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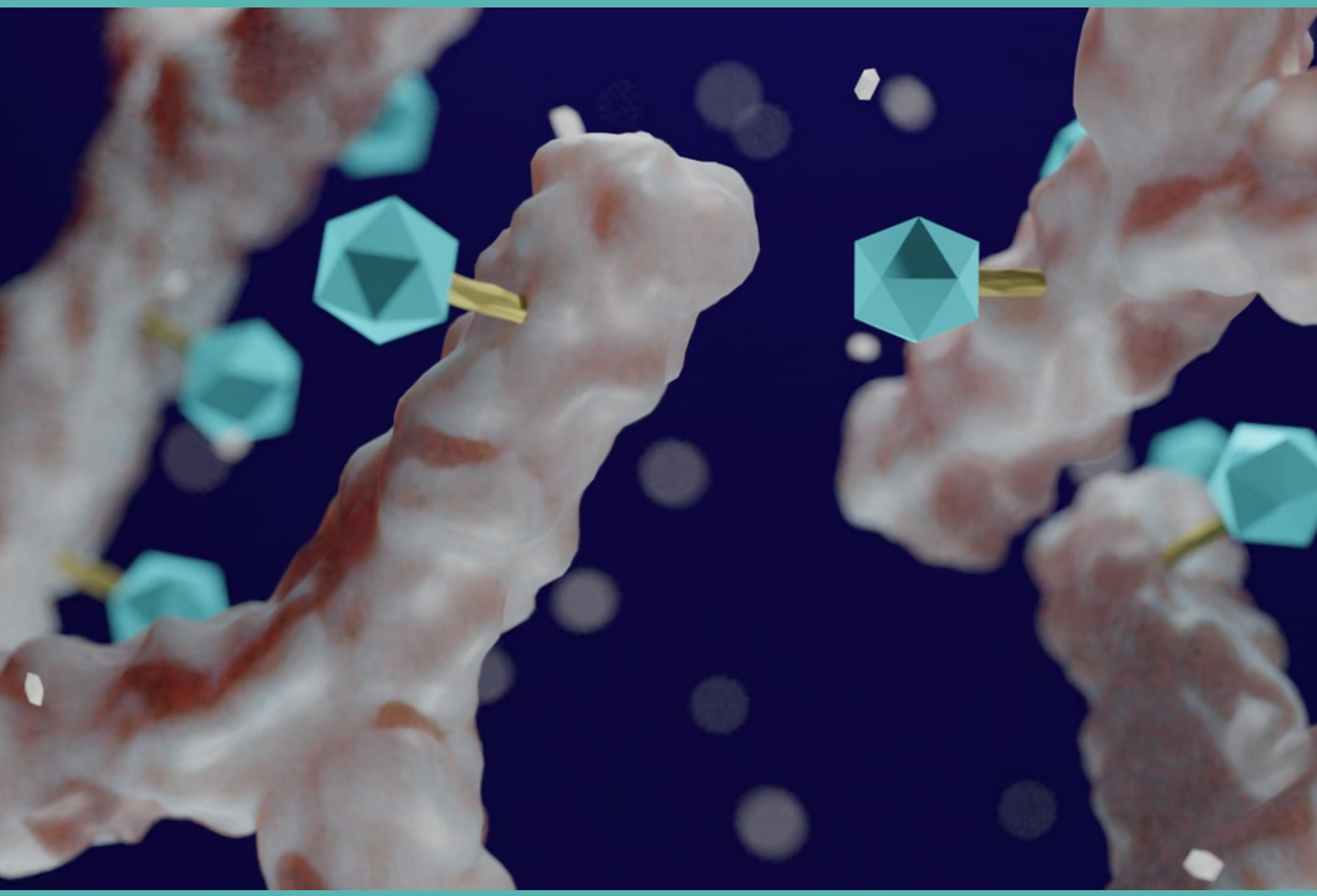

Antibody–drug conjugate bioanalysis

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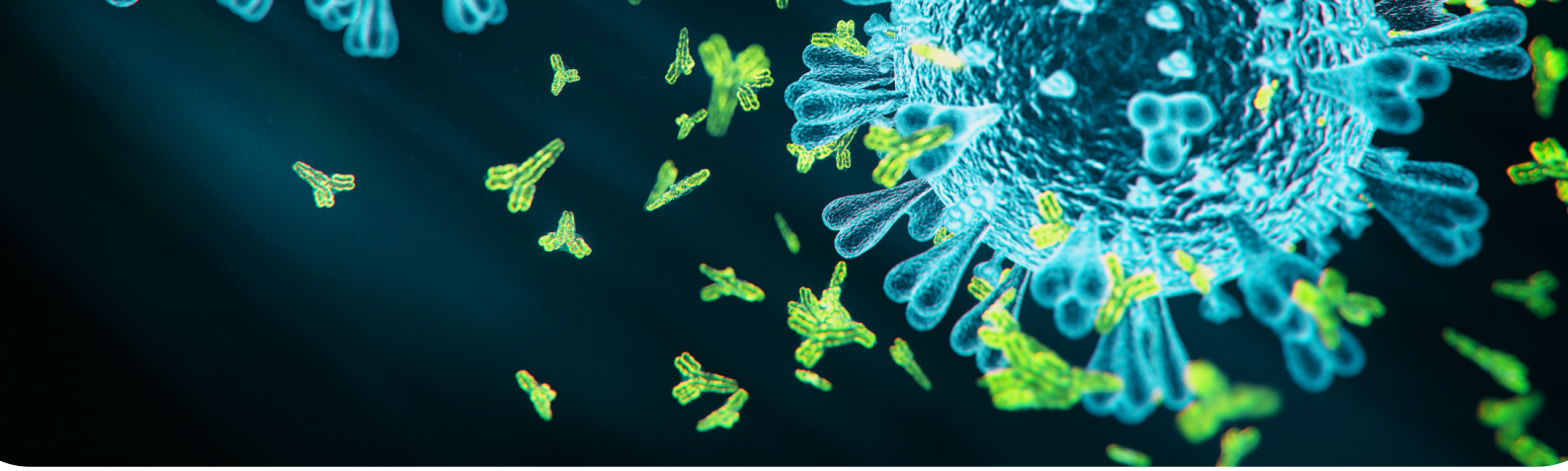

Foreword

Antibody-drug conjugates (ADCs) are a relatively new therapeutic modality compared to small molecule medicines. ADCs typically combine a monoclonal antibody (mAb) with a small molecule toxic payload. The mAb acts as a targeting protein to deliver the toxic payload to a specific protein expressed on the cell to be killed. This level of specificity is advantageous because it can increase the therapeutic index by lowering systemic exposure to the toxic payload. While this concept has been around for several decades, the practicality of designing and manufacturing these complex molecules has gained traction only in the last 10–15 years.

With the increased momentum behind the development of ADCs, there has been a corresponding increase in the bioanalytical needs for this therapeutic class. Methods capable of measuring the pharmacokinetics (PK) of and immune response against the intact ADC and the individual components (mAb, payload and linker) are all necessary in the bioanalytical support strategy.

Since ADCs have both small and large molecule components, the tools typically used to quantitate each element are required. To measure the small molecule payload, sensitive LC-MS/MS methods to detect low levels of toxic payload in a matrix are required. To quantitate the large molecule component, ligand-binding/immuno-assays are used. In both cases, assays must be able to deal with the heterogeneous nature of ADCs where many of them do not have a fixed number of payloads per mAb, instead having a range of conjugated payloads per molecule.

Finally, the stimulation of the patient's immune system by these molecules needs to be assessed, which is typically achieved by measuring both total and neutralizing antibodies (NAbs) against the intact ADC. These responses may need to be further characterized to determine whether that immune response is directed against the mAb, the payload or both.

In this eBook, we will explore the development of each of the bioanalytical assays needed to support the advancement of ADCs from the candidate selection phase through the regulatory submission process.

We hope you enjoy this eBook!

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BioAgilytix

60 seconds with David Johnson, Michelle Miller and Amanda Hays

David A. Johnson has over 20 years of experience implementing and managing preclinical drug discovery contract research services. In 2000, he joined BioAgilytix (San Diego; CA, USA), establishing the drug Metabolism group, which is responsible for preclinical drug metabolism research and non-GLP discovery bioanalytical research. Previously, David was the Drug Metabolism Product Development Manager for the Pharmazyme division of Immune Complex Corporation (CA, USA). Prior to Pharmazyme, David was a Research Assistant at Hybritech (CA, USA) where he developed methods to automate the radiolabeling of monoclonal antibodies for use in medical diagnostic kits. He earned his bachelor's degree in Chemistry from the University of California (CA, USA) and his PhD from the University of Minnesota (MN, USA).

Dr Michelle Miller has over a decade of experience working in the fields of immunology and toxicology. She has a strong background in project management, previously serving as a bioanalytical Associate Director at BioAgilytix (CA, USA). Prior to BioAgilytix, Michelle worked as a study director leading GLP toxicology and immunotoxicology studies, as a lead investigator in the toxicology field developing high throughput screening assays and as an R&D Scientist in the pharmaceutical industry. She is a current Diplomate of the American Board of Toxicology (NC, USA). In her prior role at BioAgilytix, Michelle worked closely with clients and operational staff to ensure successful on-time execution of assays, validation and sample analysis under GxP, supporting preclinical, clinical and product release studies. Michelle earned a BA in Chemistry, BS in Zoology and PhD in Immunology with a minor in Biochemistry from North Carolina State University (NC, USA).

Dr Amanda L. Hays offers more than a decade of lab experience in multiple fields, including pharmacology, drug metabolism, immunoassays, immunogenicity, biomarkers and flow cytometry. She has particular expertise leading clients from pre-clinical through phase III clinical trials and post-marketing studies. Prior to joining BioAgilytix, she served as Director of Bioanalytical Science at PRA Health Sciences (NC, USA), where she provided global scientific leadership and technical guidance for PRA's large molecule bioanalytical laboratory. Dr Hays is the Vice Chair of the AAPS Biomarkers and Precision Medicine Community and has held several volunteer leadership positions through the AAPS in the last several years. She earned her PhD in Pharmacology from the University of Kansas Medical Center in (KS, USA).

Firstly, what is an antibody–drug conjugate (ADC)?

ADCs are an innovative approach to cancer therapeutics. The backbone is an antibody that binds to a surface antigen expressed on cancer cells, thereby enabling delivery to the specific therapeutic target. The antibody is chemically linked to a drug, typically a cytotoxic small molecule, which is referred to as the 'payload' or 'warhead'. This design is intended to reduce systemic exposure and toxicity of the drug component. The most common mode of action is for the ADC to bind the tumor cell, become internalized, and then for the cytotoxic payload to kill the cell.

In your opinion, what are the main challenges when analyzing ADCs?

An ADC is a hybrid of a large molecule and a small molecule, so the bioanalytical strategy can be fairly complex, requiring multiple assays to address different portions of the molecule. PK assays must be appropriately designed to detect free or total ADC depending on the expectation for biotransformation or metabolism of the therapeutic. There are published examples of PK assay strategies describing six different assays to detect all possible forms of the ADC in circulation after dosing. When it is necessary to measure the intact ADC, the amount of payload conjugated to the mAb can be impactful on the assay so the development and implementation of assays that take into account the Drug Antibody Ratio (DAR) are also critical.

Anti-drug antibody (ADA) detection and characterization assays are also needed. For NAb detection, cell-based killing assays can be used, but the MOA of the ADC then becomes a critical component to assay design with special consideration for the internalization component.

This means that you have a broad portfolio of assays and each of these requires a variety of positive controls and reagents. So, just managing the technical aspects of getting all the assays up and performing appropriately requires a lot of time and bioanalytical expertise. Finding labs that have all the necessary capabilities under one roof can be challenging. However, there is an advantage to being able to work with a single vendor to accomplish all these measurements.

Finally, having appropriate biosecurity and ADC handling procedures in place to ensure the safety of lab staff is an important consideration when working on an ADC program. The payloads are typically potent toxins that must be handled as such and may require additional handling caution.

ADCs have been conceptualized since monoclonal antibodies became a practical reality in the 1980s. What are the main reasons this therapeutic class has only seen a dozen or so approvals so far?

The first approved ADC was in 2000. As of the end of last year, 14 have received final marketing approval from the FDA and more than 100 ADCs are currently in the clinical pipeline. These numbers aren't surprising for this type of complex therapeutic. The design of new ADCs hinges on identifying a highly specific target expressed differentially on cancer cells vs. healthy cells, and targets that are not solubilized so that the antibody binds to the tumor cell and is not released back into circulation. These can be significant challenges. Then, there is the task of balancing the clearance rate of the ADC, the stability of the linker connecting the drug to the antibody, and the binding affinity of the ADC to the target, which impacts internalization and the cytotoxic activity after internalization. All these aspects determine the therapeutic index or efficacy and safety profiles of the therapeutic and can make or break its success in the clinic.

What instrumentation do you use for ADCs analysis and what are the advantages and limitations of this approach?

As with the previous question, this really depends on the bioanalytical assay being applied. The methodologies for these assays can be quite diverse; PK or ADA assays may be developed on traditional ligand-binding assay (LBA) platforms such as ELISA or ECL, but in some cases LC-MS or even hybrid LBA: LC-MS approaches are taken for PK. DAR is typically performed through LC-MS or high-resolution MS. NAb assays tend to be cell-killing readouts, which can vary in the final platform selection.

What technological advances have been most impactful for the development of the bioanalytical assays needed for the development of ADCs?

The ADC space has grown tremendously in recent years with new antigen targets and new payloads being explored. The next generation of ADCs is focused on modifications of the antibodies to add versatility and involves a deep understanding of the mechanistic pathways involved in these therapeutics to enhance rationale design. Just the improved ability to handle the analysis of complex data sets now compared to 20 or 30 years ago has been a huge advantage in this field.

What factors will impact the pace of ADC approvals in the near future?

Leveraging the clinical data that is being generated from having 100s of ADCs in trials right now is the biggest resource for improving future ADC programs. Because there are many “common” payloads being used across multiple ADC programs, our experience with those payloads will increase and the likelihood of seeing an increase in approvals may follow. The use of combination therapies has shown some promise as well, so creative solutions to things like drug resistance may involve these types of approaches. Who knows what other advancements can be applied in the next decade? There may be new methods or systems available for improved administration and enhanced tumor infiltration that would propel these therapeutics to the next level of success. I think we are still seeing the early stages of clinical application for ADCs and there are still many areas for these therapeutics to expand and grow in the future.

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Commentary

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Bioanalysis

Immunogenicity considerations for antibody–drug conjugates: a focus on neutralizing antibody assays

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Overview of antibody–drug conjugates

The development of antibody–drug conjugates (ADCs) is one step toward the fulfillment of Paul Erhlich's 100-year-old prediction of the discovery of a 'magic bullet' for the treatment of human cancers and infectious disease [1]. ADCs are a complex class of multifunctional pharmaceuticals composed of monoclonal antibodies (mAbs) linked to cytotoxic small-molecule drugs via stable linkers with labile bonds. ADCs leverage the specificity of a mAb to deliver a potent small-molecule cytotoxic drug to the intended pharmacological target to achieve the desired therapeutic effect while reducing the exposure to normal cells [2]. Upon intravenous administration, ADCs bind to their target antigens, undergo internalization and release the drug in lysosomes, which eventually leads to cell death [3,4].

ADCs are one of the fastest growing classes of oncology therapeutics, as evidenced by a rapidly growing clinical pipeline. Although ADCs are relatively simple conceptually, they have turned out to be much more challenging than their elegant design. Despite hundreds of clinical trials, to date only three ADCs have gained entry into the market, of which only two remain: brentuximab vedotin (Adcetris®, Seattle Genetics, WA, USA) and ado-trastuzumab emtansine (Kadcyla®, Genentech, Inc., CA, USA)

ADC immunogenicity considerations

ADCs have the potential to elicit an immune response through the production of non-neutralizing and neutralizing antibodies (NAbs) that could impact their pharmacokinetics, efficacy and safety. Non-neutralizing anti-drug antibodies (ADAs) recognize their epitope outside of the active target-binding site, whereas NAbs bind to epitopes located in the target-binding site inhibiting the biological activity of the therapeutic leading to loss of drug efficacy. Should the NAbs cross-react with an endogenous counterpart, even life-threatening side effects can occur, as seen with recombinant human erythropoietin [5]. It is a regulatory expectation to monitor and characterize the ADAs against ADCs in order to evaluate the drug's efficacy and safety [6–8].

ADC risk assessment

A thorough risk assessment that estimates the probability of the biologic being immunogenic and evaluates the clinical impact of ADAs on efficacy and safety is used to classify the biotherapeutic as high, medium or low risk and will guide immunogenicity assessments [9,10]. The risk assessment is a 'living document' and is revised as data become available, which may alter the immunogenicity strategy used for an ADC program.

The risk of developing ADAs depends on many product, patient and disease-related factors [11]. ADCs have structural motifs that may carry unique immunogenicity risks. The linker and/or the small-molecule drug may act as a hapten once conjugated to form the ADC, and may result in increased immunogenicity compared with the standalone corresponding mAb. The hydrophobic nature of cytotoxic drugs can create aggregation-prone regions, increasing the likelihood of an immune response to the ADC [12]. ADAs formed against the linker-drug site can lead to formation and internalization of immune complexes by nontarget tissues and could result in toxicity [13]. Additionally, the glycosylation pattern of the mAb part can result in off-target toxicity due to mannose receptor uptake caused by the glycosylation pattern of the mAb [14]. Coupling a toxin via a linker to an mAb may induce

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neoepitopes at the site of attachment and leads to increased immunogenicity of the ADC [15]. Furthermore, an immune response reactive with one part of the molecule may eventually spread to other epitopes [16].

A trademark characteristic of a high-risk immune response is neutralization of an essential endogenous counterpart. ADAs toward ADCs are not expected to cross-react with endogenous immunoglobulin molecules and result in loss of function. Even though the generally well-tolerated human immunoglobulin component, intravenous route of administration and immunosuppressed oncology patient population are all associated with a lower immunogenicity risk, ADCs are generally considered medium risk due to the limited clinical experience and tripartite nature of the molecule [16,17].

Clinical immunogenicity for ADCs

The limited clinical data available describing the incidence and impact of immunogenicity for ADCs are gleaned from three ADCs. Of the two currently marketed, the ADA incidence for adotrastuzumab emtansine was 5.3%, with ADAs directed both against the linker-drug and/or neoepitopes in the mAb [18]. No data have been reported on the neutralizing activity of the ADAs for this ADC, and the development of ADAs in patients did not have an obvious impact on safety, pharmacokinetics (PK) and efficacy. A 37% ADA incidence was reported for brentuximab vedotin, with all ADAs directed against the chimeric mAb component; NAbs were detected in 62% of ADA-positive patients [19,20]. For gemtuzumab ozogamicin (Mylotarg®, Pfizer, MA, USA), the incidence of ADAs was 1.1% and among 182 tested patients evaluated for immunogenicity in Phase I, only two tested positive (both developed antibodies to the calicheamicin/linker portion) [21]. It is possible that ADA responses were not detected since earlier immunogenicity assay formats had low tolerance to the biotherapeutic.

Tiered immunogenicity testing strategy for anti-drug antibodies against ADCs

Immunogenicity assessments for ADCs follow the same regulatory and industry guidelines as other biologics but may require additional characterization of domain-binding specificity [6–8,22–24]. Immunogenicity can occur to any component of an ADC including epitopes present on the mAb, the linker or linker- payload, or neoepitopes formed in the mAb as a result of conjugation [16,25]. The US FDA and EMA recommend that the immunogenicity assays should be able to measure the responses to all components of the ADC utilizing a tiered approach of screening and confirmation [7,8]. Utilizing the entire ADC molecule enables ADA detection with specificity for any component of the intact ADC. All confirmed positive samples can be further characterized for domain specificity and, in later phases of development, an NAb assay may be needed to further assess the impact of ADAs. All these immunogenicity assays can be validated following existing regulatory and industry guidelines for biotherapeutics [7–8,22,26]. Data obtained during immunogenicity evaluations should be analyzed, interpreted and reported, together with information obtained for PK, safety and efficacy.

Development & validation considerations for NAb assays for ADCs

One of the biggest differences in an immunogenicity strategy between a low- and a high-risk biologic is the need for a bioassay to determine NAbs. Two assay formats have been used to measure NAb activity; cell-based bioassays and noncell-based competitive ligand-binding assays (CLB). Selection of the appropriate assay format should be reflective of the therapeutic mechanism of action (MoA) and provide clinically relevant data [27,28].

ADCs exert their MoA primarily inside the tumor cells, as the intended MoA is antigen-specific drug-dependent cytotoxicity. Cytotoxins such as auristatins and maytansinoids target rapidly dividing cells by interfering with different parts of the cell cycle whereas calicheamicins, pyrrolobenzodiazepine, doxorubicins and duocarmycins induce DNA damage ultimately resulting in either direct killing of the cell or induction of apoptosis [2,13,29–30]. In addition to payload cytotoxicity within target cells, some ADCs have inherent effector functions including antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity [31,32].

Due to the MoA of ADCs, cell-based NAb assays are recommended and are the regulatory authorities' preferred method to detect the presence of Nabs [7,8,33]. These assays use cellular responses as an assay end point to detect NAb-mediated inhibition of the biological function of the ADC and are considered more reflective of the *in vivo* situation than CLB assays. The use of CLB assays is considered appropriate only if efforts to develop a reliable cell-based assay are not successful due to susceptibility of the cells to matrix or drug interference [7].

NAbs against ADCs can effect efficacy by blocking target binding of the antibody. It is presumed that NAbs against the cytotoxin once internalized, are acidified and catabolized within the lysosome thus are expected to lose their neutralizing potential [24]. However, NAbs to the cytotoxin portion could potentially inhibit the cell-killing

activity of the ADC if they remain bound to the cytotoxin after the immune complexes are internalized and released from the lysosome. NAbs could bind to free cytotoxic drug and multiple payload molecules in the ADC resulting in immune complexes containing the payload that could reach nontarget tissues causing toxicity. Both types of ADAs may cause safety events by enhancing uptake of the cytotoxin into nontarget cells during immune complex clearance [16,24].

ADC domains (mAb and cytotoxin) are involved in sequential steps of the drug functional pathway leading to cell death. Since NAbs against the mAb or cytotoxin portion may result in blocking of the cell-killing activity of the ADC, a single cell-based NAb assay using a late step of the drug signal cascade as the assay read out, such as cellular proliferation or apoptosis would reflect the therapeutic MoA [24].

Due to their structural complexity, cell-based NAb assays require specific considerations. Development and validation of NAb assays has been thoroughly described in the literature [26,27,34,35]. Key steps in NAb assay development for ADCs include selection of a cell line expressing endogenous or transfected target antigen that responds to the ADC, choosing the proper cellular response, selection of the ADC drug dose, selection of proper controls and optimization of assay parameters.

The basis for detecting NAbs lies in the ability to observe a shift in an assay-specific cellular response. The sample is preincubated with a single concentration of ADC and if NAbs are present, an inhibition of the drug-induced assay response is observed.

Potency assays frequently provide an appropriate starting point for ADC NAb cell-based assay development and many end points are suitable, including cytotoxicity, proliferation, viability and apoptosis [36,37]. As potency assays are performed in culture media or assay buffer they need to be modified and adapted for biological matrix to be used as an NAb assay.

Various kits are available that may be adapted to be used as NAb assays for ADCs such as the CellTiter-Fluor™ cytotoxicity assay (Promega, WI, USA). Cell proliferation can be measured by indirect assays which use nucleotide analogs such as 5-bromo-2′-deoxyuridine that become incorporated into actively replicating cells. Viability can be measured indirectly based on metabolic markers that distinguish live cells from dead cells. These markers include intracellular ATP, lactate dehydrogenize (LDH), NADH and proteolytic enzymes. Assays such as the CytoTox-ONE™ and the CytoTox 96® Non-Radioactive Cytotoxicity assay (Promega) estimate the number of nonviable cells by measuring the release of LDH upon cell lysis. The reduction of tetrazolium salts such as MTT and XTT to colored formazan compounds or the bioreduction of resazurin only occurs in metabolically active cells. Actively proliferating cells increase their metabolic activity while cells exposed to ADCs will have decreased activity. In all the aforementioned assays, the presence of an NAb would inhibit the cytotoxic effect of the ADC and result in reduced cytotoxicity and increased viability and/or proliferation.

Many of these assays can also be multiplexed with other homogeneous assays.

For example, an increase in caspase activity and a decrease in cell viability can be measured within the same well using CellTiter-Blue® assay and Caspase-Glo® assay (Promega), alternalitvely, multiplex kits are available such as the Apostol-Glo™ Triplex assay (Promega) which assess viability, cytotoxicity and apoptosis in one assay or the ApoLive-Glo™ Multiplex assay (Promega) that measures viability and apoptosis in the same well. Apoptotic cells can also be identified by the presence of caspase 3 and 7 activities. Apoptosis assays require testing at a precise time point that should be optimized.

The dose response curve is evaluated prior to examining other elements of the assay and is optimized to provide the best dynamic range and adequate sensitivity using the lowest drug concentration possible. The negative control typically is a pool of drug-naive normal human biological matrix. Efforts should be made to select a negative control that is representative of the baseline reactivity in the clinical trial population being tested.

Additional factors to be evaluated include; optimization of cell density, cell passage number, incubation times and temperature, cell growth conditions and effects of biological test matrix on the assay signal. It may be necessary to use serum-free media in order to gain a more robust signal. In addition, nonspecific binding of the ADC to cells must be low.

Assessing for method specificity is particularly important, as cell-based assays are often influenced by the biological matrix and cell lines can respond to factors in the matrix that can confound the ability to distinguish between true NAb positive and negative samples. Adding test matrix directly to the bioassay in the absence of the therapeutic and testing results from baseline pre-exposure samples is informative. Diluting samples to a particular minimal required dilution to overcome matrix effect is a common approach to eliminating matrix interference, but may affect assay sensitivity.

Immunogenicity assays for ADCs require generation of specialized control reagents. Surrogate positive control antibodies that exhibit neutralizing activity against the mAb and the payload are required, and serve to monitor consistency of assay performance over time. They may be used to estimate the relative sensitivity and drug tolerance of the assay during validation. It is important to remember that the sensitivity is dependent upon the selected drug concentration and properties of the surrogate positive control typically obtained from nonclinical immunizations, which does not reflect the heterogeneous ADA response found in patient sera.

Since ADAs in circulation may be partially or fully complexed with the ADC, it is important to determine if the assay conditions are capable of detecting NAb in the presence of the ADC. As NAb assays are more susceptible to the presence of drug than immunoassays used for detecting binding antibodies, the measurement of NAbs in the presence of drug is generally not recommended. However, samples are frequently collected at time points when circulating drug is still present thus, evaluation of baseline samples and an end-of-study sample following a drug washout period may provide evidence regarding NAb induction. A pre-incubation step with acid to disassociate any immune complexes can also be used [38,39] however, a complete understanding of the linker chemistry is essential, as ADCs may incorporate acid sensitive linkers. If acid is used for pretreatment, the sample must be completely neutralized prior to incubation with cells. As the acid pretreatment conditions are performed utilizing a surrogate positive control, they may not be appropriate for treatment of actual study samples. Furthermore, it is not possible to establish a single 'drug tolerance level' as the degree of interference depends on several factors such as, the concentration of drug, the characteristics of the surrogate positive control and the assay design.

Due to the complexity of cell-based assay development, statistical multi-factor design of experiments can be implemented and is well suited to evaluate and optimize the effects of many factors on the response of the assay [40,41]. Multiple experimental factors can be varied simultaneously, as opposed to the standard practice of varying only one factor at a time (OFAT), so that both individual and interactive effects can be elucidated in fewer experiments and at a fraction of the time typically required with the OFAT approach.

Once developed, the assay must be rigorously validated to ensure that it meets several important criteria and is fit for its intended purpose. Currently, validation of NAb assays for ADC follow existing regulatory and industry recommendations [7,8,22,26]. The fundamental parameters for validation include cut point, sensitivity, specificity and selectivity, precision, reproducibility and robustness of some assay features, and stability of reagents and control samples.

Determination of cut point is a fundamental aspect of assay validation and can pose a challenge for NAb assays. As NAb assays are most commonly performed only on samples that are confirmed to have antigen-specific ADA, confirmatory approaches are not usually necessary. One point to consider is that many ADC payloads are derived from or similar to natural products and, thus, pre-existing reactivity may be observed that requires additional consideration when setting cut points [42].

Critical reagent considerations

Adequate quality and quantity of critical reagents used in ADC immunogenicity assessments is imperative for establishing robust and reliable assays with long-term supply to support the bioanalytical work during a program's life cycle. The biophysical properties, of critical reagents, have profound effects on assay characteristics [43,44]. As ADCs have already undergone one or more rounds of conjugation to a payload, it is important to understand how further labeling and assay buffer selections will affect the immunogenicity assays. For example, sites for conjugation within the complementarity determining regions may be modified thus compromising the ability of critical reagents to detect NAbs. It is important to confirm the integrity of the binding activities of the ADC using proper analytical tools following any labeling steps.

Conclusion

With increasing numbers of ADCs in clinical trials and post-marketing surveillance, more clinical data are being collected that will increase our understanding of ADCs, help identify the main species influencing immunogenic behavior and inform appropriate immunogenicity strategies moving forward. These learnings will also guide both industry and regulatory agencies to more defined guidelines and/or white papers regarding immunogenicity assessments of ADC.

The development of more potent and novel payloads, improved mAb antigen targeting and improved linker technology will lead to novel ADCs, and hold great promise for expanding the use of ADCs beyond cancer therapies. Next-generation ADC products may have different fundamental characteristics that could include

diabodies, antibody fragments, fusion proteins, kinase inhibitors and gene-targeting agents. It may even be possible to link immune-stimulating agents to mAbs to induce local immune responses against tumors.

Immunogenicity and bioanalytical assessments may become even more challenging as the immune system is exposed to these new constructs and will likely merit a case-by-case approach rather than a one size fits all for these 'armed antibodies'.

Financial & competing interests disclosure

The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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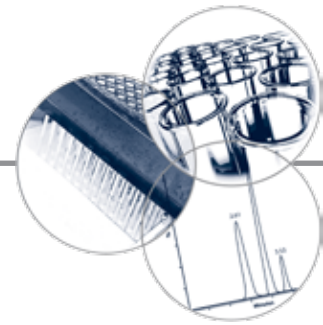

Bioanalytical assay strategies for the development of antibody–drug conjugate biotherapeutics

Antibody–drug conjugates (ADCs) are monoclonal antibodies with covalently bound cytotoxic drugs. They are designed to target tumor antigens selectively and offer the hope of cancer treatment without the debilitating side-effects of conventional therapies. The concept of ADCs is not new; however, development of these therapeutics is challenging and only recently are promising clinical data emerging. These challenges include ADC bioanalysis, such as quantifying in serum/plasma for PK studies and strategies for assessing immunogenicity. ADCs have complex molecular structures incorporating large- and small-molecule characteristics and require diverse analytical methods, including ligand-binding assays and MS-based methods. ADCs are typically mixtures with a range of drug-to-antibody ratios. Biotransformations *in vivo* can lead to additional changes in drug-to-antibody ratios resulting in dynamically changing mixtures. Thus, a standard calibration curve consisting of the reference standard may not be appropriate for quantification of analytes *in vivo* and represents a unique challenge. This paper will share our perspective on why ADC bioanalysis is so complex and describe the strategies and rationale that we have used for ADCs, with highlights of original data from a variety of nonclinical and clinical case studies. Our strategy has involved novel protein structural characterization tools to help understand ADC biotransformations *in vivo* and use of the analyte knowledge gained to guide the development of quantitative bioanalytical assays.

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Antibody–drug conjugates (ADCs) are monoclonal antibodies with covalently bound cytotoxic drugs. The ADCs are designed to bind to antigens that are overexpressed on the surface of the tumor target, but minimally expressed on normal tissue. The targeted delivery and use of highly potent cytotoxic drugs in ADCs treat the tumor with minimal systemic toxicity and offers patients the hope of cancer treatment without debilitating side-effects [1–3]. Therefore, highly potent cytotoxic agents that are otherwise too toxic to develop as therapeutics may be useful as molecular structural components of ADCs. The concept of ADCs was first validated in the clinic with gemtuzumab ozogamicin, a conjugate of an anti-CD33 antibody and the cytotoxic agent calicheamicin. In 2000, gemtuzumab ozogamicin was approved by the US FDA to treat patients with CD33-positive acute myeloid leukemia, a bone marrow cancer [4]. The product was recently withdrawn from the market after a later clinical trial raised safety concerns and failed to demonstrate benefit to patients [101]. After a hiatus, several novel ADCs are currently in preclinical, early clinical or late-stage clinical development for the treatment of solid and hematologic tumors [1–3,5–7]. In August 2011, the FDA approved Adcetris® (brentuximab vedotin), an ADC that targets CD30 on lymphoma cells, to treat Hodgkin's lymphoma and a rare lymphoma known as systemic anaplastic large-cell lymphoma, under the accelerated approval program [102]. The development of this emerging class of biotherapeutics has been challenging, particularly with regard to the development of appropriate bioanalytical strategies to characterize and measure ADCs and their catabolites in plasma and serum. The complexity of ADC analyte mixtures and the need for multiple and diverse analytical methods contribute to the challenges of ADC bioanalysis. This perspective explains why ADC bioanalysis is so complex and also describes the bioanalytical strategies we have developed to characterize **biotransformation**, PK and immunogenicity for ADCs during nonclinical and clinical development. Highlights of original data from a variety of case studies are used to illustrate the rationale for our bioanalytical approach.

ADCs have complex molecular structures, combining the molecular characteristics of small-molecule drugs, as well as those of large-molecule biotherapeutics. The drug is conjugated to the antibody via a chemical linker. A variety of

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Key Terms

Antibody–drug conjugate: Antibody with covalently attached cytotoxic drug via a chemical linker.

Biotransformation: Structural changes occurring to molecules in a biological matrix such as serum/plasma *in vitro* or *in vivo*.

chemistries and amino acid residue conjugation sites have been reported [8–11]. These include conjugation of the linker drug at lysine side-chain amines (Figure 1A), at cysteine thiols, after reduction of the interchain disulfide bonds (Figure 1B) or at engineered cysteine residues (Figure 1C). The linker may be designed to be chemically or enzymatically cleavable or noncleavable (Table 1). After binding to the tumor target, the ADCs are internalized and trafficked to lysosomes; the cytotoxic drug or cytotoxic catabolites are subsequently released within the cell. Table 1 shows examples of a variety of linkers and cytotoxic drugs at different stages of development; mechanisms of action include DNA intercalation or inhibition of tubulin polymerization.

With the exception of conjugation at engineered cysteine residues, the conjugation reaction results in a heterogeneous mixture of ADC molecules with a range of different drug-to-antibody ratios (DARs; Figure 1A–C). For example, with conjugation at lysine residues, a distribution of DARs ranging from 0 to 9 drugs has been reported (Figure 1A). In contrast, conjugation at reduced interchain disulfide cysteine residues results in mainly DAR0 and even numbered DARs 2, 4, 6 and 8 (Figure 1B) since the reduction of disulfides render pairs of cysteines to be available for conjugation. Homogeneous ADCs with a defined DAR2 have been reported for conjugation at engineered reactive cysteine residues at specific sites in antibodies (Figure 1C) [10,11]. Consequently, the physical/chemical properties and degree of heterogeneity of the ADC varies with the strategy used for conjugating the linker drug to the antibody (Figure 1A–C). An additional aspect of molecular heterogeneity not depicted in Figure 1 results from the number of potential conjugation sites available for conjugation. For example, there are typically as many as 100 lysine sites available in antibodies for potential conjugation, conferring the greatest heterogeneity. Conjugation at interchain disulfides results in significantly less heterogeneity as there are only up to eight cysteine conjugation sites available. Conjugation at engineered sites can be used to minimize the degree of heterogeneity; for example, one engineered cysteine in a light or heavy chain leads to only two sites for conjugation in the antibody.

Additional complexity of ADCs can be generated *in vivo* due to biotransformation resulting from catabolism and metabolism. Catabolism herein is defined as the breakdown of the ADC, whereas metabolism refers to

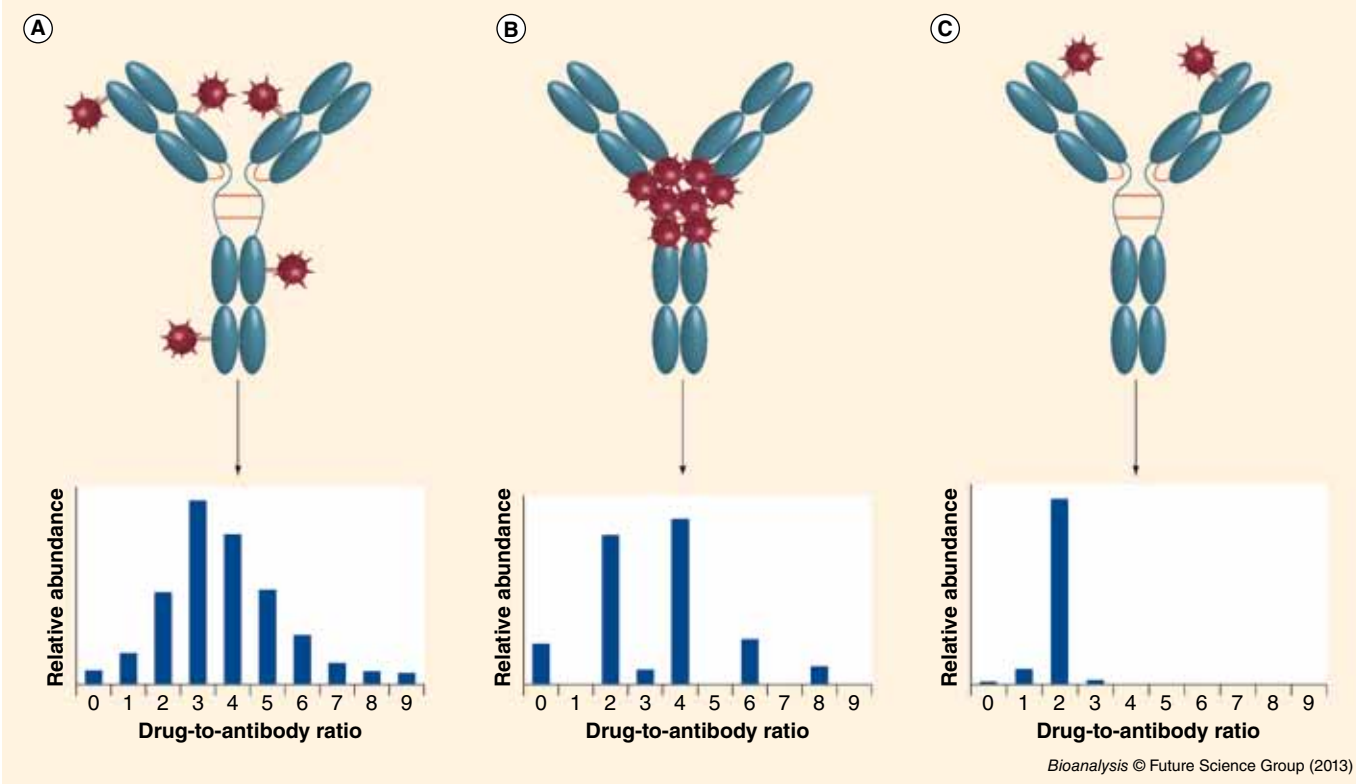

Figure 1. Antibody–drug conjugate conjugation sites and drug-to-antibody ratio heterogeneity. (A) Conjugation through lysines, **(B)** conjugation through reduced interchain disulfide bonds, and **(C)** conjugation through engineered cysteines.

Table 1. Examples of antibody–drug conjugate linkers and drug chemistries.

ADC/target	Manufacturer	Linker	Chemistry	Cleavable/ noncleavable	Drug	Ref.
Trastuzumab–emtansine/anti-HER2	Genentech/Roche/ Immunogen	Succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate	Thioether	Protease, noncleavable	Maytansinoid DM1†	[46]
Inotuzumab–ozogamicin/anti-CD22	Pfizer, Inc.	4-(4′-acetylphenoxy)butanoic acid	Hydrazide	Protease, cleavable	Calicheamicin‡	[52]
Brentuximab–vedotin/ anti-CD30	Seattle Genetics	Maleimidocaproyl-valine-citrulline-p-aminobenzyloxycarbonyl	Peptide	Protease, cleavable	Mono-methyl auristatin E†	[53]
Unnamed/anti-EGFR	State University of New York at Stony Brook	4-mercaptopentanoate	Disulfide	Protease, cleavable	Methyldisulfanyl propanoyl taxoid†	[54]

†*Mechanism of action: tubulin binding.*
‡*Mechanism of action: DNA binding.*
ADC: Antibody–drug conjugate.

chemical modification of the ADC or subsequent modification of its catabolites. Linkers are designed to be relatively stable in circulation and release the drug load after ADC internalization. Early ADCs were based on acid-cleavable hydrazone linkers that were relatively stable at neutral pH in the bloodstream (pH 7.3–7.5), whereas the more acidic environment within the cellular endosomes (pH 5.0–6.5) and lysosomes (pH 4.5–5.0) caused hydrolysis of the linker after internalization of the ADCs and release of the drug [4,12,13]. With enzymatically cleavable linkers, the drug release is designed to be more specific; for example, the linker contains a peptide bond that can be cleaved by the lysosomal protease cathepsin B [8,14,15]. Linkers that are resistant to enzymatic or chemical cleavage have also been reported [16–18]. However, even for linkers designed to be entirely stable in plasma, unanticipated chemical or enzymatic activity in plasma may result in some drug release (deconjugation), leading to additional DARs *in vivo* [19,20]. In addition to drug deconjugation, other biotransformations, such as adduct formation, generation of peptide fragments or linker drug fragments can also lead to increased complexity in circulation [19,20]. Even for a homogeneous ADC reference standard, *in vivo* processing can theoretically result in a complex ADC mixture (Figure 2). It is important to understand the DARs and catabolite products *in vivo*, in order to design appropriate quantitative assays that are capable of accurately measuring all the analytes present.

Unique ADC bioanalytical challenges

The fact that ADCs are complex mixtures that change *in vivo* creates fundamental challenges for PK bioanalysis [21]. Normally, the bioanalysis of a therapeutic, whether a biotherapeutic such as a monoclonal antibody (mAb) or a small-molecule drug, is clearly defined and involves measuring the concentration of the therapeutic over time in plasma or serum for *in vivo* studies. The same therapeutic is used as the reference standard for preparing the assay calibration curve. Although there may be some microheterogeneity in the case of a biotherapeutic, or metabolite formation in the case of small molecules, the bioanalysis is typically simply the concentration of the major parent molecular species over time. The concentration versus time profile is then used to calculate PK parameters for the therapeutic and explore potential relationships with safety and efficacy. In contrast, an ADC therapeutic *in vivo* is typically a complex and dynamically changing mixture, caused by biotransformations, differing DAR clearance rates, or a combination of these processes. In this context, even the existing PK language for 'therapeutic concentration' versus time becomes ambiguous. Furthermore, the calibration standard curve is comprised of a reference standard that may not represent the changing analytes *in vivo*, presenting a unique challenge for quantitative assays. The bioanalysis of ADCs thus requires defining the specific analytes to be measured within the *in vivo* mixture and ensuring that the corresponding assay is accurate, even though the reference standard calibration curve may not be identical to the analyte measured at various time points after dosing.

Current technologies do not quantify the individual DAR components of an ADC. Therefore, assay methods typically measure analyte mixtures that represent a subset of the DAR mixture. The theoretical number of potential

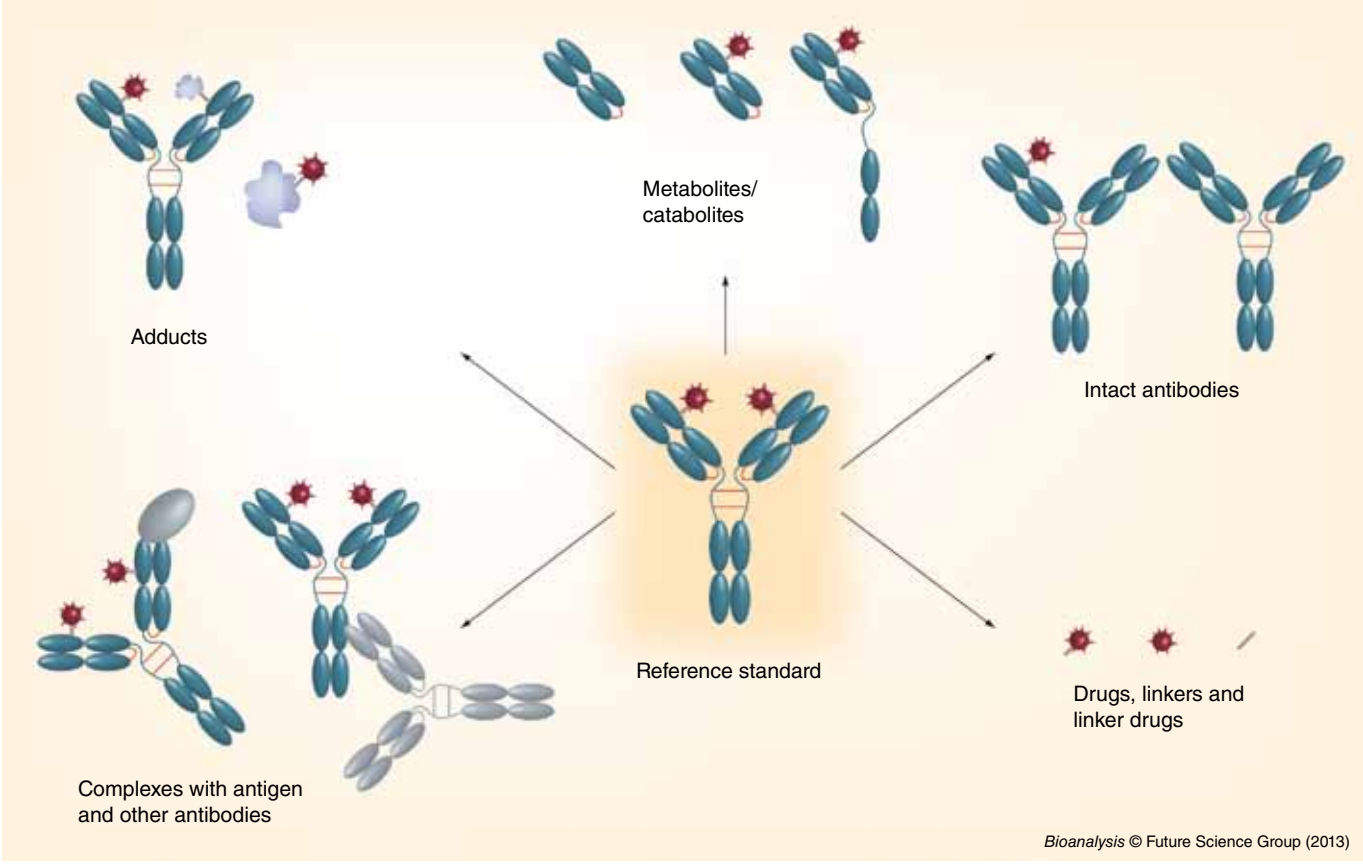

Figure 2. Antibody–drug conjugate complexity may increase *in vivo* due to catabolism or metabolism.

analyte mixtures that could be quantified for an ADC *in vivo* based on the DAR mixture compositions is large. Using a lysine-conjugated ADC as an example (Figure 3A), analyte mixtures that could theoretically be measured include: conjugated antibody, where at least one drug is present (Figure 3B); antibody-conjugated drug, measuring all drug still conjugated to the antibody (Figure 3C); total antibody, where drug may or may not be present (Figure 3D); naked antibody, where all drug has been lost (Figure 3E); or small-molecule catabolites, for example, free drug, free linker, linker drug or amino acid linker drug (Figure 3F). The analytes and key aspects of the information provided in each case are described in more detail in the relevant assay sections below. It is not possible to predict the analytes that are critical to measure for understanding ADC pharmacology based on theoretical considerations alone. At this time, given the limited information on ADCs in the clinic, it is not well understood which analytes may correlate best with safety and efficacy, or which set of analytes may provide the best overall understanding of the fate of ADCs *in vivo*. Each analyte provides insights into different aspects of the mixture; for instance the total-antibody assay is important to understand whether the ADC has the general PK characteristics of an antibody; uncharacteristically rapid clearance may indicate that conjugation of the antibody has rendered it unsuitable as a targeted delivery agent. Data for different analytes may be compared, with caveats, to obtain additional insights. Clearly, multiple assays are needed initially to build an understanding of the overall PK of ADCs. Choosing the analytes to measure represents one of the key challenges because ADCs are relatively new and experience within the industry is limited.

Some limitations of existing bioanalytical methods for ADCs

The bioanalytical methods for small- or large-molecule drug development are driven by the molecular characteristics of the therapeutic. As ADCs combine, the molecular characteristics of both small- and large-molecule therapeutics, bioanalytical methods typical for both types of therapeutics are needed. For example, large molecules have well-defined tertiary structures that

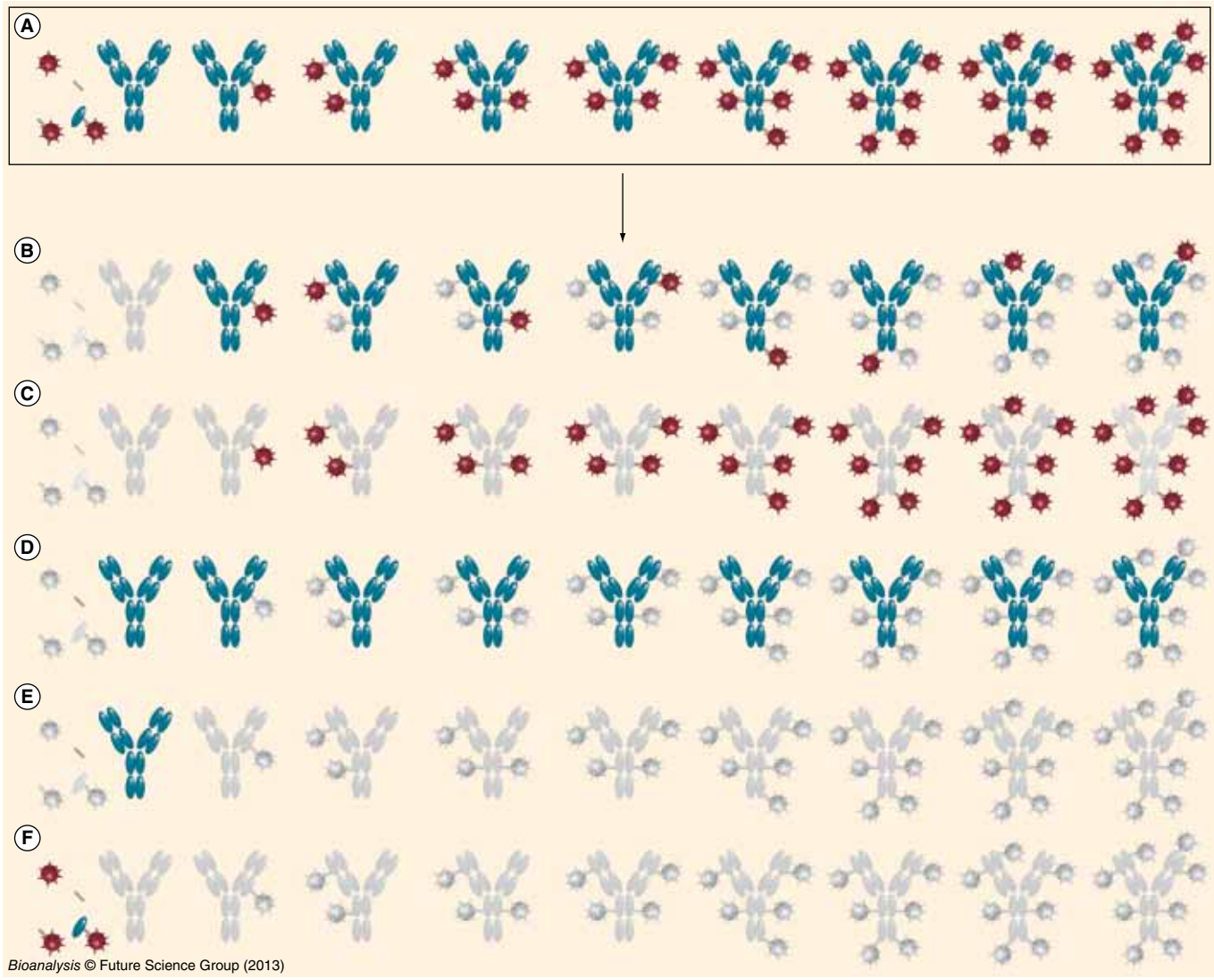

Figure 3. Antibody–drug conjugate analytes. (A) Example of a heterogeneous antibody–drug conjugate (ADC) reference standard *in vivo* and the potential theoretical analytes. **(B)** Conjugated-antibody, where at least one drug is present. **(C)** Antibody-conjugated drug. **(D)** Total antibody, includes conjugated and unconjugated-antibody. **(E)** Naked-antibody, includes fully deconjugated ADC (i.e., drug-to-antibody ratio 0) in reference standard and ADC where all drug has been lost. **(F)** Small-molecule catabolites. The analyte mixtures B–F are defined by gray areas to indicate the parts of the ADC structure that would not be fully measured by the respective assay and colored areas to indicate information that would be fully determined.

are suited for **ligand-binding assays** (LBAs), for example, ELISA [22–24]. Figure 4A shows a typical ELISA format that is commonly used for large-molecule analysis for a single analyte. Recently, hybrid binding/MS-based methods have also been shown to provide quantitative data for large molecules as an alternative bioanalytical approach where MS replaces the detection reagent of a typical ELISA [25]. While large molecules may be quantified by ELISA or MS approaches, the bioanalysis of small-molecule drugs is predominantly performed by LC–MS/MS following extraction from plasma/serum.

A variety of ELISA and LC–MS/MS methods have been reported to quantify ADCs and the cytotoxic drugs released from ADCs into the circulation [26–29]. However, these methods have some limitations. For example, conventional ELISA methods for the large-molecule component of an ADC measure the analyte concentration indirectly based on binding properties of the analyte and the assay reagents (Figure 4A). This methodology can be used to measure the total antibody analyte (Figure 4B; i.e., the sum of fully conjugated antibody, partially deconjugated antibody and fully deconjugated antibody concentrations; Figure 3D), using reagents that

Key Term

Ligand-binding assay: Method to quantify analyte concentration by the amount of binding of specific ligands.

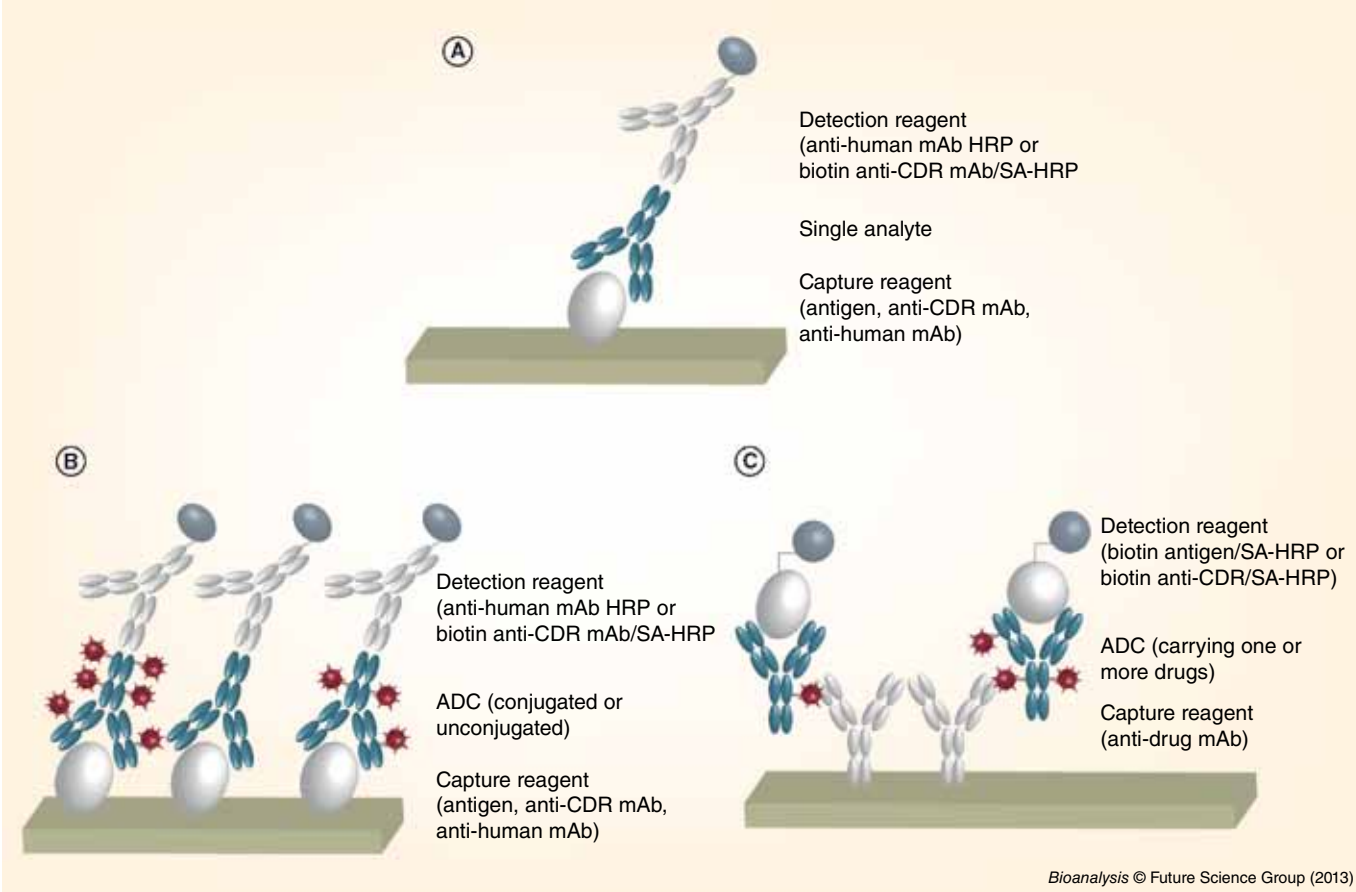

Figure 4. Ligand-binding assays. (A) Typical ELISA, **(B)** ADC total-antibody ELISA, **(C)** ADC conjugated-antibody ELISA.
ADC: Antibody–drug conjugate; Anti-CDR: Anti-complementarity determining region; HRP: Horseradish peroxidase; mAb: Monoclonal antibody; SA–HRP: Streptavidin–horseradish peroxidase.

bind to the antibody. Similarly ELISA can be used to measure conjugated antibody (Figure 4C) (antibody with one or more drugs attached, Figure 3B) using reagents that bind to the drug. However, conjugated-antibody assays do not provide measurement of the DAR or the overall drug load [28,29]. This information is important because the DARs and overall drug load may have a significant effect on the safety or efficacy of ADCs [10,30]. Conventional small-molecule LC–MS/MS methods also come with limitations in that they quantify *a priori* postulated forms of the drug released by the ADC from catabolism (Figure 3F). However the putative released drug analyte may not be the major form of the drug released; for example, it is theoretically possible that the released drug contains part of the linker, or released drug may bind to plasma peptides or proteins (Figure 3F). Thus, while conventional large- and small-molecule methods may be used for ADC bioanalysis, it is important to understand the limitations of these methods and develop additional strategies specifically for ADCs.

Clearly, conventional large-molecule LBAs and small-molecule LC–MS/MS assays used alone provide limited information for ADCs. Some methods designed specifically for ADCs have been reported recently. A **DAR distribution** characterization method we designed for ADCs involves affinity capture capillary LC–ESI-MS (Figure 5A) [19], which measures the intact molecular masses of ADCs or proteins in biological matrices. This method involves isolation of the ADC from plasma or tissues by affinity capture followed by analysis of the molecular masses of the captured ADCs by capillary LC–ESI-MS. The molecular masses provide the DAR distribution. Quantification of the relative amounts of the individual DAR ADC species can be determined by comparing the deconvoluted peak area for each DAR present. In order to compare peak areas, it is important to ensure each DAR ionizes with similar efficiency and there is no bias in

Key Term

Drug-to-antibody ratio distribution: Antibody–drug conjugate molecules with a range of drug-to-antibody ratios.

the measurement [19]. Quantitative methods developed specifically for ADCs include those that measure the total drug in plasma. For example, the total amount of antibody-conjugated monomethyl auristatin E (MMAE) and free MMAE in circulating cAC10-vc-PAB-MMAE (MMAE linked to antibody cAC10 by valine-citrulline-p-aminobenzyloxycarbonyl) ADC has been determined by incubating plasma samples with cathepsin B (to completely release the drug from the antibody). The drug (MMAE) is then detected in a competition ELISA using an MMAE conjugated to horseradish peroxidase as a reporter and an anti-MMAE monoclonal antibody coat [27]. Others have reported measuring total drug in plasma by chemical cleavage of a labile linker [31]. Novel methods such as these, designed specifically for use with complex ADCs, can provide additional information that cannot be obtained from conventional large- and small-molecule approaches alone.

Our bioanalytical PK strategy considerations

To develop our ADC bioanalytical strategy, we built a group with expertise in three key technical areas: immunoassay methods for PK evaluation and immunogenicity assessment of biotherapeutics; LC–MS/MS-based methods for small-molecule drug/metabolite identification and PK assessment; and affinity capture chromatographic and LC–MS methods to characterize protein structures in biological matrices. Our rationale has been to:

- Develop novel methods for molecular structural characterization of ADC analytes in plasma/serum to determine the different key DAR analytes in circulation (case studies 1 and 2). This approach provides an overview of the fate of the ADC *in vivo*, for instance, does the antibody still carry most of the covalently bound drug over time. The DAR information

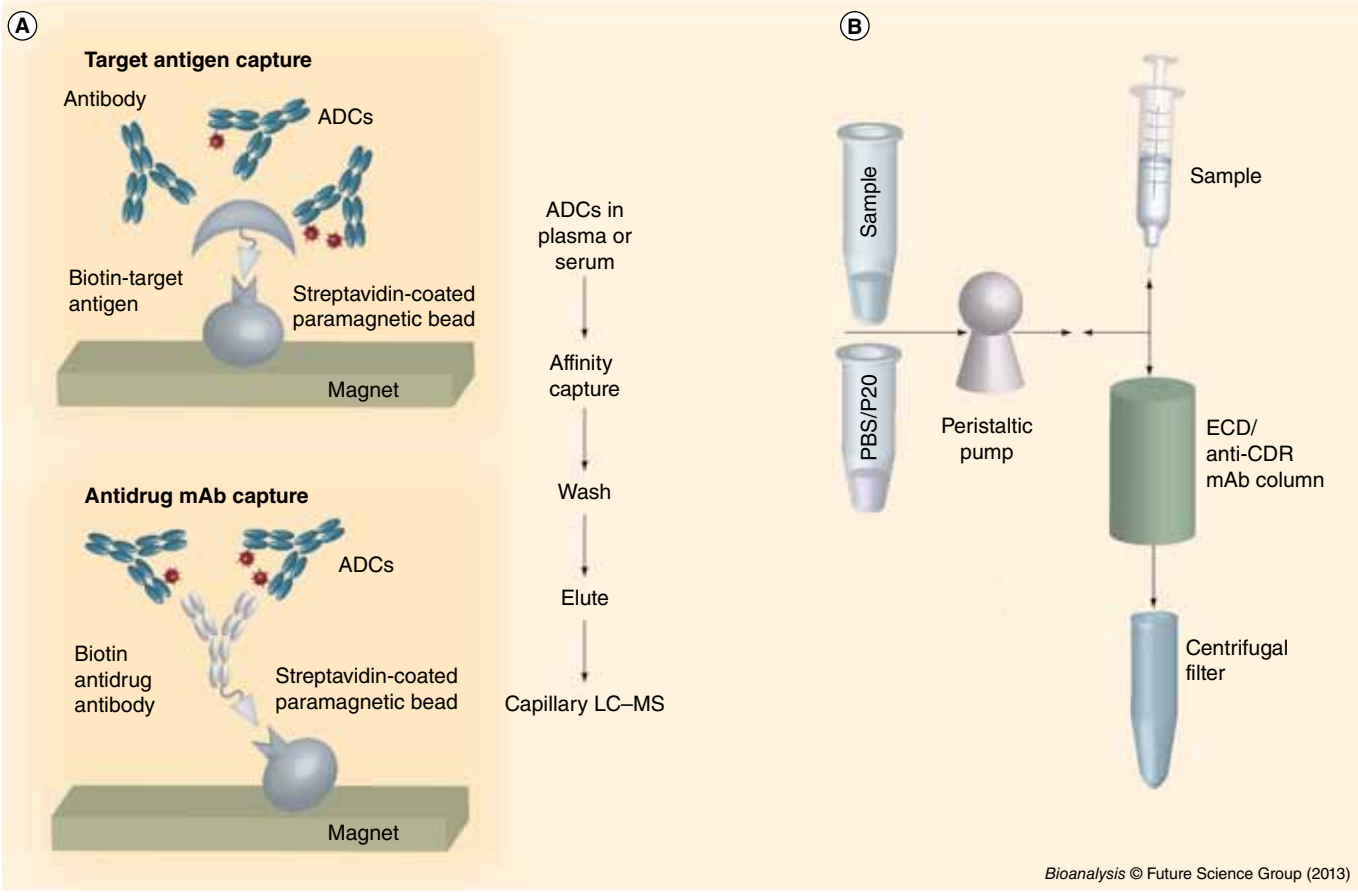

Figure 5. Methods to characterize antibody–drug conjugate drug-to-antibody ratio distributions. (A) Affinity capture LC–MS, **(B)** affinity capture HIC.
ADC: Antibody–drug conjugate; Anti-CDR: Anti-complementarity determining region; DAR: Drug-to-antibody ratio; ECD: Extracellular domain; HIC: Hydrophobic interaction chromatography; mAb: Monoclonal antibody; P20: Polysorbate 20; PBS: Phosphate-buffered saline.

Key Term

Hydrophobic interaction chromatography: Method to separate molecules on the basis of analyte hydrophobicity.

is also important to develop appropriate and accurate quantitative methods;

- A feasible number of quantitative assays can be developed using a combination of conventional large-molecule LBAs, small-molecule LC–MS/MS assays, as well as novel assay methods, such as hybrid affinity capture LC–MS/MS;
- Later in clinical development, when there may be a better understanding of the relationships of the analytes with safety and efficacy, it may be possible to reduce the number of quantitative assays through combined expertise.

All quantitative methods are evaluated to ensure accurate measurement of the DARs in the ADC reference standard and also accurate measurement of any additional DARs identified in serum/plasma *in vitro* or *in vivo* (case studies 2 and 3). Assay strategies based only on information on the analytes present in the reference standard may not accurately quantify new DARs formed *in vivo* (case study 3). Since the molecular characteristics of ADCs vary with the linker drug structures and conjugation chemistries, the specific analytes to quantify are chosen on a case-by-case basis. Rationale for the key analytes is described below.

Highlights of our PK assay strategy for ADC development (Box 1) first include using exploratory qualified methods to understand the DAR distributions of ADCs *in vitro* and *in vivo;* then developing a diverse set of validated quantitative assays to measure the key analytes: total antibody (Figure 3D), antibody conjugate (Figure 3B & C) and free drug (Figure 3F). The quantification of the total-antibody analyte (Figure 3D) is important to confirm that the PK characteristics are in a range typical for antibodies and not compromised significantly by conjugation. For the antibody-conjugated analyte (Figure 3B & C), it is noteworthy that we have used two alternate ways to define this analyte. The conjugate can be viewed from the perspective of the antibody (conjugated antibody, i.e., the concentration of antibody molecules with one or an unknown number of drugs attached), as shown in Figure 3B. Alternatively, the antibody-conjugate can be viewed from the perspective of the drug (antibody-conjugated drug, i.e., the concentration of drug that is attached to antibody) as shown in Figure 3C. When technically feasible, we have measured the latter as the preferred analyte, using the rationale discussed in the assay section below. In all cases it is important to ensure that the assays are capable of measuring the DAR analytes determined to be present in plasma over time. For nonclinical studies, we have incorporated generic assay formats, where possible, to allow ready comparison of ADC behavior *in vitro* and *in vivo* for different ADC molecules. Additional exploratory assays to measure catabolites have been developed later during development. Our strategy includes the plan to consider reducing the number of assays once an ADC is further in clinical development and the relationships between the analytes measured and safety/efficacy are better known. We have used an overall risk-based immunogenicity strategy that is typical for biologics [32–34]. The strategy involves a tiered approach for ADCs where we first develop a screening assay to detect anti-therapeutic antibodies (ATAs) to the ADC, including ATAs towards any of the ADC molecular components such as the antibody, linker, drug or epitopes involving multiple ADC components [35]. The immune response is then confirmed by competitive binding with the ADC. Further competitive binding with the antibody is used to determine whether the response is primarily to the antibody, to other ADC components (linker, drug), or to ADC-unique epitopes. Overall, we have tailored our bioanalytical approach based on consideration of the individual ADC properties. Further details of the PK and immunogenicity assay strategies and rationale are provided in the assay sections below.

Novel ADC assays to characterize DAR distributions in plasma/serum

A fundamental aspect of our bioanalytical strategy is to develop exploratory assays capable of characterizing ADC analyte structures in plasma. Biotransformation may result in ADC analytes *in vivo* that differ from those in the reference standard (case study 2). Existing methods for large and small molecules do not provide biotransformation information for ADCs. It was therefore necessary to invent novel methods such as affinity capture capillary LC–MS [19] (Figure 5A, case study 1) and affinity capture **hydrophobic interaction chromatography** (HIC; Figure 5B, case study 2). Both methods can be used to characterize DAR distributions of ADCs in plasma/serum.

The choice of DAR characterization method in serum/plasma depends on whether the antibody chains contain interchain disulfides and are covalently bound (Figure 1A & C) or whether

Box 1. Bioanalytical PK assay strategy for antibody–drug conjugate development.

- Characterize DAR changes in serum/plasma
  - DAR distribution by affinity capture LC–MS
  - DAR distribution by affinity capture HIC†
  - Test all quantitative assays with individual DARs present in serum/plasma to ensure accurate measurements
- Quantify ADC analytes in nonclinical & clinical studies using validated assays
  - Total antibody (generic or specific ELISA format)
  - Antibody-conjugated drug‡ (affinity capture/linker cleavage/LC–MS/MS)
  - Free drug catabolite (protein precipitation/LC–MS/MS)
  - Other small-molecule catabolites§ (protein precipitation/LC–MS/MS)

†For noncovalently bound ADCs conjugated at reduced interchain disulfide cysteine residues.
‡ADCs with cleavable linkers, otherwise measure conjugated-antibody analyte using ELISA.
§Selected exploratory studies in plasma, bile, urine and various tissues.
ADC: Antibody–drug conjugate; DAR: Drug-to-antibody ratio; HIC: Hydrophobic interaction chromatography.

the interchain disulfides are reduced and the antibody chains are noncovalently bound **(Figure 1B)**. This is because the affinity capture capillary LC–MS method is performed under denaturing conditions and noncovalently bound light and heavy chains separate during the analysis. Thus, only light- and heavy-chain molecular masses are obtained for ADCs that contain reduced interchain disulfides, using affinity capture LC–MS. Although a LC–MS method for the intact molecular mass measurement of noncovalently bound ADCs in simple buffers has been reported recently [36], it is not clear whether it is applicable to serum/plasma samples. Characterization of DAR distribution in plasma requires the measurement of intact ADC molecular masses and cannot be determined from individual light- and heavy-chain masses. Thus for ADCs that contain reduced interchain disulfides, LC–MS approaches provide light- and heavy-chain masses and only the average DAR can be determined, with the assumption that each ADC molecule contains two heavy chains and two light chains. In contrast, the affinity capture HIC analysis (case study 2) is performed under nondenaturing conditions and is suitable for measuring DAR distributions for ADCs that contain covalently bound chains **(Figure 1A & C)**, as well as noncovalently bound chains **(Figure 1B)**. Although both affinity capture LC–MS and affinity capture HIC methods can be used for ADCs where the antibody chains are covalently bound **(Figure 1A & C)**, the affinity capture LC–MS method is preferred due to its greater sensitivity (ng/ml vs µg/ml, respectively).

Structural characterization of ADCs in serum or plasma by affinity capture LC–MS can also provide mechanistic insights into the biotransformation *in vivo*. For example, in model ADCs, where linker drugs were conjugated to cysteines at specific engineered sites, the *in vivo* stability (i.e., deconjugation) of linker drug was observed to vary with the site of conjugation to the antibody [20]. It was hypothesized that both solvent accessibility and the charge of the local environment are important factors for stability. Identifying the importance of the site of conjugation for linker drug stability was a significant insight for ADC drug development, as deconjugation can affect both safety and efficacy [10,30].

Additional information about the mechanism of ADC deconjugation was also obtained using affinity capture LC–MS as follows. Maleimide chemistry is often used for conjugation of linker drug to cysteines and it has been proposed that maleimide can exchange with albumin in plasma *in vitro* based on data from maleimide linkers containing fluorescent tags [37]. In more recent studies using model ADCs with linker drugs conjugated to cysteines at specific engineered sites, maleimide exchange with several thiol-reactive constituents in plasma was observed both *in vitro* and *in vivo* based on identification of the molecular masses of the adducts formed, using affinity capture LC–MS. For example, masses corresponding to albumin- and cysteine-linker drug were observed [20]. The rate of exchange, based on the decrease in mass of the ADC and the formation of albumin adducts, was found to be dependent on the site of linker drug conjugation in the model ADCs. In addition, the method identified opening of the succinimide ring, characterized by an 18 Da increase in molecular mass of the ADC, which was found to stabilize the linker drug(s) and prevent further deconjugation.

In summary, methods for the structural characterization of ADCs in serum and plasma, especially DAR distributions, are a key component of our strategy. Understanding the analytes present

in vivo is important for designing appropriate PK assays (case studies 1–4). The affinity capture LC–MS and affinity capture HIC methods developed for ADC characterization also provide powerful tools for understanding the fate of ADCs *in vivo*.

LBA reagent characterization using affinity capture capillary LC–MS

The affinity capture LC–MS method is also valuable for characterizing reagents used in LBAs (case study 4), for example, to screen antidrug mAb clones that bind appropriately to a range of DARs. This can be achieved by comparing the ADC DAR distribution data obtained using the antidrug mAb clone as the capture probe in affinity capture LC–MS with the corresponding data obtained for the ADC mixture reference standard by LC–MS alone. In this case, clones that bind to all the ADC DARs in the reference standard solution and show data comparable to LC–MS alone exhibit the least bias and are most suitable for use in LBAs (case study 4). Conversely, clones that only recognize a subset of analytes in the reference standard solution, that is, the data are not comparable to analysis by LC–MS alone, show bias and are unsuitable for LBAs. Other binding characteristics of mAb clones can also be assessed by affinity capture LC–MS. For example, binding epitope information, such as, whether the mAb clone binds specifically to the ADC drug moiety or whether there is crossreactivity with the linker, can be derived by comparing data from ADCs with differing linker drug combinations. If crossreactivity with the linker is observed, it is possible the clone may bind to the ADC that contains only conjugated linker *in vivo*, even after the drug is lost. Using such a reagent in a LBA could provide misleading data. One caveat to be aware of is that the binding characteristics of assay reagents in the bead-based affinity capture LC–MS method may not be identical to those in the plate-based ELISA. Thus, any observations for the reagents, such as inappropriate binding to specific DARs, should be confirmed by testing in the ELISA.

The affinity capture LC–MS method can be used to indirectly assess the performance of LBAs. For example, LBAs should ideally be tested with individually purified DARs, for example, purified DAR1, purified DAR2, and so forth, to assess successful recovery (typically 80–120%) of the analytes to be measured in the ADC mixture present in plasma (see LBA sections below). These individual purified DAR standards can usually be obtained by chromatographic fractionation of the ADC mixture in buffer. However, in some cases, the DARs cannot be chromatographically resolved due to the nature of the linker drugs and, thus, it may not always be possible to obtain individual purified DARs (case study 4). In this case, the LBA recovery can be assessed indirectly as follows. The DAR distribution data for the ADC mixture in the reference standard solution by direct LC–MS (i.e., without an affinity capture step) can be compared with the DAR distribution data for the ADC mixture spiked into plasma and analyzed by affinity capture LC–MS. If comparable DAR distributions are observed by direct LC–MS and by affinity capture LC–MS, using capture probes containing the LBA reagents, then it provides indirect evidence that the recovery for the LBA is appropriate (case study 4).

ADC total-antibody PK assays in serum using LBAs

The total-antibody PK assay for an ADC by LBA **(Figure 4B)** is designed to measure all DARs of the ADC analyte mixture *in vivo*, including fully conjugated, partially deconjugated and fully deconjugated reference standard **(Figure 3D)**. This assay can be used to assess whether the PK parameters for total antibody (e.g., clearance or half-life) are in the range typical for an antibody. This confirmation would indicate that conjugation with drug has not compromised the characteristics of the antibody *in vivo* and support the antibody as a suitable delivery vehicle for an ADC platform. Conversely, unusual PK, such as extremely rapid clearance, would indicate that the antibody in question may not be suitable for development as an ADC. As discussed above, ADCs are dynamically changing mixtures *in vivo* and the reference standard calibration curve is typically identical to the analyte only at early PK time points. Thus, understanding the assay performance and designing appropriate assay validation experiments are more complex than for a typical single-analyte ELISA. In the absence of regulatory guidelines or established industry best practices for ADC assay validation, we have used validation approaches typical for large and small molecules and added additional experiments as indicated below. This has included using qualified assays for discovery research studies and validated assays for investigational new drug (IND)-enabling, as well as clinical studies. We evaluate typical

performance characteristics for large-molecule assay validation, for example, accuracy, precision, dilutional linearity and specificity. In addition, during assay development, we evaluate characteristics unique to ADCs such as binding to individual or enriched DAR standards (case studies 3 and 4). We also evaluate the ability of the assay to measure total antibody after incubation of the ADC in plasma at 37°C *in vitro*, to ensure that partial deconjugation does not affect the ability of the assay to measure total antibody (case study 5).

Figure 4B summarizes the total-antibody ELISA assay formats that we have used to date. For nonclinical PK and TK studies, we often used a generic assay format where the coat capture reagent and the detection reagent are polyclonal anti-human antibodies that bind to the nonvariable regions of the antibody **(Figure 4B)**. These are affinity-purified commercially available reagents that do not crossreact with endogenous IgGs in nonclinical sera. A homogeneous assay format coincubating sample and reagents instead of the step-wise procedure typical for ELISA was found to generally result in a more robust assay across a range of DARs. A generic ELISA format assay strategy is also useful during nonclinical development, since custom-ELISA reagents, such as recombinant antigens or anti-idiotypic antibodies, may not be available at this stage. More recently, we have also used an exploratory hybrid ligand-binding/MS approach to quantify ADC total antibody or to troubleshoot development of ADC ELISA. This LC–MS/MS assay involves protein A binding of antibodies in plasma followed by enzymatic digestion with trypsin, addition of a stable-labeled internal standard and quantification of a signature peptide by LC–MS/MS. Additional peptides are analyzed in a multiplexed way as a confirmation. Similar affinity capture LC–MS/MS strategies have been reported recently for antibody quantification [38].

For clinical studies, generic assay formats cannot be used due to the fact that the generic assay reagents, such as anti-human antibodies and protein A that bind to ADC-antibody nonvariable regions, would also bind to endogenous human IgGs that are present in large molar excess. Thus, for clinical assays we used custom ELISA formats incorporating recombinant antigens, anti-idiotypic or anti-complementarity determining region (CDR) antibodies to minimize background from serum proteins **(Figure 4B)**.

For all formats of the ADC total-antibody assay used for nonclinical and clinical development, it is important to ensure all DARs expected *in vivo* are quantified accurately for total-antibody concentrations. For example, it is theoretically possible that when an ADC molecule has a high DAR, the drug may sterically hinder binding of assay reagents to the antibody portion of the ADC. It is also possible that high DARs could aggregate due to hydrophobicity and not bind effectively to assay reagents. Typically, we have tested the recovery of individual DARs to confirm that there is no bias in the assay with varying DARs (case study 3). In some cases, individual DARs are not readily available, for example, the DAR mixture is too complex for chromatographic isolation of individual DARs (case studies 1 and 4) or some DARs may only be formed *in vivo* and isolating all new DARs from plasma may be challenging (case study 2). When individual DARs are not available, we have used enriched DAR fractions from crude fractionation of the ADC reference standard or from conjugation procedures designed to produce either higher or lower than reference standard DARs. One caveat to consider when using DARs isolated from reference standard for assay characterization is that the molecular structures may not be identical to the DARs formed *in vivo*. For example, where the linker drug is lost via a maleimide exchange reaction, cysteine or glutathione addition to the antibody deconjugation site could occur. Thus, we have also used an alternative approach to characterize the total-antibody assay. This involves measuring the concentration of the ADC over time, after allowing deconjugation by incubation in plasma at 37°C, with a calibration curve consisting of the ADC reference standard (case study 5). If there is no bias in the measurement of DARs, total-antibody concentration values should not change as the ADC deconjugates during the incubation.

ADC conjugate PK assays in serum/plasma using LBAs or a hybrid affinity binding & LC–MS/MS method

Although there are exploratory methods that can measure relative DAR distributions *in vivo* as described above, these methods do not provide absolute quantification of individual DAR analytes, such as individual conjugated species, for PK assessment. Thus, as in the case of the total-antibody assay above, quantitative analysis of the ADC conjugate requires measurement of a heterogeneous analyte mixture. We have used

two different formats for conjugate PK assays, based on the rationale discussed below. We have defined the conjugate analyte in two alternative ways. From the perspective of the antibody, the analyte mixture can be defined as the concentration of antibody molecules with one or more drugs attached (Figure 3B). An alternative way to define the ADC conjugate is from the perspective of the drug load, that is, the total concentration of drug that is conjugated to the antibody (Figure 3C). Overall, the antibody-conjugated drug analyte provides a more sensitive measurement of changes in drug load than the conjugated-antibody assay. In addition, the antibody-conjugated drug assay provides information that is distinctly different from the total-antibody assay (Figure 3D). Thus, the measurement of the antibody-conjugated drug (Figure 3C) and the total antibody (Figure 3D) provides a simple way to define a heterogeneous DAR mixture in terms of the total amount of the two key molecular components of the ADC, for instance the antibody and the drug. Overall, the antibody-conjugated drug is currently our preferred analyte to measure ADC conjugate PK, when technically feasible. Currently, only ADCs with cleavable linkers are amenable to analysis. The following describes considerations for the development of both types of conjugate assays.

■ Conjugated-antibody PK assay in serum using ELISA

The conjugated-antibody LBA is designed to measure all DAR analytes except naked antibody present in the reference standard or DAR0 resulting from complete deconjugation *in vivo* (Figure 3B). However, this analyte has the disadvantage that it does not provide direct information on the drug load. This is because the assay format requires that only one drug be present for binding to the ELISA coat capture reagent (Figure 4C). Thus, under the best assay conditions, the signal from a sample containing only DAR1 is the same as a sample containing higher DARs. The assay does provide some indirect information on drug load in that fully deconjugated ADC, that is, DAR0 is not detected. The fact that changes in drug load cannot be detected in a sensitive manner in the conjugated-antibody assay is a significant limitation, since changes in the ADC drug load may affect both safety and efficacy.

Figure 4C shows the ELISA formats that includes capture with an antidrug mAb and detection with either a recombinant ligand or an anti-CDR mAb. The use of specific reagents helps to minimize background, for both nonclinical and clinical assays. Similar to the case of the total-antibody ELISA above, it is also important to characterize the assay performance for individual DARs (case study 3). Again, it may not be possible to obtain individual DARs and assay performance may need to be characterized using enriched DAR fractions or assessed indirectly by evaluating the binding of DARs to assay reagents using affinity capture LC–MS (case study 4). Unlike the total-antibody assay where the binding to high DARs may theoretically be poor due to steric hindrance, for the conjugated-antibody ELISA, the binding to low DARs such as DAR1 may be poor due to low avidity. If low DARs are not accurately measured in the assay (case study 3), the PK profile would overestimate the amount of complete drug deconjugation. As in the case of the total-antibody ELISA, we used validated assays for IND-enabling and clinical studies. Assay validation experiments included those typical for large-molecule LBA with additional characterization experiments, such as individual DAR recovery.

■ Antibody-conjugated drug PK assays in plasma/serum using affinity capture, drug release & LC–MS/MS

The antibody-conjugated drug assay is designed to measure the concentration of all drug molecules that are covalently bound to the antibody (Figure 3C). This assay provides direct information on drug load and is highly sensitive to any changes in drug load. Based on the high potency of drugs used for ADCs, even a small change in the drug load could affect both safety and efficacy. Thus, the ability to detect small changes in drug load is a significant advantage for the antibody-conjugated drug assay. The assay does not provide information on the amount of antibody that is conjugated to the drug. The antibody concentration is measured directly using the total-antibody assay, as described earlier (Figure 4B).

The antibody-conjugated drug assay involves isolating the ADC from plasma using protein A binding, cleavage of the linker (Figure 6A) followed by LC–MS/MS analysis of the drug (Figure 6B). The linker cleavage may be enzymatic or chemical, depending on the linker structure. Again, it is important to characterize the assay with individual or enriched DARs to assess if there is any bias for DARs in the assay. For example, it is theoretically possible that for

high DARs, the binding to protein A during the initial affinity capture step may be sterically hindered. The assay has a generic format, using readily available reagents, for example, protein A and appropriate proteases or chemistries for linker cleavage. Assays for additional ADCs with the same linker drug but a different antibody can be rapidly developed since protein A binding and cleavage of the linker are not likely to vary for different antibodies. However, it is important to generate standard curves for each new ADC to confirm the performance of the assay. It is noteworthy that although the final analyte measured for the assay calibration standards by LC–MS/MS is the drug, the calibration standard curve is made up from the intact ADC reference standard and undergoes the affinity capture and linker cleavage step to generate the drug in the same way as the sample analytes (case study 6). It is also important to note that although this assay uses conventional LC–MS/MS for the final analysis step, the analyte (antibody-conjugated drug) is not conventional and the assay is in reality a hybrid ligand-binding LC–MS/MS assay. Thus, for assay validation we consider this a novel assay type. We have incorporated experiments from both large- and small-molecule approaches for assay validation for IND-enabling, nonclinical and clinical studies. Currently, the format of the antibody-conjugated drug assay is only suitable for ADCs with cleavable linkers. However, it should be theoretically possible to measure antibody-conjugated drug for ADCs with noncleavable linkers by affinity capture using protein A followed by exhaustive nonspecific proteolysis to yield amino acid linker drug. In the case of ADCs with engineered conjugation sites, specific proteases could be used to cleave the antibody to produce peptide linker drug.

For both types of conjugate analyte, additional information can be obtained indirectly by using data in combination with the total-antibody analyte. For example, the difference between the conjugated-antibody assay and total-antibody profiles theoretically indicates the degree of complete drug deconjugation (Figure 7A). This is because the total-antibody assay measures all DARs, including DAR0, equally (Figure 3D), whereas the conjugated antibody measures all DARs equally, except DAR0 (Figure 3B). Thus, if the linker is relatively stable and complete deconjugation (DAR0) is a minor analyte (≤20%), then the conjugated-antibody assay would provide essentially the same information as the total-antibody assay, as the only difference between the two assays is the ability to measure DAR0. On the other hand, the antibody-conjugated drug analyte provides information that is distinctly different from the total-antibody analyte (Figure 3C & D) and small changes in drug load in plasma can be readily measured (case study 6). Theoretically, the relationship between the antibody-conjugated drug and total-antibody results can reflect the change in average DAR over time (Figure 7B). Measurement of the antibody-conjugated drug (Figure 3C) and the total antibody (Figure 3D) provides a simple way to describe the complex analyte DAR mixture of the ADC (Figure 3A) in terms of its two key components, that is, the total amount of antibody and antibody-conjugated drug.

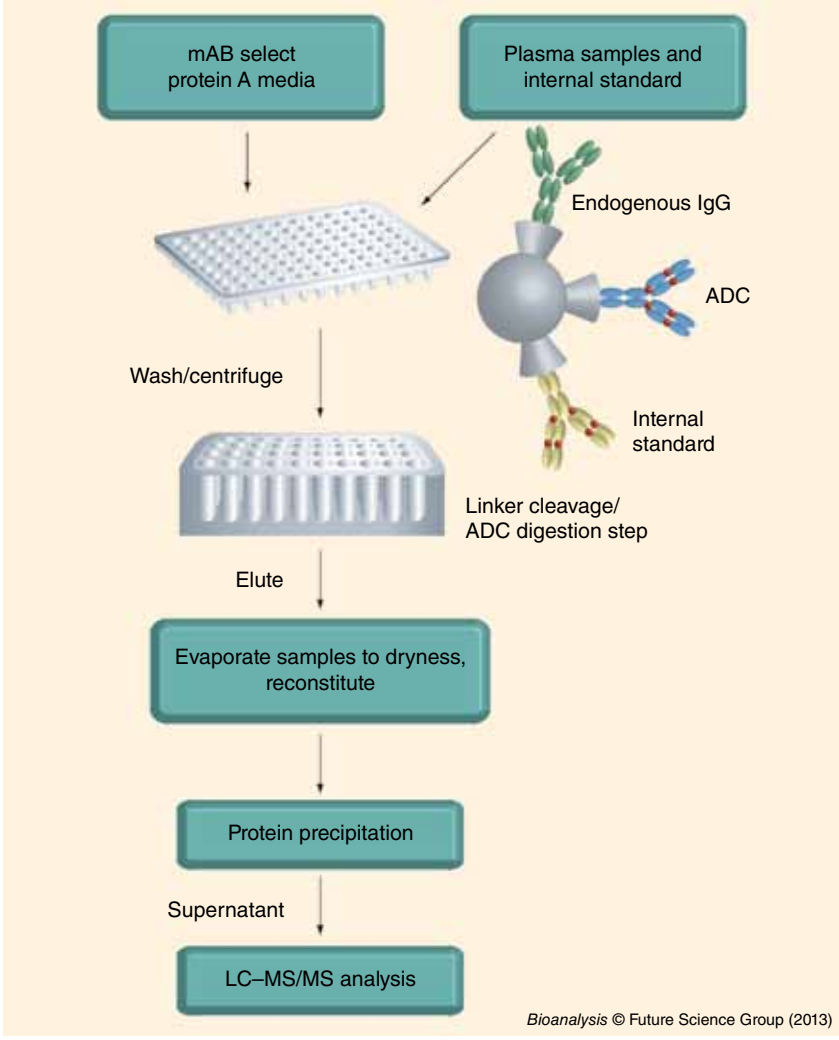

Figure 6. Antibody-conjugated drug affinity capture LC–MS/MS assay.
(A) Affinity capture and linker cleavage/ADC digestion step, **(B)** protein precipitation and LC–MS/MS analysis steps.
ADC: Antibody–drug conjugate; mAb: Monoclonal antibody.

Small-molecule catabolite quantitative assays using LC–MS/MS

Theoretically, there are many possible small-molecule catabolites for an ADC and their selection for bioanalysis is therefore challenging. Traditional *in vitro* metabolism studies and *in vivo* study designs with radiolabeled material alone are not adequate. Additional studies are typically necessary although conventional bioanalytical methods may still be used. Ideally, a dual-radiolabelled ADC that is labeled on both the antibody and the cytotoxic drug would provide the most comprehensive understanding in terms of the overall number of catabolites present and the relative abundance or overall mass balance. However, in practice, reagents for radiolabelled ADCs are challenging to obtain. Indeed, early in development, we have not had radiolabelled ADCs to study catabolism. In such instances, we have hypothesized that proteolytic degradation of an ADC would result in various catabolite structures, such as free drug, linker drug or conjugation site amino acid linker drug (Figure 3F), and used synthetically prepared materials to develop LC–MS/MS methods for each of the potential analytes. Of course, the relevance of quantitative data from this approach, in terms of relative abundance compared with all catabolites formed *in vivo*, is limited. Understanding the catabolism of ADCs requires confirmatory nonclinical studies using a radiolabelled ADC, where the drug, or both the drug and catabolites containing the drug, can be detected as peaks in the radioactivity chromatograms by HPLC analysis. High-resolution molecular masses and fragment masses can then be used to predict the molecular structures. Once the structures of the major catabolites are determined and stable-isotope-labeled internal standards or analog molecules are synthesized, conventional small-molecule quantitative LC–MS/MS assays can be developed for nonclinical and clinical studies [39].

We have typically measured the circulating free drug catabolite (Figure 3F) using a conventional small-molecule LC–MS/MS assay approach overall (Figure 6B). This has involved protein precipitation and/or SPE to remove the plasma proteins prior to LC–MS/MS quantification. In some cases, additional sample preparation steps have been added as deemed appropriate (see below). The assay is designed to measure drug that is present in circulation, but no longer covalently bound through the linker to the ADC. This could include drugs that are directly released from the ADC or drugs that are released from an ADC catabolite. In some cases, if the drug has reactive moieties such as a sulphydryl, it is possible that the drug released from the ADC could dimerize or bind covalently to plasma proteins. In such situations, our strategy has been to add an additional sample preparation step to chemically reduce the plasma sample to liberate any dimerized drug or drug that is covalently bound to plasma proteins, prior to analysis [39]. Our strategy is to obtain a conservative value for the drug lost from the ADC, since drugs used for ADCs are highly potent. Overall, the free drug assay uses conventional LC–MS/MS approaches and the assay may include sample preparation steps such as disulfide reduction followed by derivatization. We have used typical small-molecule assay validation parameters for IND-enabling, nonclinical and clinical studies. Additional experiments, unique for ADCs, have included the assessment of analyte stability in the presence of the ADC. This is important, as in addition to drug analyte concentrations

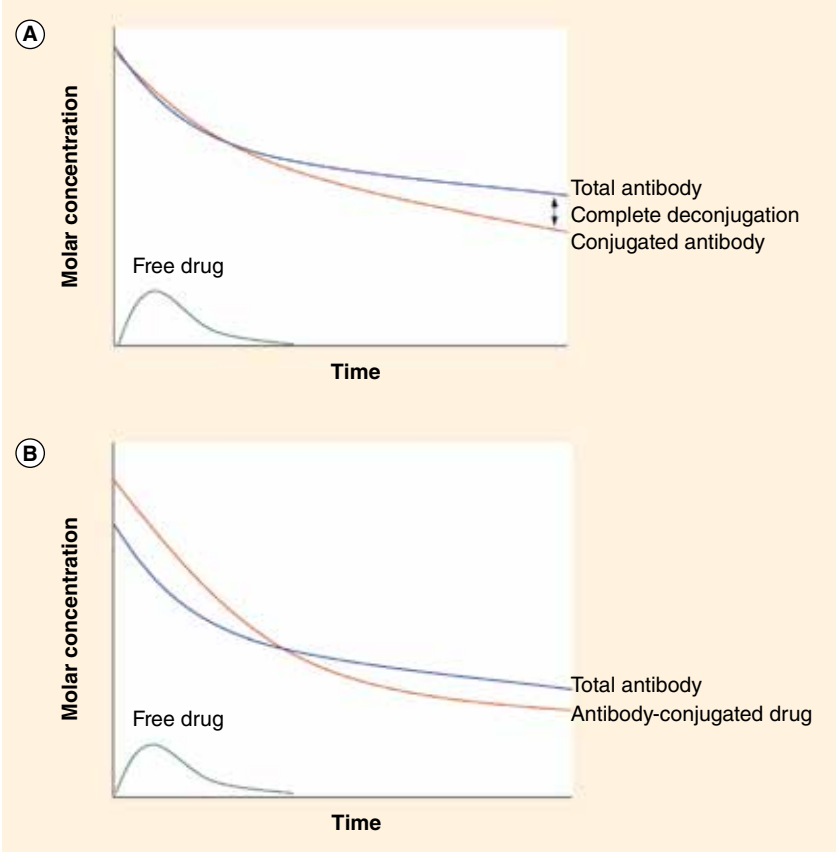

Figure 7. Theoretical inferences from comparing total antibody data and antibody–drug conjugate data. (A) Total-antibody ELISA and conjugated-antibody ELISA, **(B)** total-antibody ELISA and antibody-conjugated drug affinity capture LC–MS/MS.

decreasing during storage due to instability, drug concentrations may also increase due to the liberation of additional drug from the ADC during storage. Indeed, changes in drug concentration during storage may involve a combination of these processes. For a relatively stable linker, the molar concentration of the free drug in study samples is low compared with the antibody-conjugated drug. In our experience, the free drug measured in nonclinical/clinical studies has represented less than 1% of the molar concentration of the antibody-conjugated drug. However, even a minor release of drug from the ADC during storage can have a large impact on the free drug measurement. For example, in a sample where the free drug represents 1% of the molar concentration of the antibody-conjugated drug, a 0.5% release of drug from the ADC during storage would result in an approximately 50% increase in the free drug measurement. As an example of our assay strategy, for trastuzumab emtansine (T-DM1) we measured the free drug catabolite DM1 in systemic circulation for all nonclinical and clinical studies and measured other small-molecule catabolites, such as lysine-*N*-maleimidomethyl cyclohexane-1-carboxylate (MCC)-DM1 and MCC-DM1, in a limited exploratory manner [39,40]. In addition to nonclinical and clinical plasma samples these catabolites were also identified and quantified by LC–MS/MS in rat excreta (bile and urine).

As outlined above, the linker stability and *in vivo* stability of an ADC was assessed using radiolabeled ADC, as well as measuring exposure to free drug and linker-containing drug catabolites in both nonclinical studies as well as in patient samples. In addition, while intact ADCs will have expected routes of elimination involving receptor-mediated or nonspecific endocytosis and catabolism, they are not expected to have any significant direct involvement with cytochrome P450 enzymes. On the other hand, a small-molecule cytotoxic drug such as DM1 is expected to undergo clearance mechanisms consistent with small molecules, such as CYP450-mediated metabolism. *In vitro* hepatic metabolism studies were, therefore, also conducted and in this case determined that DM1 was primarily metabolized by CYP3A4 and, to a limited extent, by CYP3A5 [39].

ATA assays using bridging ELISA

As in the case of all biologics, ADCs have the potential to elicit an immune response. This could include ATA to the antibody, linker, drug or epitopes involving multiple ADC components. In addition to the immunogenicity concerns for all biologics [41], ADCs have unique concerns such as potential immune complexes that may deliver the cytotoxic drug to unanticipated locations. We have used a tiered strategy to screen for and characterize ATA responses to all the molecular components of the ADC (Figure 8). First, a semi-homogeneous ELISA-based bridging assay is used to detect all ATA responses, where ATAs are captured and detected using labeled ADC reagents. For example, Figure 9A depicts the detection of ATAs to the antibody and Figure 9B depicts the detection of ATAs to the drug, using biotinylated-ADC and digoxigenin-labeled ADC assay reagents. As in the case of other biologics, we have followed industry White Paper recommendations for ATA detection relative sensitivity in nonclinical and clinical assays [41]. The assays are characterized using surrogate ATA positive controls obtained from nonclinical immunizations. In addition, during ADC assay development, assay relative sensitivity is assessed using antidrug antibody clones, since the immune response could be due to either the antibody or the drug epitopes. The detection threshold is typically determined using 50–100 individuals, setting a false-positive rate of 5%. Since ATAs in circulation may be partially or fully complexed with the ADC, it is important to ascertain that the assay conditions are capable of detecting ATA in the presence of the ADC. For example, the assay reagents are in molar excess and assay incubation is overnight in order to shift the ADC–ATA complex equilibrium (if present) and allow binding to assay reagents (Figure 9). We assess relative assay sensitivity in the presence of varying amounts of ADC. Our approach is to optimize the assays to achieve high ADC tolerance and to collect samples at appropriate time points when the projected ADC concentrations are low.

Using this approach, we have developed immunogenicity assays for several ADCs using the biotin-DIG bridging ELISA (Figure 9). For nonclinical assays, the ADC-to-ATA molar ratios of drug tolerance ranged between 60:1 and 170:1. For clinical assays, the drug tolerance ratios ranged between 50:1 and 400:1. Although relative sensitivity and drug tolerance of ATA assays depend on the surrogate ATA positive control used, overall we have observed sensitivities typically in the low ng/ml range and 100s ng/ml ATA detected in the presence of tens of micrograms of ADC. As for all biotherapeutics,

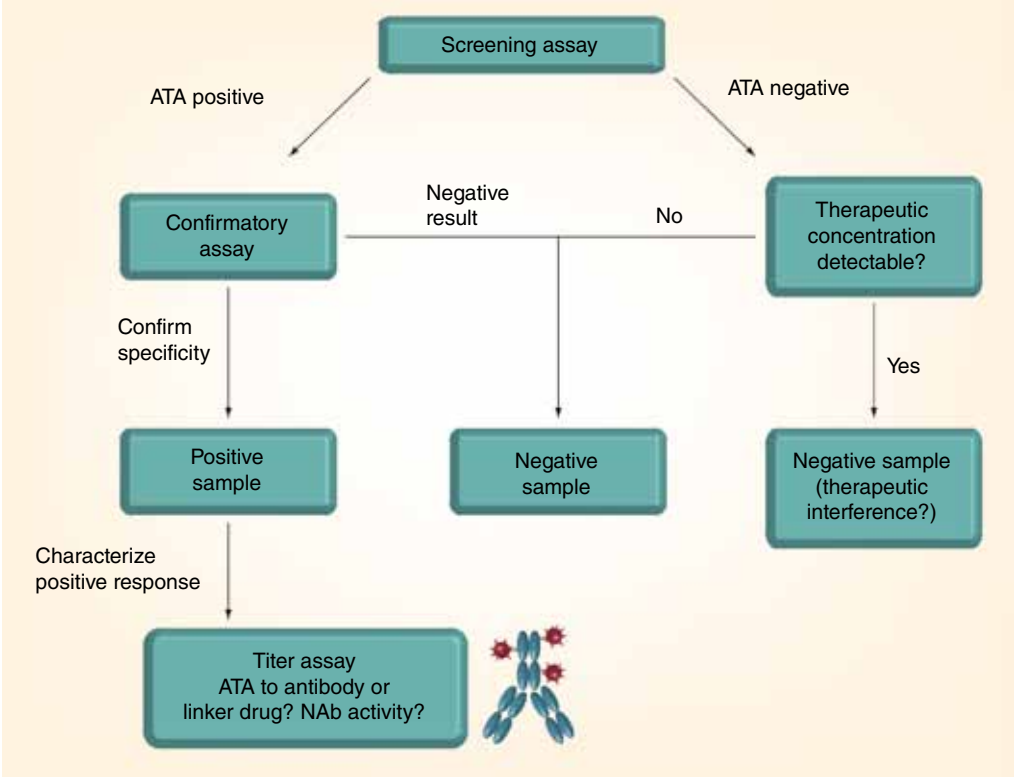

Figure 8. Anti-therapeutic antibody measurement: schematic of strategy for sample testing.
ATA: Anti-therapeutic antibody; NAb: Neutralizing antibody.
Adapted with permission from [32].

it is challenging to predict immunogenicity for nonclinical or clinical studies based on molecular structure. Futhermore, nonclinical immune response is not generally predictive of clinical immune response. In the case of ADCs, it is challenging to predict the impact of varying antibody, linker or drug. For example, the same antibody, with different linker drugs in cynomolgus monkeys, showed very different immune responses (Table 2).

Case studies to highlight bioanalytical strategies

■ Case study 1. A DAR shift to lower values identified *in vivo* by affinity capture capillary LC–MS in a T-DM1 nonclinical PK study

T-DM1 is an ADC in clinical development to treat HER2-positive tumors [40,42]. It targets tumor cells that overexpress the HER2 and releases DM1 (or DM1-containing catabolites) to inhibit microtubule polymerization and induce apoptosis [43–46]. T-DM1 contains trastuzumab, a nonreducible thioether linker (MCC) and a maytansine derivative (DM1) cytotoxic drug (Table 1). It is conjugated primarily at lysine residues and is a mixture of DAR0 to DAR8 with an average DAR of approximately 3.5 [46,47].

Four cynomolgus monkeys were administered a single intravenous injection of 30 mg/kg T-DM1 [48]. Plasma for affinity capture capillary LC–MS analysis was collected over 28 days (2 min, 4 h, 12 h, 1, 2, 3, 7, 10, 14, 21 and 28 days). Figure 5A shows the general schematic for the affinity capture LC–MS experiment. Biotinylated extracellular domain of recombinant HER2 was immobilized onto streptavidin-coated paramagnetic beads (Invitrogen™) and incubated with plasma samples for 2 h at room temperature to capture all DARs. The beads were washed, using a Kingfisher system (Thermo Scientific), to remove plasma proteins and the bead-bound DARs were deglycosylated by incubating with PNGase F (Prozyme®) in HBS-EP buffer (0.01 M HEPES; pH 7.4; 0.15 M NaCl; 3 mM EDTA; 0.005% surfactant P20; GE Healthcare) at 37°C overnight. The beads were washed extensively with HBS-EP and then water, and all DAR analytes were eluted with 30% acetonitrile in water containing 1% formic acid, for analysis. Capillary

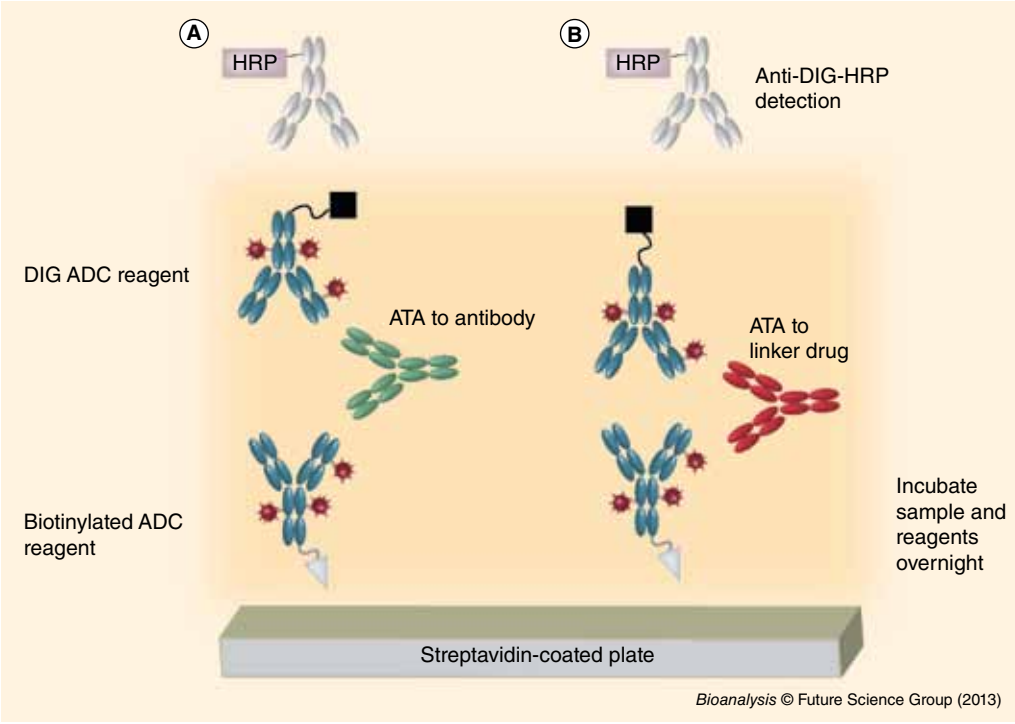

Figure 9: Anti-therapeutic antibody assay format detects immune response to antibody or linker drug. (A) ATA directed to antibody, **(B)** ATA directed to linker drug.
ADC: Antibody–drug conjugate; ATA: Anti-therapeutic antibody; DIG: Digoxigenin; HRP: Horseradish peroxidase.

LC–MS used a PLRP-S column (50 × 0.3 mm, 5 µm; 4000 Å; Agilent) with a 15 µl/min flow rate and a mobile phase containing acetonitrile and water with 0.1% formic acid. Analytes were ionized by ESI and detected by a Q-Star® XL mass spectrometer (AB Sciex) operated in the positive TOF-MS mode. Raw data were deconvoluted using Analyst QS 1.1 software, and peak areas were obtained for each DAR of interest. Relative intensities for the DARs were calculated.

The deconvoluted mass spectra for the plasma samples showed molecular masses corresponding to DAR1 to 7 **(Figure 10)**. The spectra were normalized to the most abundant DAR to allow visualization of T-DM1 DAR distribution at later time points. The DAR distribution at time 2 min was comparable to the DAR distribution in the reference standard solution, except DAR8 was not observed. The latter was present in very low abundance in the reference standard solution (<1%) and may be below the detection limits or may not be stable in plasma. The DAR distribution was relatively unchanged up to day 7. However, beyond day 7, the distribution was seen to change, where the relative abundance of higher DARs, for example, DAR4, 5, 6 and 7, decreased. This was reflected in the average DAR distribution calculated from the peak areas. It started at an average DAR of 3.16 at 2 min and decreased over time to an average DAR of 0.66 at the end of the 28-day study. After 28 days, DAR0 relative abundance was approximately 34%. Interestingly, at later time points, for example, day 10 onwards, new molecular masses were observed that were intermediates between whole DAR values. The structures of these species are not yet known. Overall, the affinity capture LC–MS provided direct measurement of DARs *in vivo* and showed a gradual shift to lower DARs over 28 days. This study allowed insights into the overall structural stability of T-DM1 *in vivo* and provided valuable information for designing quantitative assays for bioanalysis.

■ Case study 2. Formation of new odd numbered DARs identified by affinity capture HIC for an anti-STEAP1 ADC in a plasma stability study *in vitro*

The reference standard, anti-STEAP1 ADC, used in this study is an ADC in clinical development and targets the six-transmembrane cell-surface antigen STEAP1. The antigen is overexpressed in the majority of human epithelial prostate cancers, but has restricted expression in normal tissues [49].

Table 2. Immunogenicity profiles of trastuzumab antibody–drug conjugates (linker 1–drug 1 and linker 2–drug 2) in cynomolgus monkey studies.

ADC	Study	Single-dose PK	Pilot toxicity	Single-dose toxicity	Multi-dose toxicity	Cardio safety
Linker 1–drug 1	Dosing (mg/kg)	0, 0.3, 3, 30	NA	0, 3, 10, 30	0, 3, 10, 30	0, 3, 10, 30
	Anti-therapeutic antibodies and rate	17% (2 of 12) at 3 and 30 mg/kg	NA	0% (0 of 18)	0% (0 of 42)	0% (0 of 12)
Linker 2–drug 2	Dosing (mg/kg)	NA	0, 1, 4, 9	0, 1, 4, 7	0, 1, 4, 7	0, 1, 4, 7
	Anti-therapeutic antibodies and rate	NA	33% (3 of 10)	17% (3 of 18)	45% (19 of 42)	25% (3 of 12)

ADC: Antibody–drug conjugate.

The ADC consists of a humanized IgG1 anti-STEAP1 mAb, conjugated at reduced interchain disulfide cysteine residues to a cytotoxic drug, via a protease cleavable linker [49–51]. The ADC reference standard is a mixture containing DAR0, 2, 4, 6 and 8 with an average DAR of approximately 3.5. Changes in DAR distribution for the reference standard were studied in cynomolgus monkey plasma *in vitro* using affinity capture HIC **(Figure 5B)**. An anti-CDR mAb affinity capture reagent was covalently attached to a HiTrap NHS-activated HP column (GE Healthcare). Samples containing 100 µg anti-STEAP1 ADC in 1 ml of cynomolgus monkey plasma were diluted in sample diluent (phosphate-buffered saline, 0.05% P20)and loaded onto the affinity column using a peristaltic pump. The column was washed with sample diluent to remove background plasma proteins. The ADC was eluted with a nondenaturing elution buffer and concentrated. Samples were diluted with mobile phase A (1.5M ammonium sulfate, 25 mM sodium phosphate, pH 7) to a final volume of 100 µl and analyzed by HIC. The samples were injected onto a butyl-NPR column (4.6 mm × 3.5 cm, 2.5 µm [Tosoh Bioscience]) and eluted using a linear gradient from 0% to 100% mobile phase B (25 mM sodium phosphate; pH 7; 25% isopropanol) at a flow rate of 0.8 ml/min. An Agilent 1100 series HPLC system equipped with a multiwavelength detector and ChemStation software was used to resolve and quantify individual DARs.

Figure 11A shows the affinity HIC chromatogram for the reference standard spiked into cynomolgus monkey plasma at 37°C. **Figure 11B** shows the chromatogram after incubation in plasma at 37°C for 96 h. In addition to changes in the relative abundances of DAR2, 4 and 6, new peaks were observed in the chromatogram consistent with odd numbered DARs corresponding to DAR1, 3 and 5. Thus, over time, in addition to a shift to existing lower DARs, new DARs were observed during plasma incubation for anti-STEAP1 ADC at 37°C **(Figure 11)**. Formation of odd numbered DARs were also observed in plasma for a variety of other ADCs containing linker drugs conjugated at reduced interchain cysteines indicating this as a common phenomenon for this class of ADCs. The identification of the formation of new DARs, in particular DAR1, *in vitro* and *in vivo* was important because it led us to discover that immunoassays designed to measure conjugated mAb often do not capture DAR1 effectively, resulting in inaccurate quantification (case study 3).

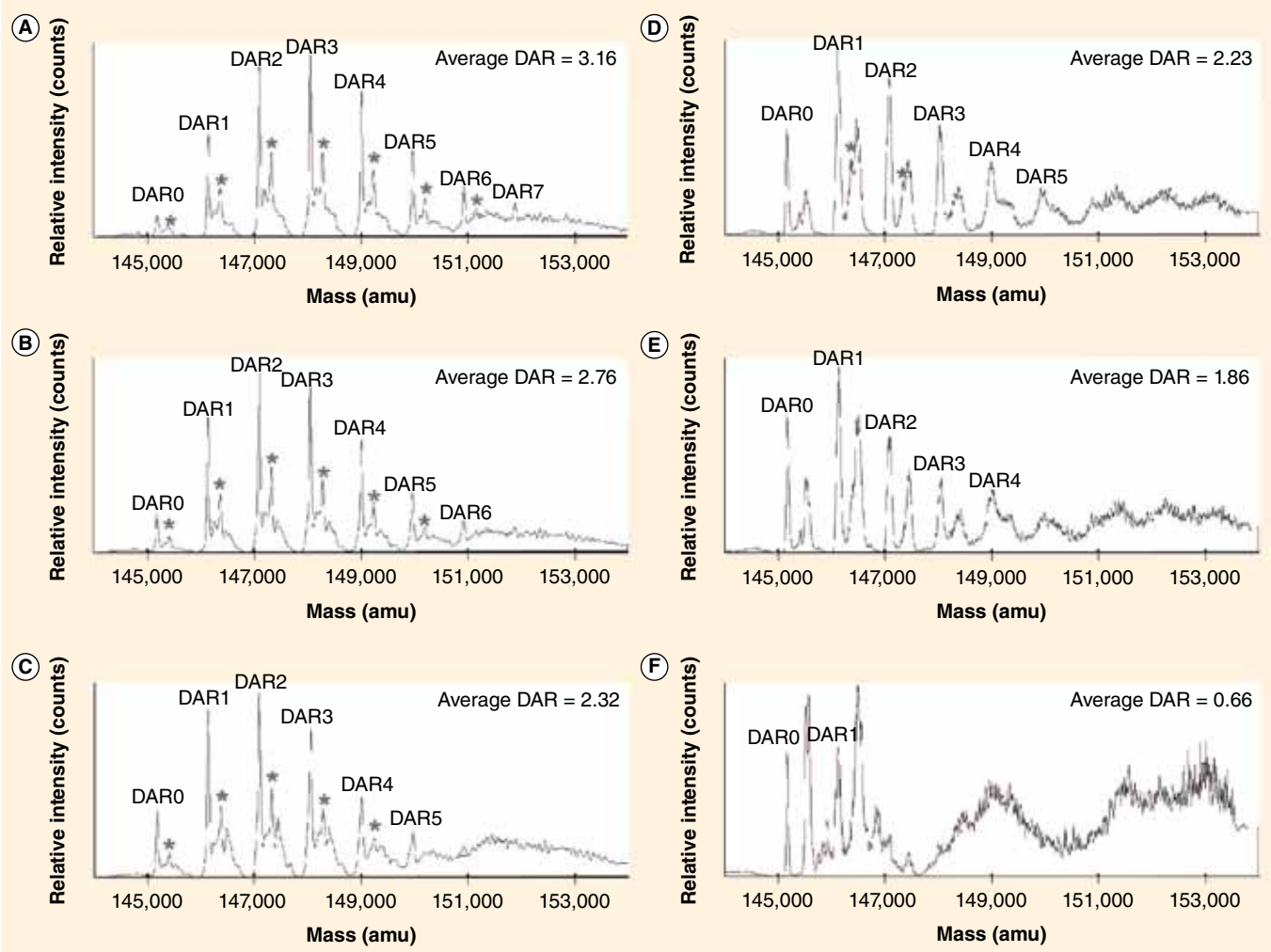

Figure 10. Characterization of antibody–drug conjugate (trastuzumab emtansine) drug distribution in a cynomolgus monkey PK study by HER2 extracellular domain affinity capture LC–MS. Shows the drug-to-antibody ratio distribution shifts to lower values over time. Spectrum is normalized to the major component at each time point. **(A)** 2 min, **(B)** 1 day, **(C)** 3 days, **(D)** 7 days, **(E)** 10 days, **(F)** 28 days.
*Extra *N*-maleimidomethyl cyclohexane-1-carboxylate linker.
DAR: Drug-to-antibody ratio.

■ Case study 3. Characterization of anti-STEAP1 LBAs using purified individual DARs showed conjugated-antibody ELISA did not measure DAR1

The anti-STEAP1 ADC reference standard is described above in case study 2. Purified individual DARs in case study 3 were tested in total-antibody and conjugated-antibody clinical LBAs (Figures 4B & C) to assess assay performance. Even numbered DARs (DAR2, 4 and 6) and DAR0 were purified directly from reference standard by HIC. In the case of DAR8, insufficient quantity was available and, thus, DAR8 was not tested. Odd numbered DARs (DAR1, 3 and 5) that are formed in plasma via incubation at 37°C (case study 2; Figure 11) were purified from human plasma extracts. In the latter case, only DAR1 was obtained in sufficient quantity for the study. The purified individual DARs were all prepared at a concentration of 1000 ng/ml in human plasma, diluted 1:100 in sample buffer and then serially diluted for analysis using the ADC reference standard calibration curves in 1% serum. The total-antibody ELISA used an anti-CDR mAb as the capture reagent and a biotinylated anti-CDR mAb and streptavidin-HRP for detection. The standard curve quantification range was 0.50 to 17 ng/ml. The conjugated-antibody assay used an antidrug mAb for capture and a biotinylated anti-CDR mAb and streptavidin- HRP for detection. The dilution schema and the standard curve composition were

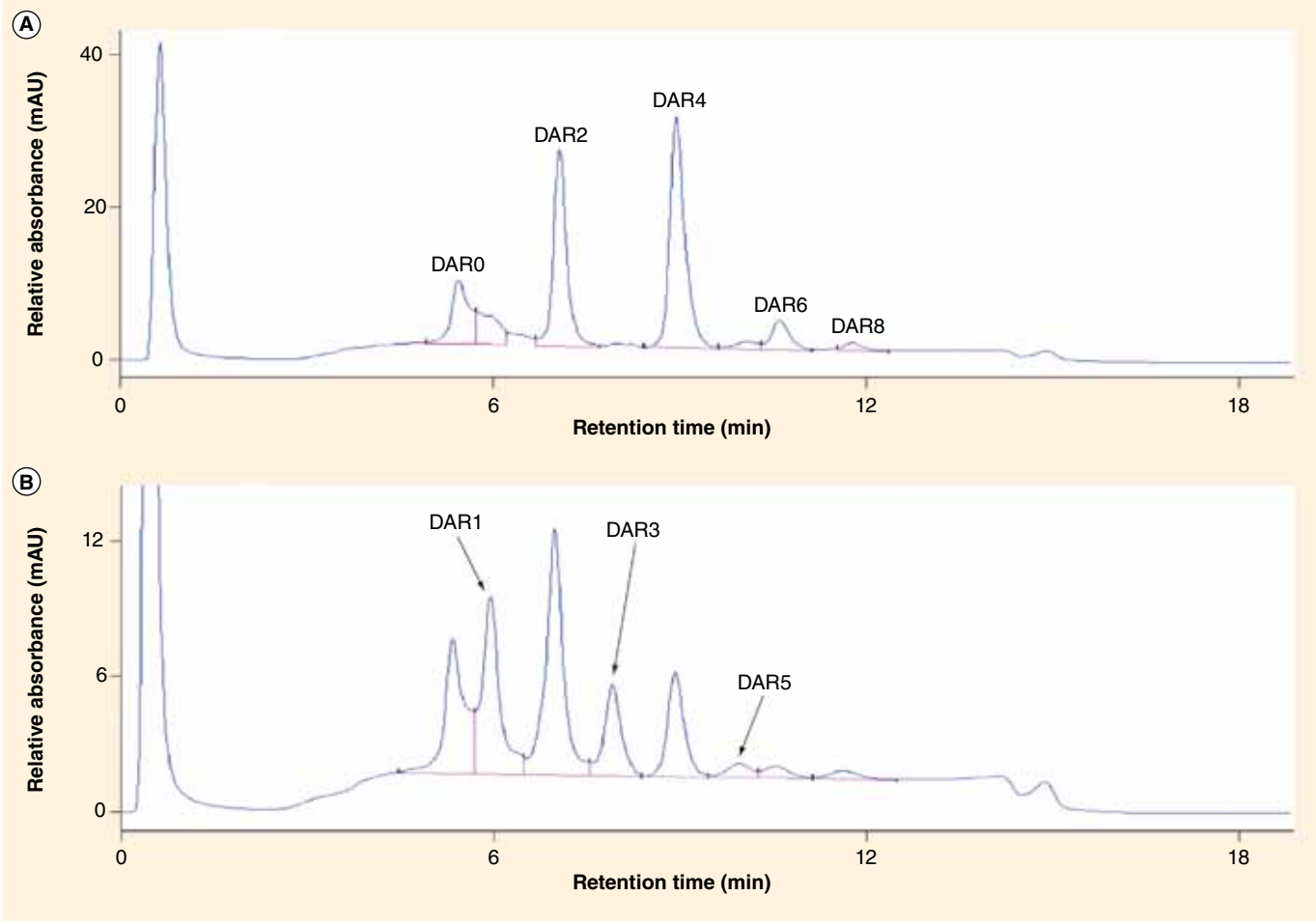

Figure 11. Affinity capture hydrophobic interaction chromatography chromatogram of 100 µg/ml anti-STEAP1 antibody–drug conjugate in cynomolgous monkey plasma stability samples. (A) Incubation for 0 h, **(B)** incubation for 96 h. DAR: Drug-to-antibody ratio.

the same as for the total-antibody ELISA. The quantification range for the conjugated-antibody assay was 0.50 to 12 ng/ml.

Table 3 summarizes the quantification of individual DARs in the two evaluated LBAs. For the total-antibody ELISA, all the individual DARs, including the DAR1 formed in plasma, were measured accurately (within ±25% of the expected value), based on nominal concentration. Quantification of individual DARs in the conjugated-antibody assay showed that DARs closer to the average DAR for the reference standard, that is, DAR2 and 4, were measured accurately (within ±25%). However, DAR1 and 6 were significantly underquantified and only 11% and 64% of the expected concentration values were measured, respectively (Table 3). It is not known why the conjugated-antibody ELISA did not accurately measure DAR1 and 6. The assay was repeated using a number of other antidrug mAb clones as the assay capture reagent. Only one antidrug mAb clone reagent tested measured DAR1 (±25%), however, the same reagent did not measure any higher DARs accurately. Thus, for the conjugated-antibody ELISA, no reagent was identified that could measure all the DARs expected to be present *in vivo* accurately. It is noteworthy that DAR1 is likely to be a relatively abundant analyte *in vivo*, based on the plasma stability data from HIC (case study 2; Figure 11B). These results for DAR quantification in total-antibody and conjugated-antibody ELISAs are generally similar to analogous studies for a number of other cysteine-conjugated ADCs. Thus, conjugated-antibody LBAs may significantly underquantify the conjugated-antibody analyte mixture *in vivo,* if DAR1 is present in a significant relative abundance. Although DAR6 was also underquantified and DAR8 was not tested, the amounts present in the reference standard solution are low and these DARs are not expected to increase with time *in vivo,* thus

Table 3. Total-antibody and conjugated-antibody ELISA and drug-to-antibody ratio recovery for anti-STEAP1 antibody–drug conjugate.

Assay	Anti-STEAP1 ADC purified DAR				
	0	1	2	4	6
Total-antibody ELISA (% recovery)†	88	78	86	92	92
Conjugated-antibody ELISA (% recovery)†	NA	11	102	99	64

†Based on expected nominal concentration of individual DARs spiked into serum.
ADC: Antibody–drug conjugate; DAR: Drug-to-antibody ratio.

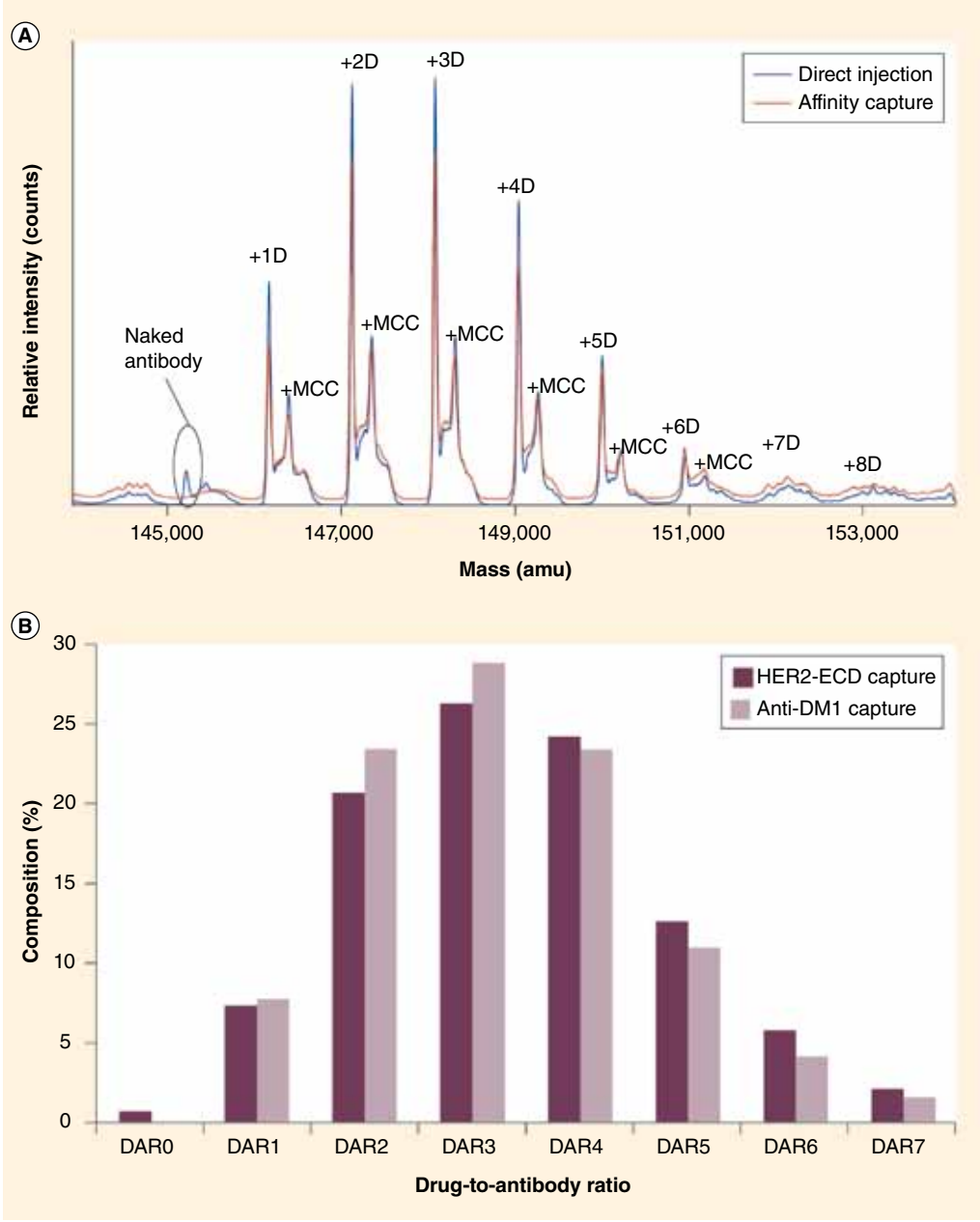

Figure 12. Direct injection versus affinity capture LC–MS of T-DM1. (A) Direct injection versus affinity capture. **(B)** HER2 extracellular domain capture versus anti-DM1 capture. DAR: Drug-to-antibody ratio; ECD: Extracellular domain; MCC: *N*-maleimidomethyl cyclohexane-1-carboxylate.

the impact on overall quantification should be minimal. This study showed the importance of testing the quantification of individual DARs during LBA development. All DARs must be measured accurately in the assay in order to produce an accurate LBA.

■ Case study 4. Characterization of ELISA reagents for total-antibody & conjugated-antibody T-DM1 assays

As described in case study 1, T-DM1 is a complex DAR mixture of DAR1 to 8. As described in case studies 2 and 3, for less complex cysteine-conjugated ADC mixtures it was possible to obtain individual DARs to characterize the performance of LBAs. However, for a more complex ADC like T-DM1 it was not feasible to obtain individual DARs for this purpose. Limited characterization of the LBAs was performed using enriched DAR fractions (DARs 2.6, 3.1, 3.4, 3.9 and 4.1). Additional characterization of the conjugated-antibody assay (Figure 4C) was performed in a multiplexed manner using affinity capture LC–MS (Figure 5A). The ability of the anti-DM1 mAb reagent to bind appropriately to all DARs effectively, including DAR1, was confirmed as follows. T-DM1 was spiked into buffer and analyzed directly by capillary LC–MS, as described above in case study 1, that is, without the affinity capture step. These data were compared with T-DM1 spiked into both serum and plasma and analyzed by affinity capture capillary LC–MS, using anti-DM1 mAb as the capture probe. Figure 12A shows the comparison of the DAR distribution data from the two analyses. Comparable DAR distributions showing DAR1–8 were detected, indicating that there were no selective losses or bias during the anti-DM1 reagent capture step (Figure 12A). As expected, DAR0 (i.e., naked trastuzumab) was not observed in the mass spectrum after affinity capture with anti-DM1 antibody as the anti-DM1 affinity probe captures the analyte via DM1. These data also confirmed that the anti-DM1 mAb reagent is capable of effectively binding low DAR species such as DAR1 and allows characterization of the conjugated-antibody ELISA capture reagent using a multiplexed approach. Figure 12B shows data from an analogous experiment where the capture probe was HER2-ECD. This showed a similar DAR distribution to a capture with the anti-DM1 mAb. Again, as expected, naked trastuzumab was observed in the latter experiment. These results indicate that the ELISA reagents for measuring T-DM1 total antibody and conjugated antibody bind appropriately to all the DARs observed *in vivo*. This study showed that a variety of analytical approaches may be needed for characterizing ADC LBAs when individual purified DARs are not available for testing in the assay of interest.

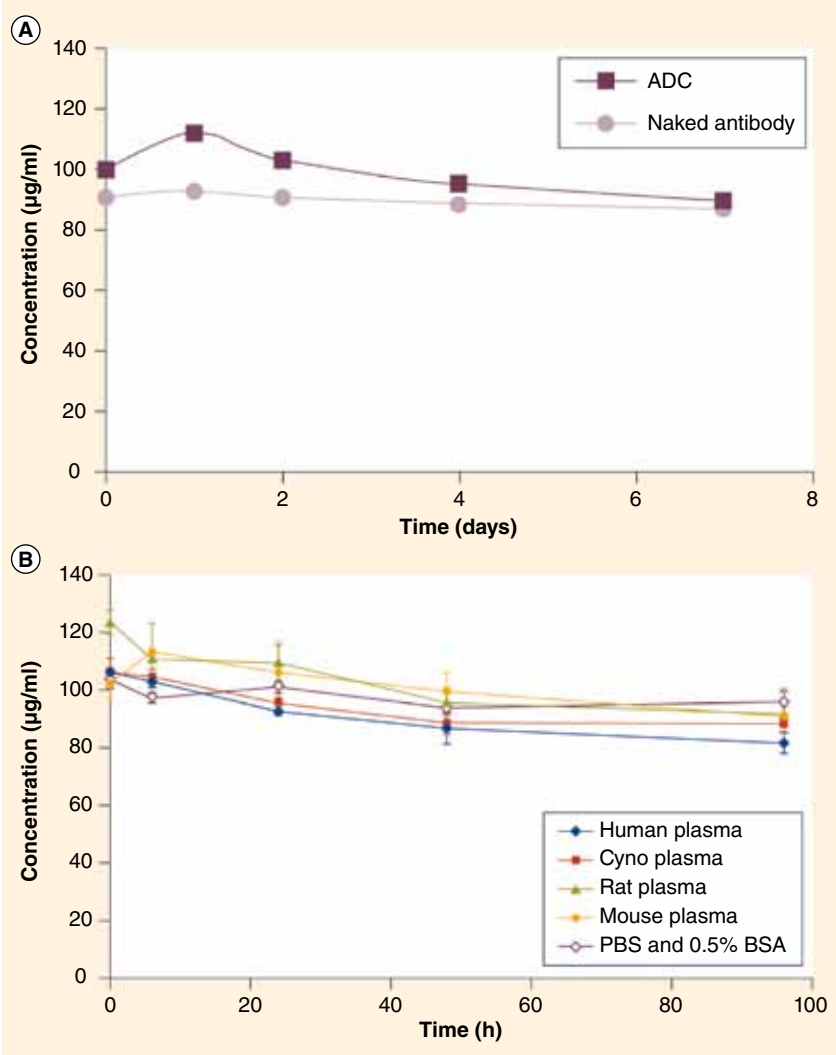

Figure 13. Total-antibody ELISA analysis. (A) Characterization of anti-STEAP1 ADC total-antibody PK ELISA (anti-complementarity determining region/anti-idiotypic format) using a plasma stability experiment with anti-STEAP1 naked antibody as a control. **(B)** Stability assessment of anti-STEAP1 total-antibody analyte in mouse, rat, monkey, human plasma and buffer control.
ADC: Antibody–drug conjugate; BSA: Bovine serum albumin; PBS: Phosphate-buffered saline.

■ Case study 5. Characterization of anti-STEAP1 total-antibody ELISA using plasma stability measurements

The anti-STEAP1 ADC reference standard used in case study 5 is described above in case study 2. In addition to testing individual DARs in ELISA

assays, as described in case study 3, we also assessed the ability of the total-antibody ELISA (Figure 4B) to measure DARs present in plasma using an alternative strategy, because purified DARs may not always be available for all the species formed in plasma. Anti-STEAP1 ADC reference standard was incubated in mouse, rat, monkey and human plasma at 37°C, as described for case study 2. Changes in DAR distributions and average DARs over 96 h were confirmed by affinity capture HIC (case study 2, Figure 11) and affinity capture capillary LC–MS (data not shown). Samples from each time point collected in the plasma stability study were analyzed in the total-antibody assay. Accurate quantification of all DARs formed during plasma incubation was expected to result in no changes in the total-antibody measurement. To correct for minor proteolysis that may have resulted in a decrease in the quantity of total antibody in the samples, a control, plasma stability experiment was conducted using naked anti-STEAP1 mAb. Figure 13A shows the concentration of the total-antibody analyte for anti-STEAP1 ADC and naked anti-STEAP1 antibody for the monkey plasma stability samples over 96 h. Results within ±20% were also obtained for mouse, rat and human plasma stability samples. Figure 13B shows the data for the ADC total-antibody analysis. The fact that the measurements are comparable (±20%) and did not change (±20%) during the plasma incubation (Figure 13A), confirmed that the total-antibody assay measured all DARs appropriately. These results are representative of a number of other ADC total-antibody assays characterized in this manner. This study showed that plasma stability experiments are a valuable tool to confirm the performance of ADC total-antibody assays.

■ Case study 6. Antibody-conjugated drug analysis to assess plasma stability & clinical PK of anti-STEAP1 ADC

The reference standard anti-STEAP1 ADC described in case study 2 was incubated in mouse, rat, monkey and human plasma at 37°C, using the conditions described for case study 2. Samples collected over 96 h were measured in the antibody-conjugated drug hybrid affinity LC–MS assay (Figure 6). The conjugated-antibody LBA was not used in the study as it did not measure DAR1 accurately during assay development (case study 3). In addition, affinity HIC analysis indicated DAR1 was present in only approximately 20% relative abundance at the end of the stability experiment (case study 2; Figure 11), therefore, data from an accurate conjugated-antibody measurement would be similar to the total-antibody measurement. Figure 14A shows the affinity capture LC–MS/MS data for the antibody-conjugated drug analyte (Figure 3C) in cynomolgus monkey plasma and represents an approximately 30% decrease in the overall concentration of antibody-conjugated drug after 96 h. This is consistent with the DAR distribution changes observed, where the majority of the antibody species were conjugated after incubation for 96 h *in vitro* (case study 2, Figure 11). As a comparison, Figure 14A

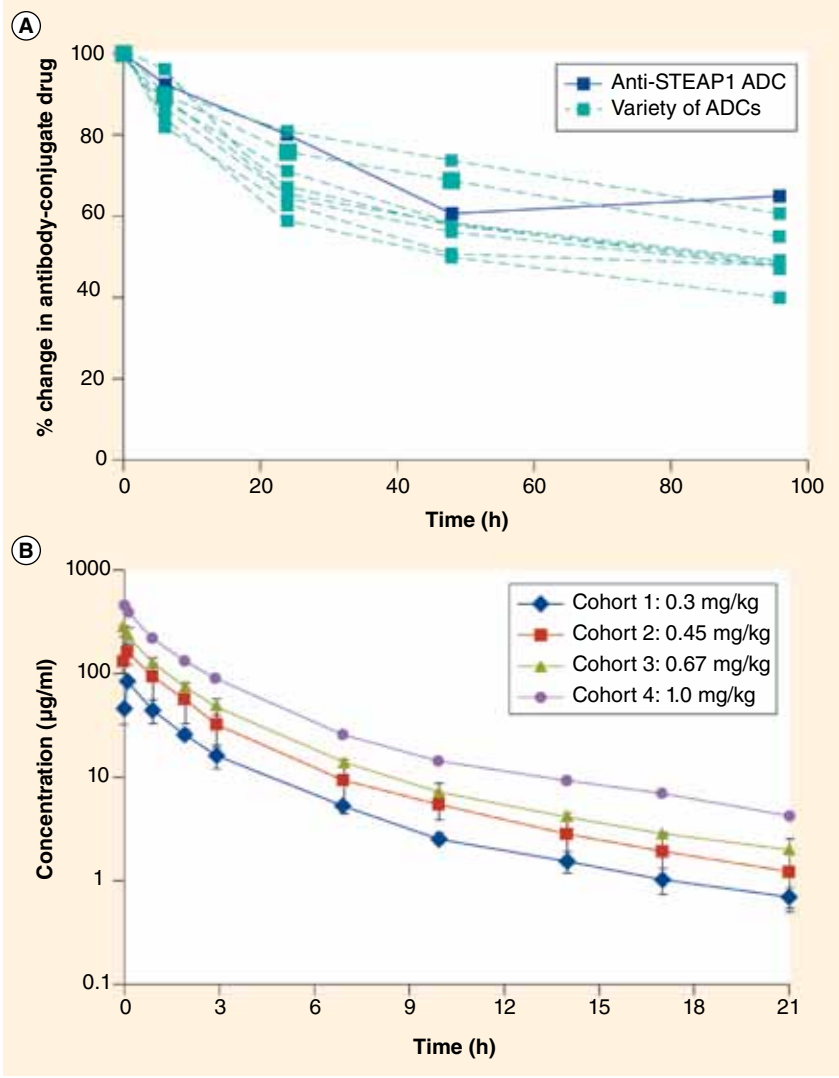

Figure 14. Anti-STEAP1 antibody-conjugated drug measurements in plasma stability studies *in vitro* and in a Phase I clinical study. (A) Plasma stability profile for anti-STEAP1 ADC (solid line) and a variety of ADCs (dashed lines) in monkey plasma *in vitro*. **(B)** Anti-Steap1 ADC clinical antibody-conjugated drug PK profiles; n = 3 patients per cohort. Error bars represent inter-patient variability.
ADC: Antibody–drug conjugate.

shows antibody-conjugated drug measurements for the same linker drug conjugated to a variety of antibodies. Overall, the data indicated that at least 50% of the drug remained covalently bound to the antibody for 96 h. Thus, data from the antibody-conjugated drug assay provided a direct measurement of the stability of anti-STEAP1 ADC in plasma with respect to overall deconjugation. In addition, the antibody-conjugated drug assay allowed ready comparisons across a number of ADCs. The antibody-conjugated drug (Figure 3C) was also a key analyte measured for clinical PK. Figure 14B shows an example of clinical PK data for the antibody-conjugated drug analyte wherein robust data were obtained over several doses. Again, the conjugated-antibody ELISA (Figure 4C) was not used for the reasons discussed above. These data were important in showing that the antibody-conjugated drug assay provides robust data for both *in vitro* and *in vivo* ADC studies.

Future perspective

The bioanalysis of ADCs is complex compared with conventional large- or small-molecule bioanalysis and the field is new and evolving. ADC analytes *in vivo* are typically heterogeneous. Given the limited information on ADCs in the clinic that is currently available, it is not well understood which ADC analytes correlate best with safety and efficacy. Moving forward, it will be critical to continue using a diversity of existing and novel bioanalytical methodologies that provide appropriate information to help answer key questions for understanding safety and efficacy across a variety of ADCs in the clinic. In addition to helping address drug-development questions typical for traditional therapeutics, it will be important for bioanalytical strategies to help address new types of questions unique to ADCs. Questions such as, the structural stability of different types of ADCs *in vivo*, the ability of the antibody to deliver the drug as intended, the amount of drug released from the antibody in circulation and the amount remaining conjugated, may help to provide insights into safety and efficacy. It may be necessary to develop bioanalytical strategies on a case-by-case basis depending on antibody–linker drug molecular

Executive summary

Background

- Antibody–drug conjugate (ADC) bioanalysis is complex and presents unique analytical challenges.
- ADCs are mixtures and the drug-to-antibody ratio (DAR) composition *in vivo* can change due to drug deconjugation or varying DAR clearance, therefore it is important to assess that the reference standard calibration curve is appropriate for all PK time points.
- Bioanalysis requires an integrated approach using novel methods designed specifically for ADCs in addition to existing large- and small-molecule quantitative methods.
- Our bioanalytical assay strategy
  - Integrating expertise in large-molecule ligand-binding methods, small-molecule LC–MS/MS methods and protein structural characterization.
  - Understanding ADC biotransformations and DAR distributions in serum/plasma using novel affinity capture LC–MS and affinity capture hydrophobic interaction chromatography methods.
  - Ensuring accurate quantification of all ADC analytes by testing assays with individual DARs identified in serum/plasma.
  - Measurement of three key PK analytes for nonclinical and clinical studies: total antibody; antibody-conjugated drug or conjugated antibody; and free drug.
  - Immunogenicity assessment using a tiered strategy to screen and characterize responses to all molecular components of the ADC.

Case studies

- A DAR shift to lower values identified *in vivo* by affinity capture capillary LC–MS in a T-DM1 nonclinical PK study.
- Formation of new odd numbered DARs (e.g., DAR1, DAR3 and DAR5) identified by affinity capture hydrophobic interaction chromatography for an anti-STEAP1 ADC in a plasma stability study *in vitro*.
- Characterization of anti-STEAP1 ADC ligand-binding assays using purified individual DARs showed conjugated-antibody ELISA did not measure DAR1 isolated from plasma.
- Characterization of ELISA reagents for total antibody and conjugated-antibody in T-DM1 assays using affinity capture capillary LC–MS showed appropriate reagent binding to all DARs.
- Characterization of anti-STEAP1 ADC total-antibody ELISA using plasma stability measurements indicated appropriate quantification of all DARs.
- Use of the antibody-conjugated drug analyte to assess plasma stability and clinical PK of anti-STEAP1 ADC.

properties. As knowledge emerges from the breadth of ADCs currently in development within the industry [103], it will help to shape best practices and regulatory guidelines for ADC bioanalysis.

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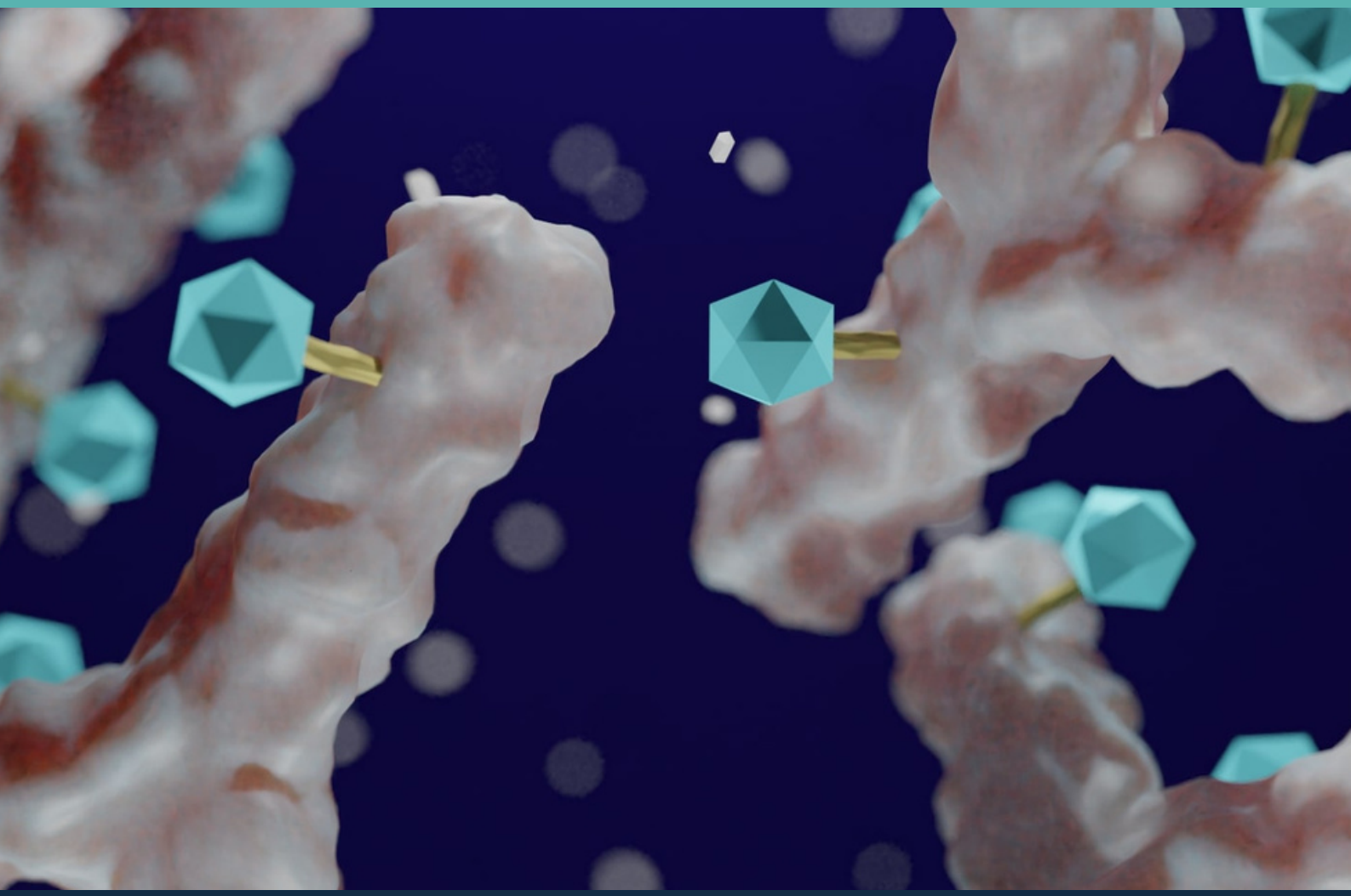

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