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MINI-REVIEW

Current status of antibody-drug conjugate bioanalysis

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Antibody-drug conjugate (ADC) consists of a cytotoxic drug covalently bound to a monoclonal antibody via a linker. Because of the complexity of ADC structure unique bioanalytical strategies are needed to identify, characterize and quantify the ADC species most relevant to safety and efficacy. ADC bioanalysis need an integrated bioanalytical approach including ligand-binding assay (LBA) and LC-MS based assays to provide comprehensive characterization of the exposure/response relationship. This mini-review will summarize recent publications on ADC bioanalytical strategies and will focus on the publications within last three years on the advancement of hybrid ligand-binding/LC-MS methods for ADC PK and stability evaluation and for intact ADC-DAR distribution measurement to profile ADC biotransformation and catabolism. Special attention is paid to the publications on selection of ADC analytes, assay platforms and DAR characteristics of LBA as well as on the transition of the bioanalytical testing strategy at different phases of clinical development.

Keywords: antibody-drug conjugate bioanalysis, ligand-binding assay, hybrid ligand-binding/LC-MS assay, drug-to-antibody ratio

Introduction

This article reviews the recent development and trends in antibody-drug conjugate (ADC) bioanalytical strategy and practices with a focus on the literature in last three years.

ADC bioanalytical strategies

With the US FDA approval of Adcetris® (brentuximab vedotin) in 2011 and Kadcyla® (ado-transtuzumab emtansine) in 2013, antibody-drug conjugate (ADC) has been a hot topic in industry. Because of the complexity of an ADC, combining monoclonal antibody and small molecule toxin, its bioanalysis has seen unprecedented amount of discussion compared to other drug modalities. Two review articles, Stephan et al. [1] and Kaur et al. [2], and an AAPS Drug Conjugate Working Group position paper [3] best describe the challenges and strategies of ADC bioanalysis. There are three key points from these milestone publications on ADC bioanalysis:

1. These articles outline the bioanalytical strategies to measure three PK analytes for non-clinical and clinical studies: total antibody, conjugated-antibody or antibody conjugated-drug, and free drug and its metabolites using ligand-binding, LC-MS or hybrid ligand-binding LC-MS assays [1,2]. They also point out that the analytes measured for a particular ADC could vary and the number of analytes could possibly be reduced late in clinical development.
2. Drug-to-antibody ratio (DAR) *in vivo* may change due to deconjugation and/or different clearance rates. The total-antibody and conjugated-antibody assay should measure different DAR species equally without DAR bias. DAR bias or DAR sensitivity has been the most challenging and debated topic in ADC bioanalytical assays.
3. Affinity capture LC-MS measurement of intact ADCs to characterize DAR distribution change *in vitro* and *in vivo* is important to understanding ADC biotransformation in developing ADCs.

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ADC bioanalysis was also outlined in a recent industry white paper on ADME (Absorption, Distribution, Metabolism, Excretion) characterization of ADCs [4] and a review of ADC analytical characterization by mass spectrometry [5]. It is fair to say that all literature and conference discussions and evolution on ADC bioanalysis in recent years have been around the topics of selection of analytes, DAR characteristics of the assay, and choice of assay platform (LBA or hybrid). Significant progress has been made in the increased applications and an improved understanding of various hybrid ligand-binding/LC-MS assays for ADC biotransformation profiling and PK (Pharmacokinetics) quantitation over last two-three years.

Hybrid ligand-binding/LC-MS assays

Conjugated-drug assay for ADC PK

Though discussed in the review articles [1,2], standalone articles fully devoted to conjugated-drug using immuno-capture LC/MS approach did not appear in publication until 2015. Over the last two years, there has been some breakthroughs in developing methodology for measuring conjugated-drug for use in ADC PK. Liu et al. [6] reported the assay development, validation and a case study for the quantitative bioanalysis of a conjugated-drug in cynomolgus monkey plasma using a hybrid immuno-capture, cathepsin B cleavage, LC/MS assay for a microtubule polymerization inhibitor with lysine random conjugation through a protease cleavable linker. The immuno-capture was conducted on AssayMAP streptavidin cartridge coated with the anti-idiotype capture reagent operated on an Agilent AssayMAP BRAVO system (Agilent Technologies, Wakefield, MA, USA). Immuno-capture and cathepsin B cleavage were optimized in the assay. The ability of the assay to differentiate species based on DAR characteristics was evaluated and the assay was determined to be DAR proportional using enriched DAR 2 and 4 reference materials measured against the standard curve of DAR 3 material. The assay has been applied to PK sample analysis in a GLP cyno toxicology study. Sanderson et al. [7] described a new version of the conjugated-drug assay for valine-citrulline-linked monomethyl auristatin E (vcMMAE) ADCs. Protein A affinity capture and solid-phase cleavage was used to assess the difference in *ex vivo* drug-linker stability of native-cysteine versus engineered cysteine ADCs. They found that papain, a more widely available and inexpensive protease, could replace cathepsin B and cleave dipeptide linkers and was used successfully in this assay. Wang et al. [8] published strategies and methodology of antibody-drug conjugate bioanalysis using ligand binding/LC-MS hybrid assays and discussed its correlation to ligand-binding assays. A series of ligand-binding and LC-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 analyte species that are traditionally measured by ligand-binding assays: total-antibody and conjugated-antibody. Specific versus generic immuno-capture methodologies were compared for conjugated-payload assays in preclinical and clinical studies. Immuno-capture hybrid conjugated-antibody assays were compared and found to be DAR insensitive using (AssayMAP) cartridge and DAR sensitive using magnetic-beads when the same anti-payload capture reagent was used.

DAR distribution-intact ADC measurement

Affinity (immuno-)capture LC-MS based assays in ADC bioanalysis were initially used to characterize intact ADCs by high resolution accurate mass measurement. The pioneering publications were by Xu et al. [9,10]. The affinity capture coupled with LC-MS or hydrophobic interaction chromatography (HIC) enabled measurement of the relative abundance of individual ADC species with different drug-to-antibody ratios for all three main

drug conjugation platforms, linked via lysine, site-specific engineered cysteine or reduced inter-chain disulfide cysteine residues. The data provided critical mechanistic insights into ADC stability *in vivo*. Following these early publications Hengel et al. [11] reported the measurement of *in vivo* drug load distribution of cysteine-linked ADC using microscale LC-MS. It was the first native LC-MS bioanalytical method of cysteine-linked ADCs for in vivo samples. ADC analytical characterization was described in a similar article published by Debaene et al. [12] on innovative native MS methodologies for ADC characterization: high resolution native MS and ion mobility (IM)-MS for average DAR and DAR distribution assessment.

New publications in 2016 demonstrated advancement in methodology and application of *in vivo* ADC DAR distribution assays. Su et al. [13] reported a custom-designed affinity capture LC-MS F(ab')2 assay for biotransformation assessment of site-specific ADCs. For the site-specific ADCs conjugated in the Fab region, the newly developed assay incorporated affinity capture of human lgGs via binding to the Fab region, followed by on-bead IdeS digestion to remove the Fc domain. The profiling of the resulting F(ab')2 fragment had improved sensitivity and resolution over previous methodologies. The reduced and optimized sample preparation time also minimized assay artifacts that resulted from *ex vivo* drug metabolism.

Excoffier et al. [14] reported a new anti-human Fc method to capture and analyze ADCs for characterization of drug distribution and the drug-to-antibody ratio (DAR) in serum from pre-clinical species. The method profiled ADC DAR distribution with inter-chain disulfide bonds reduced after anti-human Fc capture. The article claimed that the method is universal and can be used to analyze stability of virtually all ADCs in serum for pre-clinical studies. One clarification is that the method may only be used for ADCs conjugated at a site-specific engineered cysteine or at a reduced inter-chain disulfide bond. The article also claimed good correlation of average DAR profile calculated from the DAR distribution assay comparing with the profile as the ratio of ADC (conjugated-antibody) and total-antibody measured from LBAs. Caution is needed about this comparison and correlation when the DAR characteristics of the ADC and total-antibody LBAs are unclear. One interesting article from Rago et al. [15] is on calculated conjugated-drug from immunoassay and LC-MS intact protein measurements of ADC. Their method consisted of an anti-human Fc immuno-capture of inter-chain disulfide bonds conjugated ADC followed by LC-MS analysis of the light and heavy chains. The PK profile of ADC (conjugated-antibody) measured using a ligand-binding assay and that of calculated conjugated payload (DAR x total-antibody) were in good agreement. The methodology is a useful tool for PK assessment while exploring ADC metabolism and stability in discovery.

ADC catabolism and in vivo biotransformation

Saad et al. [16] evaluated different bioanalytical approaches to qualitatively and quantitatively characterize ADC catabolites using examples from Kadcyla® (T-DM1) and a THIOMAB™ ADC to illustrate the process. Tumey et al. [17] reported a survey of various types of biotransformation events that have been elucidated in recent years to demonstrate the importance of having a thorough understanding of the structural integrity of the linker, the payload and the conjugation site during biological exposure for linker-payload design and optimization.

Ligand-binding assays (LBA) for ADC PK

In addition to the discussion in the review articles [1-3] one well-accepted opinion is that the assay format, using different combination of capture and detection reagents, could affect the DAR characteristics of total-antibody and conjugated-antibody assays and hence the PK parameters [18,19]. Ideally, a DAR insensitive ADC conjugated-antibody assay is preferred [1-3,20]. Three recent articles brought a different perspective for the preference on DAR sensitivity of conjugated-antibody assays [21-23]. Kumar et al. [21] proposed to develop DAR sensitive conjugated-antibody assays during early Discovery and DAR insensitive conjugated-antibody assays from IND (Investigational New Drug) enabling toxicology studies and into clinical development stage. Myler et al. [22,23] proposed DAR sensitive conjugated-antibody assay be used throughout the entire ADC discovery and development stages when safety and efficacy are antibody mediated and DAR dependent.

Sanderson et al. [7] pointed out that the conjugated-drug assay is by nature sensitive to the drug-loading level of the ADC while the conjugated antibody assay can be configured to be insensitive to the drug-loading level or alternatively it can be configured to be sensitive to the drug-loading level. For a conjugated antibody assay to be truly quantitative for drug loading, the assay response must be exactly proportional to the drug load, which may be challenging for assay development. Wang et al. [8] concluded that fully DAR proportional conjugated-antibody LBA is equivalent to the hybrid ligand-binding/LC-MS conjugated-drug assay.

Conclusion

Despite a significant improved understanding of ADC

bioanalytical requirements and advancement of ADC bioanalytical technologies, there is no industry consensus for a few key factors in ADC PK measurement:

1. Selection of conjugated-antibody or conjugated-drug as the ADC conjugate for pre-clinical and clinical PK measurement.
2. Which conjugated-antibody assay, DAR sensitive or insensitive, correlate more closely with ADC clinical efficacy and safety and is the preferred analyte at different stages of ADC development?
3. Which assay platform is preferred when two assays are equivalent, e.g., a fully DAR proportional conjugated-antibody in an LBA or a hybrid ligand-binding/LC-MS conjugated-drug assay?

These topics and different perspectives were discussed extensively in the overviews provided by Kaur et al. [2] and Gorovits et al. [3] and the research reports from Kumar et al. [21] and Wang et al. [8] as well as in special reports [24-26]. Different bioanalytical strategies, e.g., stage specific [21, 27] or discovery and development integrated [8] for ADC PK were proposed. We are looking forward to seeing these questions be thoroughly discussed in conferences and literature in the years to come and believe an industry-wide harmonization will be achieved in the near future.

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