after dosing may not be cleared as fast as the free target. Also, total target assays are often challenging to develop in that they must detect both free and bound target. Therefore, target assays may need additional pre-treatment steps to enable accurate quantitation, such as acid dissociation of bound target from the ADC prior to testing in an LBA assay. It is important to confirm whether the assay is measuring free target or total target and confirm the appropriateness of the assay based on context of use (40). When total LBA target assay development is unsuccessful (41), total LC–MS/MS-based assay can be considered (42).

Reagents Considerations

Critical reagents form the basis of ADC bioanalytical methods and evolve from discovery through clinical stages (43). Due to ADC complexity, multiple assays and reagents are needed to generate a clear understanding of all aspects relevant for these molecules (i.e., ADC and its constituent parts). The selection and quantity of reagents are determined by the specific bioanalytical strategy and technology employed. Regardless of whether LBA or LC–MS/MS detection methods are used, it is critical that reagents are disease state agnostic and demonstrate appropriate selectivity.

During the discovery and preclinical phases, commercially available monoclonal antibodies that target common epitopes, as well as polyclonal antibodies, serve as critical reagents. Procuring these materials from reputable suppliers promotes consistency and minimizes batch-to-batch variability; however, rigorous validation of their suitability is essential. In the clinical stage, it is necessary to generate reagents specific to the ADC, which may include anti-payload or anti-ID reagents. Initiating clinical reagent development campaigns at least one year prior to the commencement of clinical trials would enable timely availability.

Ensuring batch-to-batch reproducibility and long-term functionality of reagents is crucial for maintaining assay robustness throughout multi-year clinical programs. Comprehensive reagent characterization would typically

include assessment of key attributes such as concentration, purity, and degree of labeling. Beyond physical and functional assessments, validating the reagent as part of the overall assay qualification is very important for program success. When a new reagent lot is produced, bridging validation using a single assay is recommended whenever it is feasible to compare new and existing lots. To minimize variability, bulk production and labeling of reagents are advised. Furthermore, stringent control and monitoring of storage conditions are very important, as deviations can compromise reagent integrity and performance.

Stability Considerations

It is well documented that ADC stability may be influenced by the conjugation chemistry to the antibody, and the chemical lability of the linker. Non-cleavable linkers tend to demonstrate higher *in vivo* stability than their cleavable counterparts, with relatively lower unconjugated payload levels (44). The potential also exists that sample preparation conditions may result in ex vivo deconjugation of the payload from the ADC. The rate of potential payload release may be impacted by other variables such as photosensitivity, species-specific enzymatic activity, matrix pH ranges, and susceptibility to hemolysis. Stability testing in early development can help to inform sample collection and processing in toxicology and clinical studies.

For example, for bioanalytical matrices stability consideration for the photosensitive camptothecin derivative payload (deruxtecan, DXd), understanding the special handling requirement is important for bioanalysis. DXd payloads can convert from the active lactone ring to inactive carboxylate form in a pH-dependent manner (45). The degradation rate of DXd ADCs is slower at low pH (< 6.0). For light protection, wavelengths longer than 530 nm such as LED cause minimal degradation. Amber and cyclic olefin polymer vials may also provide better protection than clear glass vial (46).

For payload stability testing, one approach involves preparing blank-, low-, and high- quality control (QC) payload matrix samples with ADC spiked at approximately C_{max} concentration. The QC values in the presence of ADC can be compared to QC's prepared without the ADC and the nominal concentrations at time zero adjusted accordingly due to potential unconjugated drug present in the ADC reference material. Both sets of QCs should be monitored under various stability conditions. If deconjugation is detected, mitigation strategies may be necessary. These can include storing samples under ultra-low temperatures (~ −70°C), using matrix additives such as acid or antioxidants, or keeping samples in an ice bath during extraction.

Stage-specific ADC Bioanalytical Strategy

PK/ADME considerations change throughout development, necessitating that bioanalytical approaches be tailored to each phase. In early drug discovery, bioanalysis of ADCs is critical to obtain pharmacokinetic, efficacy, and toxicity data in non-clinical species that inform candidate selection. The primary analytes assessed include tAb, ADC, free unconjugated payload, pharmacologically active payload-derived metabolites, and DAR distribution. When atypical pharmacokinetics indicate possible ADA-mediated clearance, ADA testing is initiated. Discovery-phase assays are typically fit-for-purpose and optimized for flexibility and rapid turnaround.

As development progresses to GLP toxicology studies, the focus shifts to increased data integrity, reproducibility, and regulatory compliance. ADC toxicology studies are generally conducted in both rodent and non-rodent species, and all bioanalytical methods supporting GLP studies must be fully validated in accordance with M10 guidelines (7). At this stage, ADC analyte panels are refined to those necessary to interpret systemic exposure and toxicological findings.

During early clinical development, bioanalysis continues to focus on ADC analytes utilizing validated methods to support safety assessment and exposure characterization. In later stages of clinical development, the bioanalytical scope may be streamlined to focus on ADC analytes most relevant to exposure–response relationships and regulatory decision-making. For instance, if a highly sensitive assay reveals undetectable levels of free payload, continued measurement of the free payload may be omitted. Likewise, if a strong correlation exists between tAb and ADC concentrations—particularly when the antibody primarily functions as a delivery vehicle—quantification of tAb may no longer be necessary (5).

In addition to analyte selection, assay platforms and critical reagents also mature across drug discovery and development phases, often transitioning from generic reagents to anti-idiotype reagents and from exploratory formats to fully validated LBA or LC–MS/MS methods. In practice, such transitions are typically driven by increasing sensitivity and specificity requirements, mitigation of matrix or catabolite interference, and the need to meet regulatory validation standards as programs move into GLP and clinical stages (2, 5, 6). When assay formats or critical reagents are modified during development, formal comparability may be necessary to ensure continuity of pharmacokinetic interpretation.

While LC–MS/MS may offer improved molecular specificity for ADCs and its constituent parts, LBA approaches may provide superior sensitivity at low clinical exposures;

therefore, platform selection remains molecule- and stage-dependent. Table I provides an overview of the typical workflow for reagent generation and stage-specific assay selection.

Cross Validation Strategies

The bioanalytical testing strategy for ADCs often includes multiple analytes in both PK and immunogenicity assessments. When a cross-validation for inter-site data comparison is performed, each method should be individually assessed for comparability between the two laboratories. When more than one technology is used (LC–MS/MS *vs* LBA) during a study, both methods should be assessed for comparability. The FDA (2018) (47) and M10 (2022) (48) guidelines provide recommendations for PK cross-validation, including when to conduct cross-validation, the selection of incurred samples and the application of statistical tools to assess agreement between two methods (49).

For both LC–MS/MS and LBA, PK cross-validation should include ≥ 30 incurred samples, if available, or sample size determination should be assessed by the statistician based on historical data from earlier studies. It is widely recognized that ADCs experience DAR changes after administration, resulting from deconjugation or biotransformation. Therefore, it is important to include study samples as well as spiked QCs in the cross-validation design. Study samples should be selected to cover the full range of potential changes and biological states, including disease state matrices, to ensure they accurately reflect modifications that could occur over time post-administration.

For ADA and NAb assays, differences in cut points, reagents, assay sensitivity, drug tolerance, positivity rate,

Table I Overview of the reagent generation workflow and stage specific assay selection, by assay type. Checkerboard experiments should be conducted to find the best capture/detection pair and order. Anti-ID and/or anti-payload synthesis is typically initiated at the transition from discovery to GLP Tox

Reagent	Bioanalytical Method	Pre-clinical (Discovery through GLP Tox)	Clinical
Capture Reagent: (LBA or LC–MS/MS)	**Conjugated ADC**:	Target or generic anti-human Fc or anti-hIgG or anti-ID (if available) or anti-payload	Anti-ID or target or anti-payload
	Total antibody:	Target or generic anti-human Fc or anti-hIgG or anti-ID (if available)	Anti-ID or target
	ADA:	ADC (ex. unlabeled, biotin, or other conjugate)	ADC (ex. unlabeled, biotin, or other conjugate)
	NAb:	typically n/a in discovery phase	ADC (ex. unlabeled, biotin, or other conjugate)
Detection Reagent: (LBA only, NA for LC–MS/MS)	**Conjugated ADC**:	Anti-payload or Target or generic anti-human Fc or anti-hIgG or anti-ID (if available)	Anti-payload or anti-ID or target
	Total antibody:	Target or generic anti-human Fc or anti-hIgG or anti-ID (if available)	Anti-ID or target or generic anti-human Fc
	ADA:	ADC (ex. HRP or Ruthenium conjugate)	ADC (ex. HRP or Ruthenium conjugate)
	NAb:	typically n/a in discovery phase	Typically determined by inhibition
ADA Domain mapping:	**Specificity Reagents**	typically n/a in discovery phase	Reagents depend on what domain is being probed: ADC, Naked Ab, linker payload attached to a carrier protein, linked payload alone, linker alone, payload alone
Positive Control	**PK (any)**	Analyte to be measured prepared in appropriate matrix	
	ADA:	Polyclonal or mAb against human framework; anti-ADC or Ab, if available	Polyclonal or mAb against the ADC, or mAb against the individual Ab and payload domains
Stable isotope label internal standard (for LC–MS/MS)		Labeled signature peptide or payload are preferred, if available	
Considerations:		1) It is common to leverage commercial and anti-human antibodies at this stage. Anti-IDs can be used if available 2) Polyclonal anti-payload Ab can be generated at this stage, if LBA is the method of choice	1) Long-term reagent use campaigns are typically initiated at least a year prior to being required in the clinical assay 2) Anti-IDs are commonly used in clinical phase, with an anti-payload, target, or anti-human Ab used as the other pair 3) While pAbs are more representative of nature's polyclonal immune response, mAbs have long-term assay reproducibility and robustness advantages

and titer should be considered in the strategy for method comparison. Cross comparison of one or multiple domain specificity tiers should be considered based on the immunogenicity risk.

Due to the complexity of ADC bioanalysis, it is suggested to perform a preliminary assessment during method transfer with a limited set of controls and pooled samples to ensure the comparability of reagents, technologies, and assay execution across labs. Each bioanalytical cross-validation/comparison should adhere to the applicable regulatory recommended standards and practices such as the M10 (7). As ADC modalities often require a larger number of bioanalytical methods including technologies not necessarily employed across other therapeutics, this presents logistical and strategic challenges regarding the capabilities and expertise of both labs involved in the cross-validations and whether a single site can be used for multiple methods.

When cross validation data exhibits significant bias, it is crucial to objectively assess the data and investigate potential biases from either or both methods to understand the root cause of discrepancies. As ADCs require multiple bioanalytical methods to quantify the ADC and its constituent parts, bias have a greater potential impact on the subsequent interpretation, such as PK exposure or exposure–response evaluations.

ADC Bioanalysis Considerations for Regulatory Submissions

Accurate quantification of ADCs and their constituent parts in biological matrices is necessary to determine key pharmacokinetic parameters and inform regulatory decisions related to the safety and efficacy of ADCs. To maintain data reliability for regulatory authorities, bioanalytical methods used for these measurements should be developed, validated, and documented. These guidelines (5, 6) outline regulatory expectations of bioanalytical data and the importance of reliability of the data included in ADC submissions.

In general, beginning with first-in-human studies, the ADC and its constituent parts should be measured using validated assays. Later in development, the ADC, its constituent parts, and its pharmacologically active metabolites that are quantifiable in systemic circulation should be measured to characterize PK of individual components and inform exposure–response analyses. All bioanalytical methods should be validated and study sample analysis should be reported as outlined in the FDA guidance entitled "M10 Bioanalytical Method Validation and Study Sample Analysis (7).

Small changes in free payload concentration can significantly impact safety profiles due to the high potency of many ADC payloads (typically cytotoxic agents with IC_{50} values in the picomolar to nanomolar range). FDA states in its guidance that "the unconjugated payload assay be sufficiently sensitive to detect small changes in systemic exposure" (5). The lower limit of quantitation (LLOQ) for the unconjugated payload assays should be established based on a thorough understanding of the payload's potency, mechanism of action, toxicity profile, and distribution characteristics. For highly potent payloads, LLOQs in the sub-ng/mL range (often 10–50 pg/mL) may be necessary to correlate with observed toxicities. This typically requires sensitivity at least tenfold below concentrations associated with adverse effects. While the LLOQ at the pg/mL presents difficulties, advances in LC–MS/MS technology, such as improved separation (e.g., chromatography) and more sensitive detectors, provide enhanced quantitation limits (50).

The FDA provides resources including guidance documents and webinars, and recommends sponsors to engage early with regulatory review divisions to seek feedback during drug development. It has been reported that recently, there has been an increase in dedicated questions regarding issues and challenges related to bioanalysis in regulatory milestone and guidance meetings (e.g., pre-IND meetings, Type C and Type D meetings) between the Sponsor and the FDA (48). It cannot be over-emphasized that robust, reliable, and reproducible bioanalytical methods are essential for successful drug development.

Conclusions

This white paper presents the current perspective on the bioanalytical strategies underpinning ADC development. This white paper outlines the most updated understanding and application of DAR dynamics, ADC stability, strategies for cross-validation, and phase appropriate testing. ADC bioanalysis considerations for regulatory submissions provided in this white paper focuses on the US FDA's recommendations and current practices regarding bioanalytical strategy for ADCs.

ADCs have become more widely explored, accepted, and applied in the last decade as 10 of the 14 FDA approved ADCs received their approval in this period. There are many more in the pipeline and the modality continues to evolve. The versatility of antibody conjugation strategies has also inspired a new generation of targeted therapeutics—including degrader-antibody conjugates (DACs), antibody-oligonucleotide conjugates (AOCs), radiolabeled ADC, immune-stimulating antibody conjugates (iSACs), and single-domain ADCs (1). They may differ from traditional ADCs in mechanism of action or structure, but they build on the core principle of payload delivery using precision targeting and offer great promise in improving patients' lives in oncology and beyond. Two things are clear: 1) the bioanalytical community will adapt and find ways to measure and characterize the

newer generations of ADCs and other targeted therapies, and 2) when it comes to ADCs, bioanalysis is a strategic pillar in drug development, not a support function.

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Declarations

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