BIOANALYSIS ZONE

Powered by fsg

Antibody–drug conjugates

TOP ARTICLES SUPPLEMENT

CONTENTS

Research Article

For reprint orders, please contact reprints@future-science.com

Bioanalysis

Antibody–drug conjugate bioanalysis using LB-LC–MS/MS hybrid assays: strategies, methodology and correlation to ligand-binding assays

Background: Antibody–drug conjugates (ADCs) are complex drug constructs with multiple species in the heterogeneous mixture that contribute to their efficacy and toxicity. The bioanalysis of ADCs involves multiple assays and analytical platforms. **Methods:** A series of ligand binding and LC–MS/MS (LB-LC–MS/MS) hybrid assays, through different combinations of anti-idiotype (anti-Id), anti-payload, or generic capture reagents, and cathepsin-B or trypsin enzyme digestion, were developed and evaluated for the analysis of conjugated-payload as well as for species traditionally measured by ligand-binding assays, total-antibody and conjugated-antibody. **Results & conclusion:** Hybrid assays are complementary or viable alternatives to ligand-binding assay for ADC bioanalysis and PK/PD modeling. The fit-for-purpose choice of analytes, assays and platforms and an integrated strategy from Discovery to Development for ADC PK and bioanalysis are recommended.

First draft submitted: 26 January 2016; Accepted for publication: 16 May 2016; Published online: 9 June 2016

Keywords: antibody–drug conjugate • antibody conjugated-payload • DAR-bias • DAR-sensitive ADC assay • drug antibody ratio • fit-for-purpose bioanalysis • generic reagents • immunocapture LC–MS/MS hybrid assay • ligand binding-LC–MS/MS hybrid assay • soluble target

Jian Wang*[,1], Huidong Gu[1], Ang Liu[1], Alexander Kozhich[1], Vangipuram Rangan[2], Heather Myler[1], Linlin Luo[1], Richard Wong[3], Huadong Sun[3], Bonnie Wang[4], Heather E Vezina[5], Shrikant Deshpande[2], Yan Zhang[1], Zheng Yang[3], Timothy V Olah[3], Anne-Francoise Aubry[1], Mark E Arnold[1], Renuka Pillutla[1] & Binodh DeSilva[1]

[1]Analytical & Bioanalytical Operations, Bristol-Myers Squibb, Princeton, NJ 08543, USA

[2]Biologics Discovery California, Bristol-Myers Squibb, Redwood City, CA 95035, USA

[3]Preclinical Candidate Optimization, Bristol-Myers Squibb, Princeton, NJ 08543, USA

[4]Drug Safety Evaluation, Bristol-Myers Squibb, New Brunswick, NJ 08903, USA

[5]Clinical Pharmacology & Pharmacometrics, Bristol-Myers Squibb, Princeton, NJ 08543, USA

*Author for correspondence:
Tel.: +1 609 252 3856
jian.wang@bms.com

This decade has seen the US FDA approvals of two antibody–drug conjugates (ADCs), Adcetris® (brentuximab vedotin) and Kadcyla® (ado-trastuzumab emtansine) for the treatment of cancer [1,2] with many more ADCs in clinical development. ADCs are drug constructs consisting of a monoclonal antibody (mAb), attached through a chemical linker to a small molecule, often cytotoxic, drug or payload. ADCs combine the target binding specificity of mAbs and the potency of cytotoxic drugs [3,4]. Many ADC drug candidates are currently at different preclinical and clinical development stages in this rapidly evolving field [5,6].

Because of the heterogeneous nature of ADC molecules, particularly for random-conjugated ADCs, multiple species may contribute to the efficacy and toxicity of ADCs. ADC PK evaluation, human dose projection, PK/PD modeling and the associated bioanalytical PK assays are complicated. ADC bioanalytical testing has, traditionally, used both ligand-binding assays (LBAs) and LC–MS/MS approaches. Currently, industry-wide standard practices, strategies, and regulatory guidelines are still developing. ADC PK and bioanalytical strategies have been one of the most hotly discussed topics industry-wide during the last 5–10 years. Two recent special focus issues have been dedicated to ADC PK and bioanalysis recently [7,8]. As a highlight, the AAPS Drug Conjugate Working Group issued a position paper on bioanalysis of ADCs in which definitions of ADC analytes and bioanalytical assays were discussed [9]. There were comprehensive reviews on ADC bioanalytical assay

FUTURE SCIENCE part of fsg

strategies and challenges [10–13], and on *in vivo* biotransformation [14] and characterization of ADC catabolism [15]. An overview of analytes and ADC bioanalytical strategies was also outlined in a recent industry white paper on ADME characterization of ADCs from the 'ADC ADME working group' of the International Consortium for Innovation and Quality in Pharmaceutical Development [16] and in reviews of ADME properties of therapeutic proteins [17], and of characterization of ADCs by MS [18]. In depth coverage of LBA in ADC bioanalysis from early to late preclinical development was the subject of one recent review [19], while the validation of an integrated series of LBAs for clinical studies was described in another article [20]. An integrated, multiplatform strategy for the bioanalysis of a microtubule polymerization inhibitor ADC [21] and details of PK assays for T-DM1 [22] have been reported. While not directly related to bioanalysis, but influential in consideration of the required assays, PK considerations and PK/PD modeling also have been the subject of review and research articles [23–27].

Based on literature and presentations from industry and regulatory authorities, four major analytes are recommended for ADC PK: total-antibody, which includes antibody conjugated to the payload and unconjugated (naked) antibody; conjugated-antibody, defined as the antibody conjugated to at least one payload; conjugated-payload, defined as payload conjugated to the antibody; unconjugated-payload, defined as payload molecules deconjugated from the antibody [9,10]. Both conjugated-antibody and conjugated-payload are considered measures of the ADC active species, describing ADCs from the perspective of either the antibody or the payload [10]. It is unclear, at this time, whether the conjugated-antibody or the conjugated-payload correlates more closely with efficacy or toxicity of ADCs. The ratio of conjugated-payload and total-antibody is defined as the average drug to antibody ratio (DAR) and its change *in vivo* is considered a good indication of the degree of ADC deconjugation and other biotransformation processes.

Historically, the primary assays for total-antibody and conjugated-antibody have been LBAs and the unconjugated-payload assay has been by LC–MS/MS, while the conjugated-payload is predominately measured by affinity capture or immunocapture LC–MS/MS hybrid assays [9–11]. Since these hybrid assays are truly the combination of ligand binding platforms and LC–MS technology, we chose to name them as LB-LC–MS/MS hybrid assays, instead, in this article. It was also reported that DAR-sensitive conjugated-antibody assays can be used to monitor *in vivo* payload change especially for ADCs with noncleavable linkers [11,19,28]. Affinity-capture followed by capillary LC–MS or hydrophobic interaction chromatography (HIC) to characterize intact ADC DAR distribution in biological matrices have been reported [10,29–32]. Immunocapture combined with cathepsin-B cleavage followed by LC–MS/MS for the quantitation of conjugated-payload has been discussed [9,10] and a case study of assay development, validation, and application has been published [33]. A recent publication reports a protein A capture LC–MS/MS hybrid assay with papain (a cysteine protease) cleavage for the quantitation of valine-citrullin-linked MMAE ADCs [34]. Method development and assay validations for the analysis of unconjugated-payload of ADCs by LC–MS/MS assays have been the topic in recent publications [35–37].

Immunocapture LC–MS/MS assays have been increasingly used in bioanalysis of protein therapeutics in recent years [38–41]. To our best knowledge, though discussed in various review articles [9–11], there have been few publications detailing LB-LC–MS/MS hybrid assay methodology, procedures and assay characteristics for ADC PK bioanalysis, particularly for total-antibody and conjugated-antibody. Meanwhile, in ADC PK and bioanalysis, questions such as "which assays and analytes are preferred, conjugated-antibody or conjugated-payload?" and "what assay format to use, LBAs or hybrid assays in preclinical and clinical PK studies?" have remained hot topics in the literature, workshops and conferences. This article describes a series of LB-LC–MS/MS hybrid assays for ADC PK analysis, which have been used in preclinical and/or clinical studies and attempts to provide some answers to these questions: Can hybrid assays play additional roles beyond measuring conjugated-payload? Are hybrid assays viable alternatives to LBA for the analysis of total-antibody? Can hybrid assays be used as an alternative or complement to LBAs for the analysis of conjugated-antibody? Can ADC bioanalysis be supported in a LC–MS based laboratory without full LBA capabilities? What is the ideal ADC bioanalytical strategy from early Discovery to late clinical stage? All hybrid assays were developed, and validated or qualified, using protocols that include tests normally required for both LBA and LC–MS/MS assays [42–46]. Method development and validation or qualification of hybrid assays and results from PK/toxicokinetic (TK) studies are discussed. This article aims at providing technical guidance for the scientists conducting various hybrid assays for ADC bioanalysis. The article will also address some of the highly discussed topics in ADC bioanalysis as mentioned above. The comparison or correlation between hybrid assays and LBAs will not only be limited to assay platform but also incorporate ADC analytes and assay DAR characteristic which brings the discussion to a wider scope and to better

clarity. Discovery and Development integrated ADC bioanalysis strategies are proposed herein as well. The ADC described in this manuscript is in early development and consists of a microtubule polymerization inhibitor as the payload attached to a specific undisclosed mAb through random lysine conjugation [21,47]. General statements made in this article are applicable to this ADC and other random-conjugated ADCs, while they may not apply to ADCs of other constructs.

Experimental

LBAs for total-antibody & conjugated-antibody

Methods utilizing a sandwich immunoassay format and the standard multiarray microplate (Meso Scale Discovery, MD, USA) platform were used in preclinical PK and TK studies. The method details have been described in Myler *et al.* [21]. The methods used in the clinical PK evaluation were modified to a Gyrolab xP Workstation (Gyros Inc, Uppsala, Sweden) format [20]. To be noted in the published methods, conjugated-antibody in which the antibody conjugates with the active payload was defined as 'Active-ADC,' and the conjugated-antibody (payload + metabolite) in which the antibody conjugates with either payload (active) or payload metabolite (inactive) was defined as 'Total ADC.' This manuscript discusses ADC bioanalytical methodology in general, so the widely used nomenclatures for ADC analytes are chosen for the convenience of communication.

LB-LC–MS/MS hybrid assays for conjugated-payload

The biotinylation and the immunocapture procedure using streptavidin cartridges with specific biotinylated anti-Id or anti-payload capture mAb for the analysis of conjugated-payload, and the subsequent cathepsin-B cleavage and LC–MS/MS detection have been described in details elsewhere [33].

Briefly, a specific anti-Id mAb was first labeled with biotin at a molar challenge ratio of 12:1, and then immobilized on high-capacity streptavidin-coated cartridges using an Agilent Bravo liquid handler (Agilent Technologies, MA, USA). Plasma samples were loaded on the cartridges at a slow flow rate of 2 µl/min, which was considered to be critical to ensure quantitative capture between immobilized capture mAb and ADC in the biological sample. After multiple washes of the cartridges and the syringes, the captured analytes were eluted into a collection plate by reducing the pH to 2. The eluted samples were neutralized immediately to a slightly acidic condition which was favorable for the following enzyme cleavage step. The ADC described in this manuscript contains a cleavable dipeptide linker that can be cleaved specifically by lysosomal enzymes. A specific lysosomal enzyme, cathepsin B, was used and the *in vivo* condition was mimicked to release the payload from the ADC. The cleavage was conducted in a buffer containing 1.34 units of the enzyme at an optimal pH of 5–6 in the presence of reducing reagent DTT. The mixture was incubated at 25°C for 3 h and the reaction was stopped by adding cold acetonitrile. LC–MS/MS was carried out using a Shimadzu (Tokyo, Japan) Nexera HPLC system interfaced to a SCIEX (CA, USA) Triple Quad 5500 mass spectrometer. A Waters (MA, USA) Acquity UPLC BEH C18 column (1.7 µm, 2.1 × 50 mm) was used for chromatographic separation and the mobile phases consisted of mobile phase A (5 mM ammonium bicarbonate containing 0.005% ammonium hydroxide in 95:5 water: acetonitrile) and mobile phase B (5:95 water:acetonitrile). Positive ion electrospray MS in multiple reaction monitoring mode was used for the detection. The transitions of *m/z* 771→98 and *m/z* 781→98 were monitored for the payload and the internal standard (IS) ($^{13}C_9$, ^{15}N-payload), respectively.

Protein A or protein G cartridges were also used for the analysis of conjugated-payload. The cartridges were equilibrated with 50 µl of PBST (Phosphate buffered saline containing 0.05% Tween 20, Sigma-Aldrich, Sweden) or DPBS (Dulbecco's phosphate buffered saline, Lonza, MD, USA) at a flow rate of 25 µl/min. Plasma samples (25 µl) were loaded on the cartridges at a flow rate of 2 µl/min. The cartridges were washed with 50 µl of washing solution, which consisted of 50 mM ammonium bicarbonate and 0.1% BSA in DPBS. The elution and neutralization steps were the same as described in the previous section.

LB-LC–MS/MS hybrid assays for total-antibody & conjugated-antibody

Immunocapture

Cartridge format

The biotinylation and the immunocapture procedure, using streptavidin cartridges with specific biotinylated anti-Id or anti-payload capture mAbs for the analysis of total-antibody or conjugated-antibody, and protein A cartridge for the analysis of total-antibody, are the same as described above for the analysis of conjugated-payload analysis.

Magnetic-beads format

Immunocapture was also conducted with Dynabeads Myone streptavidin T1 magnetic beads (Life Technologies, CA, USA) using biotinylated mouse anti-Id or mouse anti-payload mAb. Mouse anti-payload mAb bound to protein G beads were also explored.

Beads (1 ml) were washed three-times with 1 ml of PBST solution, and then resuspended with 1 ml of

PBST. Based on the bead capacities (200 µg of mAb per 1 ml of streptavidin T1 beads, and 240 µg of mAb per 1 ml protein G beads), the beads were saturated by adding about 400 µg of mAb for streptavidin T1 beads and 480 µg of mAb for protein G beads, respectively. The beads and mAb mixture was incubated at room temperature for 1 h on a rotator. After incubation, the beads were washed again three-times with 1 ml of PBST, and then resuspended with 1 ml of PBST containing 1% BSA (bovine serum albumin). The immobilized beads were stored at 4°C for future use.

PBST (10 µl) was added to 10 µl of each serum sample in a 96-well plate, and then 50 µl of capture beads was added to each sample. The plate was incubated at room temperature for 30 min in a Thermomixer R model 5355 incubator (Eppendorf, Hamburg, Germany) at a vortexing speed of 900 rpm (15 s on/off) for the immunocapture. The plate was first washed three-times with 200 µl of PBST, and then one time with 200 µl of 50 mM ammonium bicarbonate using Janus Mini automated liquid handler from Perkin Elmer (MA, USA).

Trypsin digestion for total-antibody & conjugated-antibody

To each of the samples obtained from immunocapture, using either beads format or cartridge format, 100 µl of 50 mM ammonium bicarbonate containing 10% methanol was added, the samples were then incubated at 90°C for 30 min for thermal denaturation. After the samples cooled to room temperature, 25 µl of Promega trypsin (~50 µg/ml) was added into each sample for digestion at 60°C for 2 h. The digestion was stopped by adding 10 µl of 10% formic acid in water, and 20 µl of 200 ng/ml of stable isotopic labeled signature peptide IS working solution in 20% acetonitrile and 80% water was added before LC–MS/MS analysis of total Ab or conjugated-antibody.

LC–MS/MS method

LC–MS/MS was carried out using a Shimadzu (Tokyo, Japan) Nexera HPLC system interfaced to a SCIEX (CA, USA) Triple Quad 6500 mass spectrometer equipped with a TurboIonspray™ source. Forty (40) µl of digested solution was injected into a Waters Acuity UPLC HSS T3 column (1.7 µm, 2.1 × 50 mm) with a gradient elution using mobile phases of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 0.8 ml/min. The column temperature and the autosampler temperature were 60°C and 5°C, respectively. Positive ion electrospray in multiple reaction monitoring mode was used for the detection. The optimized source temperature, turbo ionspray voltage, declustering potential and collision energy were set to 600°C, 5500 V, 50 V and 28 eV, respectively. The transitions of *m/z* 531.3 -> 785.4 and 534.3 -> 785.4 were monitored for the signature peptide [48] from the CDR region of the antibody and the IS (stable labeled signature peptide).

LB-LC–MS/MS hybrid assays developed using different immunocapture reagents on different immunocapture platforms for the analysis of conjugated-payload, total-antibody and conjugated-antibody in different biological matrices are summarized in Supplementary Table 1.

Results & discussion

Nomenclature of ADC analytes & complexity of ADC bioanalysis

The four recommended ADC analytes (total-antibody, conjugated-antibody, conjugated-payload and unconjugated-payload) [9,10] illustrate the relationship between antibody and payload in random-conjugated ADCs. The terms 'total' and 'free,' which are often used in bioanalysis of biologics [49,50], take a slightly more complex meaning in ADC bioanalysis. In protein therapeutics, 'total' refers to the sum of bound and unbound to soluble targets. In the case of ADC, 'total' can also mean conjugated plus unconjugated antibody. Then, there are two meanings of 'total' and 'free' in ADC bioanalysis. 'Free' could mean 'unconjugated' to the antibody or 'unbound' to the soluble target. To avoid confusion in communications in ADC bioanalysis, we would prefer not to use 'free,' but rather 'unconjugated' or 'unbound.' If the term 'total' has to be used for simplicity in naming or labeling ADC species for PK analysis, it has to be defined clearly.

In addition to the four assays, there are other assays that may be used to answer specific questions related to safety, efficacy or disposition. The two dimensional plots presented in Figure 1 facilitate the understanding of the complexity and requirements. The horizontal axis describes conjugation/deconjugation, and the vertical describes bound/unbound to the soluble target. For both 'antibody' and 'payload,' the analytes can exist in four different forms *in vivo*: from conjugated and bound in the lower left quarter to unconjugated and unbound in the upper right. The conjugated-antibody (assay 2 as commonly referred [10]) exists in the upper left quarter, representing antibody conjugated to the payload but unbound to the soluble target. In clinical studies, we are also often asked to measure the total conjugated-antibody concentration, unbound and bound to soluble target in patient samples, which could correlate better to total efficacy. This total conjugated-antibody (could be referred as assay 2-II), a new assay, can be measured by either LBA or hybrid assay using appropriate capture and detection approaches

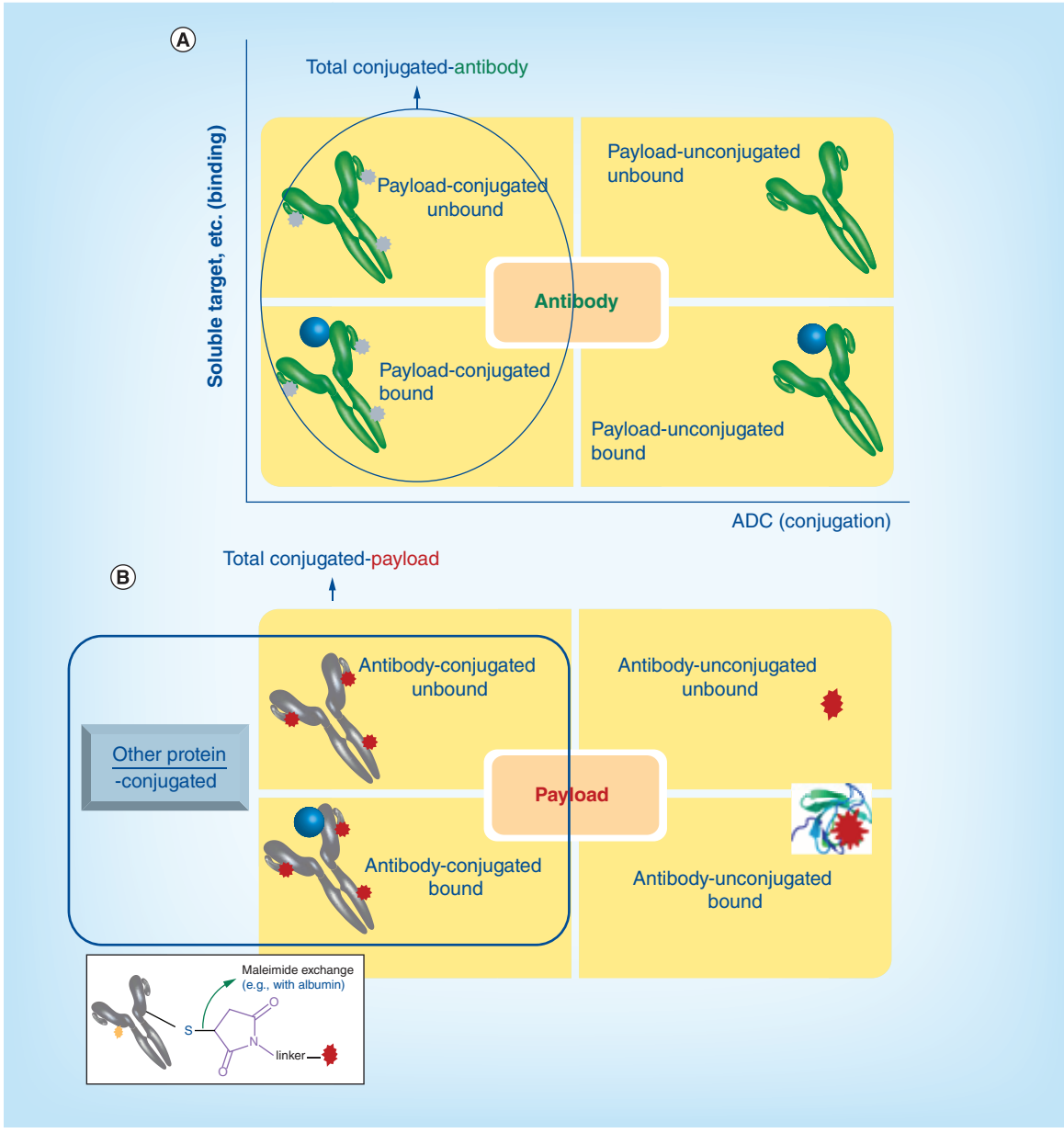

Figure 1. Complexity of antibody–drug conjugate analytes with soluble targets considered. (A) Total conjugated-antibody: conjugated-antibody unbound and bound to soluble targets. **(B)** Total conjugated-payload: payload conjugated to ADC mAb which are unbound or bound to soluble targets as well as payload conjugated to other plasma proteins. Gray dots in **(A)** and red dots in **(B)** represent payload; blue dots represent soluble target.

(reagents or MS-MS technique). Similarly, the analyte in the 'antibody conjugated-payload' (assay 3 as commonly referred [10]) is antibody linked to at least one payload and unbound to the soluble target. In some ADC chemistry, the payload can be conjugated to other plasma proteins through linker exchange [14,51–53]. Quantifying the payload conjugated to the drug antibody and all other plasma proteins could provide useful information for payload-dependent toxicity. This new analyte 'total conjugated-payload' can be measured by a new hybrid assay (could be referred as assay 3-II) in which an anti-payload capture reagent is used that can capture conjugated and unconjugated payload.

Development of LB-LC–MS/MS hybrid assays for ADC bioanalysis as complementary or alternative assays to LBAs

An immunocapture LC–MS/MS hybrid assay can be technically considered as an LBA with LC–MS/MS as the detector (or the immunocapture step can be considered as a specific extraction procedure for the highly specific mass spectrometer). No matter the perspec-

tive used, hybrid assays utilize the strengths of both approaches. Hybrid assays include three essential steps (Figure 2). The first step is immunocapture which is equivalent to the first step of a traditional LBA, the second step is enzyme cleavage or digestion and the last step is LC separation and MS/MS detection. Like in LBAs, a variety of specific capture reagents can be used including target antigens, anti-Id antibodies or anti-payload antibodies. Protein A and G can also be used as generic capture reagents. Biotinylated-antibodies can be immobilized on streptavidin coated magnetic beads or cartridges. In the case of the random-conjugated ADC of interest, enzyme digestion can be done with cathepsin-B to release the payload or by trypsin digestion to generate a signature surrogate peptide [48], which are then measured by LC–MS/MS. Various combinations of capture reagent and enzyme allows tailoring the assay for a specific analyte as described below.

Three automated immunocapture platforms in cartridge-format have been used in our routine operation: AssayMAP Bravo™ from Agilent, MSIA™ tips from Thermo and PhyTips™ from PhyNexus. Each hybrid method was optimized for immunocapture and enzyme cleavage or digestion steps. The cathepsin-B cleavage step was optimized for enzyme content, reaction time and temperature under vendor recommended pH and solution conditions. The trypsin digestion optimization and assay development are similar to that for mAbs or protein therapeutics. The difference is that a signature peptide without lysine is preferred or required for ADCs randomly conjugated at lysine residues. Validation of hybrid assays for ADCs need to fulfill the requirements for both small molecule LC–MS/MS and protein therapeutic LBAs and DAR sensitivity evaluation.

Currently hybrid assays have been developed in our laboratory for conjugated-payload, total-antibody and conjugated-antibody with different combinations of capture reagents and enzymes: anti-Id, anti-payload, cathepsin-B and trypsin (Figure 3 & Supplementary Table 1). The hybrid assays are labeled as H-1 through H-4 for ease of referencing in internal projects support. The correlations of these assays with commonly discussed assays 1–4 in literature [10] are also provided in Figure 3 in conjunction with the nomenclature of ADC analytes/assays discussed above. The anti-Id/cathepsin-B conjugated-payload assay (H-1) is the essential and primary application of hybrid assay complementary to (DAR-insensitive) conjugated-antibody measured by LBA [9,10]. The assay has been validated and used to support ADC preclinical and clinical studies [20,21,33]. Combining anti-Id capture and trypsin digestion measures total-antibody (H-3) as an alternative to the commonly used LBA.

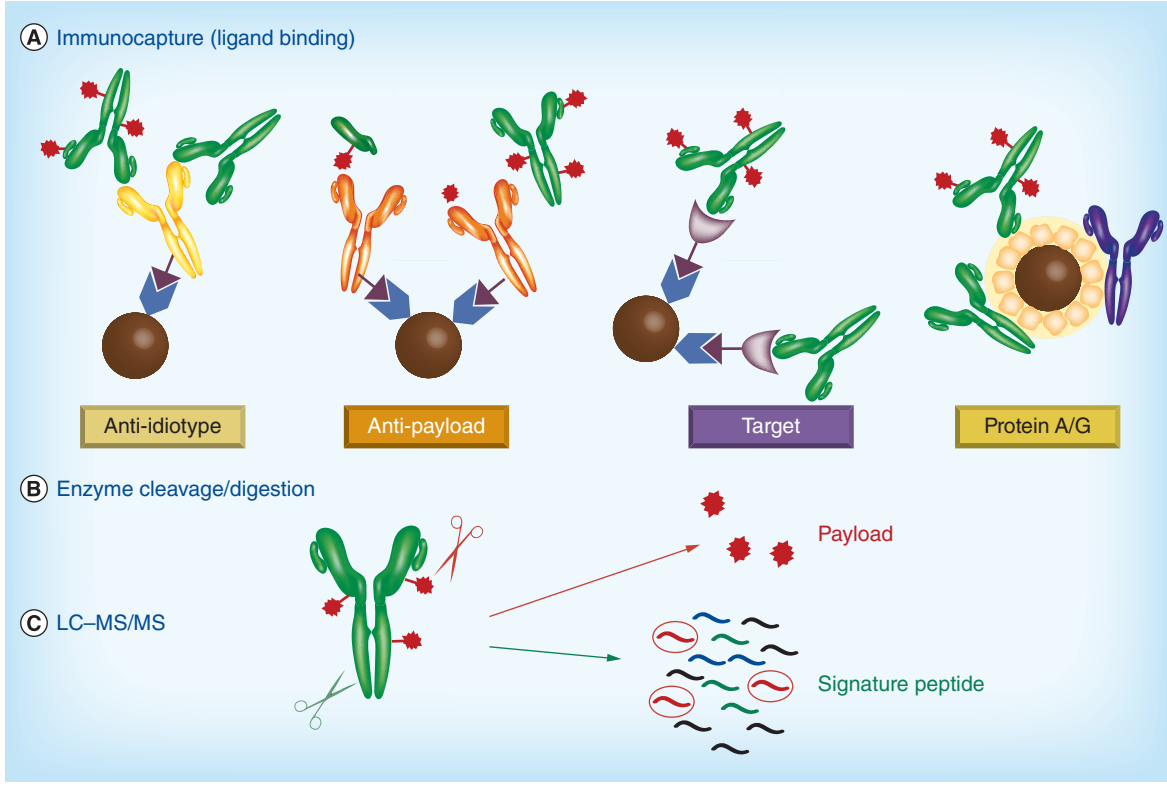

Figure 2. Procedure of LB-LC–MS/MS hybrid assays. (A) Immunocapture (ligand binding), **(B)** enzyme cleavage/digestion, **(C)** LC–MS/MS quantitation.

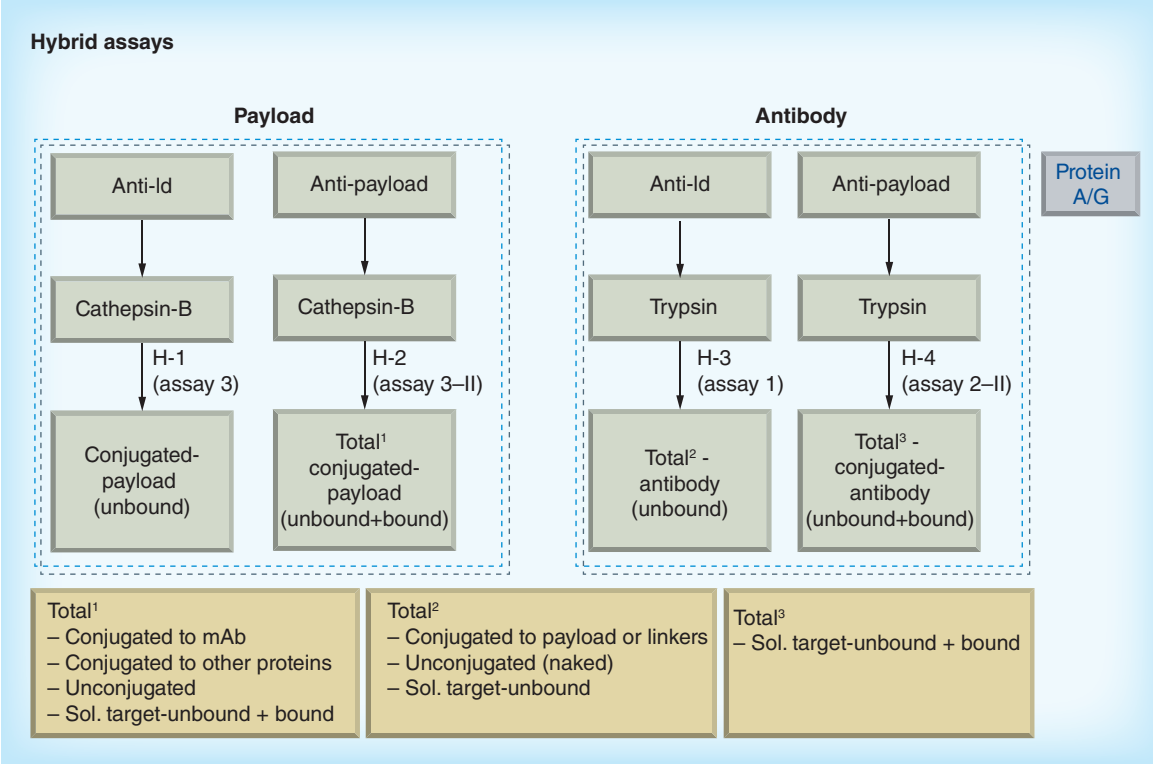

Figure 3. Hybrid assays developed with combinations of different capture reagents and enzymes. (Anti-Id, anti-payload, protein A/G, cathepsin-B and trypsin) for the analysis of conjugated-payload (H-1, assay 3), total conjugated-payload (H-2, assay 3-II), total-antibody (H-3, assay 1) and total conjugated-antibody (H-4, assay 2-II).

Anti-payload capture followed by cathepsin-B cleavage (H-2) or trypsin digestion (H-4) are advanced hybrid assays that quantify total conjugated-payload and total conjugated-antibody (bound and unbound to soluble targets), respectively. As discussed above, the total conjugated-payload assay (H-2), when needed, could provide insight to payload-dependent toxicity. Meanwhile, in an example discussed in the following section, the total conjugated-antibody hybrid assay (H-4) was used to analyze samples from a rat PK study for the ADC compound studied. Generic capture reagents such as protein A or G were also evaluated for conjugated-payload hybrid assays and results were compared with that from assays using specific reagents.

Performance & characteristics of the LB-LC–MS/MS hybrid assay for conjugated-payload

The conjugated payload was fully validated in cyno plasma, as previously reported [33], according to current FDA Guidance and EMA Guidelines on Bioanalytical Method Validation [54–57] and qualified for sample analysis in rat serum and human plasma. The experiments included the determination of the lower limit of quantification, accuracy and precision, selectivity, carryover, recovery, assay robustness and analyte stability. In addition, an ADC-specific validation experiment, the ability to accurately measure different DAR species was conducted. Here is a brief summary highlighting the key results.

- The overall recovery of the assay was 57%, with recoveries of immunocapture step and the enzyme cleavage efficiency of 78 and 73%, respectively;
- The calibration standard curve range was 0.303 to 151.442 ng/ml for conjugated payload, which was equivalent to the range of 20.0 to 10,000.0 ng/ml for the intact ADC. The assay demonstrated good linearity, accuracy and precision;
- Acceptable specificity and sensitivity were determined using ten individual lots of blank plasma unspiked and spiked at LLOQ and high QC levels;
- Conjugated payload was stable in monkey plasma for at least 24 h at room temperature, 38 days at -70°C and following five freeze–thaw cycles.
- The assay allowed accurate quantification of conjugated payload for the ADC having varying DAR. Supplementary Table 2 shows the DAR characteristics of conjugated-payload assay in cyno, human plasma and rat serum using anti-Id capture.

When anti-Id mAb capture is used, the conjugated payload assay measures ADC species that contain at

least one unbound mAb arm and is available to bind the target on tumor cells. Soluble target in circulation may block the binding sites on the ADC. In preclinical species, the soluble target may not be cross-reactive to the ADC, or the level of soluble target may be too low in relation to the ADC level to have a significant impact on the measurement, which was the case in rat in this example. Therefore, the assay tolerance to the presence of soluble target was not tested in animals. However, the levels of soluble target in the patient populations are projected to be high and variable, and, thus, the human assay was thoroughly characterized for the potential interference of soluble target across the estimated range of 10 to 1000 ng/ml. The results are shown in Figure 4. An acceptance criterion of 80% recovery was used to determine the significance of the impact. In the 'no impact zone,' soluble target levels had no impact on accurate quantitation of antibody-conjugated payload. However, relatively high levels of soluble target were found to interfere with the recovery of the analyte at low concentrations. In this 'interference zone,' a portion of ADC cannot be captured and quantified, presumably because both arms of the antibody are occupied by soluble target. Furthermore, due to this concentration-dependent impact of soluble target, a stable-isotope labeled ADC IS (SIL IS) may not be a good candidate for the measurement of conjugated payload in human samples as the IS binds to the soluble target as well, and its response vary in incurred samples and are different from that in calibration curves.

Specific versus generic immunocapture in conjugated-payload assays

Generic capture reagents such as protein A and G have been used for ADC conjugated-payload assays [10,34]. While a comprehensive comparison will be reported in a separate publication, Figure 5 compares the quantitation of conjugated-payload of the ADC of interest at ADC equivalent LLOQ concentration (20 ng/ml) in human plasma (containing lower amount of the soluble target) using different capture reagents anti-Id, protein A, Protein G in AssayMAP cartridges of 100 µg mAb capture capacity. The responses in the assays using Protein A and G are generally higher than that from the assay using anti-Id capture. This result indicates that, while in preclinical species without soluble target or human mAb cross-reactive soluble target, generic capture reagents (protein A and protein G) are comparable to the specific anti-Id capture reagent for LB-LC–MS/MS hybrid conjugated-payload assays in their ability to capture the ADC (data not shown), the higher recovery from a protein A or G in human plasma containing soluble target could be due to the fact that the conjugated-payload concentrations measured include the conjugated-payload, in which the antibody bound and unbound to soluble target (total) as discussed earlier, while anti-Id capture assay measures payload attached to the antibody unbound to the soluble target. The difference in concentrations from assays using generic and anti-Id captures provides information regarding 'free' and 'total' ADC, with regards to its binding to the soluble target or anti-drug

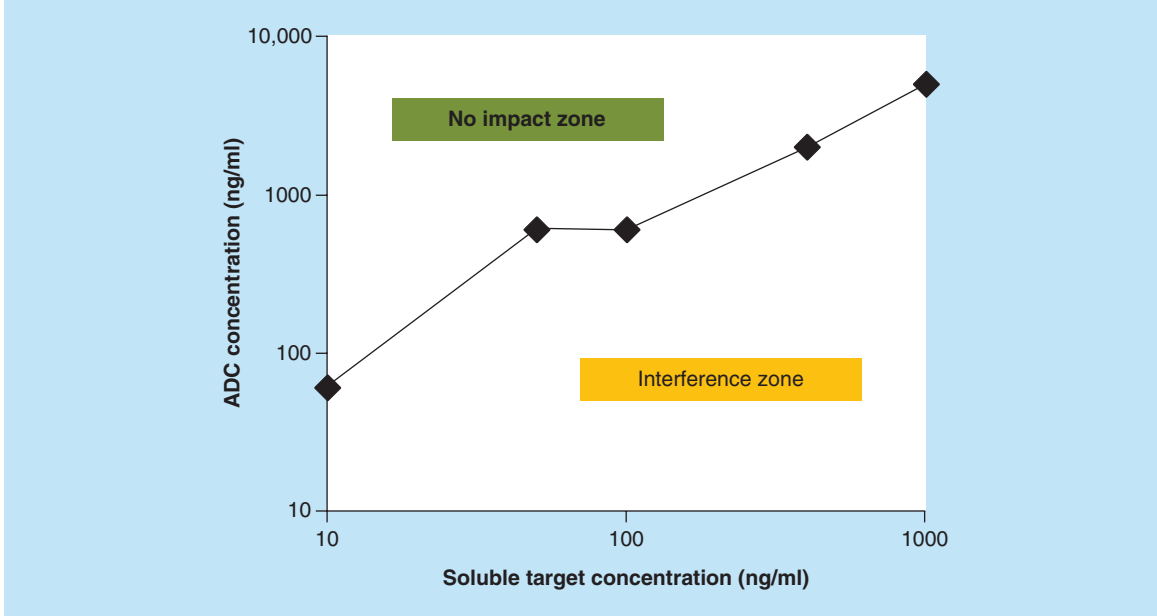

Figure 4. Tolerability to shed/soluble targets in conjugated-payload assay in human plasma.
ADC: Antibody–drug conjugate.

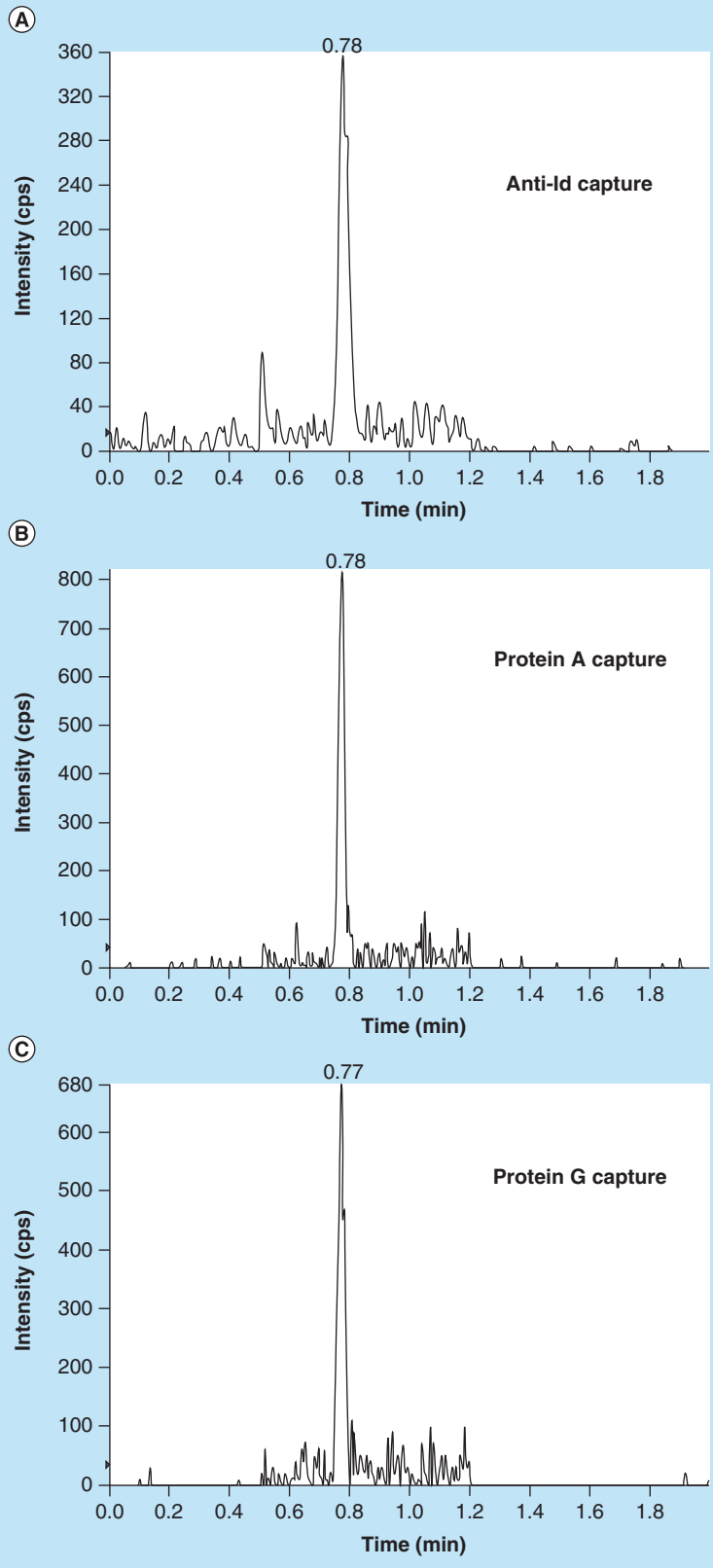

Figure 5. Chromatograms of conjugated-payload at antibody–drug conjugate equivalent LLOQ concentration (20 ng/ml) in human plasma (containing lower amount of soluble target) using different capture reagents.
(A) Anti-Id, **(B)** protein A, **(C)** protein G.

antibodies (ADAs) in clinical samples [49,50]. The recommendation is that generic capture reagents could be used in preclinical studies, especially in early Discovery, to screen antibodies and payloads when specific reagents (anti-Id and anti-payload) are unavailable; anti-Id capture reagent should be used in clinical assays if measuring soluble target unbound 'bio-active' ADC is desirable.

Performance & characteristics of LB-LC–MS/MS hybrid assays for total-antibody and conjugated-antibody

Hybrid assays with immunocapture performed on streptavidin and protein A cartridges, on an Agilent AssayMAP Bravo system, followed by trypsin digestion and LC–MS/MS analysis were developed and qualified for the quantitation of total-antibody and conjugated-antibody. During synthetic conjugation of the drug product, payloads are randomly conjugated to more than 70 lysine residues of the antibody for the ADC being studied. As lysine is one of the tryptic cleavage sties, to avoid interference of the linker to the trypsin digestion and LC–MS/MS analysis, a signature peptide without lysine, in the heavy chain CDR region, was identified and used for quantitation. Confirmatory peptides are often used in LC–MS quantitation of proteins [38–41]. They were not used in this work and will be considered in future hybrid assays for total-antibody and conjugated-antibody.

The assay performance was evaluated for both total-antibody and conjugated-antibody assays in different biological matrices, in other words, rat serum, monkey plasma and human plasma, primarily using cartridges on AssayMAP Bravo. Calibration curves ranged from 0.5 to 150 µg/ml in both assays. Supplementary Table 3 shows that the quality control samples met acceptance criteria in a conjugated-antibody assay in cyno plasma. Similar standard curves and quality control samples performance were observed for all the assays developed in other matrices. All assays achieved 0.5 µg/ml LLOQ, using 12.5 µl or less of biological sample. Assay LLOQ could be improved from 0.5 to 0.1 µg/ml by using 50 µl of biological sample. Assay specificity was tested for all assays using six different lots of matrix, and the results from the conjugated-antibody cyno plasma assay using streptavidin cartridges are shown in Supplementary Table 4. For the quantitation of total-antibody using anti-Id capture, in preclinical studies where soluble target interference is negligible, ideally, a stable-isotope labeled mAb with the labeling positions within the signature peptide would be used as the assay IS to track ADC analyte during the entire process of sample preparation, including immunocapture, trypsin digestion and LC–MS/MS analysis. As a labeled mAb was not available for the ADC of interest, a stable-isotope labeled signature peptide was used as the IS and added into the samples after trypsin digestion to track the analyte during LC separation, and MS ionization and detection. During the immunocapture and trypsin digestion process, where no IS was used, robust standard curves and QC performance was consistently demonstrated. For the total-antibody assay in human plasma using anti-Id capture, due to the presence of soluble target as discussed in the conjugated-payload assay, SIL IS of signature peptide is the choice for the assay. Meanwhile, the hybrid conjugated-antibody assay, using anti-payload capture theoretically measures conjugated-antibody unbound and bound to the soluble target which is a total conjugated-antibody assay and unaffected by the presence of soluble target.

Similarly to the approach used in the conjugated-payload assay validation, evaluation of DAR sensitivity or bias of the total-antibody and conjugated-antibody assays was accomplished by measuring enriched DAR 2 and DAR 4 standards at two concentrations against standard curves prepared with the DAR 3 reference material. No DAR bias was observed for the conjugated-antibody (Table 1) and total-antibody (Supplementary Table 5) hybrid assays using anti-payload and anti-Id capture reagents, respectively, when conducted as described above using cartridges on the AssayMAP Bravo platform.

Comparison of immunocapture using cartridges & magnetic-beads

In our experience, DAR sensitivity for LB-LC–MS/MS conjugated-antibody assays depends on the platform used for the immunocapture. For the ADC of interest, the hybrid conjugated-antibody assay using anti-payload capture was DAR-insensitive in the AssayMAP Bravo cartridge immunocapture platform but was DAR-sensitive in the magnetic-beads platform, which showed a bias for DAR 2 QC samples. While the exact mechanism is unknown and warrants further investigation, it was hypothesized that the improved recovery of different DAR species in a chromatography extraction column equivalent process during immunocapture in cartridges (vs mainly surface interaction in beads format immunocapture and plate format LBAs) could partially account for the absence of DAR bias. It is plausible that different DAR species have different thermodynamic equilibrium constant but have similar rate constant for forward binding reaction (Kon). In addition to this observed different DAR characterizes for anti-payload capture conjugated-antibody assays for the random-conjugated ADC of interest, other major differences between cartridges and magnetic-beads immunocapture for various analytes are capacity

Table 1. Drug to antibody ratio (DAR) characterization of conjugated-antibody assay in rat serum with anti-payload capture using cartridges on a AssayMAP Bravo: No DAR bias when DAR 2 and DAR 4 materials were measured against DAR 3 standard.

Conjugated-antibody (anti-payload capture)	Measured mean (μg/ml)	Adjusted mean† (μg/ml)	Adjusted nominal† (μg/ml)	CV (n = 3)	DEV%
DAR2 at 5 μg/ml	4.44	4.22	4.25	9.1	-0.8
DAR4 at 5 μg/ml	5.20	4.94	4.95	3.6	-0.3
DAR2 at 75 μg/ml	70.3	66.8	63.8	4.5	4.7
DAR4 at 75 μg/ml	68.6	65.2	74.3	8.1	-12.3

†Concentration was adjusted as DAR distribution of the standards follows Poisson distribution:
For ADC with DAR 2: 15% of naked antibody.
For ADC with DAR 3: 5% of naked antibody.
For ADC with DAR 4: 1% of naked antibody.

and the different degree of automation. A comparison of the characteristics between these two platforms of immunocapture is shown in Supplementary Table 6.

Applications of hybrid assays in preclinical PK studies

The random-conjugated ADC of interest was dosed in a discovery rat PK study designed to compare various ADC-related analytes measured by LBAs and hybrid assays. The PK profiles of five analytes measured by hybrid assays are presented in Figure 6A. These are total-antibody (red square, H3 assay), conjugated-antibody (blue diamond, H-4 assay) and conjugated-payload (blue cross, H-1 assay) which are three of the four commonly measured ADC analytes [10] (the unconjugated-payload was not measured here). As reported in earlier publications, the payload is inactivated by metabolism and there are different anti-payload capture reagents available that are specific to the payload or to both payload and the metabolite, respectively [20,21,58]. Payload and metabolite conjugated-antibody (green diamond, also an H-4 assay including the metabolite) was also measured which is labeled as conjugated-antibody (payload + metabolite) as well as conjugated-(payload + metabolite) (green cross, also an H-1 assay including the metabolite). It should be noted that, as discussed in the earlier section, that the H-4 hybrid assay using anti-payload capture can capture conjugated-antibody both unbound and bound to soluble target. However, the soluble target level in rats is negligible, and it does not cross-react with human mAb of ADC. Therefore, it is likely that the two H-4 assays only measure unbound conjugated-antibody or conjugated-antibody (payload + metabolite). The three antibody assays, total-antibody, conjugated-antibody and conjugated-antibody (payload + metabolite), were proven to be DAR-insensitive while the two payload assays were DAR-proportional. The ratio of conjugated-payload to total-antibody (average DAR, payload) and the ratio of conjugated-(payload + metabolite) to total-antibody (average DAR, payload and metabolite) across the PK time course were plotted in Figure 6B. This example demonstrated that hybrid assays are capable and sufficient to support ADC preclinical PK studies, highlighting the versatility of the LC–MS/MS platform for ADC support. The comparison of the average DAR from conjugated-payload and from conjugated-(payload + metabolite) (Figure 6B) shows that at late time points (>200 h), on average, about one third to half of the payload is deconjugated and the rest of the conjugated (payload related) species are combination of payload and metabolite in which the metabolite is about a third to half of the payload.

DAR-insensitive versus DAR-sensitive assays

One complicating factor and a center of debate in ADC bioanalysis, especially in the random-conjugated ADCs, is related to the *in vivo* DAR change. The drug substance is a mixture of ADC species of different DARs, typically 0 through 8 [9–11]. Unfortunately, most often, the individual DAR1–8 ADC reference standards are unavailable and only the drug substance with average DAR of 3 to 4 is used as the reference standard. As broadly reported in the literature, the DAR distribution *in vivo* is expected to change over time, following the administration of randomly conjugated ADCs, due to deconjugation, biotransformation and/or different clearance rates of the ADC species with different DARs [9–11]. The consequence for the quantitative bioanalysis is that the distribution of analyte species in the incurred samples is different from that in the calibrants. This is analogous to quantifying multiple metabolites of a small molecule drug using LC–MS/MS assays when the reference standards of the metabolites are unavailable and the reference standard of the parent compound is used to quantify all species. The mass spectrometric responses of the metabolites and the parent drug may not be the same leading to inaccurate measurements.

While the conjugated-payload assay is DAR-sensitive, DAR-proportional in fact, by definition, the total-

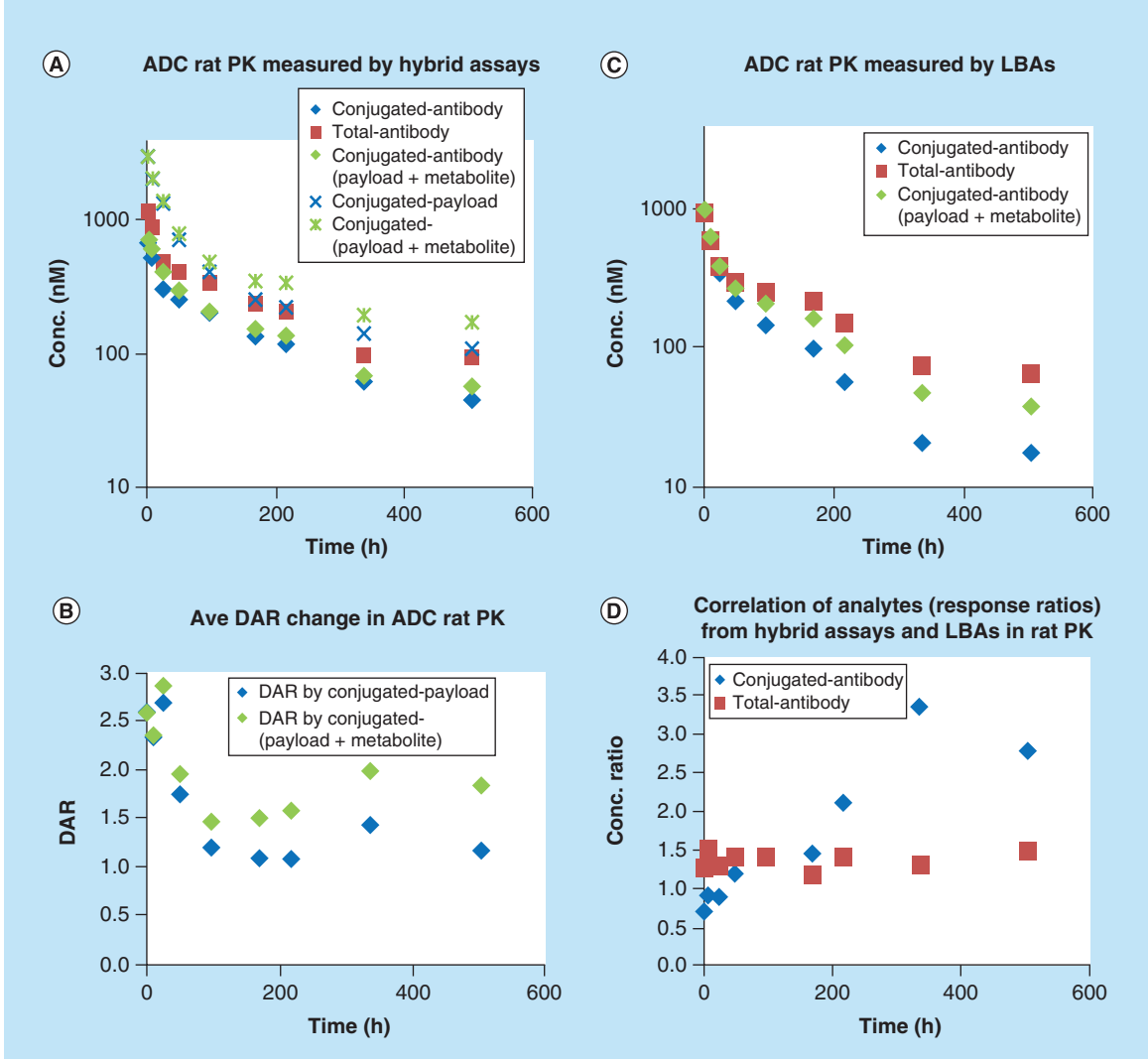

Figure 6. Hybrid assays and ligand-binding assay in antibody–drug conjugate rat PK. (A) Hybrid assays measured conjugated-antibody (blue diamond), conjugated-antibody (payload + metabolite) (green diamond), total-antibody (red square), conjugated-payload (blue cross) and conjugated-(payload + metabolite) (green cross). **(B)** *In vivo* average DAR change measured by hybrid assays, DAR by conjugated-payload (blue diamond) and DAR by conjugated-(payload + metabolite) (green diamond). **(C)** LBAs measured conjugated-antibody (blue diamond), conjugated-antibody (payload + metabolite) (green diamond) and total-antibody (red square). **(D)** Correlations of analytes (response ratios) from hybrid assays and LBAs. Conjugated-antibody (blue diamond) and total-antibody (red square).
Conjugated-antibody (payload + metabolite): payload and metabolite conjugated-antibody; conjugated-(payload + metabolite): antibody conjugated-payload conjugated to payload and the metabolite.
ADC:Antibody–drug conjugate; DAR: Drug to antibody ratio; LBA: Ligand-binding assay.

antibody and conjugated-antibody assays measure the antibody portion of ADCs and should ideally be DAR independent [9–11]. It has been widely reported and discussed that assay format, the capture and detection reagents used, and assay conditions affect the DAR characteristics of conjugated-antibody LBAs [9–11,19,28]. It is generally believed that the conjugated-antibody LBAs using anti-payload capture are more likely to be DAR-insensitive, but this may not always be the case and can be ADC molecules and reagents dependent. As an example, for the random-conjugated ADC of interest, the conjugated-antibody LBA developed using anti-payload capture reagents was DAR-sensitive. In comparison, the hybrid conjugated-antibody assays in Figure 6A using anti-payload capture in cartridge format immunocapture were proven to be DAR-insensitive as shown in Table 1.

In addition to demonstrating the capabilities of various hybrid assays in analyzing ADCs analytes (Figure 6A), the rat PK study was also aimed at evalu-

ating the correlation between LBAs and hybrid assays for ADC analytes, such as total-antibody and conjugated-antibody with assay DAR characteristics in the context. As shown in Figure 6C, the samples from the rat PK study were also analyzed for total-antibody, conjugated-antibody and payload and metabolite conjugated-antibody in the established traditional LBA methodology [20,21,58]. Several observations can be made when comparing Figure 6A & 6C and as shown in Figure 6D. One of the concerns of a DAR-sensitive conjugated-antibody assay, in comparison to a DAR-insensitive one, is the underestimation of the antibody concentrations at late time points (up to theefold when average DAR decreases from the nominal of 3 at early time points to about 1 at late time points as commonly seen for random-conjugated ADCs) [10,28]. This could affect the reported trough concentrations and exposure (AUC). Figure 6D shows that the concentration ratios of two conjugated-antibody (blue diamond) measured by DAR-insensitive hybrid in Figure 6A and DAR-sensitive LBA in Figure 6C increased from 1 at early time points to around 3 at later time points in the PK time course. The difference in AUC of the conjugated-antibody concentration versus time is about twofold (data not shown). This observation matched as reported by Stephan [28] and Kumar [19] in their LBA assay format evaluation and comparison of PK parameters. Figure 6D also shows the constant concentration ratios of total-antibody measured by the hybrid and LBA throughout the PK time course since both assays had the same DAR characteristics. It should be noted that this constant ratio ideally should be close to 1 showing the complete equivalency of the two assays measured by different assay platforms. It could be due to experimental errors or other unknown reasons that the observed constant ratio is approximately 1.3. As a result, the C_{max} from the hybrid total-antibody is higher than those from the hybrid conjugated-antibody. If the difference of concentrations at late time points of PK profiles between conjugated-antibody (payload + metabolite) (green diamond) and conjugated-antibody (blue diamond) is due to payload metabolism, the DAR-sensitive LBAs (Figure 6C) could have overestimated the degree of metabolism compared with the DAR-insensitive hybrid assays (Figure 6A).

Which conjugated-antibody assay is preferred, DAR-insensitive or sensitive? The question remains whether this threefold difference in trough concentration and twofold difference in AUC matter for the correlation between exposure and toxicity/efficacy if both DAR-insensitive and sensitive conjugated-antibody concentrations are used at different stages of ADC development [16]. Despite of the diversity of structures, heterogeneity of components present *in vivo* and their continuous change are common for ADCs [8]. In most publications on ADC bioanalysis including well cited reviews [10] and the AAPS position paper [11], DAR-insensitive conjugated-antibody assay is preferred. In a recent review paper on ADC bioanalysis LBAs, Kumar *et al.* [19] suggested to apply DAR-insensitive assays for IND toxicology studies with the rationale that this would generate better correlation between toxicology and exposure of all DAR species. We, then, may conclude that, in certain situations such as in this example, an LB-LC–MS/MS hybrid assay could be complementary to LBA, providing desired DAR-insensitive conjugated-antibody data.

Hybrid conjugated-payload assay versus conjugated-payload (DAR-sensitive conjugated-antibody) LBA

Three analytes were measured in a cyno PK study of the ADC of interest and the PK profiles are presented in Figure 7A, with conjugated-antibody (LBA) in blue, total-antibody (LBA) in red and conjugated-payload (hybrid) in green. The average DAR *in vivo* decreased from around 3, as in the dosed ADC material, to below 1 after about 1 week as is commonly seen for random-conjugated ADCs (Figure 7B). This indicates that, at later time points, on average, there is one payload attached to each antibody. As previously reported, conjugated-payload (green) and conjugated-antibody (blue) profiles were parallel throughout the PK time course with a ratio approximately equal to the starting average DAR of 3.

The results shows a good correlation between a DAR-sensitive conjugated-antibody LBA and a conjugated-payload hybrid assay for the quantitation of the active component of ADCs. The DAR-proportional conjugated-payload data validates the DAR-sensitive conjugated-antibody data [21]. Stephan stated that DAR-sensitive conjugated-antibody assays can be used to monitor *in vivo* DAR change when conjugated-payload assays are unavailable or for ADCs with non-cleavable linkers [28]. Kumar *et al.* [19] expressed that DAR-sensitive conjugated-antibody LBAs or LC–MS/MS based assays in early Discovery may be useful to better describe the changes in conjugated-payload over time and associated PK parameters. The argument is based on the hypothesis that conjugated payload is the main driver of efficacy at the site of action [19,25,59]. They further stated that ideally, a DAR-sensitive LBA would be equivalent to a conjugated-payload assay, as we observed in this example.

This suggests that the DAR-sensitive conjugated-antibody assay is not measuring the conjugated-antibody but rather is measuring the conjugated-payload. DAR-sensitive conjugated-antibody is expressed as

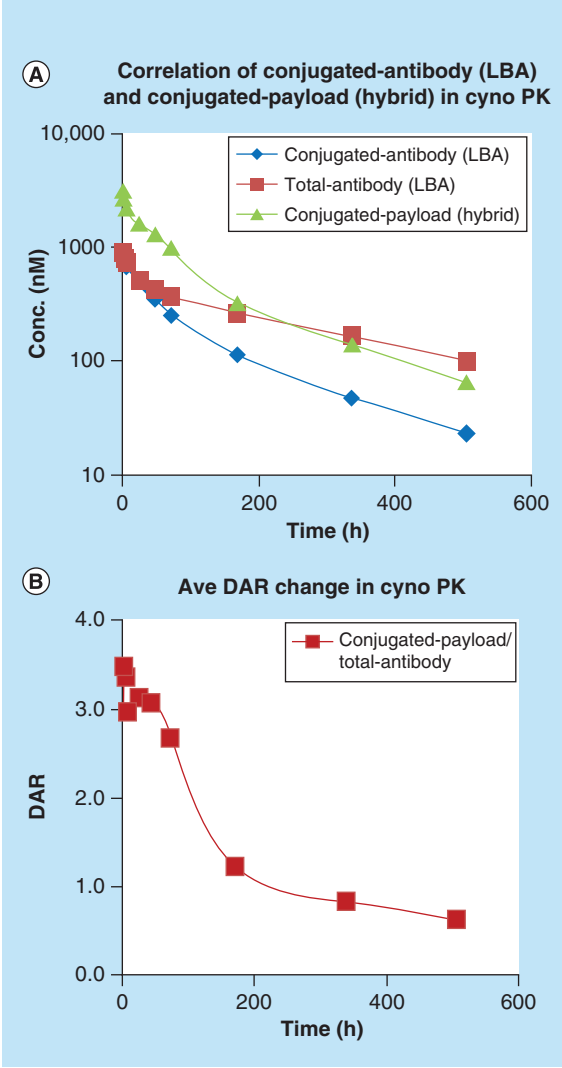

Figure 7. Correlation of conjugated-antibody (ligand-binding assay) and conjugated-payload (hybrid) in antibody–drug conjugate cyno monkey PK. (A) PK profiles of DAR-sensitive conjugated-antibody in ligand-binding assay (LBA) (blue diamond) which is equivalent to conjugated-payload in LBA, total-antibody in LBA (red square); conjugated-payload in hybrid assay (green triangle). **(B)** *In vivo* change of average drug to antibody ratio.
DAR: Drug to antibody ratio; LBA: Ligand-binding assay.

the equivalent concentration of ADC standard. Conjugated-payload measures the payload attached to the drug antibody and by definition is DAR-proportional and has clear PK and PD meaning. If a DAR-proportional assay is desired to monitor the payload change *in vivo,* then the conjugated-payload is the more direct assay. Changing the concentration unit of DAR-proportional conjugated-antibody (e.g., multiplying the nominal DAR value in the ADC dosing material) will convert the result to conjugated-payload. In that regard a perfect DAR-sensitive conjugated-antibody assay should be called a DAR-proportional assay, which is indeed a conjugated-payload assay; while DAR-sensitive assays refer to the assays which may not be able to stoichiometrically measure small molecule drug for all DAR species [19].

Since both assays are essentially the same, there is no need to conduct both assays in PK studies. Which conjugated-payload assay to use, DAR-sensitive LBA or hybrid assay? Hybrid conjugated-payload assay has less dependence on reagent availability especially in early Discovery. Generic reagents such as protein A and G can be readily available to be used to screen antibody, linkers, and payload to select the lead candidate. LBA conjugated-payload assay does require the anti-payload antibody. For the ADCs with noncleavable linkers LBA conjugated-payload assay is the assay of choice to monitor *in vivo* payload change. Post Discovery, i.e., IND enabling preclinical studies and clinical studies, if LBA conjugated-payload (aka DAR-proportional conjugated-antibody) is still desired, the assay can be processed in parallel with total-antibody and other assays in a harmonized and integrated fashion when same anti-Id capture reagent is used [20]. Alternatively, the fully established hybrid conjugated-payload assay, used since early Discovery can also be efficiently and effectively used to support ADC projects at later development stages with either generic or specific capture reagents.

Discovery & development integrated ADC PK/BA strategies: choice of ADC analytes & assays in discovery PK, development TK & clinical PK studies

Currently, there is no industry-wide consensus for ADC bioanalysis except for the fact that total-antibody, conjugated-antibody and/or conjugated-payload, and unconjugated-payload are recommended as the major analytes for ADC PK [9–11,19]. It is understood that each unique ADC molecule may require a specific combination of analytes using a fit-for-purpose approach. The key elements of Genentech's strategy [10,22] include investigating DAR distribution *in vivo* and testing all quantitative assays with individual DARs to ensure accurate quantitation of all ADC analytes in early discovery. Key PK analytes in nonclinical and clinical studies are total-antibody (LBA), conjugated-antibody or antibody conjugated-payload (hybrid assay) and unconjugated-payload (LC–MS). The conjugated-payload represents the active ADC drug component and is the preferred analyte when possible in a hybrid assay using protein A as the generic capture reagent. Pfizer proposed a stage-specific BA strategy [9,19] where a DAR-sensitive conjugated-antibody LBA was applied in early discovery and a DAR-insensitive con-

jugated-antibody LBA was preferred in development (IND-enabling and clinical studies). Pfizer scientists did acknowledge that evolution of assays could generate different PK profile and parameters at different stages of ADC development [19] as discussed above and reported by Genentech [28].

With limited experience on ADC performance in a clinical setting, it is still uncertain whether conjugated-antibody or conjugated-payload correlates more closely with efficacy and safety though it has been suggested that conjugated-payload is the key analyte for efficacy while (DAR-insensitive) conjugated-antibody is the preferred analyte for toxicology correlations [19]. To maintain the continuity of bioanalytical analytes and assays at different stages of ADC development, one strategy to be considered is that in early discovery, because of the efficiency, robustness and simplicity, a generic protein A or G capture conjugated-payload assay is ideal for screening and candidate selection. Generic capture total-antibody in LBA or hybrid format can be applied. In late discovery and early development, DAR-insensitive conjugated-antibody in LBA or hybrid format should be established if possible and both conjugated-antibody and conjugated-payload are measured. Assay performance in regard to DAR sensitivity should be evaluated at this stage using either enriched average DAR mixtures or purified individual DAR standards. It should be kept in mind that isolating individual DAR species for randomly conjugated ADCs is technically challenging and a fit-for-purpose assay strategy should be considered. Continuation with only one of the antibody-conjugate assays in late development is recommended, either a DAR-insensitive conjugated-antibody or a DAR-proportional conjugated-payload in either LBA or hybrid format depending on the technical strength of a particular bioanalytical lab and PK/PD correlations in early clinical studies. While currently there is a general belief that site-specific ADCs are the future of ADC development, and that ADC bioanalysis will be much simplified with reduced *in vivo* heterogeneity, we would like to caution that determining optimal conjugation sites for site-specific ADCs could be a challenging task. Either site-specific ADC shifts the bioanalytical focus and difficulties to different stages or randomly conjugated ADCs will continue to be one of the major directions of ADC development.

Conclusion

The complex nature of ADCs presents unique bioanalytical challenges and requires the measurement of multiple analytes. Both LBA and LC–MS/MS assays and their combination, LB-LC–MS/MS hybrid assays, are used in PK bioanalysis of ADCs. The use of 'LB' rather than 'affinity capture,' 'immunocapture' in the terminology of hybrid assays could facilitate the understanding of the nature of these assays and promote their flexible and fit-for-purpose applications. A series of hybrid assays, complementary or as substitutions for LBAs, were developed for a random-conjugated ADC, by combining anti-Id or anti-payload capture, and cathepsin-B or trypsin enzymes for the analysis of conjugated-payload, total-antibody and conjugated-antibody. Hybrid assays are capable of exclusively supporting ADC bioanalysis for PK studies. Generic reagents such as protein A and protein G have also been used in both the conjugated-payload and total-antibody hybrid assays. In general, hybrid assays using generic capture reagents are equivalent to those using specific anti-Id capture reagents in preclinical species when soluble target interference is negligible. On the contrary, in human plasms/serum samples, hybrid assays using protein A and G capture will measure analytes (conjugated-payload or total-antibody) in which the mAb unbound and bound to the soluble target while assays using anti-Id capture measures soluble target unbound ADC analytes. When considering which technology or platform to use in ADC bioanalysis and PK modeling, the comparison of LBAs and hybrid assays should include factors related to analytes being measured, assay DAR characteristic and platform availability. LBAs and hybrid assays can be complementary or alternative to each other in ADC bioanalysis. To be specific: DAR-proportional hybrid conjugated-payload assay is complementary to DAR-insensitive conjugated-antibody LBA and an alternative to DAR-sensitive conjugated-antibody LBA. DAR-insensitive hybrid conjugated-antibody assay is complementary to DAR-sensitive conjugated-antibody LBA. DAR-insensitive total-antibody hybrid assay and LBAs are alternatives to each other. The results of these assay investigations and comparisons demonstrate the possibilities of flexible and fit-for-purpose ADC bioanalytical assay strategies at different stages of ADC development and also tailored to the capabilities and preference of a particular bioanalytical laboratory with a focus on LBAs or LB-LC–MS/MS hybrid assays, and to the particular ADC constructs worked on. The strategy applied to ADC bioanalysis continues to evolve and industry-wide harmonization is desirable.

Future perspective

Diversified bioanalytical assays will continue to be used to support ADC PKs. More clinical data on a

variety of ADCs will help to decide which ADC analyte correlates more closely with safety and efficacy. We will see more applications of hybrid LB-LC–MS/MS conjugated-payload assays in Discovery to measure preclinical PK studies in addition to evaluating *in vitro* and *in vivo* linker and payload stabilities and payload metabolism using generic reagents. Either hybrid or LBA conjugated-payload (DAR-sensitive conjugated-antibody) assay can continue into Development to analyze regulated preclinical and clinical studies with a focus on efficacy correlation. As ADC toxicity can be both payload- and antigen-dependent, DAR-insensitive conjugated-antibody plays a significant role in establishing safety margins, and correlating preclinical and clinical ADC exposures. While LBAs have been the primary assays for total-antibody and (DAR-insensitive) conjugated-antibody, with more pharmaceutical companies and CROs working on ADCs and more experience industry-wide, and particularly because of the integration and close collaboration between LBA- and LC–MS/MS-based laboratories, there will be more applications of LB-LC–MS/MS hybrid assays for the analysis of these two ADC-related analytes. Immunocapture at the ADC and peptide-level coupled with micro- or nano-LCs will increase the sensitivity of these LB-LC–MS/MS hybrid assays.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/full/10.4155/bio-2016-0017

Acknowledgements

The authors would like to thank the following for contributions to the assay strategy development, execution of the studies and experiments, and discussions: J Brailsford, J Zeng, R Iyer, X Gu, W Li, M Zhu, S Piccoli, D Passmore, D Desai, J Haulenbeek, F Zambito, S Gangwar, K Kelly, P Lemaire, A Davulcu, S, Brueggemeier, M Hay, C Yeung, A Tam, J Jackson.

Financial & competing interests disclosure

The authors of this article are current or past employees of Bristol-Myers Squibb Company (BMS). All financial support for the studies reported herein was provided by BMS.

Executive summary

- Antibody–drug conjugate (ADC) molecules are heterogeneous mixtures and *in vivo* have a continuous dynamic change of composition. Randomly conjugated ADCs have higher degrees of complexity. Although each unique ADC may require a specific group of analytes, in general, four analytes are commonly measured in ADC PK studies including total-antibody, conjugated-antibody, conjugated-payload and unconjugated-payload using LBA, LC–MS/MS and ligand binding (LB)-LC/MS/MS hybrid assays.
- LB-LC–MS/MS hybrid assays were developed to measure total-antibody, conjugated-antibody and conjugated-payload in ADC PK studies. These assays are capable of fully supporting ADC bioanalytical needs and may be considered as alternative or complementary to traditional ligand-binding assays (LBAs).
- The drug to antibody ratio (DAR) sensitive hybrid conjugated-payload assay measured different DAR species accurately against an ADC reference standard with average DAR of 3. The assay results correlated well with the LBA conjugated-payload (DAR-sensitive conjugate-antibody) assay.
- Hybrid total-antibody and conjugated-antibody assays were DAR-insensitive using anti-Id and anti-payload as capture reagents, respectively.
- The immunocapture in the cartridge and beads formats generated different DAR characteristics for hybrid conjugated-antibody assays using the same anti-payload capture reagent for a random-conjugated ADC studied. More investigations and comparisons are warranted.
- Currently there is no industry-wide standard strategy or procedures for ADC bioanalysis.
- While stage specific assay strategies are currently being discussed throughout the industry, a Discovery and Development integrated ADC bioanalysis strategy with the focus of assay continuity, flexibility and fit-for-purpose is proposed;
- Due to their independency on specific reagents, DAR-proportional hybrid conjugated-payload assays have an edge in early Discovery for compound screening and candidate selection.
- DAR-sensitive conjugated-payload in hybrid or LBA format can be continued into late Discovery and early Development stages while DAR-insensitive conjugated-antibody assays in either LBA or hybrid formats should be developed.
- Either DAR-sensitive conjugated-payload or DAR-insensitive conjugated-antibody assay could be carried into later Development once efficacy/toxicity and exposure relationship (of a specific analyte) has been established.
- ADC bioanalytical strategies continue to evolve. The choice of LBA or hybrid platforms for the quantitation of the major ADC PK analytes can be made on a case-by-case, fit-for-purpose fashion and is also dependent on the strength of a particular bioanalytical lab and its ADC bioanalytical philosophy.

The authors have no other 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 apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

1 US FDA. FDA approves Adcetris to treat two types of lymphoma (2011). www.fda.gov

2 US FDA. FDA approves new treatment for late-stage breast cancer (2013). www.fda.gov

3 Goldmacher V, Kovtun Y. Antibody–drug conjugates: using monoclonal antibodies for delivery of cytotoxic payloads to cancer cells. *Ther. Deliv.* 2(3), 397–416 (2011).

4 Perez HL, Cardarelli PM, Deshpande S *et al.* Antibody–drug conjugates: current status and future directions. *Drug Discov. Today* 19(7), 869–881 (2013).

5 Beck A, Reichert JM. Antibody–drug conjugates: present and future. *MAbs* 6, 15–17 (2014).

6 Mullard A. Maturing antibody–drug conjugate pipeline hits 30. *Nat. Rev. Drug Discov.* 12, 329–332 (2013).

7 Kaur S. Bioanalysis special focus issue on antibody–drug conjugates. *Bioanalysis* 5(9), 981–983 (2013).

8 Gorovits B. Bioanlaysis of antibody–drug conjugates. *Bioanalysis* 7(13), 1559–1560 (2015).

9 Gorovits B, Alley SC, Bilic S *et al.* 2011 Bioanalysis of antibody–drug conjugates: American Association of Pharmaceutical Scientists Antibody-Drug Conjugate Working Group position paper. *Bioanalysis* 5(9), 997–1006 (2013).

• **Collaborative manuscript whereby many key opinion leaders across industry have addressed bioanalytical challenges for antibody–drug conjugate (ADC).**

10 Kaur S, Xu K, Saad OM *et al.* Bioanalytical assay strategies for the development of antibody–drug conjugate biotherapeutics. *Bioanalysis* 5(2), 201–226 (2013).

•• **High impact manuscript describing the potential ADC species and associated bioanalytical strategies employed to understand drug disposition.**

11 Stephan JP, Kozak KR, Wong WL. Challenges in developing bioanalytical assays for characterization of antibody–drug conjugates. *Bioanalysis* 3(6), 677–700 (2011).

12 Sauerborn M, Dongen W. Practical considerations for the pharmacokinetic and immunogenic assessment of antibody–drug conjugates. *Biodrugs* 28, 383–391 (2014).

13 Clark T, Han X, King L *et al.* Insights into antibody–drug conjugates: bioanalysis and biomeasures in discovery. *Bioanalysis* 5(9), 985–987 (2013).

14 Tumey LN, Rago B, Han X. *In vivo* biotransformation of antibody–drug conjugates. *Bioanalysis* 7(13), 1649–1664 (2015).

15 Saad OM, Shen BQ, Xu K *et al.* Bioanalytical approaches for characterizing catabolism of antibody–drug conjugates. *Bioanalysis* 7(13), 1583–1604 (2015).

16 Kraynov E, Kamath AV, Walles M *et al.* Current approaches for ADME characterization of antibody–drug conjugates: an industry White Paper. *Drug Metab. Dispos.* 44(5), 617–623 (2015).

17 Tibbitts J, Canter D, Graff R, Smith A, Khawli LA. Key factors influencing ADME properties of therapeutic proteins: a need for ADME characterization in drug discovery and development. *MAbs* 8(2), 229–45 (2016).

18 Beck A, Terral G, Debaene F *et al.* Cutting-edge mass spectrometry methods for the multi-level structural characterization of antibody–drug conjugates. *Expert Rev. Proteomics* 13(2), 157–183 (2016).

19 Kumar S, King LE, Clark TH. Antibody–drug conjugates nonclinical support: from early to late nonclinical bioanalysis using ligand-binding assays. *Bioanalysis* 7(13), 1605–1617 (2015).

20 Myler H, Rangan VS, Kozhich A *et al.* Validaiton of an integrated series of ligand binding assays for the quantitative determination of antibody drug conjugate in biological matrices. *Bioanalysis* 8(6), 519–531 (2016).

21 Myler H, Rangan VS, Wang J *et al.* An integrated multiplatform bioanalytical strategy for antibody–drug conjugates: a novel case study. *Bioanalysis.* 7(13), 1569–1582 (2015).

22 Dere R, Yi JH, Lei C *et al.* PK assays for antibody–drug conjugates: case study with ado-trastuzumab emtansine. *Bioanalysis* 5(9), 1025–1040 (2013).

23 Lin K, Tibbitts J. Pharmacokinetic consideration for antibody drug conjugates. *Pharm. Res.* 29, 2354–2366 (2012).

24 Shah D, Barletta F, Betts A *et al.* Key bioanalytical measurements for antibody–drug conjugate development: PK/PD modelers' perspective. *Bioanalysis* 5(9), 989–992 (2013).

25 Bender B, Leipold DD, Xu K *et al.* A mechanistic pharmacokinetic model elucidating the disposition of trasutuzumab emtansine (T-DM1), an antibody–drug conjugate (ADC) for treatment of metastatic breast cancer. *AAPS J.* 16(5), 994–1008 (2014).

26 Sukumaran S, Gadkar K, Zhang C *et al.* Mechanism-based pharmacokinetic/ pharmacodynamic model for THIOMAB™ drug conjugates. *Pharm Res.* 32(6), 1884–1893 (2014).

27 Khot A, Sharma S, Shah D. Integration of bioanalytical measurements using PK-PD modeling and simulation: implications for antibody–drug conjugate development. *Bioanalysis* 7(13), 1633–1648 (2015).

28 Stephan JP, Chan P, Lee C *et al.* Anti-CD22-MCC-DM1 and MC-MMAF conjuagtes: impact of assay formation on pharmacokinetic parameters determination. *Bioconj. Chem.* 19, 1673–1683 (2008).

29 Xu K, Liu L, Saad OM *et al.* Characterization of intact antibody–drug conjugates from plasma/serum *in vivo* by affinity capture capillary liquid chromatography-mass spectrometry. *Anal. Biochem.* 412, 56–66 (2011).

30 Xu K, Liu L, Dere R *et al.* Characterization of the drug-to-antibody ratio distribution for antibody–drug conjugates in plasma/serum. *Bioanalysis* 5(9), 1057–1071 (2013).

31 Luo Q, Chung HH, Borths C *et al.* Structural characterization of a monoclonal antibody-maytansinoid immunoconjugate. *Anal. Chem.* 88(1), 695–702 (2016).

32 Redman EA, Mellors JS, Starkey JA, Ramsey JM. Characterization of intact antibody drug conjugate variants using microfluidic CE-MS. *Anal. Chem.* 88(4), 2220–2226 (2016).

33 Liu A, Kozhich A, Passmore D *et al.* Quantitative bioanalysis of antibody-conjugated payload in monkey plasma using a hybrid immuno-capture LC–MS/MS approach: assay development, validation, and a case study. *J. Chromatogr. B* 1002, 54–62 (2015).

34 Sanderson RJ, Nicholas ND, Baker Lee C *et al.* Antibody-conjugated drug assay for protease-cleavable antibody–drug conjugates. *Bioanalysis* 8(1), 55–63 (2016).

35 Birdsall RE, McCarthy SM, Janin-Bussat MC *et al.* A sensitive multidimensional method for the detection, characterization, and quantification of trace free drug species in antibody–drug conjugate samples using mass spectral detection. *MAbs* 8(2), 306–317 (2016).

36 Li Y, Gu C, Gruenhagen J, Yehl P, Chetwyn NP, Medley CD. An enzymatic deconjugation method for the analysis of small molecule active drugs on antibody–drug conjugates. *MAbs* 8(4), 698–705 (2016).

37 Heudi O, Barteau S, Picard F, Kretz O. Quantitative analysis of maytansinoid (DM1) in human serum by on-line solid phase extraction coupled with liquid chromatography tandem mass spectrometry -Method validation and its application to clinical samples. *J. Pharm. Biomed. Anal.* 120, 322–332 (2016).

38 Neubert H, Muirhead D, Kabir M *et al.* Sequential protein and peptide immunoaffinity capture for mass spectrometry-based quantification of total human β-nerve growth factor. *Anal. Chem.* 85, 1719–1726 (2013).

39 Lund H, Lovsletten K, Paus E *et al.* Immuno-MS based targeted proteomics: highly specific sensitive and reproducible human chorionic gonadotropin determination for clinical diagnostics and doping analysis. *Anal. Chem.* 84, 7926–7932 (2012).

40 Whiteaker JR, Zhao L, Zhang HY *et al.* Antibody-based enrichment of peptides on magnetic beads for mass-spectrometry-based quantification of serum biomarkers. *Anal. Biochem.* 362, 44–54 (2007).

41 Schlosser G, Vekey K, Malorni A *et al.* Combination of solid-phase affinity capture on paramagnetic beads and mass spectrometry to study non-covalent interactions: example of minor groove binding drugs. *Rapid Commun. Mass Spectrom.* 19, 3307–3314 (2005).

42 Dufield D, Neubert H, Garofolo F *et al.* 2014 White Paper on recent issues in bioanalysis: a full immersion in bioanalysis (Part 2 – hybrid LBA/LCMS, ELN & regulatory agencies' input). *Bioanalysis* 6(23), 3237–3249 (2014).

43 Knutsson M, Schmidt R. LC–MS/MS of large molecules in a regulated bioanalytical enviroment-which acceptance criteria to apply. *Bioanalysis* 5(18), 2211–2214(2013).

44 DeSilva B, Smith W, Weiner R *et al.* Recommendations for the bioanalytical method validation of ligand-binding assays to support pharmacokinetic assessments of macromolecules. *Pharm. Res.* 20, 1885–1900 (2003).

45 Duggan JX, Vazvaei F, Jenkins R. Bioanalytical method validation considerations for LC–MS/MS assays of therapeutic proteins. *Bioanalysis* 7(11), 1389–1395 (2015).

46 Jenkins R, Duggan JX, Aubry AF *et al.* Recommendations for validation of LC–MS/MS bioanalytical methods for protein biotherapeutics. *AAPS J.* 17(1), 1–16 (2015).

47 Rao C, Rangan VS, Deshpande S. Challenges in antibody–drug conjugate discovery: a bioconjugation and analytical perspective, *Bioanalysis* 7(13), 1561–1564 (2015).

48 Lee JW. Generic method approaches for monoclonal antibody therapeutics analysis using both ligand binding and LC–MS/MS techniques. *Bioanalysis* 8(1), 19–27 (2016).

49 Lee JM, Kelley M, King LE *et al.* Bioanalytical approaches to quantify "total" and "free" therapeutic antibodies and their targets: technical challenges and PK/PD applications over the course of drug development. *AAPS J.* 13, 99–110 (2011).

50 Mayer AP, Hottenstein CS. Ligand-binding assay development: what do you *want* to measure versus what you *are* measuring? *AAPS J.* 18(2), 287–289 (2015).

51 Shen BQ, Xu K, Liu L *et al.* Conjugation site modulates the *in vivo* stability and therapeutic activity of antibody–drug conjugates. *Nat. Biotechnol.* 30(2), 184–189 (2012).

52 Alley SC, Benjamin DR, Jeffrey SC *et al.* Contribution of linker stability to the activities of anticancer immunoconjugates. *Bioconjug. Chem.* 19(3), 759–765 (2008).

53 Tumey LN, Charati M, He T *et al.* Mild method for succinimide hydrolysis on ADCs: impact on ADC potency, stability, exposure, and efficacy. *Bioconjug. Chem.* 25(10), 1871–1880 (2014).

54 US Department of Health and Human Services, Food Drug Administration, Center for Drug Evaluation and Research. Guidance for Industry: Bioanalytical Method Validation. Rockville, MD, USA (2013). www.fda.gov

55 European Medicines Agency. Guideline on Bioanalytical Method Validation. London, UK (2011). www.ema.europa.eu

56 Japan Ministry of Health and Labor Welfare. Guideline on Bioanalytical Method (Ligand Binding Assay) Validation in Pharmaceutical Development (2014). www.nihs.go.jp

57 China Pharmacopoeia (ChP). Draft Guidance on Bioanalytical Method Validation (2014). http://knex2014.europeanbioanalysisforum.eu

58 Vangipuram SR, Myler H, Kozhich A *et al.* Biotransformation and stability of antibody–drug conjugates: payload metabolism and linker cleavage delineation. *Bioanalysis* 7(11), 1319–1323 (2015).

59 Wada R, Erickson HK, Lewis Phillips GD *et al.* Mechanistic pharmacokinetic/ pharmacodynamic modeling of *in vivo* tumor uptake, catabolism, and tumor response of trastuzumab maytansinoid conjugates. *Cancer Chemother. Pharmacol.* 74(5), 969–980 (2014).

Research Article

For reprint orders, please contact reprints@future-science.com

Bioanalysis

Validation of an integrated series of ligand-binding assays for the quantitative determination of antibody–drug conjugates in biological matrices

Background: The bioanalytical strategy for antibody–drug conjugates (ADC) includes multiple integrated measurements of pharmacologically relevant ADC. **Methods & results:** Three ligand-binding assays were validated for the measurement of total antibody, active ADC and total ADC. Accuracy and precision demonstrate%bias from -8 to 14%, %CV from 3 to 11% and total error from 3 to 21%, with >98% samples meeting incurred sample reanalysis criteria. Each assay met stability, selectivity, dilutional integrity, carry over and specificity criteria with no interference from associated metabolite/impurity. Given the active ADC assay sensitivity to payload, active ADC was used to assess drug to antibody ratio. **Discussion & conclusion:** Implementation of a microfluidic automated platform enabled high throughput sample analysis of multiple analytes with minimal sample processing.

First draft submitted: 16 November 2015; Accepted for publication: 19 January 2015; Published online: 26 February 2016

Keywords: antibody–drug conjugate • drug antibody ratio • ligand-binding assays • selectivity • specificity • total antibody • payload

Heather Myler*[,1], Vangipuram S Rangan[2], Alex Kozhich[1], Brian Hoffpauir[3], Donna Dail[1], Jennifer Cummings[1], Mark Saewert[1], Amy Manney[1], Ang Liu[4], Chetana Rao[2], Jian Wang[4], Renuka Pillutla[1] & Binodh DeSilva[1]

[1]Bioanalytical Sciences-Biologics, Bristol-Myers Squibb, Lawrenceville, NJ, USA
[2]Biologics Discovery California, Bristol-Myers Squibb, Redwood City, CA, USA
[3]Immunochemistry, PPD Laboratories®, Richmond, VA, USA
[4]Bioanalytical Sciences-LCMS, Bristol-Myers Squibb, Lawrenceville, NJ 08648, USA
*Author for correspondence:
Tel.: +1 609 252 3600
heather.myler@bms.com

Antibody–drug conjugates (ADC) are a re-emerging class of cancer therapeutics that have shown promise in treating cancer patients with the goal of maximizing therapeutic exposure at the tumor site while minimizing systemic exposure and the severe side effects of conventional chemotherapeutics. The growing database for ADC-based targeted therapies, including monoclonal antibodies conjugated to DNA alkylators, DNA strand breakers, tubulin inhibitors and tubulin stabilizers [1–3], continues to broaden our knowledge base and provides advantages to the development of additional targeted therapy options including peptide drug conjugates, nanocarriers and viral drug delivery systems.

Randomly conjugated ADC, resulting in highly heterogeneous mixtures of drug product with varying numbers of payload conjugated to each antibody molecule at variable conjugation sites, have provided significant challenges for the analytical community. One of the challenges to the bioanalytical community is whether to develop assays that are sensitive or insensitive to the drug antibody ratio (DAR). There are a wide variety of opinions currently in debate, but there is yet to be a consensus on which is more correlative with safety and efficacy parameters. Furthermore, this may be different for different ADC. There are many valuable case studies in the literature that we can use to guide our evolving bioanalytical strategies for quantifying ADC [4–8], however, the complex nature of ADC along with the potential for activity modulating biotransformation events currently necessitates a multianalyte bioanalytical strategy comprised of measuring the intact ADC (Ab conjugated to payload), total Ab and unconjugated payload.

This manuscript describes the validation of the three ligand binding bioanalytical assays utilizing highly specific reagents piv-

FUTURE SCIENCE part of fsg

otal to our current bioanalytical strategy to measure total antibody, active ADC and total ADC. The total antibody (Ab) assay measures total circulating therapeutic antibody irrespective of the presence or absence of conjugated payload or payload metabolites.

[Total Ab] = [Active ADC] + [Inactive ADC] + [unconjugated Ab]

The active ADC assay measures free or partially free antibody (one or both arms of the antibody are not bound to soluble target [9]) conjugated to active payload. When developed for quantitative payload sensitivity, the active ADC ligand-binding assay (LBA) can be used to determine DAR and should be highly analogous to the antibody conjugated payload assay measured via immunocapture LC–MS/MS (IC-LC–MS/MS). The total ADC assay measures free or partially free antibody [9] conjugated to active payload or conjugated to inactive payload metabolites.

[Total ADC] = [Active ADC] + [Inactive ADC].

It is likely that site specific technologies will further impact bioanalytical strategies. As ADC and other multiple domain therapeutics become more homogeneous through the implementation of site specific conjugations [10,11], analytical methods for characterizing those therapeutics become less complex. Limited information is available in the literature for ADC that have payload that undergo modification exclusive from deconjugation as is the case discussed in this manuscript, preceding manuscripts [12,13] and manuscripts in preparation [Wang *et al.* Antibody–drug conjugate bioanalysis using lb-lc–ms/ms hybrid assays: strategies, methodology and complementarity to lba (2016), Submitted]. These biotransformation events can have significant impact on the potency of the molecule, making it important to differentiate between the resulting active and inactive analytes. Antipayload antibodies that specifically recognize the parental active version of the payload and the metabolized/inactive version of the payload greatly facilitate the measurement of pharmacologically relevant ADC.

Experimental

LBAs: total Ab, active ADC & total ADC

All of the LBAs were technically and operationally harmonized to improve efficiency and data integration as described in Myler *et al.* [12] and in the methods sections below. In brief, the same standards, quality controls (QC), patient samples, capture Ab and buffers were used for all analytes measured in the LBA assays. Distinct detection Abs were used to provide analyte specificity for the active ADC, total Ab, or total ADC analytes. A single, 20-µl aliquot was used to generate results for all three analytes. Additionally, the antibody conjugated payload immunocapture-LC–MS/MS assay described in Liu *et al.* [13] uses the same capture antibody as the three LBA to provide further continuity.

Nonclinical toxicokinetic methods

The nonclinical methods used to support the GLP toxicology studies utilized a sandwich immunoassay format and the standard MULTI-ARRAY® microplate (Meso Scale Discovery [MSD], Gaithersburg, MD) platform. Nonclinical method details are described in Myler *et al.* [12].

Clinical pharmacokinetic methods

The following instruments, chemicals, noncritical reagents and matrices were used: EZ-Link™ NHS-LC–LC-Biotin (Thermo Scientific, MA, USA), Alexa Fluor647 NHS ester (Succinimidyl Ester; Thermo Scientific, MA, USA), Human Serum, CD-1 mouse serum (Bioreclamation), 10% Tween-20 (Thermo Scientific, MA, USA), Glycine (JT Baker, PA, USA), 10% sodium dodecyl sulfate (SDS; Life Technologies, NY, USA), Immunoassay Diluent (ANP Tech., DE, USA), Milli-Q 'Synthesis' Water Treatment/Purification System (EMD Millipore, MA, USA), Stericup-GP, 0.22 µm, polyethersulfone, 1000/1000 ml, radiosterilized filters (EMD Millipore).

A mouse anti-idiotype (anti-Id) monoclonal antibody was biotinylated [12] and used as the capture reagent for all ADC analytes. The same anti-id Ab was also conjugated to Alexa-647 and used as a detection reagent for the total Ab assay. An antipayload mAb that binds specifically to only the active form of the payload (described above) was conjugated to Alexa-647 (Thermo Scientific, MA, USA) and used to detect the active ADC analyte. An antipayload mAb that binds to both the active and inactive form of the payload was conjugated to Alexa-647 NHS ester (Thermo Scientific, MA, USA) and used to detect the total ADC analyte.

The clinical method for quantitation of active ADC, total Ab and total ADC utilized Bioaffy 1000 microfluidic CDs containing streptavidin-coated columns with a Gyrolab xP Workstation (Gyros Inc, Uppsala, Sweden). Each automated Gyros run was executed using the Gyrolab control software (version 5.4). Three Gyrolab runs were utilized to measure the unique ADC analytes. Two wash solutions were used for all Gyrolab runs. Wash solution 1 consisted of 0.05% Tween 20 in PBS and was connected to station 1 and also used as the hydraulic solution for sample and reagent needles. Wash solution 2 consisted of 50 mM glycine, 0.5% SDS, pH 9.5 and was connected to wash station 2. All wash solutions were filtered prior to use. The minimum required dilutions (MRD) for all analytes were

prepared with immunoassay diluent containing 2% CD-1 mouse serum. The MRD for the active ADC assay was 1:20, total Ab assay was 1:50 and total ADC assay was 1:100. Total Ab and total ADC MRD were prepared from the active ADC MRD.

All Gyros runs were executed as laboratory information management system (LIMS) runs, and work lists were generated by PPD Laboratories® proprietary LIMS (Assist LIMS, Version 5.4). Gyros runs for the active ADC and total ADC analytes utilized a three-step Gyrolab method (Capture-Analyte-Detection) with a PMT gain of 5%. The capture Ab for the active ADC and total ADC analytes was diluted to 100 µg/ml in Immunoassay Diluent. Rexxip F Buffer (Gyros Inc.) was used to dilute the active ADC detection Ab (4 µg/ml) and the total ADC detection Ab (0.5 µg/ml). For the total Ab, a master mix containing 50 µg/ml biotin- and Alexa-647-conjugated anti-Id Ab was mixed with an equivalent volume of the total Ab MRD and then incubated for 30–120 min prior to loading sample plates and initiating the run on the Gyrolab xP workstation. The Gyros run for the total Ab analyte used a one-step Gyrolab method with PMT gain of 5% to deliver samples containing the bridged complexes to the CDs. A four parameter logistic regression curve with $1/Y^2$ weighting was used to calculate the concentrations of active ADC, total Ab, total ADC in the samples.

Method validation experimental design

Bioanalytical methods employed to support regulated studies such as IND-enabling toxicology studies and clinical studies are validated according to specified criteria as defined in various Bioanalytical Method Validation Guidance's and Guidelines [4,14–17]. Standard parameters to be assessed include: intra- and interassay accuracy and precision, selectivity, specificity, dilutional integrity and/or carryover, and stability. Acceptance criteria for these parameters can be found in the cited guidance's but generally consist of precision requirements of ≤20% coefficient of variation (%CV) and accuracy within ±20% bias (or difference from theoretical). Experimental design and criteria details described below pertain specifically to the clinical assay validation but are highly comparable to that used for the nonclinical assay validation.

Accuracy & precision

Intra- and inter-assay accuracy and precision are evaluated as described in DeSilva *et al.* [4]. All validation samples were prepared in 100% matrix prior to performing the MRD. Standard levels included: 100 (low anchor point), 200 (LLOQ), 400, 800, 1600, 3200, 6400, 12,800 (ULOQ) and 25,600 ng/ml (high anchor point). QC levels included: LLOQ at 200 ng/ml; low QC (LQC) at 600 ng/ml; mid QC (MQC) at 2000 ng/ml; high QC (HQC) at 10,000 ng/ml and ULOQ at 12,800 ng/ml. QCs at each level were analyzed ($n \geq 3$) in six separate runs each assay. The dilution QC at 128,000 ng/ml, was diluted 1:50 in 100% human serum prior to performing the MRD. The intra-assay and inter-assay precision (%CV) of the LLOQ was required to be ≤25.0%; precision at all other levels were required to be ≤20.0%. The intra-assay and interassay accuracy as assessed by % difference from theoretical (DFT) or bias at the LLOQ was required to be within ±25.0% and within ±20.0% at all other levels. Total error (precision + |accuracy|, or TE) was required to be ≤ 40.0% at the LLOQ and ≤30.0% at all other levels.

Selectivity

Matrix samples from at least ten individual human donors from each of the applicable patient populations including healthy human donors, ovarian cancer patients, mesothelioma patients, pancreatic cancer patients, non-small-cell lung cancer patients and gastric cancer patients were analyzed at blank, LLOQ and HQC levels to evaluate assay selectivity.

Eighty percent of the blank samples from each population were required to quantitate less than the LLOQ. For each population indicated above, at least 80.0% of the samples at the LLOQ level were required to quantitate within ±25.0% of the theoretical value, and at least 80.0% of the samples at the HQC level were required to quantitate within ± 20.0% of the theoretical value.

Runs containing selectivity samples must also contain pooled matrix controls prepared at equivalent concentrations (LLOQ and HQC). For each run containing selectivity samples at the LLOQ level, at least two of the three pooled matrix control replicates at the LLOQ were required to quantitate within ±25.0% of the theoretical value and have a %CV ≤25.0%. For each run containing selectivity samples at the HCQ level, at least two of the three pooled matrix control replicates were required to quantitate within ±20.0% of the theoretical and have a %CV ≤20.0%. Selectivity samples analyzed in runs containing pooled matrix control samples (at the same level) that failed to meet the above acceptance criteria were re-evaluated in a run with acceptable controls.

Selectivity testing was also done in hemolytic and lipemic samples. To evaluate the effect of sample hemolysis on study sample quantitation, low and high QCs were prepared in matrix hemolyzed to approximately 1100 mg/dl free hemoglobin and analyzed (n = 6 each level). To evaluate the effect of lipemia on study

sample quantitation, low and high QCs were prepared in lipemic matrix with >300 mg/dl triglycerides and analyzed (n = 6 each level). The %CV and mean accuracy was required to be ≤20.0% and within ±20.0%, respectively. The blank hemolyzed matrix was required to quantitate below the LLOQ.

Specificity

Anticipated supraphysiological levels of soluble target (0, 10.0, 30.0, 90.0 270, 810, 2430, 7290 ng/ml), inactive ADC (0.00, 400, 1200, 1800 ng/ml), unconjugated Ab (0.00, 400, 1200, 1800 ng/ml) and anti-drug antibody (ADA; 0.00, 16.0, 80.0, 400, 2000 and 10,000 ng/ml) were evaluated minimally at the blank, LQC and HQC levels to assess interference in the total antibody, total ADC and active ADC assays. Each interference sample (drug plus potential interfering factor), prepared in pooled serum, was incubated at 37°C for at 1–2 h and then frozen at -80°C ± 10°C prior to analysis. Noninterference of the compounds was demonstrated if the interference sample was within ±20.0% of the theoretical value. The ADA used in this experiment was an affinity purified polyclonal Ab derived from hyperimmunized rabbit serum. Rabbits were immunized with drug substance.

The inactive ADC reagent was a discovery grade reagent used to provide qualitative results and facilitate a better understanding of the specificity of each of the total Ab, total ADC and active ADC assays. Although quantitative acceptance criteria were not applied to the inactive ADC interference evaluations, inactive ADC concentrations were expected to be additive for the total Ab and total ADC assays. To demonstrate specificity of the active ADC assay for active analyte only, inactive ADC was required to not be measurable in blank control samples or to positively bias LQC and HQC samples. In order to determine a lack of interference of the inactive ADC (metabolite or impurity) in the active ADC assay, inactive ADC was required to not interfere in the quantitation of the active ADC high and low QCs.

The results for unconjugated Ab were expected to be additive for the total Ab assay only. In order to determine a lack of interference of the unconjugated Ab (metabolite or impurity) in the total ADC and active ADC assays, unconjugated Ab was required to not interfere in the quantitation of the total ADC and active ADC high and low QCs.

Stability

The following stability conditions were evaluated: long-term stability in frozen matrix, freeze–thaw stability, analyte stability in thawed matrix and processed sample stability. Stability samples were spiked at the HQC, LQC and Dil QC levels in pooled normal human serum. For all stability tests five values were required to calculate stability statistics for each QC level tested and the %CV and the mean accuracy of the replicate determinations were required to be ≤20.0% and within ± 20.0%, respectively.

The effect of freezing the analyte in biological matrix and storing samples in this condition over an extended period of time was evaluated by analyzing low, high, and Dil QCs (n = 6) stored in a cryofreezer to support cryofreezer storage and stored in a freezer to support freezer storage. Long-term stability ≥12 months is ongoing.

The influence of the physical processes of freezing and thawing on analyte stability in biological matrix was evaluated by subjecting low, high, and Dil QCs (n = 6) to eight freeze–thaw cycles (8FT). For the first freeze–thaw cycle, samples were maintained at -80°C for a minimum of 24 h and for subsequent cycles, the samples are held in the freezer for at least 12 h. Following each freezer cycle, the samples are removed from the freezer until completely thawed.

To evaluate whether holding samples in a thawed state at room temperature for at least 6 h or on wet ice for at least 24 h adversely affects analyte stability in biological matrix, low, high and Dil QCs (n = 6) were maintained frozen for a minimum of 24 h and then thawed and maintained at room temperature for at least 6 h or on wet ice for at least 24 h.

To evaluate whether holding samples that have been diluted in the minimum required dilution (MRD) buffer adversely affects analyte stability, low, high and Dil QCs (n = 6) were diluted to the MRD with MRD buffer and then maintained at least 16 h at room temperature. Additional sets of processed stability samples (n = 6) were diluted to the MRD in MRD buffer and maintained for 24 and 48 h at 2–8°C.

Dilutional integrity & hook effect

The ability to dilute study samples >ULOQ was evaluated by preparing a Dil QC at or above the estimated maximum concentration (C_{max}) of ADC in study samples and diluting it into assay range. The Dil QC was prepared by spiking reference material into pooled normal human serum to achieve a concentration of 128,000 ng/ml drug in at least 98% serum. Serial dilutions were prepared by diluting the Dil QC in 100% pooled normal human serum prior to diluting to the MRD. The dilution series extended above and into the range of quantitation and were analyzed at 128,000 ng/ml (Dil 1), 32,000 ng/ml (Dil 4), 8000 ng/ml (Dil 16), 2000 ng/ml (Dil 64) and 500 ng/ml (Dil 256).

The %CV and the mean accuracy for each dilution QC replicate was required to be ≤20.0% for those dilu-

tions within the range of quantitation. For dilutions where the expected response after dilution is above the highest calibration curve point, the result was required to read above the highest calibration standard or greater than the ULOQ. Samples with concentrations of analyte greater than the ULOQ that quantitate within the range of quantitation are indicative of a prozone or hook effect.

Carryover

To evaluate the potential carryover of transfer needles, a QC pool prepared at the LLOQ and diluted to the MRD in MRD buffer was transferred from the sample plate to the CD by all eight sample needles (one replicate; 2 CD structures) before and immediately after each needle had transferred one replicate (two CD structures) of a Dil QC sample (128,000 ng/ml) from the sample plate to the Gyros CD. Thee-fourths of the LLOQ samples transferred before and after the Dil QC were required to have %CV and mean accuracy values ≤25.0% and within ± 25.0%, respectively.

DAR sensitivity

Due to the discovery grade quality of the DAR reagents, DAR sensitivity testing was done outside of the formal validation as part of method development to further characterize the active ADC assay which is the assay thought to best correlate with safety and efficacy. The standards and QCs were prepared from reference material (mean DAR 3.0) and drug substance with mean DAR of 3.9, 2.0, 1.7 and 0.8 were evaluated at the low, middle and high ends of the standard curve. Purified DAR species testing is in progress and will be included in a subsequent manuscript.

Results & discussion

The bioanalytical strategy consisted of several independent measures of the ADC including: total antibody, active ADC, total ADC, Ab-conjugated payload, unconjugated payload, soluble/shed target, a panel of biomarkers indicative of programmed cell death and a series of immunogenicity assays designed to elucidate antidrug antibody specificity and is described in Myler *et al.* [12]. The three LBA used to assess PK were technically and operationally harmonized to maximize functionality and data integration. To further increase efficiency in the clinical setting, the platform was changed from a plate-based electrochemiluminescense platform to a CD-based microfluidics platform that required only a single, 20 µl aliquot to generate results for the three analytes. The same standards, QCs, patient samples, capture Ab and buffers were used for all analytes. Distinct detection Abs were used to provide specificity for the active ADC, total Ab, or total ADC analytes. Each sample was diluted to the analyte-specific MRD and was run in accordance with Figure 1. The diluted samples were loaded onto the Gyrolab and analyzed for active ADC, total Ab and total ADC in series. The bridging incubation for the total Ab occurred during the active ADC run time so as to eliminate down time due to incubation steps. Each CD has a run time of 1 h, enabling the generation of approximately 200 results in 7 h by a single analyst (6-h run time and 1-h sample preparation).

Validation results

The assays described herein were validated in accordance with current global regulatory guidance's [14–17] and are consistent with applicable white papers [7].

Accuracy & precision

The assay range for the three clinical assays (LLOQ at 200 ng/ml to ULOQ at 12,800 ng/ml) was established and validated so that clinical sample dilution was minimized, thereby maximizing operational efficiency.

Table 1 provides a summary of the clinical validation accuracy and precision data for the active ADC, total Ab and total ADC assays. For all 3 LBA, total error ranged from 4.4 to 21.4, %Bias ranged from -2.6 to 13.8 and %CV (interassay) ranged from 3.8 to 10.7. For the active ADC alone, total error ranged from 6.6 to 16.8, %Bias ranged from 2.0 to 8.9 and %CV (interassay) ranged from 3.8 to 7.9, well within the acceptable limits per regulatory guidelines. Supplementary Table 1 provides a validation summary for the nonclinical validation of the total Ab and active ADC assays in rat and monkey serum. For all LBA in both nonclinical species matrix, total error ranged from 3 to 15, %Bias ranged from -8 to 10 and %CV (interassay) ranged from 3 to 9. All parameters tested including accuracy and precision, robustness, hook effect, dilutional integrity, selectivity, specificity and stability met acceptance criteria. Additionally, 54/54 (100%) of the total Ab and 52/54 (96%) of the active ADC samples met incurred sample reanalysis (ISR) criteria, were within 30% of the original value.

Selectivity

Over 80% of samples prepared at LLOQ & HQC levels in healthy human donor, ovarian cancer, mesothelioma, pancreatic cancer, non-small-cell lung cancer and gastric cancer patient serum met acceptance criteria, within ±25% bias at LLOQ and ±20% bias at HQC; 100% of blank samples < LLOQ (Figure 2). There was no effect from hemolysis, ≥1100 mg/dl of hemoglobin, or lipemia, >300 mg/dl triglycerides, on the quantitation of the active ADC, total Ab, or total ADC (data not shown).

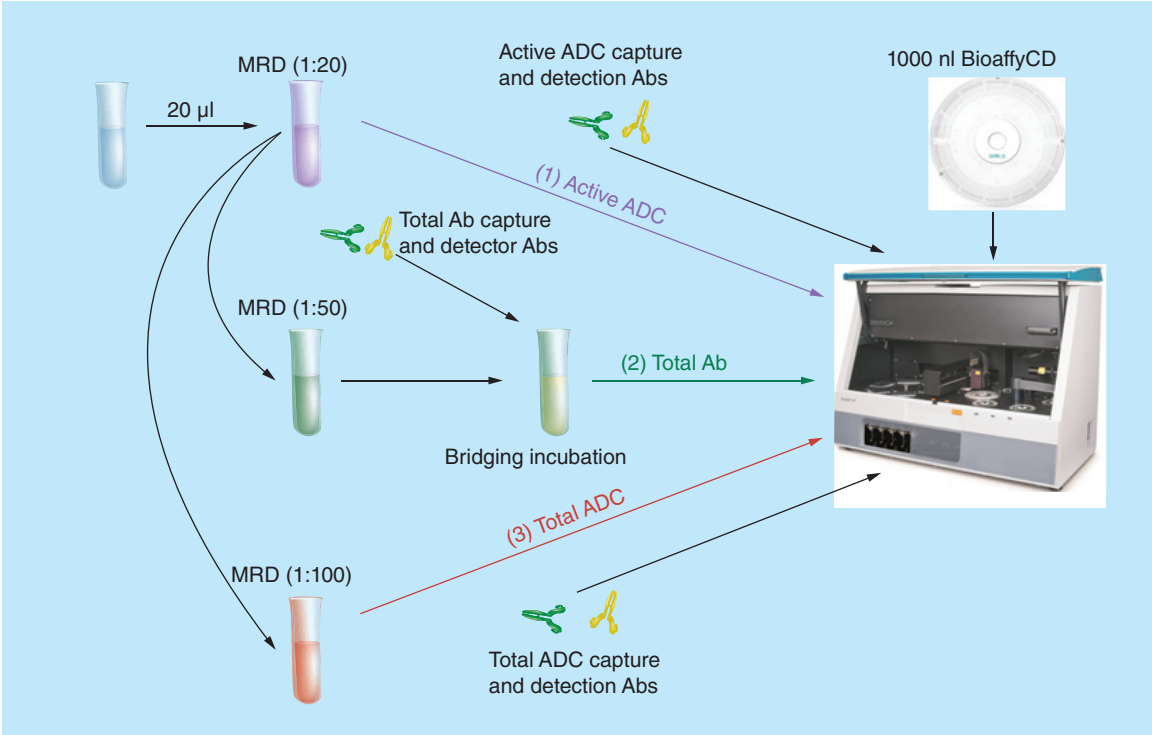

Figure 1. Automated Gyrolab™ xP workstation enabled development of single, multianalyte PK Method: 192 results in 7 h by one analyst.

Specificity

Target sensitivity

The relevance of soluble and/or shed target levels in the ADC PK assays are described in greater detail in Myler *et al.* [12]. The clinical assay validation results for this parameter are described herein. Shed target has no impact on samples that do not contain ADC. In the active ADC and total Ab assays, no interference was observed for the LQC with soluble target ≤90 ng/ml (10, 30 and 90 ng/ml); negative bias was observed for LQC spiked with soluble target ≥270 ng/ml (270, 810, 2430 and 7290 ng/ml). No interference was observed for HQC spiked with soluble target ≤810 ng/ml (10, 30, 90, 270 and 810 ng/ml); negative bias observed for HQC spiked with soluble target at 2430 and 7290 ng/ml. In the total ADC assay no interference was observed for the LQC spiked with soluble target ≤270 ng/ml (10, 30, 90 and 270 ng/ml); negative bias was observed for LQC spiked with soluble target ≥810 ng/ml (810, 2430 and 7290 ng/ml). No interference was observed for HQC spiked with soluble target ≤2430 ng/ml (10, 30, 90, 270, 810 and 2430 ng/ml); negative bias was observed for HQC spiked with soluble target at 7290 ng/ml. As discussed previously, the measurement of free (at least one Ab arm not bound to target) active (nonmetabolized payload) ADC is the desired measurement as this allows for a more accurate determination of bioactive ADC.

Anti-drug antibody interference

ADA impact on PK is assessed during method validation by spiking an ADA positive control into high and low quality controls. The impact of ADA on exposure response analysis is described in the literature and was recently addressed by the global bioanalytical consortium [18]. No interference was observed at the blank or HQC spiked with ADA up to 10,000 ng/ml (16, 80, 400, 2000 and 10,000 ng/ml). In the active ADC assay, no interference was observed for LQC spiked with ADA ≤400 ng/ml (16, 80 and 400 ng/ml); negative bias was observed for LQC spiked with ADA at 2000 and 10,000 ng/ml. In the total Ab and total ADC assays, no interference observed for LQC spiked with ADA ≤2000 ng/ml (16, 80, 400 and 2000 ng/ml); negative bias was observed for LQC spiked with ADA at 10,000 ng/ml.

Analyte specificity & impurity/metabolite tolerance

To test the specificity of the active ADC, total Ab and total ADC assays, drug product with a mean DAR of 3.0 was spiked with increasing levels of inactive ADC that had chemically inactivated payload or unconjugated Ab that had no payload. Inactive ADC should be detectable in the total Ab and total ADC assays only. Unconjugated Ab should only be detectable in the total Ab assay. Impurity and or metabolite levels were

Table 1. Clinical assay accuracy and precision.

Analyte	Sample	Nominal conc. (ng/ml)	Precision		Accuracy	Total error
			Intra-assay %CV	Interassay %CV	Mean %Bias	(Mean %Bias) + Interbatch %CV
Active ADC	LLOQ	200	3.8	3.8	3.4	7.2
	LQC	600	5.9	6.2	2.6	8.7
	MQC	2000	4.5	4.5	2.1	6.6
	HQC	10,000	6.4	6.8	5.3	12.0
	ULOQ	12,800	4.5	5.2	2.0	7.2
	Dil QC	128,000	2.8	7.9	8.9	16.8
Total Ab	LLOQ	200	6.4	9.4	-2.6	12.0
	LQC	600	4.8	6.6	2.6	9.2
	MQC	2000	5.1	5.1	2.9	8.0
	HQC	10,000	3.2	4.7	3.1	7.9
	ULOQ	12,800	1.9	3.9	0.5	4.4
	Dil QC	128,000	2.1	6.8	5.8	12.5
Total ADC	LLOQ	200	3.1	4.3	3.2	7.5
	LQC	600	3.4	4.9	3.6	8.5
	MQC	2000	4.9	5.7	1.7	7.4
	HQC	10,000	4.2	7.6	13.8	21.4
	ULOQ	12,800	5.1	10.7	10.0	20.7
	Dil QC	128,000	3.5	9.8	10.5	20.3

n = 18, 6 runs, three replicates each run.
%CV = (standard deviation/mean) ×100;
%Bias = (mean calculated concentration-nominal concentration)/nominal concentration × 100 or;
%Bias = (mean calculated concentration/ nominal concentration)-1] × 100 (calculation used in above data table).

selected based upon the nonclinical primate model where active ADC was shown to comprise 20–25% of the heterogeneous mixture 504 h after dosing [12].

The presence of inactive ADC or unconjugated Ab was not expected to interfere with or positively bias the quantitation of the active ADC analyte, because the detection Ab for active ADC assay is specific for the active form of payload. The results presented in Table 2 show that all blank samples spiked with increasing levels of inactive ADC or unconjugated Ab impurity or metabolite quantitated below the LLOQ, and that all LQC and HQC samples spiked with increasing levels of impurity or metabolite quantitated within ± 20% of the nominal ADC concentration, demonstrating specificity for the active payload and insignificant interference from unconjugated Ab and inactive ADC when spiked at up to 75% of the heterogeneous mixture.

Inactive ADC and unconjugated Ab were both expected to be measurable in the total Ab assay. The expected result for the total Ab assay spiked with unconjugated Ab was expected to be equal to the sum of the nominal concentration of ADC and unconjugated Ab. The results presented in Table 3 show that all samples spiked with increasing levels of unconjugated Ab quantitated within ± 20% of the expected result [ADC + unconjugated Ab], demonstrating equal quantitation of unconjugated Ab and Ab conjugated to payload (mean DAR of 3.0) in the total Ab assay at up to 75% unconjugated Ab impurity or metabolite. The concentration of unconjugated Ab was not accounted for in the analytical specifications for the inactive ADC reagent. Thus, over-recovery was anticipated in the total Ab assay and only qualitative data was reported for this impurity. Results presented in Table 3 indicate that increasing concentrations of the inactive ADC in samples prepared at blank, low and high QC levels resulted in cumulative increases in measured concentrations of total Ab. It is understood that higher levels of unconjugated Ab impurity exist in this formulation than were accounted for in the specifications and/or calculations, thus resulting in over-recovery in the total Ab assay.

The presence of unconjugated Ab was not expected to interfere with or positively bias the quantitation of the total ADC analyte, because the detection Ab for total ADC assay is specific for the payload. The results

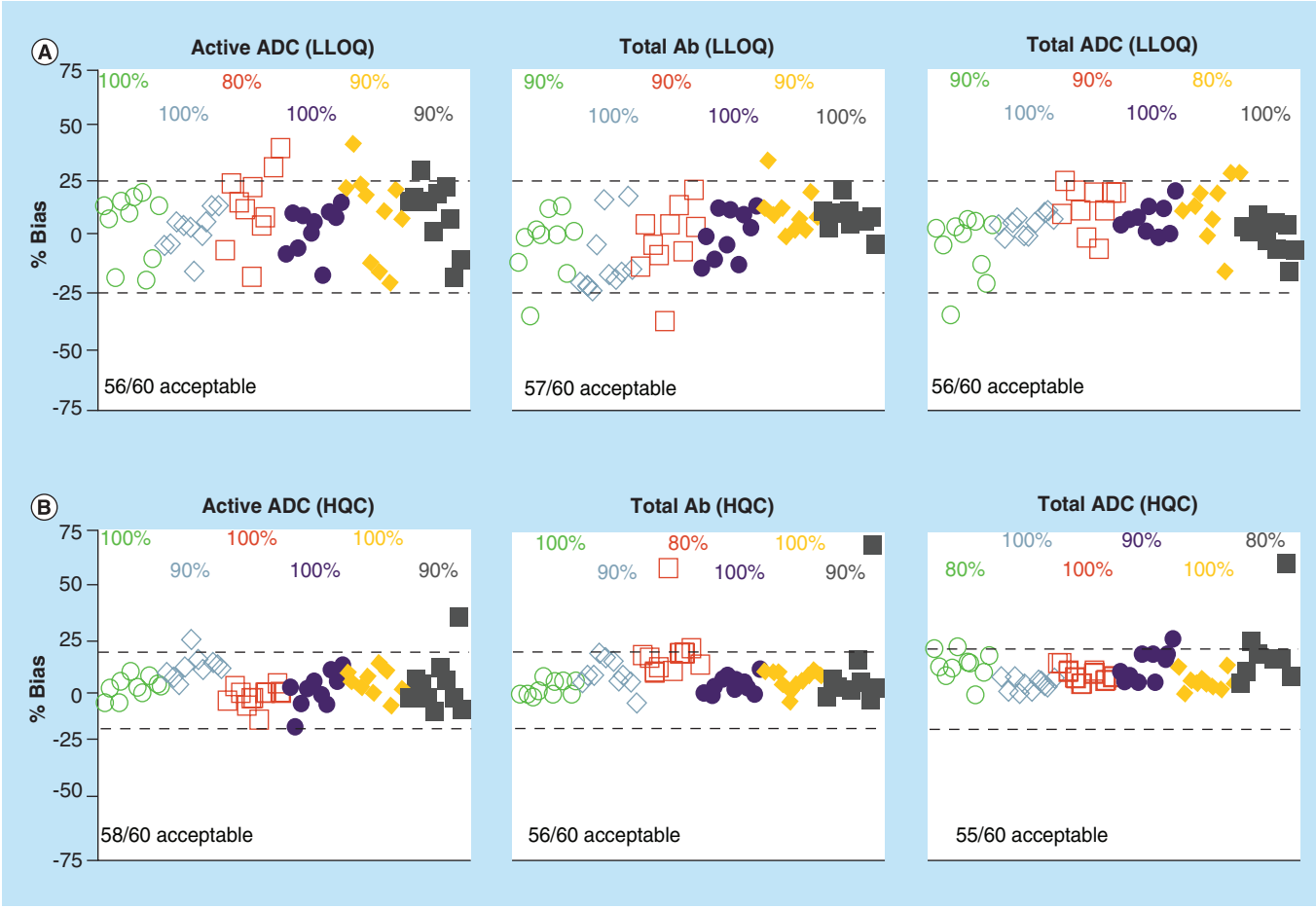

Figure 2. Clinical assay selectivity. Open circles indicate ovarian cancer; open diamonds indicates mesothelioma; open squares indicate non-small-cell lung cancer; closed circles indicate pancreatic cancer; closed diamonds indicate gastric cancer; closed squares indicate healthy human serum. In each panel, the color-matched percentages listed above each population denote the percent of individuals with acceptable %Bias. Overall results for all individuals are listed at the bottom of each graph.

presented in Table 4 show that all blank samples spiked with increasing levels of unconjugated Ab quantitated below the LLOQ, and that all LQC and HQC samples spiked with increasing levels of this impurity/metabolite quantitated within ±20% of the nominal ADC concentration, demonstrating specificity for Ab conjugated to payload and insignificant interference from unconjugated Ab impurity/metabolite at up to 75% of the heterogeneous mixture. Although quantitative acceptance criteria were not applied to inactive ADC interference evaluations due to the discovery grade nature of this reagent, as described above, the result of increasing inactive ADC concentrations was expected to be additive in the total ADC assay. Results presented in Table 4 indicate that increasing concentrations of the inactive ADC in samples prepared at blank, low, and high QC levels resulted in cumulative increases in measured concentrations of total ADC. The DAR normalized data are shown and indicate acceptable % bias; however, these data are only included for informational purposes as quantitative criteria were not applied in the validation. DAR normalization is discussed in greater detail in the DAR sensitivity section below.

In summary, the total Ab assay, Table 3, is DAR independent and thus does not require normalization based upon DAR. The active ADC and total ADC assays are DAR dependent or DAR sensitive, and thus do require normalization based upon DAR (Table 4). The active ADC and total ADC assays are insensitive to unconjugated Ab and thus the presumed additional unconjugated Ab in the inactive ADC reagent has no impact on the these assays. The total Ab assay, however, is sensitive to unconjugated Ab thus the over-recovery due to presumed levels of unconjugated Ab impurity.

Analyte stability

A set of low, high and Dil QCs (n = 3 vials each) were tested in each assay, active ADC, total Ab and total ADC, under a variety of conditions to establish analyte stability in the applicable matrix including: frozen serum stability (ongoing at -70°C and -20°C),

Table 2. Active ADC assay specificity: impact of impurities and metabolites.

Spiked conc. (ng/ml)			Expected result (ng/ml)	Observed result (ng/ml)	%Bias from expected
ADC (DAR 3.0)	Unconjugated Ab	Inactive ADC			
0	400	0	0	<LLOQ	N/A
0	1800	0	0	<LLOQ	N/A
0	0	400	0	<LLOQ	N/A
0	0	1800	0	<LLOQ	N/A
600	400	0	600	573	-4.5
600	1800	0	600	597	-0.5
600	0	400	600	647	7.9
600	0	1800	600	581	-3.1
10,000	400	0	10,000	9044	-9.6
10,000	1800	0	10,000	8686	-13.1
10,000	0	400	10,000	9349	-6.5
10,000	0	1800	10,000	8339	-16.6

The active ADC assay measures Ab conjugated to active payload only with insignificant interference from up to 75% unconjugated Ab and inactive ADC metabolites and/or impurity.

thawed serum stability (6 h at RT and 24 h on wet ice), processed sample stability (post MRD; 16 h at RT and 48 h at 2–8°C), and freeze–thaw stability (eight cycles). All sample data met the criteria, within ± 20% bias, for demonstrating stability under these conditions. The %Bias data is summarized in Table 5. Stability in the active ADC assay shows that the payload is not hydrolyzed nor is the linker cleaved under these conditions. Similarly, stability in the total ADC assay supports that the linker is not cleaved under these conditions. Stability in the total Ab assay indicates that the target binding region is still intact. These stability data have been cumulatively graphed in Supplementary Figure 1 to show the comparability of stability in each of the assays for each of the analytes.

Table 3. Total antibody assay specificity: impact of impurities and metabolites.

Spiked conc. (ng/ml)			Expected result (ng/ml)	Observed result (ng/ml)	%Bias from expected
ADC (DAR 3.0)	Unconjugated Ab	Inactive ADC			
0	400	0	400	475	18.9
0	1800	0	1800	2051	13.9
0	0	400	400	574	Detectable
0	0	1800	1800	2258	Detectable
600	400	0	1000	1025	2.5
600	1800	0	2400	2557	6.6
600	0	400	1000	1250	Detectable
600	0	1800	2400	2907	Detectable
10,000	400	0	10,400	11,788	13.3
10,000	1800	0	11,800	12,970	9.9
10,000	0	400	10,400	11,762	Detectable
10,000	0	1800	11,800	13,534	Detectable

The total antibody assay measures all Ab related species, Ab conjugated to active payload (active ADC), Ab conjugated to inactive payload (inactive ADC) and Ab w/ no payload (unconjugated Ab).
Over-recovery of the inactive ADC is attributed to levels of unconjugated Ab impurity that were not accounted for in the specifications. Quantitative criteria were not applied to this analyte during validation.

Table 4. Total ADC assay specificity: impact of impurities and metabolites.

Spiked conc. (ng/ml)			Expected result (ng/ml)	Observed result (ng/ml)	%Bias from expected
ADC (DAR 3.0)	Unconjugated Ab	Inactive ADC (DAR 2.2)			
0	400	0	<LLOQ	<LLOQ	N/A
0	1800	0	<LLOQ	<LLOQ	N/A
0	0	400	293	287	-2.0
0	0	1800	1320	1066	-19.2
600	400	0	600	576	-4.0
600	1800	0	600	645	7.5
600	0	400	893	911	2.0
600	0	1800	1920	1630	-15.1
10,000	400	0	10,000	11,255	12.6
10,000	1800	0	10,000	11,216	12.2
10,000	0	400	10,293	11,445	11.2
10,000	0	1800	11,320	12,116	7.0

The total ADC assay measures Ab conjugated to active and inactive payload with insignificant interference from up to 75% unconjugated Ab metabolites and/or impurity.
Quantitative criteria were not applied to the inactive ADC analyte during validation due to the discovery grade nature of this reagent. The expected results are normalized for DAR 2.2 calibrated to the DAR 3.0 calibration curve. The impact of DAR on ADC quantitation is discussed in greater detail below.

Dilutional integrity & Hook effect

The Dil QC containing 128,000 ng/ml was assessed undiluted and at 4-, 16-, 64- and 256-fold dilutions. All results within the assay range, that is diluted at least 16-fold in 100% human serum prior to the MRD, were within ± 20% bias in all three assays. Dilutions where the concentration after dilution were above the highest calibration curve point, were >ULOQ, thus there was no 'hook effect' observed at concentrations up to 128,000 ng/ml (data not shown).

Carryover

The potential carryover of transfer needles was evaluated by transferring LLOQ QC samples from the sample plate to the CD by all eight sample needles before and immediately after each needle transferred one replicate of Dil QC (128,000 ng/ml) from the sample plate to the CD. As shown in online available Supplementary Table 2, there was no evidence of carryover in any of the three assays (active ADC data shown).

DAR sensitivity

DAR sensitivity was tested in the active ADC assay by spiking nominal low or high concentrations of various DAR species into the assay including mean DAR 0.8, 1.7, 2.0 and 3.9, normalizing the results for DAR and assessing the %Bias. As shown in Table 6, the active ADC assay is shown to be quantitatively proportional for each DAR species. For example, DAR 0.8 is 27% of DAR 3.0, the calibrator DAR, thus the normalized expected result for a DAR 0.8 species would be 27% of the nominal concentration. At a nominal concentration of 1570 ng/ml DAR 3.0, DAR 0.8 would be expected to yield 27% of that or 419 ng/ml. The observed result for these conditions was 432 ng/ml resulting in a 3% bias. DAR species, 0.8 through 3.9, were tested at nominal concentrations of 1570 ng/ml and 6820 ng/ml, translating to 419 through 8866 ng/ml once DAR normalized. All except one of the conditions tested had %bias within ±20%. Due to the discovery grade quality of these DAR species, these evaluations were done in method development and were not performed during method validation. The characterization of DAR species, including the distribution around the mean DAR value is likely to impact the quality of associated results, so caution should be taken when carrying out these experiments and analyzing associated data.

In addition to the *in vitro* testing of individual DAR species discussed above, *in vivo* testing also shows the active ADC assay to be quantitatively DAR proportional as demonstrated by the parallelism to the Ab-conjugated payload immunocapture LC–MS/MS (Supplementary Table 3). More detail on this can be found in Myler *et al.*

Conclusion

The inherent complexity of randomly conjugated ADC, exacerbated by *in vivo* processing, necessitates

Table 5. Active ADC, total Ab and total ADC stability.

	%Bias								
	Active ADC			Total Ab			Total ADC		
	LQC	HQC	Dil QC	LQC	HQC	Dil QC	LQC	HQC	Dil QC
Freeze–thaw, eight cycles	1.0	9.1	19.9	14.5	3.0	12.2	5.0	3.1	12.8
Thawed serum, wet ice, 24 h	1.4	10.5	13.6	6.5	17.9	17.9	2.1	7.1	3.5
Thawed serum, RT, 6 h	-1.3	7.6	12.6	12.1	0.8	11.4	2.4	2.8	4.3
Processed sample, ≥16 h, RT	4.8	2.0	6.8	12.2	8.1	12.1	5.1	7.2	10.1
Processed sample, ≥48 h, 2–8°C	-1.6	-0.9	8.7	5.5	12.0	8.5	3.5	0.1	8.8
Frozen serum, 39 days, -25°C ± 5 C	6.2	-7.0	7.4	0.8	7.2	12.3	3.6	8.6	12.7
Frozen serum, 40 days, -80°C ± 10°C	3.8	-5.0	7.6	-1.8	5.7	11.7	2.0	1.8	8.7

the implementation of a series of bioanalytical methods to adequately describe the pharmacological and PK properties. Each assay described herein provides information about a particular ADC species present in circulation at any given time. It is important to understand the quantitative relationships among the different ADC species which, in combination with the bioanalytical data, can be accomplished through PK modeling and simulation approaches [19,20].

The automated microfluidic Gyrolab platform allowed for the development and validation of a single method that could be run by a single analyst to quantitate all three ADC analytes, active ADC, total Ab and total ADC, in series in under 4 h using a single 20 µl sample (Figure 1). This semiautomated, single method, multianalyte approach offered efficiency gains over other more laborious methods by reducing the documentation and perhaps more significantly reducing the sample processing burden while substantially increasing analyst throughput. The assays were determined to meet all validation criteria as described in the health authority guidance's on bioanalytical method validation [14–17].

Future perspective

As the number of ADC in the clinic is increasing, bioanalytical strategies to identify and quantitate ADC analytes for PK analysis continues to evolve. At different stages of ADC drug development, the need for essential bioanalytical methods varies. We are still identifying what is needed when but in general, the focus of bioanalytical assays in the early drug discovery stage is to provide exposure information, metabolism information, toxicokinetic (TK) characterization, and to predict toxicity and efficacy in humans. Understanding these parameters can assist in the selection of a lead ADC molecule whereas in the later nonclinical and clinical drug development stages, bioanalytical focus shifts to providing regulatory compliant PK/TK data for a comprehensive understanding of the pharmacology and toxicology of the therapeutic.

Due to the highly heterogeneous nature of randomly conjugated ADCs, a combination of LBA and LC–MS/MS-based bioanalytical assays are used to support ADC programs. If random conjugation transitions to site-specific conjugation for ADC, some of the bioanalytical considerations related to DAR that we have addressed herein, such as DAR dynamics, may become unnecessary. LBA assays due to their simplicity, throughput, accuracy and reproducibility will continue to play a major role in ADC bio-

Table 6. DAR sensitivity

DAR	0.8	1.7	2.0	3.9
% of DAR 3.0	27%	57%	67%	130%
Nominal conc. (ng/ml)			1570	
DAR normalized conc. (ng/ml)	419	890	1047	2041
Result (ng/ml)	432	928	1165	2385
%Bias of normalized result	3	4	11	17
Nominal conc. (ng/ml)			6820	
DAR normalized conc. (ng/ml)	1819	3865	4547	8866
Result (ng/ml)	1680	4210	5095	11200
%Bias of normalized result	-8	9	12	26

DAR normalized concentration indicates that the concentration was normalized to the DAR 3.0 calibrator. For example, DAR 0.8 is 27% of DAR 3.0 (calibrator DAR) and 419 ng/ml (DAR 0.8) is 27% of 1570 ng/ml (DAR 3.0).

analysis. Compared to the conventional colorimetric (ELISA) platforms, second and third generation platforms including chemiluminescence, ECL and fluorescent microfluidics offer better sensitivity, a broader dynamic range, and decreased sample volume; some come with automation and/or multiplexing options. As new classes of payload are being evaluated, we will continue to generate highly specific reagents, such as that described herein, to support the PK and immunogenicity assessment of these new classes. These reagents are used across the pipeline where a specific payload is employed. This reuse potential makes these reagents quite cost effective.

Tracing the biotransformation of payload associated with ADC in circulation continues to be of utmost importance. Immuno capture based LC–MS conjugated payload assays are going to continue to be critical to evaluate the *in vitro* and *in vivo* serum stability of ADC and to help identify pertinent metabolites. As more fully discussed in Myler *et al.*, an integrated set of assays can be used to quantify the most prevalent and relevant analytes [12].

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/full/10.4155/bio.16.13

Financial & competing interests disclosure

The authors have 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.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Unique bioanalytical strategies are needed to identify, characterize and quantify the ADC species relevant to safety and efficacy.
- Reagents that allow for the derivation of active and inactive ADC are a crucial part of our bioanalytical PK strategy which includes the measurement of total antibody, active ADC and total ADC by LBA, conjugated payload by IC-LC–MS/MS and unconjugated payload by LC–MS.
- All LBA were validated according to current regulatory guidelines for bioanalytical method development to support nonclinical IND-enabling toxicology studies and clinical studies.
- All validation criteria were met and additional characterization data were generated to enhance assay and PK understanding.
- Given the accuracy of the active ADC assay in proportionally measuring payload conjugated to antibody, active ADC/total Ab can be used to assess DAR.
- The number of assays used to characterize ADC PK may be reduced to only include the most value added endpoints following the evaluation of sufficient clinical data.
- Clinical ligand-binding assays were automated using a miniaturized platform to optimize robustness and throughput.
- Minimal sample processing, reproducible results.
- One analyst can generate 192 results (64/assay) each 7-h shift, equivalent to 1 result every 2 min.

References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

1 Diamantis N, Banerji U. Antibody–drug conjugates – an emerging class of cancer treatment. *Br. J. Cancer* doi: 10.1038/bjc.2015.435 (2016) (Epub ahead of print).

2 Peters C, Brown S. Antibody–drug conjugates as novel anti-cancer chemotherapeutics. *Biosci. Rep.* 35(4), 1–20 (2015).

3 Perez HL, Cardarelli PM, Deshpande S *et al.* Antibody–drug conjugates: current status and future directions. *Drug Discov. Today* 19(7), 869–881 (2014).

4 DeSilva B, Smith W, Weiner R *et al.* Recommendations for the bioanalytical method validation of ligand-binding assays to support pharmacokinetic assessments of macromolecules. *Pharm. Res.* 20, 1885–1900 (2003).

•• **High-impact manuscript describing requirements for biotherapeutic bioanalytical method validation.**

5 Myler HA, Given A, Kolz K *et al.* Biotherapeutic bioanalysis: a multi-indication case study review. *Bioanalysis* 3(6), 623–643 (2011).

6 Kaur S, Xu K, Saad OM *et al.* Bioanalytical assay strategies for the development of antibody–drug conjugate biotherapeutics. *Bioanalysis* 5(2), 201–226 (2013).

•• **High impact manuscript describing the potential ADC species and associated bioanalytical strategies employed to understand drug disposition.**

7 Gorovits B, Alley SC, Bilic S *et al.* 2011 Bioanalysis of antibody–drug conjugates: American Association of Pharmaceutical Scientists Antibody–Drug Conjugate Working Group position paper. *Bioanalysis* 5(9), 997–1006 (2013).

• **Collaborative manuscript whereby many key opinion leaders across industry have addressed bioanalytical challenges for ADC.**

8 Hamblett KJ, Senter PD, Chace DF *et al.* Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate. *Clin. Cancer Res.* 10(20), 7063–7070 (2004).

9 Lee JW, Kelley M, King LE *et al.* Bioanalytical approaches to quantify "total" and "free" therapeutic antibodies and their targets: technical challenges and PK/PD applications over the course of drug development. *AAPS J.* 13(1), 99–110 (2011).

• **Describes the importance of fit-for-purpose assay design, specifically with respect to target-therapeutic binding status.**

10 Cho H, Daniel T, Buechler YJ *et al.* Optimized clinical performance of growth hormone with an expanded genetic code. *Proc. Natl Acad. Sci. USA* 108(22), 9060–9065 (2011).

11 Sukumaran S, Gadkar K, Zhang C *et al.* Mechanism-based pharmacokinetic/pharmacodynamic model for THIOMAB™ drug conjugates. *Pharm. Res.* 32(6), 1884–1893 (2014).

12 Myler H, Rangan V, Wang J *et al.* An integrated multiplatform bioanalytical strategy for antibody–drug conjugates: a novel case study. *Bioanalysis* 7(13), 1569–1582 (2015).

•• **Preceding manuscript describing the potential ADC species and associated bioanalytical strategies employed to understand drug disposition.**

13 Liu A, Kozhich A, Passmore D *et al.* Quantitative bioanalysis of antibody-conjugated payload in monkeyplasma using a hybrid immuno-capture LC–MS/MS approach: assay development, validation, and a case study. *J. Chromatogr. B* 1002, 54–62 (2015).

14 US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research. Guidance for Industry: Bioanalytical Method Validation. Rockville, MD (2013). www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm368107.pdf

15 European Medicines Agency. Guideline on Bioanalytical Method Validation. London, England (2011). www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf

16 Japan Ministry of Health and Labor Welfare. Guideline on Bioanalytical Method (Ligand Binding Assay) Validation in Pharmaceutical Development (2014). www.nihs.go.jp/drug/BMV/BMV-LBA_draft_140124_E_rev.pdf

17 China Pharmacopoeia (ChP). Draft Guidance on Bioanalytical Method Validation (2014). http://knex2014.europeanbioanalysisforum.eu/site/ebf_knex2014/assets/slides/Dafang-Zhong--Guidelines-on-BMV-in-China.pdf

18 Sailstad JM, Amaravadi L, Clements-Egan A *et al.* A white paper–consensus and recommendations of a global harmonization team on assessing the impact of immunogenicity on pharmacokinetic measurements. *AAPS J.* 16(3), 488–498 (2014).

19 Gibiansky L, Gibiansky E. Target-mediated drug disposition model and its approximations for antibody–drug conjugates. *J. Pharmacokinet. Pharmacodyn.* 41, 35–47 (2014).

20 Lu D, Gibiansky L, Jin JY *et al.* Integrated pharmacokinetic model for antibody–drug-conjugate (ADC) in patients with B-cell malignancy: implications for optimal sampling. *J. Pharmacokinet. Pharmacodyn.* 41, S44 (2014).

Bioanalysis

Antibody–drug conjugates nonclinical support: from early to late nonclinical bioanalysis using ligand-binding assays

Seema Kumar is a Principal Scientist at Pfizer. She leads a group that provides regulated bioanalytical support including assay development, validation and sample analysis for the PK and immunogenicity assessment for preclinical and clinical development of Pfizer's biotherapeutics portfolio. She is also responsible for scientific oversight of regulated studies outsourced at CROs. Prior to Pfizer, Dr Kumar held a similar role as Director of CLIA certified Clinical Bioanalytical Laboratory at XBiotech USA, Inc. She holds a PhD in Biophysical Chemistry from Johns Hopkins University, and has published several publications in peer-reviewed journals, and contributed to book chapters.

Seema Kumar
Author for correspondence:
Department of Pharmacokinetics, Dynamics & Metabolism, Pfizer Global R&D, One Burtt Road, Andover, MA 01810, USA
Tel.: +1 978 247 1856
seema.kumar@pfizer.com

Lindsay E King
Department of Pharmacokinetics, Dynamics & Metabolism, Pfizer Global R&D, Eastern Point Road, Groton, CT 06340, USA

Tracey H Clark
Department of Pharmacokinetics, Dynamics & Metabolism, Pfizer Global R&D, Eastern Point Road, Groton, CT 06340, USA

Boris Gorovits
Department of Pharmacokinetics, Dynamics & Metabolism, Pfizer Global R&D, One Burtt Road, Andover, MA 01810, USA

The objective of antibody–drug conjugate (ADC) bioanalysis at different stages of drug development may vary and so are the associated bioanalytical challenges. While at early drug discovery stage involving candidate selection, optimization and preliminary nonclinical assessments, the goal of ADC bioanalysis is to provide PK, toxicity and efficacy data that assists in the design and selection of potential drug candidates, the late nonclinical and clinical drug development stage typically involves regulated ADC bioanalysis that delivers TK data to define and understand pharmacological and toxicological properties of the lead ADC candidate. Bioanalytical strategies and considerations involved in developing successful ligand-binding assays for ADC characterization from early discovery to late nonclinical stages of drug development are presented here.

Antibody–drug conjugates (ADCs) are an emerging class of biotherapeutics that hold potential over conventional chemotherapies to provide targeted delivery of cytotoxic drug to tumor cells with enhanced biological activity (improved efficacy, selectivity and therapeutic index), and limited systemic exposure. ADCs have complex multicomponent structures and are inherently heterogeneous in nature. The heterogeneity arises due to the different number of small molecule drugs conjugated to the antibody moiety of ADC (also defined as drug–antibody ratio [DAR]), and the different sites of conjugation on the antibody. It is a by-product of conjugation chemistries employed for linking small molecule drugs to the antibody moiety of ADC.

The DAR heterogeneity in starting reference material may evolve further in systemic circulation due to enzymatic or chemical-induced deconjugation of the small molecule drug from the ADC and due to differences in the clearance rate of different DAR species [1–7]. The physicochemical characteristics of ADCs such as chemistry of conjugation, lability (cleavable vs noncleavable) of the linker, and the actual sites of conjugation govern the mechanism of deconjugation, the rate of small molecule drug release and the overall *in vivo* stability of ADC [1,7].

Owing to their multicomponent structure, inherently heterogeneous and dynamic nature, multiple analytes are utilized to determine PK and fate of ADCs *in vivo* [8]. The commonly used ADC-related analytes

FUTURE SCIENCE part of fsg

Key terms

Drug–antibody ratio: Amount of drug conjugated per antibody molecule.

Total antibody assay: Measures total antibody–drug conjugate (ADC) concentration (sum of conjugated and unconjugated forms) irrespective of whether small molecule drug is attached to the antibody moiety or not.

Conjugated antibody assay: Measures total ADC concentration with at least one small molecule drug attached to the antibody moiety.

DAR-sensitive assay: Measures ADC concentration based on the valences of small molecule drugs on the ADC.

DAR-insensitive assay: Measures ADC concentration irrespective of the valences of small molecule drugs on the ADC.

include the conjugated antibody (DAR greater than or equal to one), the total antibody (DAR greater than or equal to zero), the antibody-conjugated small molecule drug, the released small molecule drug (resulting from *in vivo* deconjugation), the DAR distribution over time and the antidrug antibodies (ADAs) against any component of multicomponent ADC molecule. However, the precise collection of analytes needed depends on the detailed physicochemical characteristics of the ADC molecule and the endpoints required for understanding of the ADME properties and PK characteristics of the ADC molecule.

Depending on the PK information sought, bioanalytical methods such as ligand-binding assays (LBA), LC separation coupled with MS detection (LC–MS), and combination of both methods are employed for analyzing the diversity of ADC analytes. LBA-based methods are often employed for monitoring the total antibody, conjugated antibody and antidrug antibody analytes [8]. LC–MS-based methods are mainly utilized for the detection and quantitation of the antibody-conjugated small molecule drug, released unconjugated small molecule drug and its metabolites, and DAR distribution over time [9–11]. However, the exact set of methods applied for understanding PK and immunogenicity of ADCs may evolve as more information is gained on ADC pharmacology from clinical trials and postmarketing data. This review focuses on challenges associated with ADC bioanalysis using LBA-based methods and the bioanalytical approaches and strategies that might be adopted for PK characterization of diverse ADC analytes from early discovery to late nonclinical stages of drug development. The LBA-based ADA bioanalysis is outside the scope of this review article.

ADC bioanalysis at different stages of drug development

The early to late stages of drug development may have different PK questions (such as ADC stability, exposure and safety) that need to be answered and thus may require different endpoints to address these questions [9,12,13]. During early drug discovery stage that involves candidate selection, optimization and early nonclinical assessments, the goal of ADC bioanalysis is to provide insights into the overall stability, safety and efficacy characteristics of the drug candidates. Such information allows comparison of ADC candidates with various linker–small molecule drug combinations and facilitates selection and ranking of optimal candidates that can progress to lead candidates with the best safety and efficacy profile. The commonly measured analytes at early discovery stage are tabulated in Table 1 and may include the total antibody, conjugated antibody and/or antibody-conjugated small molecule drug, released unconjugated small molecule drug and its metabolites, and *in vitro* and *in vivo* changes in DAR distribution over time [9]. In addition, ADA might also be evaluated during early drug discovery stage, particularly if abnormal PK profile due to ADA-mediated clearance is suspected in initial nonclinical assessments [14].

During late nonclinical stage of drug development, the objective of ADC bioanalysis is to gain better

Table 1. Analytes for antibody–drug conjugate bioanalysis from early drug discovery to late nonclinical stages of antibody–drug conjugate development.

ADC analytes	Drug discovery	Nonclinical†
Total antibody	✓	✓
Conjugated antibody and/or antibody-conjugated small molecule drug	✓	✓
Released/free small molecule drug and its metabolites	✓	✓
In vitro and *in vivo* DAR distribution	✓	
ADA	✓‡	✓

†Nonclinical herein refers to the post-lead candidate selection stage involving regulated TK and toxicity studies such as investigational new drug enabling studies.
‡ADA evaluations in early discovery are done if ADA related changes in PK profile are observed.
ADA: Antidrug antibodies; ADC: Antibody–drug conjugate; DAR: Drug–antibody ratio.

understanding of the nature of pharmacological and toxicological properties of the lead drug candidate. The observed toxicity and TK data provides insights about the exposure–response relationship, aids in predicting human PK, and facilitates translation of nonclinical data to clinical outcomes. The additional analyte measured at this stage typically includes ADAs along with the other multiple analytes measured in early drug discovery stage (Table 1) [9,12,13].

ADC bioanalysis using ligand-binding assays & associated challenges

Ligand-binding assays offer unique advantages for quantitation of large molecule component of ADCs including high assay sensitivity and throughput, the broad range of quantitation, the requirement of minimal sample volume and their ability to measure the analyte of interest in the biological matrix without additional sample extraction steps. But the complex multicomponent structure, inherently heterogeneous and dynamically evolving *in vivo* behavior of ADCs presents unique challenges in ADC bioanalysis using LBAs:

- While single LBA is typically utilized for quantitation of large molecule therapeutics, multiple LBAs such as the **total antibody assay** and the **conjugated antibody assay** are needed for the quantitation of diverse ADC-related analytes;
- Due to their heterogeneous nature and dynamically evolving *in vivo* behavior, the starting ADC reference material may not accurately represent the ADC composition present in the incurred samples, particularly at later time points [8,9]. Thus the selection of correct reference standard for quantitative LBAs for ADC bioanalysis presents a major challenge. Current bioanalytical validation guidance does not address this ADC specific challenge;
- Depending on the question to be answered, **DAR-sensitive** or **DAR-insensitive** LBAs may be needed for ADC bioanalysis (discussed in detail in the later section). A DAR-sensitive assay attempts to measure ADC analyte concentration based on the number of small molecule drugs attached to the ADC, but may not be able to measure small molecule drug for all DAR species stoichiometrically. Ideally a DAR-sensitive LBA would be equivalent to the conjugated small molecule assay. The reverse is true for DAR-insensitive assays that attempt to measure various DAR components of the ADC equally, and hence are not biased toward the varying DAR values of the ADC.

Since DAR sensitivity of an assay could be governed by the critical (capture and detection) reagents and assay formats used [1,9,15,16], the design and development of DAR-sensitive and DAR-insensitive assays may require screening multiple assay formats, assay conditions and critical reagents. In addition, for a given drug candidate, due to differences in critical reagents and assay formats used, the DAR-sensitive and DAR-insensitive assays may deliver significantly different observed PK profiles and associated critical PK parameters such as clearance and drug exposure [15,17], which in turn may present significant challenges when early discovery PK data for the same drug candidate is compared with its late stage regulatory TK data. The variable sensitivity of the assays to the DAR values may also complicate direct comparison of the exposure results between various ADC candidates;

- Interference from unconjugated antibody (Ab) may reduce the specificity of bioanalytical assay designed for exclusive quantitation of intact ADC (fully conjugated and partially conjugated). The specificity of an assay format exploiting target tumor antigens or antibodies against antibody components of ADCs as capture reagents to detect intact ADC can be affected by competition from the Ab resulting from complete deconjugation *in vivo*. Theoretically, this competitive binding of Ab may impact the observed concentration of intact ADC, particularly at later time points when owing to *in vivo* deconjugation, the concentration of Ab in the system may rise relative to the ADC concentration. The consideration for Ab interference in the intact ADC quantitation could be taken in the context of target interference in the LBA assay. Similar to the target interference, the interference information may not be known upfront but evaluating it early would eliminate the risk of developing assays that may exhibit interference.

Table 2 shows an example for the impact of Ab interference on the performance of an assay designed for exclusive quantitation of intact ADC. In the assay format, the intact ADC was captured using target protein and was detected using antibodies against small molecule drug. The accuracy of the assay was determined by spiking known concentrations of antibody component of ADC (i.e., Ab) into QC samples prepared with ADC dosing material. The QC samples quantitated against the ADC reference standard exhibited significantly reduced recovery at higher concentrations of Ab. Even a five-fold increase in concentration of capture reagent showed no substantial improvement

Table 2. Unconjugated antibody interference in intact antibody–drug conjugate assay.

Spiked unconjugated antibody conc. (µg/ml)	Accuracy for LQC (%RE)	Accuracy for HQC (%RE)
0	-19	-22
2.5	2.0	-18
5.0	-11	-27
25	-65	-79

HQC: High QC; LQC: Low QC.

in the recovery of ADC QC samples when spiked with Ab (data not shown).

Accuracy is defined as the closeness of agreement between the observed concentration (E) and the nominal concentration (T) and is expressed as percent relative error (%RE).

$$\%RE = \left(\frac{E - T}{T}\right) \times 100$$

The assay performance was evaluated in rodent serum for an ADC containing lysine-based conventional conjugation chemistry. The assay format on a Mesoscale Discovery (MSD) platform involved capture of intact ADC via its antibody framework using target protein as the capture reagent and detection using antibodies against the small molecule drug. High (1000 ng/ml) and low (25 ng/ml) QC ADC samples spiked with varying concentrations of Ab (0–25 µg/ml) were quantitated against the ADC reference standard. The range of quantitation (ROQ) for standard curve was 10–1300 ng/ml in 100% matrix;

- Due to highly potent cytotoxic activity of small molecule component of ADCs, the potential toxicity concerns are usually higher for ADCs. As a result, compared with large molecule therapeutics, relatively lower dose ranges may be selected for nonclinical and clinical studies for ADCs. LBAs for ADC bioanalysis thus may require high assay sensitivities (in the low ng/ml range) than typically expected for large molecule therapeutics [18–21];

- The selection of an appropriate biological matrix also presents unique challenge during ADC bioanalysis. The preferred matrix for bioanalysis of small molecule therapeutics using LC–MS platform and large molecule therapeutics using LBA platform are typically plasma and serum, respectively. However, since ADCs contain both small and large molecule therapeutics, the selection of optimal matrix for ADC bioanalysis becomes debatable.

Depending on the linker lability and ADC stability, the processing of serum may cause deconjugation and release of cytotoxic small molecule drug that may interfere in quantitation of small molecule analyte of some ADCs. The plasma matrix is thus preferred for LC–MS-based quantitation of the antibody-conjugated small molecule drug and/or the released unconjugated small molecule drug and its metabolites [9]. But the plasma processing is also associated with its own set of challenges such as the selection of appropriate anticoagulant from the list of available anticoagulants such as EDTA, heparin or sodium citrate; the appropriate volume of blood collected in collection tubes to ensure optimum blood/anticoagulant ratio; and the freeze–thaw stability of plasma [22,23];

- Though not commonly observed, LBA performance may be impacted by the binding of known matrix components to either large molecule or small molecule component of multicomponent ADC [24]. Such interferences may reduce desired sensitivity of the assay.

Table 3 shows an example for the impact of known matrix component interference on the assay performance in serum samples from rodents and nonhuman primates (NHP). Though similar assay format and similar experimental conditions were employed for quantitation of intact ADC in both species, the assay performance reflected by significantly reduced recovery of QC samples at the lower end of the ROQ was impacted in NHP serum samples. Further analysis revealed that the specific binding of a known matrix protein to one of the component of multicomponent ADC in NHP serum caused the observed decrease in recovery of QC samples (data not shown).

The assay performance was evaluated in two individual species, rodents and NHP, for an ADC with lysine-based conventional conjugation chemistry. The assay format on an MSD platform involved capture of intact ADC via its antibody framework using target protein as the capture reagent and

detection using antibodies against the small molecule drug. The accuracy of the assay was determined by quantitating ADC QC samples (ULOQ: 1300 ng/ml, High QC: 1000 ng/ml, Mid QC: 500 ng/ml, Low QC: 25 ng/ml, LLOQ: 10 ng/ml) spanning the range of quantitation against the ADC reference standard in both species. The ROQ for standard curve was 10–1300 ng/ml in 100% matrix.

Strategies & considerations to mitigate challenges associated with LBA-based ADC bioanalysis

How PK parameters are used may be different at different stages of drug development, and so are the associated bioanalytical challenges. The strategies and bioanalytical approaches employed for assay development may thus need to be adapted at each stage. This may require exploring multiple bioanalytical platforms, assay formats, critical reagents and biological matrix (plasma vs serum) to ensure that the most appropriate bioanalytical tools are used. In addition, the information about the biology (such as mechanism of action, targeted tumor antigen) and physicochemical characteristics (such as conjugation chemistry, type of linker, average DAR, small molecule drug properties) of ADC can aid in the design and development of robust and reliable LBAs for analyzing diverse ADC analytes.

In general, during the early drug discovery stage, where research teams are working with large number of similar compounds for a given target, the availability of critical reagents, time and resources may be limited. As a result, flexible 'fit-for-purpose' assay development approaches and generic reagents against human IgG, or Fc region or $(Fab')_2$ region are often employed [14,25]. In contrast, the late nonclinical drug development stage involving investigational new drug (IND) enabling studies typically require validated assays that follow regulatory guidance on criteria used to define the ROQ, precision, accuracy, specificity, selectivity, robustness and ruggedness [26]. In order to meet these expectations, specific reagents such as target antigen proteins, monoclonal anti-idiotype (Id) antibodies, anti-complementary determining regions (anti-CDR) antibodies, etc. are frequently used in the validated assays. The usage of specific reagents may also raise questions whether the assay measures 'free' versus 'total' analyte concentration [27]. In the context of ADC bioanalysis, the 'free' analyte concentration reflects concentration of ADC analytes that have at least one unoccupied target binding site (i.e., both antigen-free and -partially free analytes). Whereas the 'total' analyte concentration provides all forms of target antigen bound and unbound concentration of ADC analytes.

The differences in critical reagents and assay formats adopted for large molecule bioanalysis at different stages of drug development may have an impact on the observed analyte concentration and the associated PK profile and calculation of critical PK parameters. Though there are no issued regulatory guidelines or industry-wide best practices for assays validations specific for ADCs, currently the assay validation guidelines for large and small molecules are applied for ADC bioanalysis. Thus, the challenges associated with large molecule bioanalysis at various stages of drug development are applicable for ADC bioanalysis as well.

In addition, during early non-regulated discovery stage, when PK data is needed for design and selection of optimal ADC candidates that can advance to lead candidates, DAR-sensitive LBAs may be useful to better describe the changes in conjugated small molecule over time and associated PK parameters. At this stage in drug development, toxicology and efficacy studies conducted with multiple antibody, small molecule, linker and conjugation chemistry (and conjugation site) combinations often aid in selection and ranking of multiple ADC candidates. The need to obtain some measure of conjugated small molecule by either LC–MS or through the use of a DAR-sensitive conjugated antibody assays is based on the mechanism of action based hypothesis that conjugated small molecule drug is the main driver of efficacy at the site of action [28,29], and that the potency is directly propor-

Table 3. Matrix component interference in ligand-binding assay-based quantitation of intact antibody–drug conjugates.

QC samples	Accuracy in rodents (%RE)	Accuracy in NHP (%RE)
ULOQ	8.6	-2.0
HQC	-1.8	-1.0
MQC	-15	-16
LQC	-18	-63
LLOQ	-14	-59

HQC: High QC; LQC: Low QC; MQC: Mid QC; NHP: Nonhuman primates.

tional to DAR [1]. To better understand the change in DAR species over time, LC–MS measurement of average DAR is also often measured and the DAR distribution data are combined with the total antibody and conjugated antibody data to select optimal candidates.

During the late drug development stage, when the IND enabling studies are conducted for the lead ADC candidate, validated DAR-insensitive LBAs may be needed for collection of PK data so that all DAR species are measured equally to aid in defining and understanding potential toxicity and TK of the lead candidate. This is because of a need to meet regulated validation expectations and because of our limited current understanding on which DAR species can provide best correlation for exposure–response relationship for safety of ADCs.

The recommendations for assay development and validation for total antibody and conjugated antibody analytes of ADCs has been discussed elsewhere [8]. The heterogeneous mixture of ADCs containing various DAR species and diverse conjugation sites may have different binding affinities for the critical reagents used in the total antibody and conjugated antibody assays. Thus depending on the reagents used, the low avidity and the steric hindrance observed during critical reagent binding may lead to inaccurate estimation of low and high DAR species, respectively. For DAR-insensitive assays, it is recommended that the assay sensitivity to DAR values be evaluated early on during assay development by comparing the recovery of enriched or individually purified DAR species against the reference standard containing average DAR to ensure that the chosen assay format and critical reagents recover all DAR species equally [8,9].

The enriched DAR species represent the heterogeneous mixture of DAR species that has relatively higher abundance of a certain DAR species in the mixture. They are used when the DAR heterogeneity in ADC is too complex to isolate individual DAR species, and are prepared by either crude fractionation of the ADC reference standard or employing conjugation procedures designed to produce DARs either higher or lower than the reference standard DAR [9,15]. The availability of enriched or purified DAR species may depend on the chemistry of conjugation. For instance, isolation and purification of heterogeneous ADC mixture to individual DAR species may be challenging for conventional lysine-based conjugation chemistry that yields DAR values of 0–8 and generates greater than 1 million different ADC species due to conjugation sites located at approximately 20 different lysine residues on both the heavy and light chain [30] compared with novel site-specific conjugation chemistries that yield site-specific ADCs with DAR values of two or four [31].

An overview of bioanalytical considerations and challenges associated with LBA-based assessment of total antibody and conjugated antibody analytes are discussed below.

Total antibody assay

Total antibody assay as the name implies measures total antibody (DAR greater than or equal to 0, sum of conjugated and unconjugated forms) analyte of ADC. It monitors antibody component of ADC irrespective of whether cytotoxic small molecule drug is conjugated to the antibody or not. The total antibody concentration is commonly used to assess the antibody-dependent PK characteristics (half-life, clearance) and the overall *in vivo* stability of the ADC. The conjugation of small molecule drug to the antibody moiety may negatively influence its PK characteristics such as shorter half-life and faster clearance *in vivo* compared with the parent antibody. For this reason, during early drug discovery stage, the total antibody assay is the primary choice of analyte for comparing ADC candidates with various linker–cytotoxic small molecule drug combinations. In addition, during early discovery stage, in the absence of a conjugated antibody LBA or antibody conjugated small molecule drug LC–MS assay, the data from total antibody assay in combination with DAR measurements could be used for assessing conjugated small molecule drug concentration.

The critical reagents typically employed in the total antibody assay bind to the antibody component of ADC regardless of its DAR value (Figure 1). Though these reagents do not directly bind to the small molecule drug, due to steric hindrance the small molecule drug can indirectly influence binding of these reagents to the antibody component of ADC. This interference might be more prominent for higher DAR species. As a result, the assay may not accurately estimate all expected DAR species in systemic circulation, thereby, affecting the observed overall PK characteristics of the ADC. It can be particularly challenging to address this potential issue in early discovery when multiple ADCs with different conjugations sites are analyzed from a single study with the same total antibody assay.

Industry recommends determining if the total antibody assay is DAR-insensitive by evaluating recovery of the samples prepared with the Ab as well as the samples prepared with the enriched or individually purified DAR species against the ADC reference standard with average reported DAR [8]. If a total antibody assay is DAR-insensitive, both the Ab and the enriched or purified DAR species should yield percentage recovery within the acceptable range of the assay (e.g., ±20% of nominal).

Both specific reagents such as targeted tumor antigens or anti-Id or anti-CDR monoclonal antibodies,

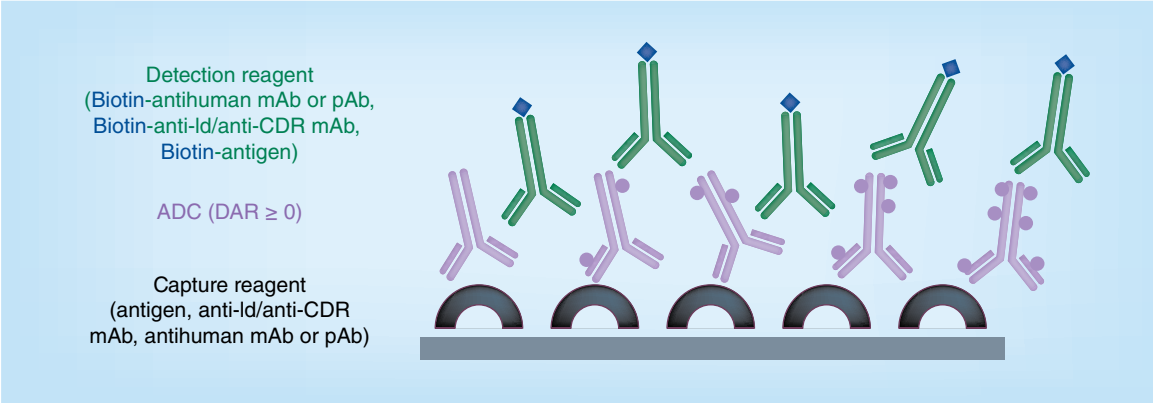

Figure 1. Ligand-binding assay for antibody–drug conjugate total antibody analyte.
ADC: Antibody–drug conjugate; CDR: Complementarity determining region; DAR: Drug–antibody ratio; Id: Idiotype; mAb: Monoclonal antibody; pAb: Polyclonal antibody.

and generic reagents against human IgG or $(Fab')_2$ region or Fc region, or light chain (LC), or heavy plus light (H+L) chain regions could be used as critical reagents for total antibody assay. All of these reagents offer their own advantages and limitations. Theoretically, the assay formats utilizing specific reagents owing to their higher binding affinity and specificity should provide better specificity and selectivity, extended dynamic range, higher assay sensitivity and should be relatively easily transferred from nonclinical to clinical matrices. But the availability of specific reagents, particularly targeted tumor antigen may be limited. Even if the antigen protein is available, the application of antigen protein as the critical reagent may render assay susceptible to the target interference. Similarly, though the use of anti-Id or anti-CDR antibodies may offer high assay specificity and sensitivity over generic reagents, their binding to antibody component of ADC may be impacted by the conjugation site on the ADC [16]. Thus, the critical reagent employed for total antibody analyte quantitation depends on the availability of the reagents as well as on the requirement of DAR-sensitive or DAR-insensitive PK profile and PK parameters to evaluate safety, efficacy and *in vivo* stability of the drug candidate. It has been reported that while for some ADCs, only generic reagents could yield DAR-insensitive total antibody assays, for other ADCs both the generic or specific reagents could yield DAR-insensitive assays [15,17]. The physicochemical characteristics of the ADC such as conjugation chemistry, linker type and small molecule drug type and conjugation site govern whether the generic or specific or both types of reagents would yield the DAR-insensitive assay. For instance, if a DAR-insensitive assay is needed for an ADC that has conjugation sites largely located on a certain region of the antibody moiety, the usage of a critical reagent that binds to this region may not be the right choice, particularly for higher DAR species.

An example of the impact of change in critical reagent on the DAR sensitivity of a total antibody assay is illustrated in Table 4. For an ADC with conventional lysine-based conjugation chemistry, when the assay format involving target protein as capture reagent and anti-human Fc as detection reagent was employed for total antibody quantitation, it yielded an overestimation in concentrations for samples prepared with Ab relative to the average DAR standard. The observed over-recovery of samples prepared with Ab suggested that the binding of either the capture or the detection reagent to the antibody component of ADC had been compromised by the small molecule drug conjugation. It has been reported that conventional lysine-based conjugation occurs primarily on regions of the heavy and light chains that offer areas of large solvent accessibility and structural flexibility, and no conjugation sites were observed in CDRs of the antibody moiety [30]. Based on this information, when the detection reagent was changed from the generic antihuman Fc antibody to the LC specific polyclonal antibody in the total antibody assay format, a DAR-insensitive assay was generated. This new assay format yielded acceptable recovery for both Ab and average DAR standard as shown in Table 4.

Alternative assay formats involving coincubation of samples and critical reagents have also been reported to offer better assay performance against various DAR species [9,16,17]. However, since not all ADCs are same, there is no single DAR-insensitive assay format for the total antibody analyte that could fit all ADCs independent of their physicochemical characteristics. The assay strategy for total antibody quantitation would thus need to be adapted on a case-by-case basis.

Total antibody assay performance was evaluated in rodent serum for an ADC containing lysine-based conventional conjugation chemistry. The assay format on an ELISA platform involved capture of the total (uncon-

Table 4. Impact of change in critical reagents on the drug–antibody ratio sensitivity of the total antibody assay.

QC samples	Accuracy for Fc specific detection (%RE)	Accuracy for LC specific detection (%RE)
HQC - ADC	13	8.0
MQC - ADC	7.0	-2.0
LQC - ADC	8.0	2.0
HQC - Ab	130	-5.0
MQC - Ab	70	-10
LQC - Ab	17	0.5

Ab: Unconjugated antibody; ADC: Antibody–drug conjugate; HQC: High QC; LC: Light chain; LQC: Low QC; MQC: Mid QC.

jugated, fully conjugated and partially conjugated) antibody using target protein as a capture reagent, and either Fc- or LC-specific anti-human IgG as detection reagents. The accuracy of the assay was evaluated by quantitating QC samples prepared with ADC or prepared with Ab against the ADC reference standard. HQC, MQC and LQC represent the high, mid and low concentration range of the standard curve, respectively with HQC at 750 ng/ml, mid QC at 500 ng/ml and low QC at 100 ng/ml concentrations in 100% matrix.

Conjugated antibody assay

Conjugated antibody assay is used for monitoring antibody concentration bearing at least one small molecule drug (i.e., DAR is greater than or equal to 1). Because the intact ADC is the active therapeutic analyte, the conjugated antibody assay is used to measure active ADC concentration and to determine ADC PK parameters. In systemic circulation, the conjugated antibody concentration may change owing to the elimination of intact ADC and due to the complete deconjugation of ADC to the Ab.

In order to detect intact ADC, the conjugated antibody assay typically employs critical reagents that bind both the small molecule drug component as well as the antibody component of ADC (Figure 2). Similar to the total antibody assay, conjugated antibody assays also exhibit sensitivity to the site of conjugation and the DAR values of the ADC. The binding of anti-small molecule drug antibodies to the small molecule drug component of ADC might be hindered by the solvent accessibility of the conjugation site. In addition, proportional binding may not be possible due to the steric hindrance from multiple adjacently conjugated small molecule drugs. Thus, the conjugated antibody assay may provide inaccurate measurement of higher and lower DAR species in systemic circulation [9,16].

The assay format and critical reagents chosen for the conjugated antibody assay may also influence DAR sensitivity of the assay, which in turn may impact observed intact ADC concentration *in vivo*. Theoretically, an assay format employing anti-small molecule drug antibodies as the detection reagent might exhibit more DAR sensitivity because the observed assay signal might be proportional to the total number of small molecule drug conjugated to the ADC (Figure 2). In other words, higher DAR species may exhibit higher assay signal compared with the lower DAR species. Whereas, an assay format utilizing anti-small molecule drug antibodies as capture reagent might not be as DAR-sensitive because such antibodies might capture intact ADC through its small molecule drug component irrespective of the number of small molecule drug conjugated to the ADC [15].

An objective criterion to determine if the assay format selected for the conjugated antibody assay has rendered the assay performance DAR-sensitive, is to evaluate recovery of the samples prepared with the enriched or individually purified DAR species against the ADC reference standard with average reported DAR [8]. If all samples exhibit percentage recovery within the acceptable range of the assay (e.g., ±20% of nominal), it reflects that the chosen assay format is the optimal choice for DAR-insensitive assay. However, if the recovery of samples prepared using various DAR species falls outside of the acceptable range for the assay, further evaluation of assay format and/or reagents is warranted.

The impact of assay format on the DAR sensitivity of a conjugated antibody assay for an ADC with conventional cysteine-based conjugation chemistry is illustrated in Table 5. In this case, when the assay format involving target protein as capture reagent and the anti-small molecule drug antibodies as detection reagent was employed for intact ADC quantitation, it yielded an overestimation of individually purified high DAR species and underestimation of low DAR species relative to the average DAR standard. However, changing the assay format to using anti-small molecule drug antibodies as capture reagent and target protein as detection reagent, significantly improved percentage recovery of both low and high DAR species.

For some ADCs, irrespective of whether the assay format employs small molecule drug specific detection or the small molecule drug specific capture, the DAR sensitivity of the assay may not be alleviated. Thus, like total antibody assay, the DAR sensitivtity of the conjugated antibody assay may also be governed by the physicochemical characteristics of ADC. In addition, due to the high cytotoxic potency of the small molecule drug, generation of high quality antibody reagents with high binding affinity and avidity against the small molecule drug poses a challenge. Table 6 displays DAR sensitivity of a conjugated antibody assay for an ADC containing lysine-based conventional conjugation chemistry. In this case, irrespective of whether the assay format employed either small molecule drug specific detection or the small molecule drug specific capture, the conjugated antibody assay performance for the ADC was affected by DAR values based on the DAR reference standards used. Thus in this situation, it is important to understand that irrespective of the assay format chosen, the critical reagents employed will deliver DAR-sensitive assay, so the observed results should be interpreted accordingly.

Other considerations that may aid in overcoming challenges associated with development and validation of LBAs for ADC bioanalysis include:

- Using the ADC dosing material as a reference standard in both the total antibody and conjugated antibody assays. Development of multiple assays, with each assay utilizing reference standard with a specified DAR to determine impact of ADC heterogeneity on the accuracy of assay is not practical. In addition, since clinical and nonclinical end points are measured following administration of the dos-

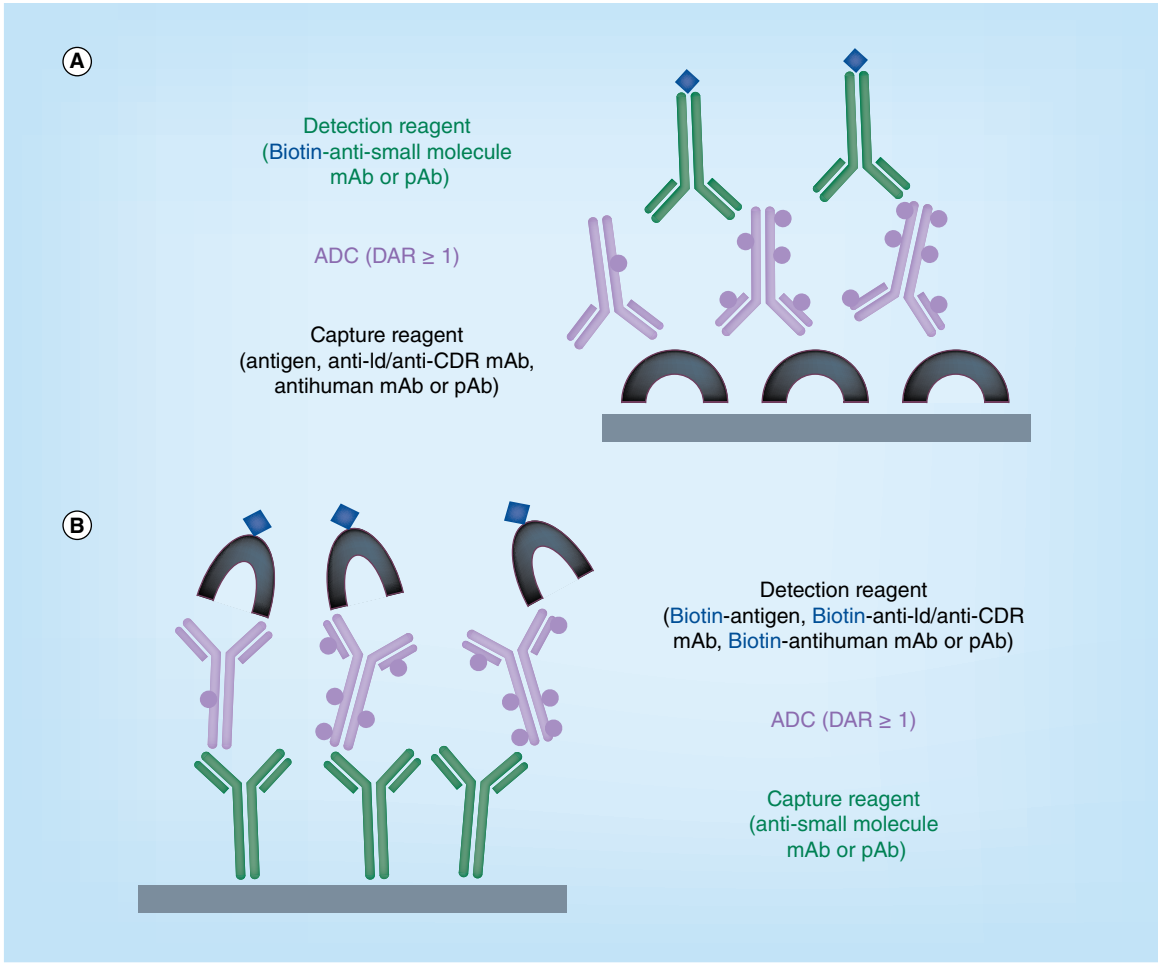

Figure 2. Ligand-binding assay formats for the conjugated antibody analyte. (A) Capture and detection of the intact ADC using reagents specific for antibody framework and small molecule component of ADC, respectively. **(B)** Capture and detection of the intact ADC using anti-small molecule antibodies and antibodies against antibody component of ADC, respectively.
ADC: Antibody–drug conjugate; CDR: Complementarity determining region; DAR: Drug–antibody ratio; Id: Idiotype; mAb: Monoclonal antibody; pAb: Polyclonal antibody.

Table 5. Impact of change in assay format on drug–antibody ratio sensitivity of the conjugated antibody assay for an antibody–drug conjugate containing conventional cysteine-based conjugation chemistry.

Purified DAR species/QC	Accuracy for anti-small molecule antibodies-based detection (%RE)	Accuracy for anti-small molecule antibodies-based capture (%RE)
DAR 2	-70	-47
DAR 6	100	9.0
DAR 8	127	-24
DAR 4†	2.5	-4.0

DAR sensitivity of the conjugated antibody assay was evaluated in nonhuman primate (NHP) serum. The assay format on an ELISA platform involved either capture of intact ADC using target capture reagent and detection using antibodies against small molecule drug, or *vice versa*. Samples prepared with individually purified DAR components (DAR 2, 6, 8) at 175 ng/ml concentration (equivalent to mid QC concentration) in 100% NHP matrix were quantitated against the ADC reference standard (average DAR of 4) curve. The range of quantitation for the assay format employing antibodies against small molecule drug as detection reagent was 18.6–500 ng/ml in 100% NHP matrix. The assay format employing antibodies against small molecule drug as capture reagent had a range of quantitation of 30.0–500 ng/ml in 100% NHP matrix.
†DAR 4 sample at mid-QC level was prepared using the ADC reference standard material.
ADC: Antibody–drug conjugate; DAR: Drug–antibody ratio.

ing material, the use of ADC dosing material as a reference standard is appropriate;

- Mitigating Ab interference in the conjugated antibody assay by changing the assay format in such a manner that the reagents against the small molecule drug are used as capture reagents. Theoretically, since such reagents do not directly bind to the antibody component of ADC, they may increase the assay specificity of conjugated antibody assay against intact ADC;

- Compared with the conventional ELISA platform, novel MSD and Gyrolab-based immunoassay platforms that claim to offer higher sensitivity, broad dynamic range, low sample volume and/or reduced matrix interference [32] can be used for attaining higher assay sensitivity for LBAs employed for ADC bioanalysis;

- Endogenous protein interference in the assay can be alleviated either by the use of critical reagents that are not impacted by the binding of such proteins or by optimizing assay buffer composition by introducing appropriate reagents that can block such interference in the assay [24,25,33]. In cases where it may not be feasible to eliminate endogenous protein interferences, an increase in the minimum required dilution may be required.

Conclusion

The complex multicomponent structure and dynamic and heterogeneous nature of ADCs require bioanalysis of multiple analytes for PK assessment of ADCs. This review has highlighted unique challenges associated with bioanalytical characterization of ADCs and the strategies to mitigate them. Since the stage of ADC development dictates what PK questions needs to be answered, the bioanalytical challenges associated in addressing those questions may also be unique to each stage. The bioanalytical approaches and strategies therefore would need to be adapted depending on the desired endpoint.

The assay formats and assay reagents for ADC bioanalysis may evolve as ADC drug candidates progress from

Table 6. Lack of impact of change in assay format on drug–antibody ratio sensitivity of the conjugated antibody assay for an antibody–drug conjugate containing conventional lysine-based conjugation chemistry.

Enriched DAR species†	Accuracy for anti-small molecule antibodies-based detection (%RE)	Accuracy for anti-small-molecule antibodies-based capture (%RE)
DAR 4	-21	-25
DAR 4.4	-10	-29
DAR 5.1	30	31

DAR sensitivity of the conjugated antibody assay was evaluated in rodent serum. The assay format on an Mesoscale Discovery platform involved either capture of intact antibody–drug conjugate (ADC) via its antibody framework using target protein as the capture reagent and detection using antibodies against small molecule drug, or *vice versa*. Samples prepared with enriched DAR fractions (with average reported DAR of 4, 4.4 and 5.1) at 1 μg/ml concentration (equivalent to high quality control concentration) in 100% matrix were quantitated against the ADC reference standard (average DAR of four). The range of quantitation for the standard curve was 10.0–1280 ng/ml in 100% matrix.
†The DAR heterogeneity in ADC was too complex to chromatographically isolate individual DAR species. Thus, enriched DAR species were prepared by crude hydrophobic interaction chromatography fractionation of the ADC reference standard.
DAR: Drug–antibody ratio.

early to late stages of drug development. Based on the limited industry experience, the evolution of assays could result in differences in observed PK profile and calculation of key PK parameters over the course of ADC development. However, by applying rational scientific understanding of what each assay format and assay reagent is measuring, an appropriate interpretation of observed data can be made. Moreover, once enough understanding of the most relevant ADC analyte and DAR species is built, keeping the assay formats and assay reagents similar in late stages of drug development such as between IND-enabling and first-in-human studies might help simplify interpretation of the observed results.

Future perspective

The number of ADC programs across industry is growing rapidly. The increased number of ADCs in the clinic may help better define what ADC analytes are most useful in understanding fate of ADCs *in vivo*. Novel site-specific ADCs may have less heterogeneity as well as greater *in vivo* stability than conventional conjugates. The use of novel small-molecule drug classes, linker types and conjugation chemistries will also require increased investment in appropriate bioanalytical strategies to support their progress throughout the drug development. A combination of LBA and LC–MS-based methods will be needed to support future ADC programs. But the balance between platform investments would be defined by the specifics of the ADC program and the need to measure all the relevant analytical species.

Novel small-molecule drug classes may be challenging for generation of appropriate reagents against them and may need an early increased investment to define absorption, distribution, metabolism and elimination before specific LBA-based PK assays could be developed.

The relationship between systemic exposure and cellular response remains complex with a multiday transduction process involving extravasation into tissues, cellular binding, internalization, intracellular trafficking, small molecule release and finally intracellular target engagement. Thus, tissue PK and cellular biodistribution studies can be expected to play an important role in understanding this relationship and would require additional bioanalytical investment.

Acknowledgements

The authors would like to thank N Duriga, T Taylor and E Hamel for their contributions to the presented data.

Executive summary

- Antibody–drug conjugates (ADCs) hold potential to deliver improved overall safety and reduced nonspecific off-target toxicity of chemotherapeutics agents.
- ADC bioanalysis at different stages of drug development may vary and so are the associated bioanalytical challenges.
- Novel bioanalytical approaches and strategies including combination of ligand-binding assays (LBA), LC–MS-based platforms are needed to overcome challenges unique to each stage of drug development.
- LBAs offer unique advantages for quantitation of ADCs:
  - High assay sensitivity and throughput;
  - Broad range of quantitation;
  - Minimal sample volume;
  - Ability to measure in biological matrix without additional sample extraction steps.
- The complex multicomponent structure of ADC also presents unique challenges in ADC bioanalysis using LBAs:
  - ADCs are heterogeneous mixtures of various drug–antibody ratio (DAR) species. The DAR heterogeneity comes from conjugation chemistry, linker lability and actual site of conjugation;
  - ADC heterogeneity is dynamically changing *in vivo* due to spontaneous or environment-induced deconjugation and due to differences in the clearance rate of various DAR species;
  - Multiple assays are needed for monitoring diverse ADC analytes;
  - LBAs may be sensitive to the amount of conjugated small molecule drug present on the ADC molecule.
- ADC-related analytes include the conjugated antibody, the total antibody, antibody-conjugated small molecule drug, released unconjugated small molecule drug and its metabolites, changes in DAR over time and ADAs against any component of the multicomponent ADC.
- In early drug discovery, the goal of ADC bioanalysis is to allow comparison of multiple ADC candidates with various antibodies, linker–small molecule drug combinations to select an optimal candidate.
- During pre-clinical regulated studies, the exposure–safety relationship of the lead candidate is defined using analytically validated assays.
- There is no single bioanalytical assay strategy that fits all ADCs, rather strategy needs to be adapted on a case-by-case basis depending on the physicochemical characteristics of the ADC, and the drug development stage.

Financial & competing interests disclosure

All authors (S Kumar, LE King, TH Clark and B Gorovits) are employees of Pfizer, Inc. (MA, USA), which is involved in the development of compounds relevant to those discussed in this article, and receive salary- and equity-based remuneration. The authors have no other 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 apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

1 Hamblett KJ, Senter PD, Chace DF *et al.* Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate. *Clin. Cancer Res.* 10(20), 7063–7070 (2004).

2 Xie H, Audette C, Hoffee M *et al.* Pharmacokinetics and biodistribution of the antitumor immunoconjugate, cantuzumab mertansine (huC242-DM1), and its two components in mice. *J. Pharmacol. Exp. Ther.* 308(3), 1073–1082 (2004).

3 Junutula JR, Raab H, Clark S *et al.* Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. *Nat. Biotechnol.* 26(8), 925–932 (2008).

4 Baldwin AD, Kiick KL. Tunable degradation of maleimide-thiol adducts in reducing environments. *Bioconjug. Chem.* 22(10), 1946–1953 (2011).

5 Shen BQ, Bumbaca D, Saad O *et al.* Catabolic fate and pharmacokinetic characterization of trastuzumab emtansine (T-DM1): an emphasis on preclinical and clinical catabolism. *Curr. Drug Metab.* 13, 901–910 (2012).

6 Shen BQ, Xu K, Liu L *et al.* Conjugation site modulates the *in vivo* stability and therapeutic activity of antibody–drug conjugates. *Nat. Biotechnol.* 30(2), 184–189 (2012).

7 Strop P, Shu-Hui L *et al.* Location matters: site of conjugation modulates stability and pharmacokinetics of antibody drug conjugates. *Chem. Biol.* 20(2), 161–167 (2013).

8 Gorovits B, Alley S, Bilic S *et al.* Bioanalysis of antibody–drug conjugates. AAPS ADC working group position paper. *Bioanalysis* 5(9), 997–1006 (2013).

•• **Antibody–drug conjugate (ADC) working group position paper that provides a comprehensive review of ADC bioanalysis.**

9 Kaur S, Xu K, Saad OM *et al.* Bioanalytical assay strategies for the development of antibody drug conjugate biotherapeutics. *Bioanalysis* 5(2), 201–226 (2013).

• **Describes different bioanalytical methods required during different stages of ADC development.**

10 Dufield D, Neubert H, Garofolo F *et al.* 2014 White Paper on recent issues in bioanalysis: a full immersion in bioanalysis (Part 2 – hybrid LBA/LCMS, ELN & regulatory agencies' input). *Bioanalysis* 6(23), 3237–3249 (2014).

11 Keyang Xu, Luna Liu, Randall Dere *et al.* Characterization of the drug-to-antibody ratio distribution for antibody–drug conjugates in plasma/serum. *Bioanalysis* 5(9), 1057–1071 (2013).

12 Lin K, Tibbitts J. Pharmacokinetic considerations for antibody drug conjugates. *Pharm. Res.* 29(9), 2354–2366 (2012).

13 Sauerborn M, van Dongen W. Practical considerations for the pharmacokinetic and immunogenic assessment of antibody–drug conjugates. *BioDrugs* 28(4), 383–391 (2014).

14 King LE, Leung S, Ray C. Discovery fit-for-purpose ligand-binding PK assays: what's really important? *Bioanalysis* 5(12), 1463–1466 (2013).

15 Stephan JP, Chan P, Lee C *et al.* Anti-CD22-MCC-DM1 and MC-MMAF conjugates: impact of assay format on pharmacokinetic parameters determination. *Bioconjug. Chem.* 19(8), 1673–1683 (2008).

• **Compares assay formats for conjugated antibody assay formats and their impact on PK parameter determinations.**

16 Stephan JP, Kozak KR, Wong WLT. Challenges in developing bioanalytical assays for characterization of antibody–drug conjugates. *Bioanalysis* 3(6), 677–700 (2011).

17 Kozak KR, Tsai SP, Fourie-O'Donohue A *et al.* Total antibody quantification for MMAE-conjugated antibody–drug conjugates: impact of assay format and reagents. *Bioconjug. Chem.* 24(5), 772–779 (2013).

18 Dowell JA, Korth-Bradley J, Liu H *et al.* Pharmacokinetics of gemtuzumab ozogamicin, an antibody-targeted chemotherapy agent for the treatment of patients with acute myeloid leukemia in first relapse. *J. Clin. Pharmacol.* 41, 1206–1214 (2001).

19 Dijoseph JF, Armellino DC, Boghaert ER *et al.* Antibody-targeted chemotherapy with CMC-544: a CD22-targeted immunoconjugate of calicheamicin for the treatment of B-lymphoid malignancies. *Blood* 103(5), 1807–1814 (2004).

20 Dere R, Yi JH, Lei C *et al.* PK assays for antibody–drug conjugates: case study with ado-trastuzumab emtansine. *Bioanalysis* 5, 1025–1040 (2013).

21 Hussain A, Gorovits B, Leal M *et al.* PK of immunoconjugate anticancer agent CMD-193 in rats: ligand-binding assay approach to determine *in vivo* immunoconjugate stability. *Bioanalysis* 6(1), 21–32 (2014).

22 Tuck MK, Chan DW, Chia D *et al.* Standard operating procedures for serum and plasma collection: early detection research network consensus statement standard operating procedure integration working group. *J. Proteome Res.* 8(1), 113–117 (2009).

23 Mitchell BL, Yasui Y, Li CI *et al.* Impact of freeze-thaw cycles and storage time on plasma samples used in mass spectrometry based biomarker discovery projects. *Cancer Inform.* 1, 98–104 (2005).

24 Kelley M, Ahene AB, Gorovits B. Theoretical considerations and practical approaches to address the effect of anti-drug antibody (ADA) on quantification of biotherapeutics in circulation. *AAPS J.* 3, 646–658 (2013).

25 Stevenson L, Amaravadi L, Myler H *et al.* White Paper on recent issues in bioanalysis: a full immersion in bioanalysis

(Part 3 - LBA and immunogenicity). *Bioanalysis* 6(24), 3355–3368 (2014).

26 US FDA. *Guidance for Industry: Bioanalytical Method Validation.* Center for Drug Evaluation and Research. www.fda.gov

27 Lee JW, Kelley M, King LE *et al.* Bioanalytical approaches to quantify 'total' and 'free' therapeutic antibodies and their targets: technical challenges and PK/PD applications over the course of drug development. *AAPS J.* 13(1), 99–110 (2011).

28 Wada R, Erickson HK, Lewis Phillips GD *et al.* Mechanistic pharmacokinetic/pharmacodynamic modeling of *in vivo* tumor uptake, catabolism, and tumor response of trastuzumab maytansinoid conjugates. *Cancer Chemother. Pharmacol.* 74(5), 969–980 (2014).

29 Bender B, Leipold DD, Xu K *et al.* A mechanistic pharmacokinetic model elucidating the disposition of trastuzumab emtansine (T-DM1), an antibody–drug conjugate (ADC) for treatment of metastatic breast cancer. *AAPS J.* 16(5), 994–1008 (2014).

30 Wang L, Amphlett G, Blattler WA *et al.* Structural characterization of the maytansinoid-monoclonal antibody immunoconjugate, huN901-DM1, by mass spectrometry. *Protein Sci.* 14(9), 2436–2446 (2005).

31 Panowksi S, Bhakta S, Raab H *et al.* Site-specific antibody drug conjugates for cancer therapy. *MAbs* 6(1), 34–45 (2014).

32 Leary BA, Lawrence-Henderson R, Mallozzi C *et al.* Bioanalytical platform comparison using a generic human IgG PK assay format. *J. Immunol. Methods* 397(1–2), 28–36 (2013).

33 Sailstad JM, Amaravadi L, Clements-Egan AA *et al.* White paper-consensus and recommendations of a global harmonization team on assessing the impact of immunogenicity on pharmacokinetic measurements. *AAPS J.* 16(3), 488–98 (2014).