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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.

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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

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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

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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

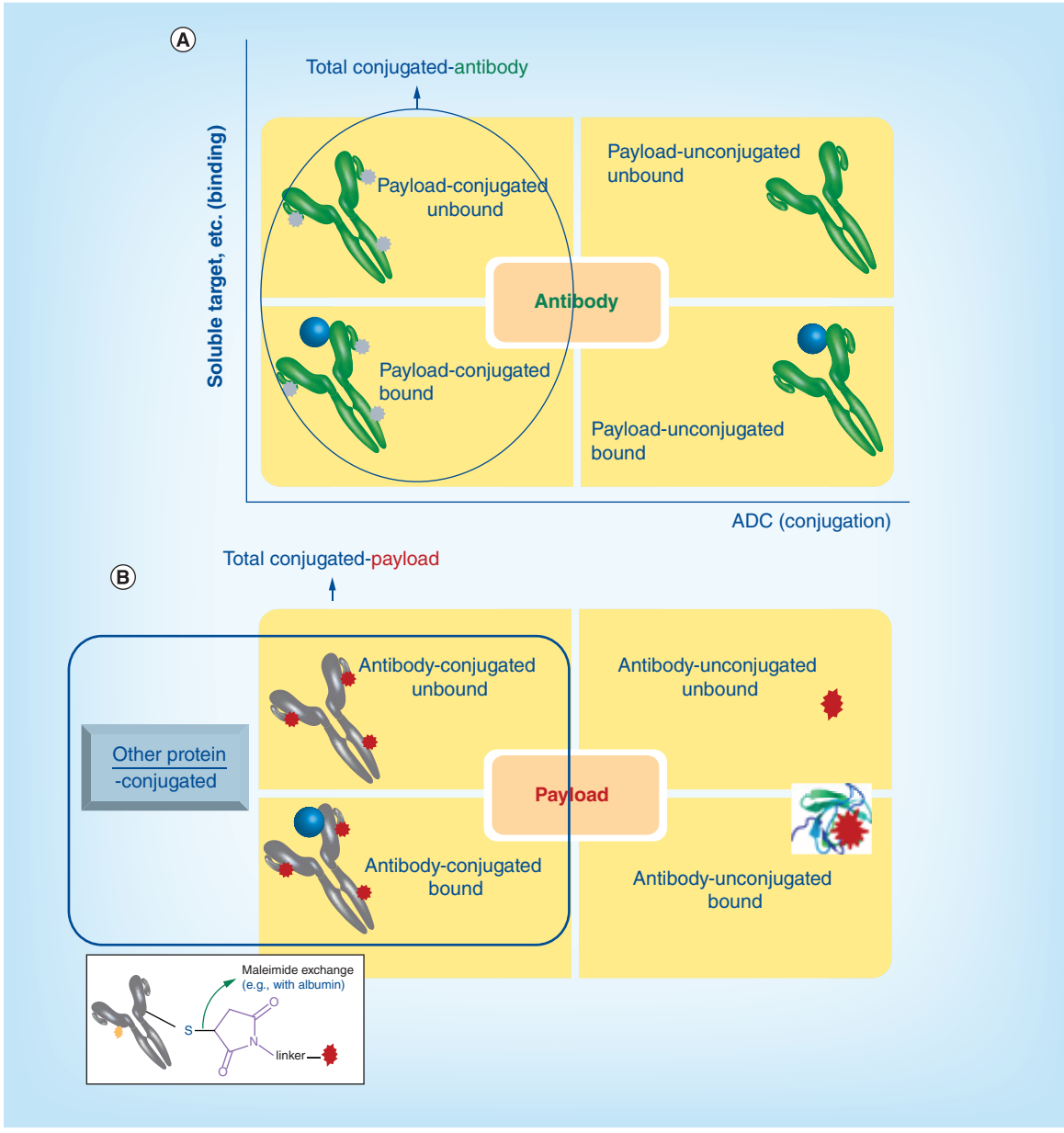

Figure 1. Complexity of antibody–drug conjugate analytes with soluble targets considered. (A) Total conjugated-antibody: conjugated-antibody unbound and bound to soluble targets. **(B)** Total conjugated-payload: payload conjugated to ADC mAb which are unbound or bound to soluble targets as well as payload 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.

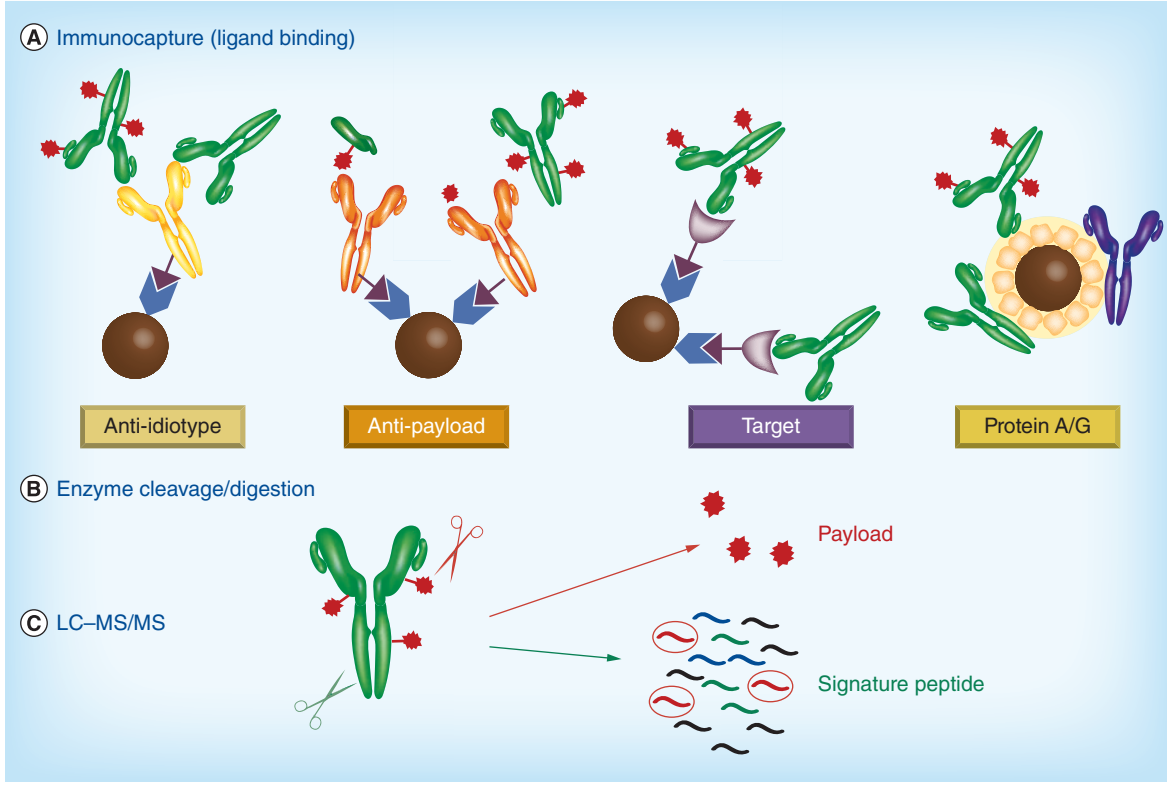

Figure 2. Procedure of LB-LC–MS/MS hybrid assays. (A) Immunocapture (ligand binding), **(B)** enzyme cleavage/digestion, **(C)** LC–MS/MS quantitation.

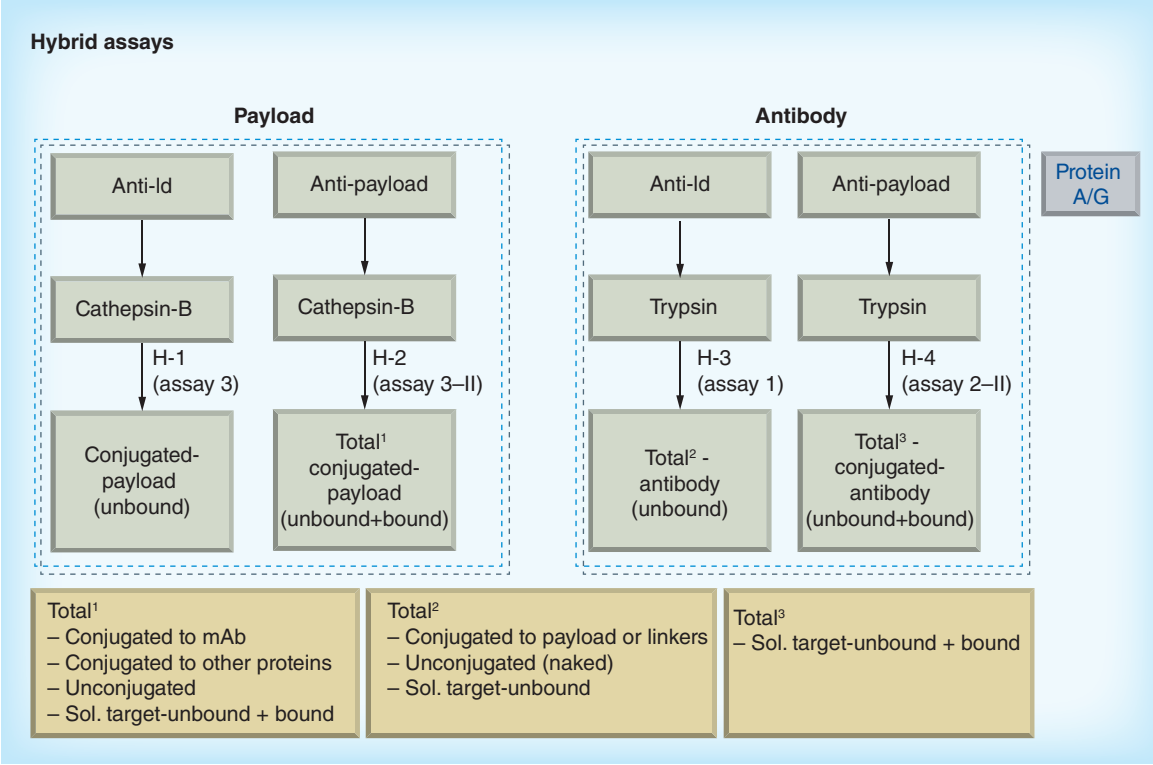

Figure 3. Hybrid assays developed with combinations of different capture reagents and enzymes. (Anti-Id, anti-payload, protein A/G, cathepsin-B and trypsin) for the analysis of conjugated-payload (H-1, assay 3), total conjugated-payload (H-2, assay 3-II), total-antibody (H-3, assay 1) and total conjugated-antibody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

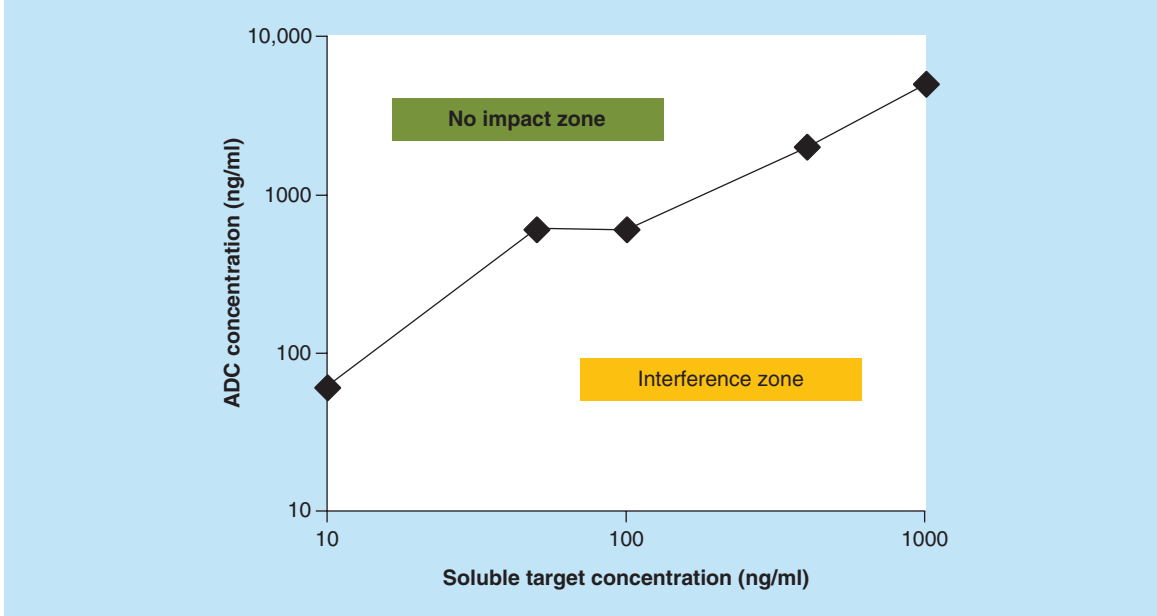

Figure 4. Tolerability to shed/soluble targets in conjugated-payload assay in human plasma.
ADC: Antibody–drug conjugate.

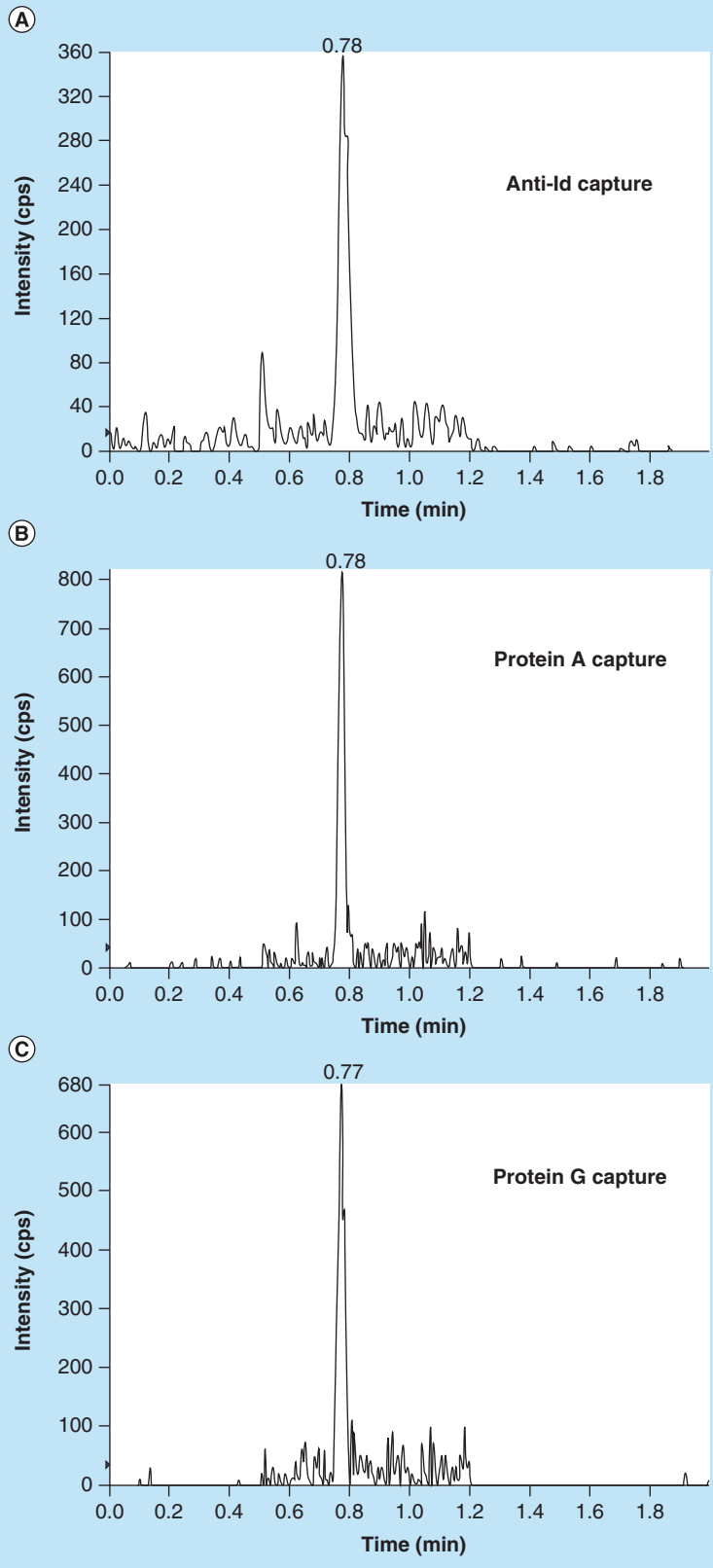

Figure 5. Chromatograms of conjugated-payload at antibody–drug conjugate equivalent LLOQ concentration (20 ng/ml) in human plasma (containing lower amount of soluble target) using different capture reagents.
(A) Anti-Id, **(B)** protein A, **(C)** protein G.

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-

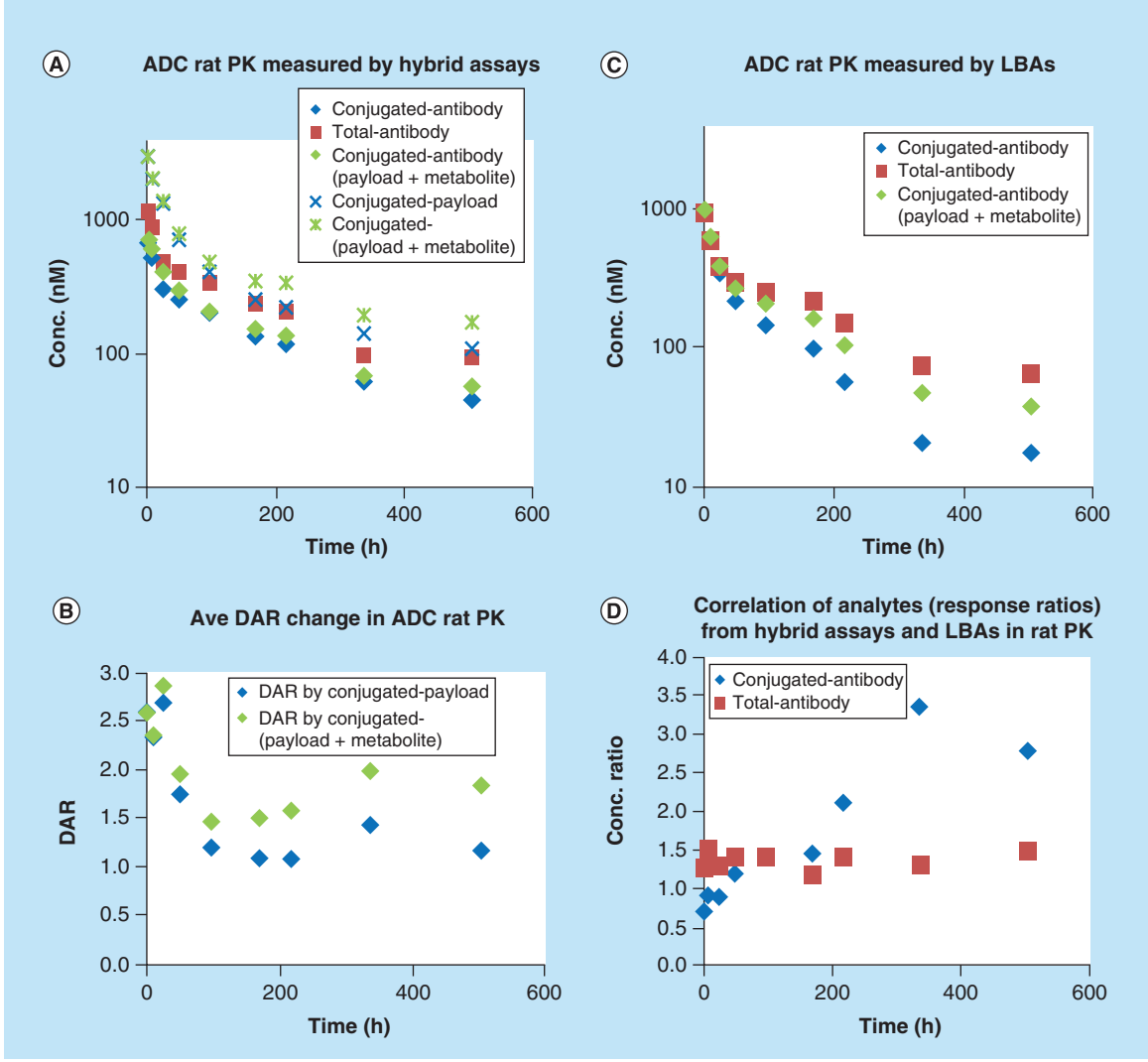

Figure 6. Hybrid assays and ligand-binding assay in antibody–drug conjugate rat PK. (A) Hybrid assays measured conjugated-antibody (blue diamond), conjugated-antibody (payload + metabolite) (green diamond), total-antibody (red square), conjugated-payload (blue cross) and conjugated-(payload + metabolite) (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

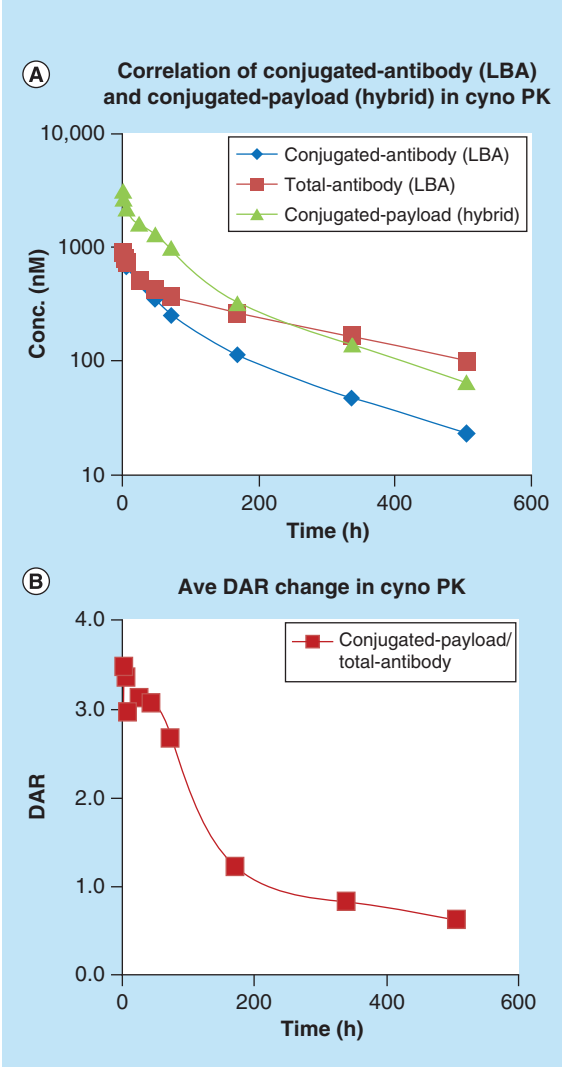

Figure 7. Correlation of conjugated-antibody (ligand-binding assay) and conjugated-payload (hybrid) in antibody–drug conjugate cyno monkey PK. (A) PK profiles of DAR-sensitive conjugated-antibody in ligand-binding assay (LBA) (blue diamond) which is equivalent to conjugated-payload in LBA, total-antibody 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

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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.

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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.

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Review

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Bioanalysis

Techniques for quantitative LC–MS/MS analysis of protein therapeutics: advances in enzyme digestion and immunocapture

LC–MS/MS has been investigated to quantify protein therapeutics in biological matrices. The protein therapeutics is digested by an enzyme to generate surrogate peptide(s) before LC–MS/MS analysis. One challenge is isolating protein therapeutics in the presence of large number of endogenous proteins in biological matrices. Immunocapture, in which a capture agent is used to preferentially bind the protein therapeutics over other proteins, is gaining traction. The protein therapeutics is eluted for digestion and LC–MS/MS analysis. One area of tremendous potential for immunocapture-LC–MS/MS is to obtain quantitative data where ligand-binding assay alone is not sufficient, for example, quantitation of antidrug antibody complexes. Herein, we present an overview of recent advance in enzyme digestion and immunocapture applicable to protein quantitation.

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Ligand-binding assays (LBA) have traditionally been used to quantify protein therapeutics in support of drug discovery and development [1,2]. The selection/detection of the protein therapeutic (or protein analyte) in a complex matrix (e.g., serum) is accomplished by the specific binding of the protein analyte to the capture antibody/detection antibody. Recently, LC–MS/MS has been investigated as a complementary technique to quantify protein therapeutics in biological matrices because of its unique mass selectivity, as selection/detection of protein analyte is accomplished by its unique *m/z* ratio. Due to the limited sensitivity of analyzing intact protein by MS, unlike in LBA, the protein analyte is usually digested by an endoprotease such as trypsin to generate one or more surrogate peptides. These surrogate peptides are then analyzed by LC–MS/MS [3–10]. For quantitative purpose, one surrogate peptide is used as the 'surrogate' of the protein. Therefore, it is important to generate a surrogate peptide that is unique to the protein. In the case of antibodies, it is preferable that the surrogate peptide is located in the complementarity determining region (CDR) of the protein.

Another challenge for analyzing protein therapeutics in biological matrices is separating the protein analyte from a large number of proteins with similar physical–chemical properties. For LBA, this is accomplished by utilizing a highly selective capture antibody. For LC–MS/MS, the traditional sample preparation methods such as SPE or liquid–liquid extraction usually are not sufficiently selective and likely result in significant loss of analyte. It is highly desirable to minimize the number of proteins going into the enzyme digestion mixture in order to reduce interference. One methodology reported is the differential precipitation by organic solvent in which the different solubility of pegylated proteins and nonpegylated pro-

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teins in an organic solvent are exploited. For example, Wu *et al.* [11]. reported that pegylated proteins were solubilized in 0.1% formic acid in 2-propanol while other endogenous proteins were not. The serum samples were then treated with 0.1% formic acid in 2-propanol to precipitate the endogenous proteins. Another methodology for isolating the protein analyte is to precipitate the protein analyte and all other proteins with an organic solvent, for example, methanol, while leaving other endogenous components (e.g., small-molecule entities that are soluble in the organic solvent) in solution. The precipitated proteins are then resuspended in digestion buffer for enzyme digestion [12,13]. It is noted that this method does not result in clean extracted samples for subsequent enzyme digestion.

Another methodology that is gaining traction is immunocapture (or immunoaffinity capture), in which a capture agent, usually an antibody, is used to capture the protein analyte, or the surrogate peptide after enzyme digestion, essentially augmenting the selectivity of LC–MS/MS with the orthogonal selectivity of ligand-binding assay. The analyte (protein or surrogate peptide) is then eluted for LC–MS/MS analysis (Figure 1).

Herein, we discuss recent advance in the techniques used for quantitative LC–MS/MS analysis of protein therapeutics, especially in the areas of enzyme digestion and immunocapture.

Advances in enzyme digestion

Trypsin has been the endoprotease of choice for quantitative work, while other endoproteases such as chymotrypsin, Asp-N, Glu-C, Lys-C, protease K and pepsin have also been used in proteomics and quantitative work [14–20]. Trypsin specifically hydrolyzes peptide bonds at the carboxyl side (or so called C-terminal) of lysine and arginine residues and tends to yield surrogate peptides typically in the 5–40 amino acid range. Other endoproteases hydrolyze peptide bonds at other specific amino acids. A list of endoproteases and their specific cleavage sites is presented in Table 1. It is, therefore, possible to target a specific region of the protein, for example, the CDR in an antibody, by choosing the appropriate endoprotease (or a mixture of endoproteases) to generate a surrogate peptide that encompasses the region of interest. For example, Hager *et al.* [6] used Asp-N to generate peptides at the C-terminus of various FGF21 modalities to study FGF21 C-terminus clipping *in vivo*, while trypsin failed to generate a suitable surrogate peptide. By making the appropriate choice of surrogate peptide and endoproteases, it is feasible to obtain high selectivity against a complex mixture of proteins in typical biological matrices. It is noted that the choice of enzyme(s) can be guided by using online tools such as ExPASy [21], and should be confirmed experimentally for reasons detailed below. It is the authors' experience that the best surrogate peptides for good retention on reversed-phase LC columns and reasonable MS sensitivity are between 10 and 30 amino acids in length and basic in nature for optimal ESI.

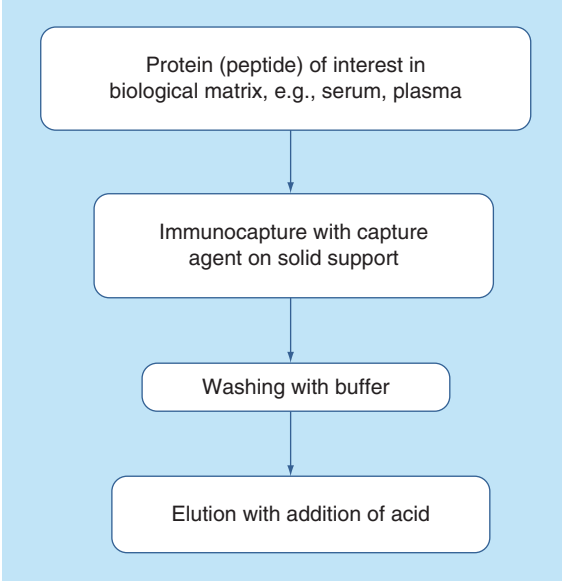

Figure 1. A flowchart depicting the workflow of immunocapture.

Recently, cysteine proteinase from *Streptococcus pyogenes* (IdeS) has been adopted to cleave IgG at a single site below the hinge region, yielding $F(ab')_2$ and Fc fragments for protein characterization, making it attractive in the area of antibody–drug conjugates (ADCs) development [22,23]. It could potentially be combined with other endoproteases in quantitative work. Aside from these enzymes, other new enzymes are being utilized for proteomic work, which can be readily adopted for quantitative protein analysis. For example, Kadek *et al.* reported the production of aspartic protease Nepenthesin-1 using recombinant technology as an alternative to the endoprotease pepsin [24].

For protein quantitation by LC–MS/MS, the enzyme digestion step is crucial to the reproducibility and sensitivity of the analytical assay, especially when a good internal standard that can compensate for variability of digestion between samples, in the form of stable-isotope labeled protein is not always readily available. Enzymatic digestion is a series of complex chemical reactions with the enzyme serving as catalyst, and there are different parameters that can affect the reproducibility of enzyme cleavage. Trypsin, being the mostly commonly used endoprotease in quantitative work and proteomics, has been extensively investigated. While these investigations have been conducted

Table 1. A list of commonly used endoproteases.

Enzyme	Biological source	Cleavage site	Comments
Arg-C	*Clostridium histolyticum*	Cleaves peptide bonds at the C-terminal of arginine, including sites next to proline. Cleaves also at lysine residue	Requires dithiothreitol, cysteine or another reducing agent, and $CaCl_2$ to activate
Asp-N	*Pseudomonas fragi*	Cleaves peptide bonds at the N-terminal side of aspartic acid and cysteic acid residues	Not applicable
Chymotrypsin	Bovine pancreas	Cleaves peptide bonds at the C-terminal of tyrosine, phenylalanine, tryptophan and leucine. Methionine, alanine, aspartic acid and glutamine may be cleaved at a lower rate	Not applicable
Glu-C	*Staphylococcus aureus*	Cleaves peptide bonds at the C-terminal of glutamine and aspartic acid	Not applicable
Lys-C	*Lysobacter enzymogenes*	Cleaves peptide bonds at the C-terminal of lysine	Not applicable
Pepsin	Porcine stomach	Cleaves peptide bonds at the C-terminal of phenylalanine, leucine, tyrosine and tryptophan.	Not applicable
Proteinase K	*Tritirachium album* Limber	Cleaves peptide bonds adjacent to the carboxylic group of aliphatic and aromatic amino acid	Useful in general digestion of proteins
Trypsin	Bovine or porcine pancreas	Cleaves peptide bonds at the C-terminal of lysine and arginine residues	Not applicable

with the goal of applying to proteomics, the same principles are applicable to quantitative analysis of proteins. Trypsin is a relatively well-behaved endoprotease, yet, there is published report on 'missed' or nonspecific cleavage with the use of trypsin [25]. In other words, it cleaves peptide bonds at residues other than the C-terminal of lysine and arginine residues. Since most of the peptides are generated by at least two or more (the exceptions are the ones at the C-terminus and N-terminus) cleavages, any 'missed' or 'nonspecific' cleavage can affect the generation of the desired surrogate peptide (and hence quantitation of the protein analyte) especially if the 'other' cleavage is located within the surrogate peptide. Although online *in silico* tools such as ExPASy are invaluable to identify the potential cleavage positions, bioanalysts are encouraged to confirm the cleavage experimentally by identifying the formation of the desired surrogate peptides with tools such as high-resolution mass spectrometer.

For some proteins, it was reported that the use of surfactants, reduction of the disulfide bonds on proteins followed by alkylation of free thiol groups are needed to achieve efficient trypsin digestion by exposing the desired cleavage site to trypsin. Obviously, these additional chemicals, especially surfactants may introduce undesired matrix effects to the mass spectrometric analysis and the additional sample cleanup would likely result in sample loss and lower sensitivity. A number of recent studies have been focused on identifying surfactants that are compatible with mass spectrometric analysis [26–28].

Another important, albeit not intuitive, parameter is the quality of trypsin, given that the native trypsin is susceptible to autolysis in which the trypsin cleaves itself generating pseudotrypsin, which exhibits a broadened specificity including a chymotrypsin-like activity. Such autolysis products, together with contaminants [29] present in a trypsin preparation, would result in additional peptide fragments that could interfere with the detection of the target surrogate peptide. In addition, the autolysis of trypsin could result in lowering of trypsin concentration over time. This can potentially affect the digestion efficiency and specificity and hence, the reproducibility of the quantitation work. Some commercial trypsin suppliers modify the lysine residues in the porcine trypsin by reductive methylation, yielding a highly active and stable molecule that is not susceptible to autolysis. The specificity of purified trypsin can also be further improved by tosyl phenylalanyl chloromethyl ketone (TPCK), a protease inhibitor treatment, which inactivates chymotrypsin. Multiple groups [29–32] have evaluated the quality of the commercially available trypsin and it was generally agreed that it had significant impact on enzymatic digestion. Burkhart *et al.* [32] proposed a procedure to evaluate the digestion efficiency and specificity of the trypsin. The primary drawback of

using modified trypsin is the higher cost compared with native trypsin, especially for quantitation of protein therapeutics in pharmacokinetic/toxicokinetic samples in which a large number of samples are processed.

Additional parameters such as digestion buffer composition/pH, ratio of protein to enzyme and combination with other endoproteases such as Lys-C have been evaluated [26–33]. While it is possible that some of these parameters are protein-specific (or peptide-specific), it is certainly worthwhile to investigate the effect of these parameters on the tryptic digestion of the protein analyte and carefully optimize as needed during method development. In addition, though most of the investigative work has been performed extensively with trypsin because it is the most widely used endoproteases in proteomics and protein quantitation, the same parameters should be carefully considered when other endoproteases are used.

In an effort to improve reproducibility and efficiency, research has been undertaken to immobilize endoproteases such as trypsin, pepsin and protease K on solid support such as magnetic beads. The immobilized enzymes have been reported to have improved reproducibility and efficiency by reducing nonspecific cleavage, and making online digestion feasible and amenable to automation [14–15,34].

Besides enzyme digestion, it is possible to generate surrogate peptides with chemical means such as cyanogen bromide and dilute formic acid. Fung *et al.* and Wang *et al.* [35,36] reported protein quantitation with the use of dilute formic acid at elevated temperatures. The advantages of using chemical means include the relative ease of use and low cost. The major drawback is lack of specificity compared with endoproteases. Nonetheless, it is a valuable tool for protein quantitative work. Just like endoproteases, the potential cleavage positions can be identified by using online *in silico* tools such as ExPASy. As in the case of enzymatic digestion, bioanalysts are encouraged to confirm the cleavage experimentally, and evaluated the optimal parameters such as concentrations of cyanogen bromide, formic acid and temperature for the protein analyte.

With the advance in protein engineering and purification, and the wider use of LC–MS in proteomics, protein characterization and protein quantitation, there are opportunities for manufacturers to further improve the quality and specificity of the endoproteases, and identify new endoproteases with specific cleavages.

Advances in immunocapture

Protein therapeutics in general have similar physiochemical properties as other endogenous proteins and very different physiochemical properties from small molecules, therefore, traditional sample cleanup techniques for small molecules such as liquid–liquid extraction with water-immiscible solvents such as ethyl acetate and protein precipitation may not be suitable.

Recently, immunocapture has been more widely employed as a highly selective sample cleanup method by taking advantage of the unique immunoaffinity of the target analyte (either protein therapeutic or its surrogate peptide) and the capture agent, and thus provides unique selectivity. It is similar to the capture step used in LBA. The capture agent is usually an antibody specific for the target analyte and binds to solid support. In this procedure, the target analyte binds specifically to the capture agent, which is immobilized on a solid support (e.g., magnetic beads, agarose beads or column packing material), and thus is separated from other endogenous proteins and peptides, which do not bind very tightly to the capture agent. The mixture is then washed with a buffer to remove unbound proteins and other endogenous components. The analyte is then eluted from the capture agent by the addition of acid, and followed by digestion with an endoprotease, or hydrolyzed by dilute acid at elevated temperature. The resulting surrogate peptide is then analyzed by LC–MS/MS. As expected, this sample cleanup produces a very clean extract and greatly reduces the matrix effect to the LC–MS/MS analysis. Besides removing other endogenous proteins and components, the immunocapture step can also serve as an enrichment step, and hence, improve the sensitivity of the assay as detailed by Wang *et al.* [10].

As the name implies, the crucial component to successful immunocapture is the capture agent, be it an antibody, a protein or a fragment of a protein. The ideal capture agent, as in LBA, binds the protein analyte alone with high affinity (but not irreversible binding to allow dissociation of protein from the capture agent) and with minimal affinities to other potential interfering components at much higher concentrations than the protein analyte such as peptides, endogenous proteins and co-administered protein therapeutics. Commercial availability and low cost are additional attributes to an ideal capture agent. In practice, a less than ideal capture agent can be used successfully with LC–MS/MS detection because unlike LBA with nonspecific detection antibody and detection techniques such as fluorescence, LC–MS/MS provides a high-level of specificity as the detection of surrogate peptide is based on its intrinsic *m/z* ratio and in theory, as little as 1 amu difference can be detected. This allows the use of less specific capture agent in which small amount of other proteins with various affinities to the capture agent are copurified and digested by endoproteases, and the unique surrogate peptide from the protein analyte is then analyzed by LC–MS/MS. Table 2 summarizes different capture agents used for immunoaffinity enrichment.

Commercially available Protein A, G, A/G and L, immobilized on agarose beads or magnetic beads, have been used as the capture agents. These proteins binds to different areas (Fc, Fab, or κ light chain in the case of protein L) of the immunoglobulins, especially IgG of many species with different affinities. They are very useful for the protein analytes containing appropriate fragments (Fc, Fab, κ light chain etc.) of IgG as demonstrated by Chenau *el al.* [17]. and Bronesma *et al.* [37] Another commercially available class of capture agents, anti-human IgG (Fc-specific) antibodies from goat or mouse have also been successfully deployed [7]. These antibodies bind proteins analytes containing human IgG, and specifically the Fc domain of IgG. They offer the advantages of being commercially available in immobilized, high-throughput format, with established protocol, ease of use and amenable to automation. These antibodies are especially useful during the discovery phase of drug development when multiple protein therapeutic candidates are evaluated and limited resources are available to generate the antibodies specific for the protein analytes in a timely manner. The major drawback is that they also bind to other endogenous proteins containing IgG. Cross-reactivity with other endogenous proteins should, therefore, be carefully evaluated when these capture agents are used.

The best capture agents are the ones that specifically bind the protein analytes. They have been suc-

Table 2. A list of commonly used capture agents for immunocapture.

Capture agent	Target	Pros	Cons
Protein A	IgG of many mammalian species, specifically the heavy chain within the Fc region of most immunoglobulins and also within the Fab region of the human VH3 family	– Commercially available – Binds to many proteins that contain IgG – Good capture agent after immobilized on agarose beads or magnetic beads	– Not highly selective due to cross-reactivity with other proteins that contain IgG
Protein G	Binds to the Fc and Fab region of immunoglobulins	– Commercially available – Binds to many proteins that contain IgG, with different affinity than Protein A – Good capture agent after immobilized on agarose beads or magnetic beads	– Not highly selective due to cross-reactivity with other proteins that contain IgG
Protein A/G	Protein A/G is a recombinant fusion protein that combines IgG-binding domains of both Protein A and G. It combines the binding affinity of Protein A and G, and is lesser pH-dependent than Protein A	– Commercially available – Combines the affinity of Protein A and G – Good capture agent after immobilized on agarose beads or magnetic beads	– Not highly selective due to cross-reactivity with other proteins that contain IgG
Protein L	Protein L binds antibodies through light chain interactions, specifically those with κ light chain. Protein L binds to representatives of all antibody classes, including IgG, IgM, IgA, IgE and IgD. Single chain variable fragments (scFv) and Fab fragments also bind to Protein L	– Commercially available – Binds to antibodies with κ light chain, thus, offer alternative to Protein A and G – Good capture agent after immobilized on agarose beads or magnetic beads	– Not highly selective due to cross-reactivity with other proteins that contain κ light chain – Comparatively higher cost
Anti-human IgG Fc specific	Targets human IgG and does not bind other human immunoglobulins	– Commercially available – Binds specifically with proteins containing human IgG (Fc) with no significant reactivity with human IgG (Fab2), IgM or other serum proteins. It is therefore, potentially useful to fusion proteins containing human IgG	– May need to test cross-reactivity with other species IgG
Target capture	Antibodies targets uniquely the analyte of interest	– Highly specific with desired binding affinity – Minimal crossreactivity with other proteins in the biological matrices	– Time consuming and costly to produce, especially for analytes at discovery stage

cessfully used in many cases, for example, the use of anti-neuron-specific enolase to capture neuron-specific enolase [3,5,6,8,9]. These capture agents in general are custom-made, and can be either polyclonal or monoclonal. They produce the cleanest extract. They are usually used in later stage of drug development because it is in general time-consuming and costly to generate them. The capture agents can bind to the CDR of the therapeutic antibody or a unique region of the protein therapeutic. The key to the selection of a suitable capture agent is a thorough understanding of difference between the protein therapeutic and other proteins (or interfering components) in the biological matrix. For example, Fung *et al.* [35] used an antibody that targeted the adnectin region of an FGF-21-adnectin fusion protein even though the surrogate peptide was in the FGF-21 portion of the protein. Xu *et al.* [3] used anti-PEG antibody to capture a pegylated protein therapeutic by binding to the PEG component of the protein therapeutic. It is recommended to screen for a number of different antibodies to select the one with highest recovery of the protein therapeutic.

In recent years, the specificity of immunocapture has been further explored to detect/quantify analytes of biological interest that could not have been accomplished otherwise by LBA alone, for example, quantitation of antidrug antibody (ADA) complexes. Bronsema *et al.* [37] reported the use of immunocapture with Protein G as the capture agent to quantify ADA-human α-glucosidase complex in human plasma. In this work, the ADA-human α-glucosidase complex is captured by Protein G because of its unique binding affinity to the constant region of the immunoglobulin of the ADA, and thus separating from the unbound human α-glucosidase (with no bound ADA).

Another area for applying immunocapture is in the pharmacokinetic assays of ADCs. Dere *et al.*, Kaur *et al.* and Myler *et al.* [38–40] reported the use of immunocapture-LC–MS/MS to quantify the active payload (the active drug in the modality) and its metabolite conjugated to the antibody in an ADC. The ADC (with the active payload and its metabolite) was bound to the capture agent (an anti-ID antibody) and separated from the unconjugated payload. The active payload and its metabolite were then analyzed by LC–MS/MS after cleavage from the ADC by enzymes such as cathepsin B. Besides proteins, immunocapture can be adopted to capture other types of analytes. For example, Chenau *et al.* [17] reported the use of immunocapture to detect *Bacillus anthracis* spores, in which an antibody that was specific to *B. anthracis* spores was used to capture the spores, which was then analyzed by LC–MS/MS, following trypsin and Glu-C digestion.

Besides taking advantage of the unique immunoaffinity between the capture agent and the protein therapeutic to isolate the protein therapeutic before enzyme digestion, it is also possible to explore the unique immunoaffinity between the capture agent and the surrogate peptide(s) to perform immunocapture after enzyme digestion, and achieve additional sample cleanup and improved sensitivity [10]. Neubert *et al.* [41] reported performing two immunocaptures to quantify total human β-nerve growth factor, the first immunocapture step was to capture the human β-nerve growth factor before trypsin digestion, followed by the second immunocapture step to capture the surrogate peptide produced by the trypsin digestion with a different capture agent. Palandra *et al.* [42] also successfully employed the same strategy to quantify human and monkey IL-21.

Despite the unique selectivity of immunocapture, there are occasions where a more universal approach is desired, especially during the discovery phase in which a single drug candidate has not been finalized and there is interest in quantifying a class of proteins instead of a single one. Li *et al.* and Zhang *et al.* [43,44] reported the use of anti-human fragment (anti-Fc) antibody that recognized human monoclonal antibody protein therapeutics but not the endogenous immunoglobulins in the preclinical samples (e.g., monkey serum). Another possibility is to utilize more than one capture agent to capture different multiple analytes [45].

As expected, one of the major drawbacks (or bottleneck) of employing target-specific capture agent is the availability of the appropriate capture agent. In order to overcome the long lead time in generating the capture agent, research work has been pursued to expedite the generation of capture agents. Säll *et al.* [46] reported the use of AFFIRM – a multiplexed immunoaffinity platform that utilized recombinant antibody fragments (in this case, scFv), generated by phage display technology to produce capture agents against different target proteins, while Whiteaker *et al.* demonstrated that Fab alone can be used as the capture agents instead of monoclonal antibodies [47]. In addition, Bostrőm *et al.* [48] investigated the applicability of antibodies generated with Human Protein Atlas as the capture agents.

On another front, agarose beads and magnetic beads have been widely used to solid support for the immobilization of capture agents [3,5–9,37,43–44]. Agarose beads require the use of a centrifugation step or chromatographic setup for isolating the protein therapeutic and less amenable for high-throughput sample processing. In recent years, magnetic beads are gaining popularity due to its ease of isolating/removing the magnetic beads, comparability of high-throughput sample processing and shorter processing time. Their major disadvantages are time-consuming and labor-intensive

washing step and the associated cost. Yang *et al.* [49] investigated the use of ELISA microplate as a cost-effective alternative to magnetic beads. Another possibility is to reuse the antibody and additional work will need to be done on this end.

Another area that can impact the quantitation of protein therapeutic is the choice of internal standard to correct for the variability of the immunocapture between different samples. The internal standard would need to be a protein of very similar properties to the analyte so it binds to the capture agent in the same manner as the analyte. It is not likely that an analog surrogate peptide (stable-isotope labeled or otherwise) would have similar binding affinity to the capture agent as the protein analyte and thus compensate properly for the variability. Nonetheless, based on the published results [3,5–9,37,41,43–44], acceptable accuracy (within ±20%) and precision (≤20%), good linearity could still be achieved even in the absence of a stable-isotope labeled protein internal standard [50].

Conclusion

Nowadays, protein therapeutics make up for a significant portion of the portfolio of many pharmaceutical/biotechnology companies. With these new modalities, new analytical technologies are needed to properly characterize and quantify them. LC–MS/MS, especially in combination with immunocapture, has emerged as a viable technique to quantify protein therapeutics in biological matrices. With LBA being the cost-effective gold standard of quantifying proteins, one area of great potential is to apply immunocapture-LC–MS/MS to answer biological questions where LBA data alone are not sufficient, for example, quantitation of ADA–protein complexes, conjugated payload in ADC. As summarized in this work, tremendous advancement and understanding of the important parameters that can significantly impact the enzymatic digestion have been made in recent years, be it the quality of the trypsin or identification of new endoproteases. As for immunocapture, selecting a suitable capture agent requires a thorough understanding of difference between the protein therapeutic and other, often interfering proteins in the biological matrix, stage of drug development and appreciation of the biological questions that need to be answered. The commercially available capture agents such as Protein A, anti-human Fc IgG and custom-made target capture agents, with their pros and cons are all important tools in the endeavor to answer important biological questions about the absorption, distribution, metabolism and excretion of the protein therapeutics. These quickly become valuable tool kits in the toolbox of bioanalysts.

At the time of publication, most of the work published (with few exceptions) has not been conducted in regulated environment, or used in Biologics License Applications filings yet, since the field is still at a relatively early stage. There is need for continuous dialogues with the regulators before submitting pharmacokinetic data generated by immunocapture-LC–MS/MS and how they correlate with the data generated by LBA, if it is deemed possible. Toward that end, one potential area of future development is the generation of internal standards that can compensate for the variability of immunocapture and enzyme digestion, and therefore improve the reproducibility and ruggedness of the bioanalytical methods, which is important for filing purpose. Stable-isotope labeled proteins are the ideal internal standards because they have same immunoaffinity to the capture agents, enzyme digestion efficiency and mass spectrometric properties as the protein analytes, but it is time-consuming and costly to generate them. Advancement in protein engineering can result in cost reduction and decrease in production time. Another possibility is to carefully control the immunocapture procedure to minimize variability, for example, improvement in instrumentation, and it is certainly another area of focus for bioanalysts.

Future perspective

In the next 5–10 years, immunocapture-LC–MS/MS will continue to mature and will likely be more widely adopted and routinely used for protein bioanalysis. We expect further advancement in the biology of endoproteases and improvement in immunocapture technology, both in the instrumentation and the generation of capture agents, thus resulting in reduction of the cost of sample analysis and lead time for method development. With the advancement of instrumentation used for automation, the immunocapture and enzyme digestion steps can be further improved to reduce the analysis time and increase throughput. Addition dialogues with regulators will facilitate the inclusion of data generated by this technology platform in filing applications.

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Executive summary

Background

- LC–MS, especially in combination with immunocapture has emerged as a viable technique to measure protein therapeutics in biological matrices. One area of great potential is to apply immunocapture-LC–MS/MS to answer biological questions where ligand-binding assays data alone are not sufficient, for example, quantitation of antidrug antibody–protein complex, conjugated payload in antibody drug conjugate.

Advance in enzyme digestion & immunocapture

- Tremendous advancement has been made in recent years to both the enzyme digestion and immunocapture.

Conclusion

- At the time of publication, most of the work published (with few exceptions) has not been conducted in regulated environment, or used in Biologics License Applications filings yet since the field is still at a relatively early stage. There is need for continuous dialogues with the regulators before submitting pharmacokinetic data generated by immunocapture-LC–MS/MS and how they correlate with the data generated by ligand-binding assays, if it is deemed possible.

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Perspective
Special Focus Issue: Bioanalysis of Large Molecules by LC–MS

Bioanalysis

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The integration of ligand binding and LC–MS-based assays into bioanalytical strategies for protein analysis

Both LBAs and LC–MS-based assays are reviewed and summarized for applications in quantitative protein analysis. A strategy for platform selection is proposed based on several factors that contribute to the complexities of bioanalysis of biologics. Protein types, multiple co-existing forms, post-translational modifications, and affinities to ADA, targets, and endogenous proteins need to be considered when selecting the most appropriate platform. Other factors, such as intended use of data, assay sensitivity, available reagents, and multiple analytes also impact on the choice of bioanalytical platform. At BMS, strategies for the seamless integration of both platforms are being implemented to provide not only PK/PD data of the molecules but also useful information of the amino acid structure and functional relationship of the proteins.

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Background

Pharmaceutical research focused on discovering and developing viable therapies for the treatment of diseases continues to branch out in new directions to identify safe and effective drugs in a cost-effective manner. Although small-molecule drugs continue to provide an effective means for the management of many medical conditions, alternative therapeutic agents such as engineered protein constructs have also been shown to be safe and efficacious [1,2]. With this growing interest to identify diseases that could be treated with protein therapeutics, drug-discovery pipelines are filling with potential candidates that need to be rapidly characterized and selected for advancement. Additionally, gaining a better understanding of drug binding at the endogenous target, its effect on biological processes, as well as the need to better identify and select patients who would benefit from this type of therapy, are all important factors to consider. Ligand Binding Assays (LBA) [3] and Liquid Chromatography Mass Spectrometry (LC–MS)-based methods [4–7] are well-established bioanalytical platforms used throughout the pharmaceutical industry. In order for critical decision to be made during the selection and characterization of drug candidates, high-quality quantitative methods are required throughout the drug discovery and development process. However, deciding which method is appropriate for specific types of studies, as well as strategies for implementation must be established for their seamless integration into various stages of programs. This article provides details, strategies, and examples for integrating LBAs and LC–MS-based assays into bioanalytical strategies for protein analysis at different stages of the drug discovery and development process.

Bioanalytical assays for protein therapeutics & targets

Ligand binding assays

A ligand binding assay (LBA) is based on the binding of an analyte to an assay reagent such as an antibody or a receptor protein. This strategy has been successfully applied to the quantification of proteins in a given sample. Since the invention of radioimmunoassay (RIAs) for peptide hormones more than a half century ago by Rosalyn Yalow, who was awarded by the Noble Prize for her achievement [3,8,9], LBA techniques for protein bio-

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analysis, whether for evaluating PK, biomarkers or immunogenicity, have greatly progressed.

While enzyme-linked immunosorbent assay (ELISA) is the most frequently cited method in literature, increased use of electrochemiluminescence for biotherapeutic bioanalysis has been reported during the last decade, largely due to the enhanced sensitivity and dynamic range of the technology [10]. The use of Gyrolab® immunoassays has recently gained increasing interest. The technology combines the microfluidic method and fluorescence detection in a fully automated system, with the advantage of higher throughput and substantially reduced reagent consumption. Multiplex technologies, such as Luminex [11] and MSD [12] based assays, which allow multiple analytes to be assayed simultaneously in a single sample, have been widely used for biomarker evaluation and profiling. Label-free and real-time analysis technologies such as Biacore [13] and ForteBio Octet [14], in addition to being used for bioanalytical assays, have played important roles in reagent screening and characterization by producing measured affinity and binding kinetics of an assay reagent for an analyte.

Other technologies for different applications of protein bioanalysis include AlphaLisa [15], Singulex Erenna Immunoassay [16], Imperacer®Immuno-PCR [17], Optimiser™ [18], and ANP Nano-Intelligent Detection System (NIDS®) [19]. Overall, advancement of the platform technologies has enhanced the sensitivity, dynamic range and robustness of LBAs.

LBAs are the most commonly used methods to measure therapeutic proteins and protein biomarkers. These techniques are considered the standard for assessing clinical pharmacokinetics (PK). For example, only LBA was discussed in the 2007 European Medicines Agency Guideline on the Clinical Investigation of The Pharmacokinetics of Therapeutic Proteins [20]. More than 95% of clinical PK data on therapeutic proteins published so far have been obtained with LBA techniques [21]. The importance and widespread usage of LBA in protein bioanalysis are attributed to their inherent specificity, high-throughput, and high sensitivity for the analysis of wide range of analytes, especially large molecules at the femtomole to attomole level in complex biological matrices.

As the majority of therapeutic proteins function by binding to the target protein as either an antagonist or agonist, LBAs which are able to measure the binding activities of the molecules have the advantage of measuring the concentration of only molecules that have binding activity. The most often used assay formats of LBAs for PK and pharmacodynamics (PD) assessment are 'free' (unbound) and 'total' assays. A free (unbound) assay is designed to measure the analyte in which the binding site is free and, thus, able to bind the specific binding partner, whereas a total assay is designed to measure both free and bound analyte. As such, a free assay is generally considered to be the best choice when measurement of the functional molecules is needed (additional discussion is provided in the "Measurement of 'free' and/or 'total' concentrations in samples" section). An assay measuring a partially free and partially bound analyte (one binding site free and the other sites bound) was often referred to as a free assay in many publications. Figure 1 shows a schematic design of typical free/partially free assays and total assays to measure the therapeutic protein. In a free/partially free assay (Figure 1A), a drug target or a surrogate target (i.e., anti-idiotype (id) antibody that competes with the target for binding the drug) is used as a capture reagent. On the other hand, in a total assay (Figure 1B), reagents that bind to the scaffold region of the molecule and do not compete with the target binding are used for both capture and detection. A major advantage of LBAs is the possibility to analyze either total drug/target concentrations or specifically only the fraction of drug/target that is still able to bind target/drug. The discussions on the free assay in the following sections of this article also apply to the partially free assays.

Despite a long history and significant technique advancement during the recent years, LBAs still face many challenges. As these methods are based on the interaction of the biologic analyte with assay reagents such as antibodies or receptor proteins, they highly depend on the quality of assay reagents. The assay specificity and accuracy can be questionable if the reagents do not achieve the desirable affinity or specificity. In addition, endogenous components in biological matrices may interfere with reagents interacting with the analyte, resulting in potential biased results or variability.

LC–MS assays & ligand binding–LC–MS hybrid assays

For over 20 years, LC–MS detection has been the cornerstone technology in the pharmaceutical industry for definitive, quantitative analysis of small-molecule drugs, drug metabolites and endogenous entities in biological samples [7]. The success of LC–MS as the predominant technology in quantitative bioanalysis stems from its ability to detect multiple analytes with high specificity and sensitivity within a single sample which enhances the magnitude of information that can be gained in selected experiments. The growing interest and expanded use of LC–MS-based methods for the quantitative analysis of proteins has grown from the experiences gained in small-molecule bioanalysis.

Some of the same advantages garnered in the analysis of small molecules: rapid development of methods on existing platforms, demonstration of wider linear dynamic ranges for assays, and discrimination of multiple target analytes on a molecular level have firmly positioned LC–MS as a widely applicable technology platform for protein bioanalysis, as well as a viable complementary technique to established ligand binding assays. Nevertheless, there are also significant challenges in isolating, concentrating, detecting and quantifying specific proteins by LC–MS in the presence of the overwhelming abundance of endogenous proteins found in biological matrices. Optimizing procedures that first isolate and then specifically detect either intact protein or selected peptides following enzymatic digestion of the protein of interest is critical to successful method development. Achieving sufficient sensitivity by mass spectrometric detection of intact proteins is challenging due to the charge distribution that occurs when a protein is ionized. Therefore, the quantification of proteins larger than 10 kDa molecular weight is commonly performed by digesting the protein followed by the LC–MS/MS analysis of one or more surrogate peptide(s) using a triple quadrupole instrument with optimal sensitivity. The digestion step is critical to the success of the quantitative method and significant research has gone into determining optimal conditions [22]. Additional steps include the selection of targeted protein or surrogate peptide ions from *in silico* modeling, LC–MS parameter optimization for the detection of multiple peptides, sample preparation to isolate and/or enrich analyte proteins, optimization of proteolytic digestion conditions, sample analysis, and data processing and reporting on multiple analytes/peptides.

Plasma and tissue matrices can present significant challenges for low level detection of proteins. Affinity enrichment procedures, described as ligand binding (LB), immunocapture (IC) or immunoprecipitation (IP), have been increasingly applied in quantitative bioanalysis to remove interfering matrix components from plasma and tissue samples in protein quantification methods [23,24]. As described earlier for LBA, affinity enrichment utilizes a reagent to selectively 'capture' analytes (e.g., protein or peptide) from the biological sample through affinity binding interactions. Following the binding step, unbound matrix components (e.g., endogenous proteins, lipids, carbohydrates, salts) are washed away. In LC–MS-based detection methods, the analyte is then either released from the capture surface, then digested or the bound analyte can be directly digested *in-situ* prior to LC–MS/MS analysis which offers the potential to specifically detect

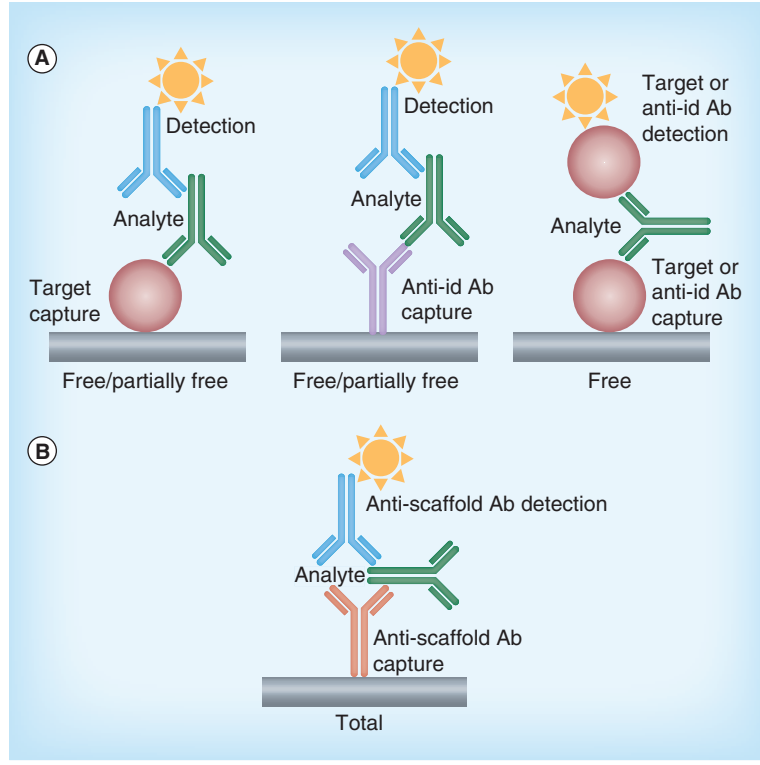

Figure 1. Examples of ligand binding assay free and total assay formats. **(A)** Free/partially free assays, the anti-idiotype antibody shown in the diagrams only takes account of the anti-idiotype antibody that competes with target for binding to the analyte. **(B)** Total assay.

which analytes where actually captured. This is useful in assessing the specificity of the capture reagent, but may limit the sensitivity of the assay because of the lower MS response factors when compared with signal amplification factors used in ELISA detection. Affinity enrichment procedures provide the cleanest samples for LC–MS analysis among all available sample pretreatment techniques and sample enrichment capabilities. However, it also requires the availability of high quality reagents and may be subject to the same matrix interference as experienced by LBAs. Ackermann and Berna [25] published the definitive review article describing LB–LC–MS techniques.

LC–MS methods based upon the simultaneous detection of multiple peptides derived from the protein of interest may offer several advantages. These include the opportunity to obtain specific molecular information on protein modifications resulting from metabolism, assessment of the protein stability in biological matrices and the capability to simultaneously measure multiple proteins (target and therapeutic) in the same sample, as a means to assess the accuracy, precision and specificity of the assay. However, LC–MS methods using digested peptides as surrogates for the protein may show potential bias due to chemical/enzymatic

modification of surrogate peptides. Although still early in the application development phase, LC–MS has already begun to demonstrate utility as a viable bioanalytical technique for quantification of proteins in discovery laboratories.

Factors contributing to increased complexity for bioanalysis of biologics

With the expansion of protein engineering capabilities for drug design and an increase in the number of drug targets that are being pursued, there is an increasing demand placed on bioanalysis, especially in early discovery to support PK and PD studies. In comparison to the bioanalysis of small-molecule drugs, the bioanalysis for large protein therapeutics has its own unique complexity, which requires the selection of the best technologies and methodologies.

Many factors contribute to increased complexity for bioanalysis of biologics. First, there is increasing diversity in the types of therapeutic proteins under development. These include monoclonal antibodies, domain antibodies [26], and antibody–drug conjugates (ADC) [27]. Additionally, there are molecules with alternative scaffolds, such as Adnectin [28], DARpin [29,30] and Centyrin [31] as well as fusion proteins, PEGylated molecules and bi-specific molecules. Each of these modalities requires different bioanalytical strategies and methods that are suited for the physiochemical properties.

Secondly, multiple 'species' or 'forms' can co-exist in biological matrices for many therapeutic proteins and their targets. Biotransformation or post-translational modifications can result in catabolites or modified forms in addition to the intact and unmodified molecule. Due to biological interactions, a therapeutic molecule and its target protein can exist as bound, partially bound, free and high-order complex forms. Different forms often have distinct functions. As a result, multiple end points may be needed for PK/PD analysis of each therapeutic candidate, such as total and free drug, and target concentrations.

Additionally, unlike analysis in a buffer system, matrix interferences from biological matrices add complexity to bioanalysis. Those matrix interfering factors include but are not limited to soluble targets, anti-drug antibodies, disease and treatment-specific factors and other endogenous proteins. They could either negatively interfere with or positively enhance assay reagent binding to analytes or skew detection signals. In unique cases, some interference can be desirable, such as the interference of a soluble target protein to the assay measuring the 'free' therapeutic protein; however, many are not desirable and must be minimized.

Lastly, target specificities for biologics are often different between species, more so than observed for small-molecule targets. In the cases of animal PK/PD testing, different surrogate protein constructs are required to bind to animal targets to assess the same mechanisms of action as theorized for human targets. These surrogate proteins for the animal PK and toxicity testing will have different amino acid sequences and different ligand binding affinity than the human equivalents. Therefore, different assays are often needed for different species due to not only different biologic matrices but also to different target specificities. Also, as mentioned earlier, ligand-binding assays highly depend on the availability of quality of assay reagents which can take months to generate. In the discovery space, fast turn-around of data are needed for quick decision making on candidate molecules. Therefore, it is not always feasible, in terms of cost and time, to develop all of the assay reagents for LBA needed for early candidate selection. Mass spectrometry has played an increasingly important complementary role in many of these cases.

Considerations on what forms of therapeutic proteins & their targets should be measured in serum or plasma samples

In order to select the best bioanalytical platform for analysis of samples from specific studies, it is important to understand which 'form' of the analyte needs to be measured. As mentioned in the previous section, therapeutic and target proteins can exist in multiple forms in samples collected during the course of a study through either biological interactions with their targets/partners or through biotransformation modification. Understanding factors that impact on forms to be measured is critical in selecting the right bioanalytical assay. Two important considerations are "what is the purpose of the study?" and "how is the data intended to be used?" For example, a study that focuses on off-target binding likely requires a different set of data from one that assesses the exposure and efficacy relationship. Other factors which affect the measurement forms include therapeutic protein modality, target characteristics, and post-translational modification risks. Deciding "which form(s) to measure?" and "how to measure them?" have complicated the selection of bioanalytical strategies. The following discussion and recommendations represent general bioanalytical concerns for therapeutic proteins and target measurements and are not intended to be all-encompassing.

Measurement of 'free' and/or 'total' concentrations in samples

Most therapeutic proteins are designed to interact with specific circulating or cell membrane bound target proteins via noncovalent, high affinity, reversible

binding that elicit a pharmacological effect. Once administered to animals or to humans, therapeutic proteins can bind to various components such as circulating target proteins, anti-drug antibodies, and other endogenous entities in circulation. Defined here, "free" (unbound) forms which have their target binding sites free of binding partners, "partially bound" forms which have at least one target binding site free, and "fully bound" forms which have no target binding site free, may co-exist. The free and partially bound forms are considered bioactive forms in most cases due to their target-binding potential. The levels of these circulating binding partners may change with time in relation to the drug PDs, immunogenicity, or disease state. Drug-free and drug-bound forms of the therapeutic target proteins also co-exist in relation to drug exposure, target engagement, and biological responses. As a result, reliable methods to measure either the free or the total concentration of biologics and their targets are needed to fully evaluate the drug PK/TK and their relationship with PD/TD [32,33].

The significance of measuring free levels of therapeutics continues to be an ongoing topic of discussion in the bioanalytical community [32–36]. Measurement of free drug concentration was considered to provide a better estimate of efficacious concentrations and safety margins [33]. The AAPS Ligand-Binding Assay Bioanalytical Focus Group initiated discussions of free and total drug and target measurements at the 2008 National Biotechnology Conference. They then published a white paper on challenges and issues of measuring free and total drugs and targets, and how data should be used to support drug discovery and development [32]. The paper recognized the challenges in developing free drug and target assays and the method caveat; therefore, questions were raised on when free assays are needed. The diagram in Figure 2 provides some guidance to assess the need for such determination.

The characteristics of target proteins are one of the important factors for determining whether free assays are needed. Therefore, our decision tree in Figure 2A begins with questions on whether the target is a circulating or membrane-bound receptor.

The recommendations for assay choice are discussed below for each of the three categories of the molecules based on the target characteristics. This recommendation is limited to therapeutic proteins that function by binding to target proteins as an antagonist or agonist. For therapeutic compounds that are analogues of endogenous proteins, the discussion in the section of "Assessment of Structure Variants of Proteins in Samples" will be more appropriate.

Circulating soluble targets

Circulating molecules, such as cytokines, have been attractive therapeutic targets. Their endogenous concentrations range from picogram per milliliter to nanogram per milliliter. In addition, total target levels often increase in a disease state and following drug treatment due to reduced clearance, as a result of being bound to the therapeutic protein. Since the concentrations of both free and total target concentrations provide key evidence of target engagement, the measurements of both free and total target levels for studies in discovery and early development are often recommended. Whether a free assay is required for measuring the therapeutic protein depends on several factors including the concentration of targets, drug levels after dosing and drug–target affinities. The mathematical model to estimate the impact of those factors has been described previously [36]. In the discovery or early development space, determining low levels of drug concentrations is often important for comprehensive PK analysis including the calculation of terminal half-life. Free assays for therapeutic proteins, in general, are preferred, especially when soluble targets are at comparable levels to those of therapeutic proteins or when the differences between total and free therapeutic concentrations are expected to be large. In cases where the drug–target affinity equilibrium dissociation constant (K_D) is known, the concentration of free therapeutic protein can be deduced in theory from the concentrations of total therapeutic protein and total target. However, how best to accurately measure the K_D in biologic samples presents another bioanalytical challenge.

Cell surface targets that have shed or soluble form in circulation

The targets for many therapeutic proteins are membrane-bound. These targets may shed their ectodomain (ECD) physiologically or have a soluble form in systemic circulation. The level of shedding is regulated and could be significantly elevated in disease states, or affected by different stimuli including therapeutic treatment. The shed or soluble form of target protein in circulation may reduce the effectiveness of the drug therapy by occupying and thus reducing the number of binding sites available to bind to cell surface target. In such cases, the recommendation of assay selection for the therapeutic protein is similar to that for the soluble target as described earlier (see circulating soluble target). The concentration of the shed or soluble forms of the target, although not as a direct target engagement biomarker, has been used as a disease or predictive biomarker in some cases. The decision to measure the shed or soluble form of the target depends on the utility of the biomarker for each specific target.

Cell surface targets that have no (or minimal) shed or soluble forms in circulation

If the membrane-bound cell surface target has negligible levels of shed or soluble forms of the target protein, the therapeutic protein is generally thought to exist in the target free form and, therefore, target interference on drug quantitation is not anticipated. Either free or total drug assays, in general, can be considered 'fit-for-purpose'.

However, since biotherapeutic proteins can elicit immunogenicity, the generated anti-drug antibodies (ADAs) in these samples are also likely to either bind to the therapeutic protein's target binding site or change the conformation of the protein and thus block it from interacting with the target. Therefore, concentrations of the therapeutic protein which are free from ADA binding at the target binding site are more relevant to estimate overall drug function. Immunogenicity responses generally start 10–14 days after dosing and are more predominant if the species, from which the therapeutic protein is derived, is different from the one that is being dosed in the study.

Assessment of structure variants of proteins in samples

Following *in vivo* dosing, therapeutic proteins may undergo biotransformation, such as proteolysis, deami-

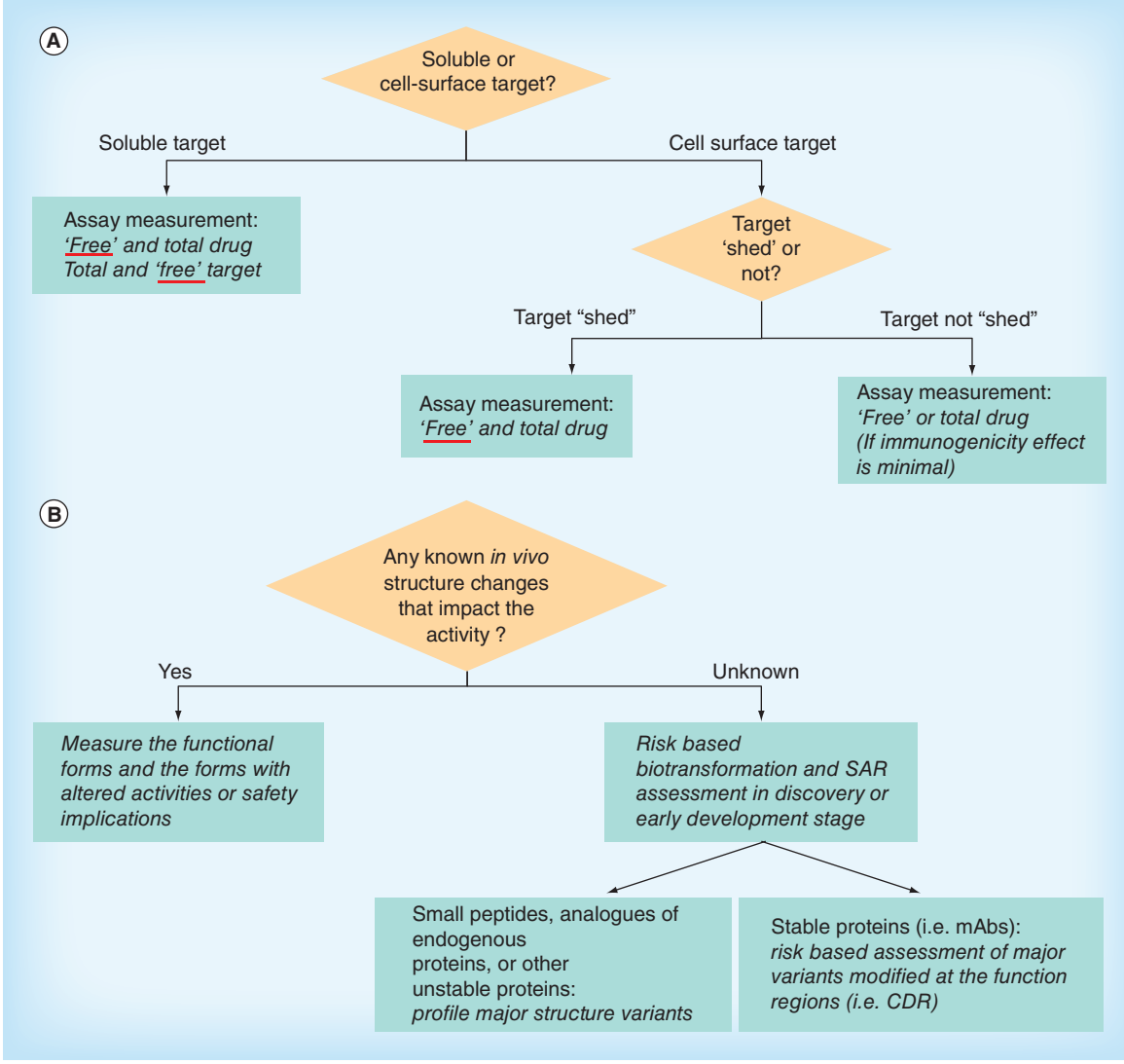

Figure 2. Work flow to determine which forms of the analyte to measure in serum or plasma samples. Work flow with consideration on **(A)** target characteristics and **(B)** protein biotransformation liabilities. These work flows are useful in setting bioanalytical strategies in discovery and early development stages where more comprehensive data are desirable to make informed decisions on compound selection. At later stages, however, bioanalytical methods are focused on fewer selected analytes in specifically designed studies.
*Underlined: Higher priority; 'Free' drug: Free or partially free of target binding but may be bound to other proteins; 'Free' target: Free or partially free of drug binding but may be bound to other proteins.

dation, glycolytic hydrolysis, isomerization, oxidation, phosphorylation, glycosylation and/or disulfide bond disruption, resulting in structural modifications. Such modifications to the drug may impact stability as well as function including efficacy. Metabolites from both endopeptidase and exopeptidase activities have been observed for peptides conjugated to Fc, such as in the cases of romiplostim and Fc-FGF21 conjugates [37,38]. Endogenous target proteins can also co-exist in multiple forms with different structures as the result of post-translational modifications and/or metabolism. Due to the greater structural complexity of proteins compared with small-molecule drugs, it is more challenging to quantitatively determine the structure–activity relationships (SARs) for therapeutic protein candidates. In general, it has been considered sufficient to measure the concentrations of 'total functional' therapeutic molecules related to an administered therapeutic protein that bind to a target for PK/PD modeling. Risk-based assessment based on amino acid sequences and *in vitro* data is recommended to determine whether structural variants may have safety and/or efficacy liabilities that would require specific monitoring of that entity (Figure 2B). In the discovery space, biotransformation and SAR assessment is recommended for therapeutic proteins with likely structural modifications that might impact safety and efficacy to provide guidance on drug design and selection to minimize the liability. For small peptides, analogues of endogenous proteins, and/or other unstable proteins, profiling major structural variants such as cleaved or modified forms is highly recommended. For proteins with more stable structures, such as antibodies, in general, the assessment can focus on effects at the target binding site (i.e. CDR) or other function relevant regions as needed (i.e. FcγRs binding site). For target measurements, which form to measure is determined on a case-by-case basis, depending on the utility of the data.

Considerations on platform choice

Many factors influence the decision on the preference of platforms between LBA and LC–MS assays to address bioanalytical needs. The key questions to ask include: "what is the question at hand – efficacy, safety or target engagement?", "what forms of an analyte can provide this answer?" and "what are the bioanalytical challenges associated with a platform to measure those forms?" In addition, the types of proteins, availability of reagents, sensitivity requirement and development stage need to be taken into consideration. The decision trees and recommendations in the following sections are aimed at providing guidance on how to best select and integrate LBA and LC–MS assays into bioanalytical strategies for measuring therapeutic and endogenous proteins through drug development in Bristol-Myers Squibb.

Factors to consider

If a free form of a protein to be quantified, a LBA using the target protein or target surrogate (i.e. anti-id antibody) as the capture reagent is a preferred choice, with affinity enrichment (with a target protein or an anti-id Ab) LC–MS assay as a backup. However, if the total concentration of all forms of a protein are needed, either LBA or LC–MS-based assay can be used based on availability of the reagents and other factors discussed in the following paragraphs (Figure 3A) [39,40]. It can be challenging to measure a free form of a therapeutic protein in presence of ADA, as the desirable assay should tolerate ADA interference at the non-target binding but not at the target binding site. If the detection reagent used in a LBA binds to a non-target-binding site of the drug, such binding may be interfered by the ADA. Consequently, the assay may underestimate the drug concentration. If ADA interference is a big concern, a LBA, in which both capture and detection reagents bind to the target-binding sites, can be considered. Alternatively, an affinity enrichment (with a target protein or anti-id antibody) LC–MS assay can be a good option since a second binding step is not needed. An example showing the effects of ADAs or other matrix factors on LBA and LC–MS assay results and thus PK profiles is described in the case studies.

When biotransformation and/or SAR assessment are needed for therapeutic proteins at risk for structural changes that may impact safety and efficacy, platform selection depends on the risks and the forms of the analyte that need to be measured [38,41] (Figure 3B). If measurements for site-specific modification variants are needed, such as deamidation and oxidation variants, LC–MS is often the preferred approach [42,43]. A LBA platform is implemented only as needed or if the 'right' reagent in terms of specificity is available. If the risks are due to cleavage, higher order complex, or conformational changes, parallel LBA and LC–MS approaches, if available, are recommended during discovery or early development stages to provide comprehensive data for better understanding of the effect of these variants on the drug exposure–response relationship. After sufficient understanding is achieved, either LBA or LC–MS approach can be used to support later studies based on the earlier data. When concentrations of a full-length protein are desired, an LC–MS approach is preferred if the protein size is small (i.e. MW <10 kDa); otherwise, either an LBA or LB–LC–MS hybrid method to capture one specific site of the molecule and detect another specific portion of the protein is recommended.

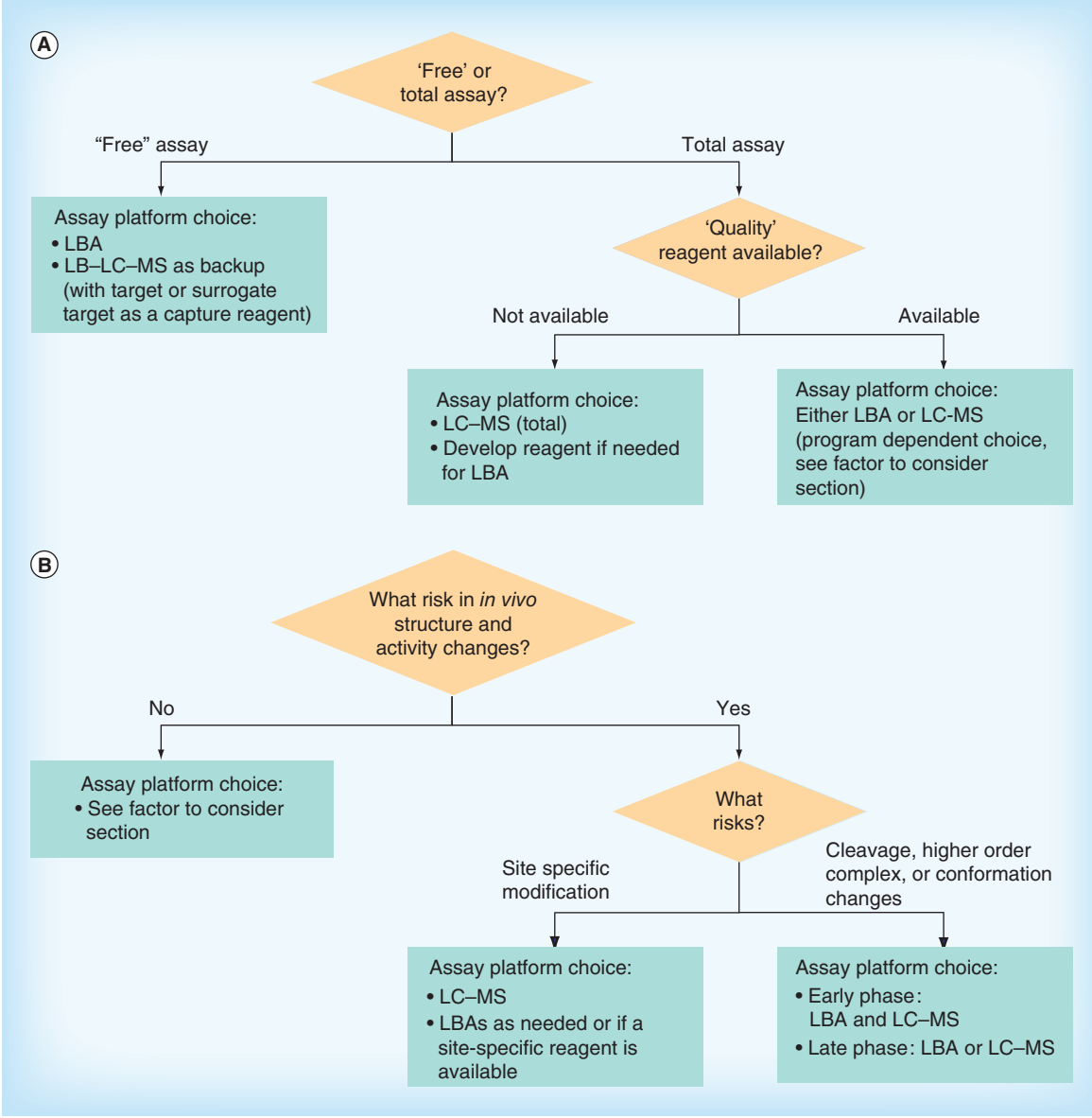

Figure 3. Assay platform considerations. Assay platform when **(A)** a free or total assay, or **(B)** measuring a specific structural variant is preferred.

In the discovery stage, the measurement of multiple drug candidates, surrogates and comparators for the same target are often needed during candidate selection and optimization. LBAs or LB–LC–MS hybrid assays using target proteins as capture reagents are often the preferred approaches to assess PK parameters. Such approaches allow assays with the same or similar formats to measure and compare the functional PK of those molecules, especially in cases where the sequence information of those molecules are not available. Alternatively, generic LBA methods using reagents that bind to sequences in the scaffold region or generic LC–MS methods that detect scaffold peptides are often quick initial assays that are used in early discovery, if the scaffold regions of the drug are derived from different species than the dosing animals [44–47]. The downside of using a generic assay is that the assay in general measures the concentration of the total analyte, which may not always reflect the exposure levels of the functional molecule.

Some studies require measurements of multiple analytes with similar structures in the same samples, such as a peptide analog drug and its endogenous counterpart or peptide drug and its enzymatic cleavage metabolite [48,49]. High-quality reagents may not always be available early in the process to distinguish those analytes. In these cases, LC–MS is often the preferred choice to selectively measure them. For example, therapeutic peptide davalintide has the same amino acid sequence as its active proteolytic metabolite, except for a single lysine at the N-terminus. Generation of an antibody

capable of distinguishing both davalintide and des-Lys davalintide species for a specific LBA is particularly challenging. In contrast, LC–MS can readily distinguish both species, since they differ by 146 mass units, and quantify them simultaneously [49].

When measuring an endogenous target protein, both assay platforms face additional challenges which include the availability of a true reference standard, the fundamental understanding of baseline levels versus nonspecific matrix background, and the true recovery of the analyte from the biological matrix in the assays. One of the common practices of assessing the assay recovery of an endogenous analyte is use of a recombinant protein as the reference standard and testing it in a surrogate matrix (e.g., synthetic serum, buffer). While such approach adds confidence in evaluating assay accuracy and precision, whether the assay results truly reflect the endogenous protein levels in real biological matrixes is still questionable. For the LBAs, the assay reagents selected may not always meet the requirements for optimal selectivity, binding affinity and avidity. In addition, the detection response for the analytes in the method may not always achieve the needed specificity and sensitivity. On the other hand, a LC–MS-based assay may have difficulty in measuring low level endogenous target protein and the detection may be interfered with by other abundant proteins in the samples. In this case, the assay selection will be decided case by case based on selectivity, sensitivity and availability of reagents. LB–LC–MS hybrid assay can be a good option when only one antibody reagent is available or the specificities of the reagents are not optimal.

It is becoming more common to quantify co-administered protein therapeutics in selected studies. In general, multiple LBAs have been developed to quantify those co-administered therapeutics. However, if reagents are available, LBA multiplex assays such as those based on Luminex or MSD platforms, which have been widely used for simultaneous analysis of multiple biomarkers in the same assay, have the potential to be used to measure multiple protein therapeutics in the same assay especially in the discovery and nonregulated space. If reagents are not available, LC–MS technology, can be used to simultaneously quantify multiple proteins and/or their metabolites [44,45,50].

As understanding the drug exposure at the site of action is one of the pillars of the fundamental PK/PD [51], there is an increase in the number of requests to quantify biotherapeutics concentrations in tissues. While both LBAs and LC–MS assays can be used; when harsh extraction conditions are needed to liberate the drug analyte, LC–MS without affinity capture is the preferred choice due to its higher tolerance to harsh chemical conditions.

Other factors may also influence the decision on the choice of the platforms. For example, LBAs are often the choice if high sensitivity is required. Furthermore, when the function of the protein molecule is more susceptible to matrix interference, an LBA or a LB–LC–MS hybrid assay with target or a target surrogate (i.e., anti-id antibody) as a capture reagent can better estimate the concentration of the functional molecule (i.e., the molecules competent in binding the target). However, if the matrix factors interfere with reagent binding to the analyte in a LBA, a LC–MS method is often preferred.

While driving towards making fast decisions at a specific stage of development is important, alignment with future platform needs (e.g., preclinical vs clinical support) and ensuring the transferability of the method for future studies are also critical. Furthermore, the optimal use of available resources and capacity in either LBA or LC–MS teams play another important role in assay platform selection to meet tight timelines in both discovery and development.

Cases when both LBA & LC–MS assay are recommended

In principle, only one bioanalytical assay platform should be used to support the majority of studies for protein therapeutic drug development when considering available resources and time for decisions. There is no need to always have both LBA and LC–MS assays developed in parallel even though bioanalysts tend to do that during the initial stages of method development. It has been reported that the results generated by both types of assays have good agreement with each other in most cases from a statistical point of view [39,50,52]. In some cases, however, due to the complexity and diversity of emerging biologics drug portfolios, as discussed before, it is becoming a necessity to have both sets of data from LBA and LC–MS assays to provide complementary information to aid better decision making. A number of examples are listed here:

- Evaluation of molecules with risk of *in vivo* modifications in structure which impact pharmacological activities including on- or off-target binding, clearance and distribution during discovery and early development (see the discussion in the section of 'Structure variants'). The data from both LBA and LC–MS assays can provide more comprehensive understanding of both SARs and liability of the molecules;

- Investigating PK/PD disconnect. Comparison of data/information from both LBA and LC–MS assays can help identify issues such as potential data bias from either technology platform;

- Providing data on multiple bioanalytical end points to establish drug exposure and responses for complex modalities such as ADC, for which the concentrations of total antibody, conjugated antibody (or conjugated drug) and unconjugated drug are needed. Traditionally, the total antibody concentrations are measured by LBAs and unconjugated drug concentrations are measured by LC–MS. Either LBAs or LC–MS assays have been reported for measuring ADC depending on the quality of the available reagents and whether the conjugated antibody or conjugated drug concentrations better meet study need [53,54];
- Verification of the effect of ADAs on drug clearance, when needed. The reduction in drug concentrations measured by LBA may due to either enhanced drug clearance or ADA interference with assay reagent binding. The data for total drug concentration by LC–MS can add clarity to the observed ADA effects.

Case studies

Example of PK/PD assessment of a therapeutic protein that binds a soluble target

The drug candidate is an engineered fusion protein composed of a human IgG1-Fc domain fused to an adnectin molecule [28] that targets a human soluble protein in circulation as an inhibitor. This drug candidate was investigated for nonclinical PK/PD and safety assessments. LBAs were developed for analysis of the free drug, free target (free of drug binding) and target–drug complex, and a LC–MS/MS bioanalytical method was developed for analysis of the total drug in serum samples. The recombinant target protein was used as a capture reagent for the 'free' drug assay, and the drug molecule was used as the capture reagent for the 'free' target assay. In the assay measuring target–drug complex, an anti-target antibody, that did not compete with the drug in target binding, was used as capture reagent and another antibody that binds to the drug scaffold region was used as the detection reagent. As matrix interference with LBA reagent for the total drug assay was suspected, the LC–MS/MS assay was selected for the total drug measurement. The Figure 4 shows a typical example of concentration versus time profile of total drug (LC–MS), drug–target complex (LBA), and free target (LBA). Greater than 90% reduction in free target was observed up to 24 h in serum following administration of the drug candidate, demonstrating strong target engagement. The target–drug complex was detected shortly after the administration. After reaching the maximum level, the target–drug complex concentrations followed a

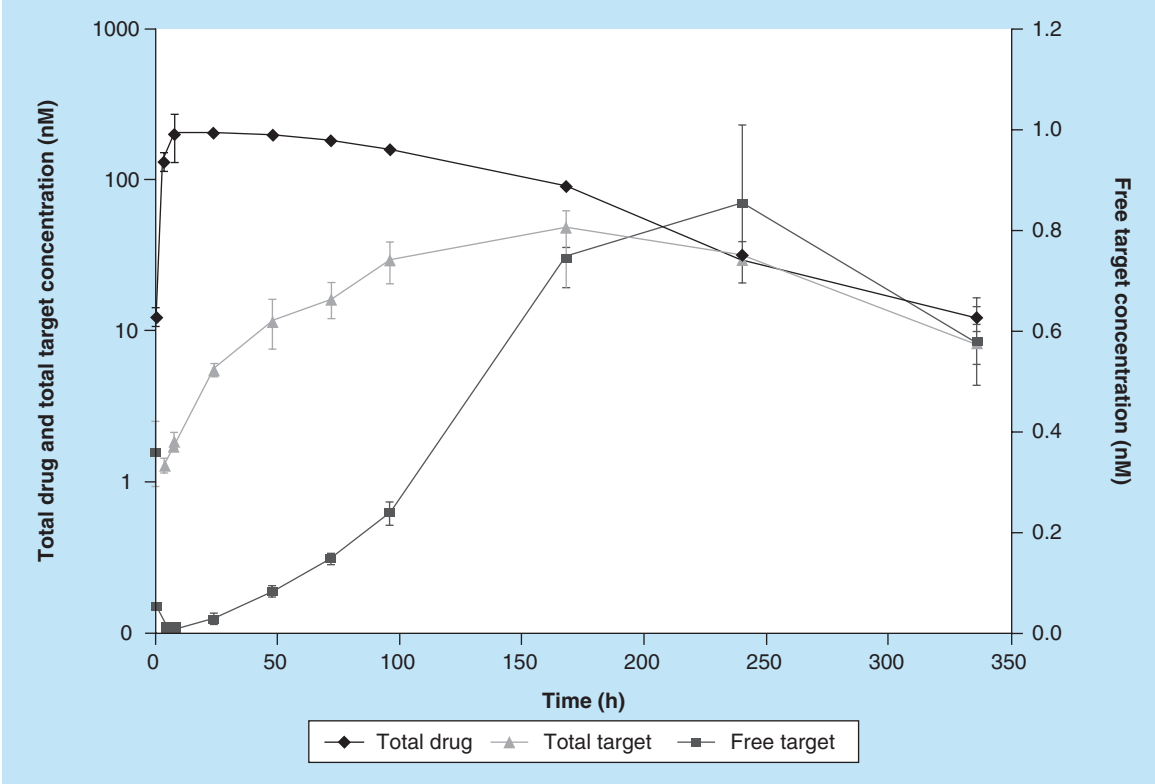

Figure 4. Phamacokinetic/pharmacodynamic assessment of a therapeutic protein that targets at a soluble molecule.

mono-exponential decline. Lower concentrations of free drug were observed in comparison to the total drug (data not shown), correlating to of formation of the target–drug complex. As in this special case, the target–drug complex also plays an inhibitory role on the target-mediated pathway to the same extent as the drug candidate, the concentrations of the total drug (including both free and bound drug) were considered to best estimate the functional drug and thus used for the PK/PD analysis. The concentration data on the free target and the target–drug complex provide quantitative information on target engagement. The PK and PD data in conjunction with other efficacy and safety data enable PK/PD modeling and human dose projection. Integration of both LBA and LC–MS assay enable such comprehensive PK/PD analysis.

Example of simultaneously measuring a therapeutic protein & endogenous protein with minor difference in amino acid sequences

A therapeutic protein, recombinant-methionyl human leptin, derived from endogenous leptin is being developed for treatment of human lipodystrophy, a rare disease caused by leptin insufficiency. The molecular weight for the therapeutic protein is 16,155 Da and its amino acid sequence is the same as the endogenous leptin except one extra methionine at the N-terminal. A sandwich ELISA assay was developed to measure the total leptin concentration in human plasma which includes both drug and endogenous leptin level due to lacking of site-specific reagents [48]. In some clinical studies, the total leptin concentration was significantly higher than expected for some subjects. It was desired to understand what forms of leptin contributed to the elevation of total leptin concentration in order to better understand both safety and efficacy. A LC–MS/MS assay was developed to quantify both recombinant and endogenous leptin simultaneously using an API4000 Q-trap LC–MS/MS system. The assay measured the intact proteins after immunoaffinity sample cleanup without enzymatic or chemical digestion. The LC–MS/MS assay was able to specifically detect both forms since there was a 131 Da mass difference. The assay reached a lower limit of quantification of 15.63 ng/ml, which was close to endogenous leptin concentration (0.5 to 30 ng/ml). The total concentration results from the ELISA assay and the LC–MS/MS assay (sum of recombinant and endogenous leptin) correlated well with a R^2 value of 0.9011. The assay was used to analyze samples from 16 subjects in a clinical study which revealed that some subjects had significant elevated levels of leptin. Figure 5 shows the concentration data generated from the ELISA and LC–MS/MS assays for total leptin (ELISA), recombinant human leptin

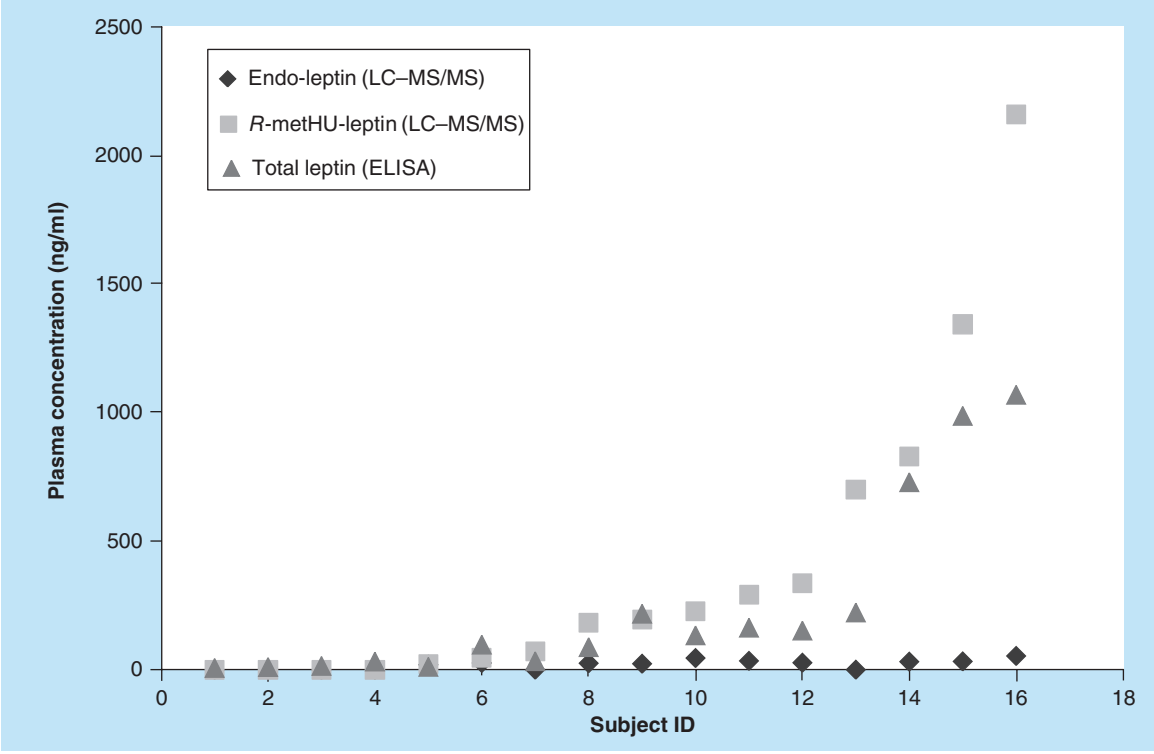

Figure 5. The LC–MS/MS assay provided the concentration for both recombinant and endogenous proteins in circulation and facilitated the rapid decision making for the program.
Data taken from [48].

and endogenous leptin. The results clearly demonstrate that the accumulation of the total leptin after dose was caused by recombinant human leptin while endogenous leptin remain at predose levels.

Example of assessing the effects of ADA & other matrix factors on PK profiles

LC–MS/MS and ELISA methods were developed for the quantification of a PEGylated protein drug in support of discovery studies in cyno monkeys. The LC–MS/MS-based detection method was developed using an extraction procedure for the therapeutic protein via a specific water-miscible organic solvent followed by trypsin digestion and subsequent detection of several surrogate peptides. The assay was linear between 10–3000 ng/ml. The ELISA method used a therapeutic target-binding format in which the recombinant target antigen captured the drug, and was followed by detection with an anti-PEG monoclonal antibody. The assay range was 30–2000 ng/ml. A correlation study was conducted to measure drug concentrations in plasma samples obtained from a single dose (5 mg/kg subcutaneous) PK study in cynomolgus monkeys (n = 4). The drug concentrations obtained by the LC–MS/MS method agreed very well with those obtained by the ELISA method in the early time points of the PK profile. However, at later time points, the drug concentrations measured by the LC–MS/MS method were consistently higher than those measured by the ELISA method. (Figure 6). PK parameters calculated from the plasma-concentration levels showed that both methods showed equivalent peak exposure (Cmax) at 24–48 h. However, the LC–MS/MS results calculated to be approximately 1.53-fold higher total exposures (AUC_{tot}) than the ELISA results. The difference between the LC–MS/MS and ELISA results was investigated through immunogenicity testing, ADA epitope mapping, and Western blot analysis of samples coupled with protein G separation. The results showed the presence of ADA that were specific to the antigen-binding region of the scaffold protein drug and interfered with drug binding to the target antigen in the ELISA method. In the presence of the ADA, it was postulated that the ELISA method measured only free circulating drug (target-binding), while the LC–MS/MS method measured total circulating drug levels. The work presented here indicates that the bioanalysis of protein drugs is more complicated than for small-molecule drugs because of the presence of drug-binding endogenous components or ADAs in the post-dose (incurred) samples. The clear understanding of the behavior of different bioanalytical techniques vis-à-vis the potentially interfering components found in incurred samples, and which forms of drug (i.e. free or total) are critical in selecting bioanalytical strategies for measuring protein drugs.

Conclusion & future perspective

The complexity and the diversity of biologics portfolios require a variety of analytical tools to address the scientific and technical issues associated with these molecules. It is critical to integrate ligand binding and mass spectrometry-based assays into bioanalytical strategies to help provide more meaningful data for decision making in a timely fashion. In this article, both LBAs and LC–MS-based assays are reviewed and summarized

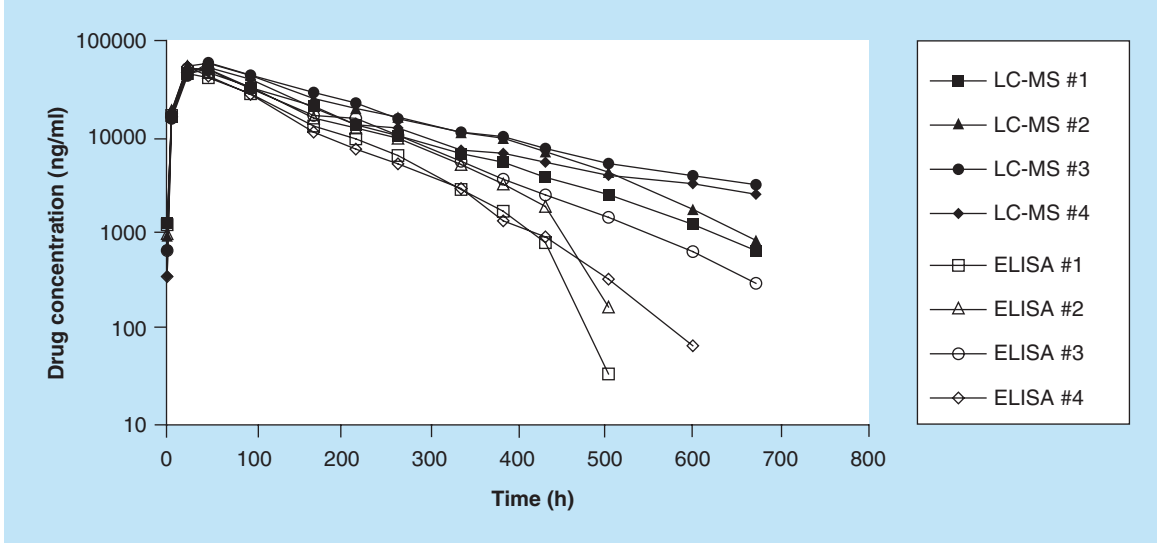

Figure 6. The plasma concentration versus time profiles obtained by LC–MS/MS (solid symbols) and ELISA (open symbols) in four cynomolgus monkeys. Attribution of the discrepancy between ELISA and LC–MS/MS assay results of a PEGylated scaffold protein in post-dose monkey plasma samples due to the presence of anti-drug antibodies. Reprinted from [40].

for their applications in protein analysis. A strategy on selection of platforms to support biologics development is proposed based on factors contributing to complexities of bioanalysis of biologics. Protein types, multiple co-existing forms, post-translational modifications, and interactions to ADA, targets and endogenous proteins need to be considered when selecting a bioanalytical platform. Multiple end points for PK/PD analysis require a variety of bioanalytical tools. Other practical factors, such as assay sensitivity, available reagents, multiple analytes and resources also impact on the choice of bioanalytical platform. Although the LBAs are currently the gold standard for protein analysis in biological samples because of their sensitivity and high-throughput capacity, with new instrumentation and better sample cleanup processes being developed, LC–MS-based assays are becoming more readily applicable in biologics drug development. In fact, we find that on many occasions, both assays are needed to answer the development questions. In the near future, LBAs and LC–MS-based assays will be more seamlessly integrated to provide not only PK/PD data of the molecules but also useful information of the amino acid structure and functional relationship of the proteins. A better understanding of the structural characteristics of therapeutic proteins *in vivo* will improve our overall biomedical understanding to facilitate drug development.

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Executive Summary

- Ligand binding assays (LBAs) are currently used to quantify the majority of protein biotherapeutics, endogenous targets and biomarkers.
- LC–MS assays and ligand binding–LC–MS hybrid assays have been increasingly used in both discovery and development.
- The expanding diversity of biotechnology compounds, multiple forms co-existing in biological matrices, post-translational modifications, need for multiple end points for phamacokinetic/pharmacodynamic analysis, and interaction with anti-drug antibodies, targets and other endogenous proteins, all contribute to the increased complexity of bioanalysis of biologics which often requires integration of both LBA and MS assays to support.
- Determination of which form of the analyte (i.e., free, total, site-specifically-modified and cleaved) to measure is a key step to selecting appropriate bioanalytical platform.
- Other practical factors, such as assay sensitivity, available reagents, multiple analytes and resources also impact on the choice of bioanalytical assay platform.
- A strategy on selection of bioanalytical platforms to support biologics development is proposed based on above discussed factors.
- It is critical to have integrated bioanalytical strategies which include both LBAs and LC–MS assays to aid fast and informative decision making.

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