Commentary

Theoretical Considerations and Practical Approaches to Address the Effect of Anti-drug Antibody (ADA) on Quantification of Biotherapeutics in Circulation

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Abstract. Continuous improvement in bioanalytical method development is desired in order to ensure the quality of the data and to better support pharmacokinetic (PK) and safety studies of biotherapeutics. One area that has been getting increasing attention recently is in the assessment of “free” and “total” analyte and the impact of the assay format on those assessments. To compliment these considerations, the authors provide a critical review of available literature and prospectively explore methods to mitigate the potential impact of anti-drug antibody on PK assay measurement. This challenge is of particular interest and importance since biotherapeutic drugs often elicit an immune response, and thus may have a direct impact on quantification of the drug for its PK and safety evaluations.

KEY WORDS: anti-drug antibody interference; dissociation methods; ligand-binding assays; monoclonal antibody biotherapeutics; total and free PK assays.

INTRODUCTION

An article, recently authored by Lee *et al.* (1), focused on the theoretical and practical considerations for measuring total/free analyte to support pharmacokinetic (PK) assessments of monoclonal antibody biotherapeutics from the perspective of circulatory ligands that are either secreted as soluble forms or shed from membrane receptors. Because of the complexity of the topic, Lee’s paper did not address the challenges of introducing anti-drug antibodies (ADA) into the discussion. The present commentary introduces the concept of investigating PK assessments of free and total drug in the presence of ADA. Most examples included in this commentary are, not surprisingly, monoclonal antibody drugs since they represent the majority of new drug entities for protein biotherapeutics. It is conceivable however that the suggestions presented here for assessing the PK of biotherapeutics could be applied to other classes, such as fusion proteins, recombinant proteins with endogenous counterparts, bi-specifics, and alternate antibody-based scaffolds. It is recognized that there may be limited experience or

literature on some of these newer modalities. Additionally, the majority of the published reports related to this topic seem to be written from the viewpoint of removing free drug to assess ADA presence. Thus, at the present time, the ability to quantify drug in the presence of ADA mostly remains a theoretical consideration. Nonetheless, it is logical that the methods for increasing drug tolerance in ADA assays could be re-purposed for assessing or increasing ADA tolerance in PK assays, usually with a preparatory step to break up the immune complex and extract the drug. It must be noted that implementation of such challenging manipulations would not be considered routine for late-stage clinical bioanalysis, but would provide valuable information early on in the investigative stage of method development to pharmacokineticists for their interpretation.

Biotherapeutic drugs often elicit an immunological response characterized by circulating ADA that may or may not neutralize the target binding of the drug, alter the clearance rate, and interfere in the PK assays used to measure the drug concentration in circulation. Thus, ADA can significantly impact PK or toxicokinetic (TK) calculations and may result in insufficient exposure to calculate safety margins from toxicology studies. Additionally, there may be pre-existing antibodies to some biotherapeutics which could have similar impact. While we refer primarily to mAbs, our approach would also apply to other biotherapeutic classes. Therefore in the context of this article, “free drug” is drug not bound to an ADA (ADA-free drug), “bound drug” is drug bound to ADA (ADA-bound drug), and “total drug” would include the sum of both forms. Soluble target bound to drug is not in scope for this article. Ultimately, any extraction process used to help quantitate drug would likely result in a “total” assessment.

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In order to better understand the impact of ADA on the quantitation of free and total drug, and as an aid in interpreting the results within the context of the methods employed, several questions or considerations can be highlighted. While these questions may remain rhetorical at times, they still serve to focus the analysts' attention on the challenges at hand in order to better develop bioanalytical strategies for the evaluation of the PK of the drug.

What mitigation strategies can be developed and implemented to avoid or minimize the ADA impact on PK assay performance based on the format of the PK assay? For a given project, is it even possible to construct a PK assay that is totally unaffected by the presence of ADA? Would such a PK assay format satisfy project requirements by detecting appropriate analyte, *e.g.*, free *vs.* total drug and when should it be implemented?

What strategies are available to differentiate the effect of ADA on a quantitative method to support PK compared with the observation of PK clearance? Is it possible to assess if an abnormal PK profile is due to ADA-mediated clearance specifically or due to another cause (*e.g.*, target-mediated drug disposition)?

Based on different PK assay formats, is it possible to differentiate the potential impact of various binding but non-neutralizing ADA (binding framework) from neutralizing ADA (active site)?

Based on the perceived impact of ADA on PK assay outcome, what considerations should be made regarding study protocol, for example, ADA *vs.* PK sample collection time based on the expected drug PK characteristics? Communication with the pharmacokineticist and toxicokineticist is key so that application of free or total drug can be applied appropriately to their statistical and modeling efforts.

ASSAY FORMATS

Methods to support the quantification of drug for PK analysis (PK assays) are required to meet accuracy and precision criteria for regulated bioanalysis (2). Although LC-MS may be used more in the future to measure biotherapeutics and would be unlikely to be as affected by ADA, most current assays to quantify drug are ligand-binding assays. Unlike for an ADA assay, PK assays require standard curve calibrators and quality control samples that are prepared from well-defined reference standards. The original Free and Total paper (1) discussed the various PK assay formats in detail with reference to the target and clearly defined free, partially bound and fully bound drug in this context so this paper will merely summarize some of these in the form of graphics. As well as the target, there are other molecules, such as shed target receptors and/or endogenous binding protein that may also be present and contribute to the potential complexity of the form of drug species present. However, in this article we have focused on ADA-drug complex specifically. Also while this paper encompasses most biotherapeutics, these graphics illustrate a monoclonal antibody since this remains the most common class of biological drug in development. When visualizing these common PK formats as graphical representations, it becomes clear that if antibodies to the administered drug should be elicited, the

ADA could interfere with the capture and/or detection of the drug in these assays (see Figs. 1, 2, 3, and 4).

The first diagram (Fig. 1) illustrates both an anti-idiotypic and an antigen capture format. It is easy to see that an ADA could interfere with the detection of the drug in both assay formats through direct competition or steric hindrance of reagent binding with either the framework or the target binding site (neutralizing ADA). Assays designed to detect total analyte (regardless of whether the drug is free or bound to target) are depicted in Fig. 2. Obviously, ADA targeting the framework could interfere with binding of capture and/or detection reagents through both direct competition when epitopes overlap, or through steric hindrance when epitopes are sufficiently proximal to each other in these formats. In the bridging format (Fig. 3), framework-specific ADA are unlikely to interfere although steric hindrance remains a possibility, however neutralizing ADA most likely would interfere. While it is evident from these illustrations that ADA may interfere in the measurement of the drug through the blocking of the binding of specific reagents, it is also possible that these assays can detect drug-ADA complexes when the ADA does not interfere with reagent binding. In all cases, these assays are constructed and assessed without considering the potential for ADA interference or in making efforts to increase their robustness to ADA interference. It is recognized that interference testing with surrogate ADA polyclonal samples provides no surety of robustness to incurred sample ADA. However, reagent excess is typically required for the assay formats illustrated in Figs. 1, 2, and 3 and in principle excess high-affinity reagent may increase assay robustness.

Additionally, there may be unique situations, such as that observed in the clinic for adalimumab, where it appears that the complete ADA responses in the patients examined were directed against the same or overlapping epitopes (4). In these cases, it may be possible to measure adalimumab in the presence of ADA with a total PK assay. Similarly, for infliximab clinical ADA was predominately against the mouse sequence and a PK assay reagents could be selected to allow for measurement of infliximab in the presence of such ADA (5). However, assays that *a priori* can be expected to detect free drug *vs.* those that detect bound drug or total drug from an ADA perspective have not to our knowledge been developed. Thus, given the range of individual animal responses to a specific biotherapeutic which would cover epitopes from framework to target binding site (complementary determine region), it is difficult to conceive an assay format/reagent set, that on its own, would allow such measurements. However, sample pretreatments and/or alternate bioanalytical methods such as LC-MS may be useful in this context and we discuss these later in the paper.

Two types of ligand-binding assays are commonly employed to measure anti-drug antibodies: (a) the direct enzyme immunoassay format and (b) bridging assays. Various modifications have been suggested in the sample preparation to permit the detection and quantification of ADA with reduced interference from drug. It is likewise conceivable to employ these approaches to detect the drug without interference of ADA in the context of PK assays.

Free and Partially Bound PK Assays

ADA can interfere with capture (neutralizing ADA) and detection (anti-frame work ADA) step

Fig. 1. Free and partially bound PK assays. These assay formats measure biotherapeutic antibodies that are partially bound or fully unbound (free) with respect to target. ADA can interfere in measurement of drug in both assay formats. Neutralizing ADA could reduce or prevent binding of the drug in the capture step. The high local concentration of capture reagent at the surface of the solid phase may favor dissociation of ADA and binding to the capture reagent. Framework ADA could reduce or prevent binding of detection reagents to the frame. The red arrow indicates graphically where ADA would interfere in the assays. The framework of all the antibodies is illustrated in a darker color than the hypervariable region which contains the target binding site

NONCLINICAL AND CLINICAL STUDY SAMPLES

In studies where there is a suspicion of ADA based on the PK time-concentration profiles of individual subjects, it may be possible to mitigate the impact on the exposure assessment through application of methods that can distinguish whether the apparent reduction in drug concentration is due to increased drug clearance by the presence of ADA, or interference of ADA in the PK assay, or both.

There are situations early in the post-drug administration time course where exposure profiles may suggest target-mediated drug disposition (6) pre-existing ADA (7) and/or early onset treatment-emergent ADA. For example, an observation of a sharp increase in the slope of the PK curve some time after dosing might indicate the development of a treatment-emergent immune response. However, it could also reflect that the target-mediated route of clearance is saturated at higher drug concentrations but becomes more apparent as drug concentrations fall closer to the saturating level.

Unless directly investigated, these three causes cannot be differentiated. Investigations may include comparisons of pre-dose with post-dose sample ADA reactivity for a given study subject, direct experimental confirmation of the presence of ADA in the sample as well as characterization of ADA specificity and evaluation of the isotype of the drug-specific immunoglobulins.

In nonclinical studies, ADA evaluations are either assessed in all samples collected for immunogenicity testing or based on PK profile abnormalities. ADA that shows no impact on the expected PK profile may not trigger an automatic investigation for the presence of ADA. Thus frequently the true scope of the ADA response may not be fully characterized. Moreover, the same sample may provide different results when tested in different PK assay formats, depending on the sensitivity of the method and the nature of ADAs since some formats may be more ADA tolerant than others.

Due to the need to address drug safety, every patient in a clinical study is tested for ADA so the true scope of the ADA response is better characterized than in nonclinical studies. However, PK parameters remain an integral component of clinical development so it is important to select ADA and PK time points at pre-dose and trough circulating drug concentrations, and preferably after an appropriate drug washout period (8), to truly understand the impact of ADA on the PK and ensure there is minimum interference in the PK assay. Although PK sample time points are typically more numerous than ADA sampling, it is important to have some PK and ADA sampling points at the same time to truly understand the impact of the ADA on PK.

For drugs with long half lives, it remains a special challenge to accurately report PK since ADA may impact the PK profile. The measurement of ADA-drug complex

Total PK Assays

Generic and Non-competing anti-idiotype assays

Frame work specific ADA can interfere with the capture and the detection step

Fig. 2. Total PK. Generic and noncompeting anti-idiotype assays can measure all forms of biotherapeutic antibodies with respect to target (fully bound, partially bound, or fully unbound (free)). ADA can interfere in measurement of drug by both assay formats. Framework ADA could reduce or prevent binding of capture and detection reagents. The *red arrow* indicates graphically where ADA would interfere in the assays. The framework of all the antibodies is illustrated in a *darker color* than the hypervariable region which contain the target binding site

Free PK Assay:

Bridging assay

Frame work specific ADA unlikely to interfere.

Neutralizing ADA expected to interfere

Fig. 3. Free PK assay: bridging assay. This assay format measures biotherapeutic antibodies that are fully unbound (free) with respect to target. Neutralizing ADA can interfere in measurement of drug IN this assay format; it could reduce or prevent binding of the drug to reagents in the capture step and detection step. The *red arrow* indicates graphically where ADA would interfere in the assay. The framework of the antibody shown is illustrated in a *darker color* than the hypervariable region which contains the target binding site

Acid Dissociation to Detect ADA and Drug

ADA pre-treatment and Assay described by Patton *et al.*, 2005

Fig. 4. Acid dissociation to detect ADA and drug. 1, Illustration of a sample containing ADA complexed to biotherapeutic antibody. 2, Illustration of the use of acid to dissociate ADA from biotherapeutic antibody. This step combined with the steps summarized in (3) comprises the ADA assay as described by Patton *et al.* (3) and detected the ADA after removal of dissociated drug. The acid dissociated sample from (2) could also follow the same steps as (3) using target capture PK assay to measure the released biotherapeutic antibody as illustrated in (4). This example illustrates graphically how an ADA assay designed to remove interfering drug could be adapted to remove interfering ADA

offers one strategy. If adequately identified, one may be able to, at best, eliminate the ADA-impacted samples when drawing conclusions about drug PK.

DISRUPTION METHODS

Concern about patient safety has been a prime driver for industry to place emphasis on the evaluation of immune responses to large molecule drugs *in vivo*. The focus has turned to assays that can detect the ADAs even in the presence of drug, so that all subjects eliciting an immune response can be accurately identified. In fact, efforts to reduce drug interference in ADA assays are expected by regulators (9). For these reasons, there is a push to develop ADA assays that demonstrate increased drug tolerance, hence, the reason for the recent increased interest in different approaches to identifying the presence of ADA in the presence of drug. The assessment of the impact of the drug on the ADA assay performance has become routine for most labs during ADA assay development or validation (often referred as ADA assay drug tolerance). Immunogenicity methods, unlike their quantitative PK assay counterpart, have no true reference standard and therefore, do not attempt to quantify the immune response. Instead, they define the positive result based on statistical analysis of samples obtained from drug treatment-naïve subjects, and further support the reported positive result by confirming and characterizing the response specificity. They typically include drug and or target interference testing in the method

validations as recommended in the current regulatory guidance and white papers (9–11).

The converse scenario for PK assays has not yet been fully realized. It seems reasonable to consider the possibility of assessing the ADA impact on the quantification of drug concentration early in development. Technologies that can enhance the accuracy of drug concentration quantification in the presence of ADA would be of great benefit to PK/PD modeling, drug exposure, efficacy, and toxicological studies. On the other hand, depending on the PK assay format, it is understood that the presence of ADA may lead to underestimation of the free-drug concentration because ADA interferes with the capture or detection reagent. Therefore, analytical investigators have started to address the presence of ADA in method development of PK assays. The assessment of interference of ADA surrogate (or even bound target) on quantitative PK assays may be included in method development by adding in positive control ADA or soluble target. Surrogate ADA's may not behave similarly to all potential ADA produced against a drug *in vivo*. However, since there is no way to obtain a "complete" ADA reference standard that represents all likely ADA forms, the surrogate would still be useful to determine whether a PK method is potentially ADA tolerant. The challenge remains that accuracy and precision must still be met for PK assays if the values are to be used for PK assessments. Additionally, rarely is an effort made to determine the proportion of free drug (considered the fully active moiety) compared with target/drug and ADA/drug complexes. Furthermore, the relative amounts of free,

bound, or total drug are not usually routinely assessed. Even if the resulting method does not provide absolute accuracy, some understanding of the actual presence of interferants would help with data interpretation and/or lead to an investigation.

A review of the literature for case studies to define the impact of ADA on quantitative PK assays and for efforts that reduce those impacts yields few examples. One case in point was the example by Ahene (12) where samples were analyzed in a free PK assay, then treated with an acetic acid/1 M Tris combination to acidify and neutralize the samples prior to reanalysis in the same assay. The data revealed an increase in apparent drug concentration especially in the elimination phase of the PK curve which was not visible before the acid dissociation.

However, there are numerous citations for methods that reduce the interference of drug in assays designed to detect ADA. Most methods rely on the disruption of the immune complex so that the ADA (or in some cases, soluble target) can be “free” to be captured by an assay reagent(s) (3,13–18). Alternatively, a generic ADA assay able to detect ADA–drug complexes formed *in vivo*, has been described by Stubenrauch *et al.* (19).

As mentioned, these methods could potentially be applied to the challenge of reducing ADA impact on PK assays. The pretreatment steps used to separate immune complexes are challenging to perform with accuracy and precision in the PK assay setting. Additionally, there is no ADA reference standard that unequivocally represents the *in vivo* ADA scenario. Hyperimmunized polyclonal antibodies, exhibiting high affinity may not be representative of the myriad of *in vivo* ADA responses. Thus, the validation of a method including such disruption pretreatment steps can only approximate the real-life situation when study samples are being assessed. QC samples prepared to represent the immune complexes of known concentrations of drug and ADA would be surrogate. Nonetheless, they could be useful in the development of methods to break apart the immune complexes. Since known concentrations are used, they could be reflective of the accuracy imparted by the pretreatment steps. Thus, for purposes of the discussion, this paper will focus on the methods used to disrupt ADA/drug complexes found in the ADA literature. The authors propose that those methods may be used in a converse fashion and thus be applied to PK assays.

Examples of these ADA disruption methods that might hold promise for measuring the drug concentration in the presence of ADAs are described below. Of note is the study by Myler *et al.* (20). Although the endogenous binding agent identified in the article was not an antibody, the technique used to enhance the detection of the drug could also be applicable to quantification of the drug in the presence of ADA. In that study, glycine HCl buffer, pH 2 was used to disrupt the binding of human growth hormone-binding protein to pegylated human growth hormone in the samples. The acidified samples were neutralized with HEPES buffer prior to determination of human growth hormone concentrations in a sandwich electrochemiluminescence assay. In comparing the results from samples that were treated as above with nontreated samples, 92% of mid-dose samples were quantifiable with

acid dissociation compared to 38% without acid dissociation.

Literature references discuss the use of chaotropic agents, such as guanidine (18) or acids (3,14) to disrupt the binding of anti-drug antibodies to their target. Relatively mild disruptive agents that preserve the tertiary conformation of antibodies are preferred. Thus, harsh reagents, such as guanidine have been used only when the tertiary conformation is not required for the subsequent assay, such as in the example where the disruption of ADA by guanidine allows for more accurate determination of taspoglutide (21). Acids are the most common agents used in dissociating the antibody from the drug. Generally, after the dissociation step, the samples are neutralized to be compatible with binding reagents in the analytical procedure that is used to quantify the ADA. The following are some examples from the literature.

1. Acid dissociation: Patton *et al.* (3) acidified serum samples containing mAb therapeutic and ADA with 300 mM acetic acid for an hour to dissociate the drug–antibody complex. The samples were neutralized with 1 M Tris. The dissociated ADA was detected in an ELISA *in situ* without separation of the drug antibody and the ADA using a traditional bridging format. Capture of ADA was enhanced *via* covalent high-density immobilization of the drug onto the ELISA plate. A further increase in sensitivity was obtained using a high concentration of biotinylated drug to compete with free drug in the sample.
2. Affinity capture elution (13): In this nonbridging format procedure, the samples were treated with 300 mM acetic acid, then added to 96-well ELISA plates coated with drug and neutralized to capture the ADA on the plate. The liberated free mAb drug was washed away. The bound ADA was then eluted with acid and added to a fresh antigen-coated plate containing neutralizing buffer. The captured ADA was then detected by addition of biotinylated antigen, streptavidin-horseradish peroxidase and 3,3',5,5' tetramethylbenzidine (TMB) substrate. The authors claim detection of ADA was possible in the presence of 1,000-fold molar excess of drug.
3. Solid-phase extraction with acid dissociation: In this method, Smith *et al.* (14) physically extracted the ADA–drug complex and the ADA from the serum. A high concentration of biotinylated drug was added to samples to react with ADA, which was then captured on a streptavidin coated plate. 300 mM acetic acid was added to liberate the ADA from the biotin–drug and neutralized with 1 M Tris. This extract was used to coat ELISA plates and the ADA was detected using HRP-labeled drug and TMB substrate. The assay was able to detect 0.5 µg/mL of ADA in the presence of 80 µg/mL of drug. Even though the example cited in the publication used a monoclonal antibody as the drug, the authors posited that the method could be applied to other protein therapeutics.
4. pH Shift anti-idiotypic method (15): This method involved the treatment of post-dose adalimumab serum samples with 0.1 M glycine–HCl (pH 2.5) for 30 min. An excess of anti-idiotypic F(ab) was then added to compete with the ADA for binding to the drug, therefore preventing reformation of drug–ADA complexes. The acidic pH was

neutralized with 1 M Tris. The ADA was then measured using a radiolabeled assay.

5. Acid dissociation with bridging ECL on MSD (16): This paper focuses on reducing the effect of a soluble target in patient serum which can potentially generate false-positive results in the ADA assay. Serum samples containing AMG 386, a peptide-Fc fusion protein, were pretreated with 300 mM acetic acid for up to 2 h. High concentrations of anti-target mAbs were also added to block re-binding of target to the drug; 50- μ L aliquots were neutralized with 1 M Tris, pH 9.5. The biotinylated and the ruthenylated drug were preincubated with the acid-treated samples before transferring to an avidin-coated MSD plate for detection. The addition of the competing anti-target mAbs increased the detection of ADA in the assay when drug was present in the samples.
6. Acid dissociation and Biacore (17): To allow for reliable immunogenicity determinations, Sickert *et al.* acidified both control rabbit ADA-chimeric mAb drug complex and human mAb-ADA complex to pH 2.5 and 3.0, respectively with 1 M HCl. The pH was optimized for each species. The samples were neutralized to pH 6.0 or 6.5 before determination of ADA by surface plasmon resonance using the Biacore platform. ADA was able to be measured in the presence of a 10- to 200-fold molar excess of drug.
7. Extreme pH dissociation/denaturation (18): Therapeutic mAbs can interfere with the recovery of total soluble target concentration in a sample. Base (pH>13) or acid/guanidine (pH<1) treatments were used to preferentially denature therapeutic mAbs to soluble targets, enabling the quantitation of total target concentration by ELISA in a serum sample in the presence of the drug. Interferences were completely eliminated using these approaches. This same principle could be applied to non-mAb drugs in the presence of ADAs. If a non-mAb drug can survive high pH treatment and if its ADAs can be denatured during the process, this can provide a strategy to measure total non-mAb drug concentration in a sample in the presence of ADA.

These methods are also summarized in Table I along with how they might theoretically be applied to measure drug instead of ADA. To further illustrate the potential to adapt these ADA sample disruption methods to measure drug, the method of Patton *et al.* (3) is graphically summarized in Fig. 4 (1–3) for ADA as well as how the same sample after acid dissociation and neutralization might theoretically be assessed for drug levels using a target capture assay (Fig. 4 (4); see also Fig. 1).

In addition to disruption of complexes by chemical means there are several methods that used other approaches. Examples of those are listed below and also contained in Table II for ease of reference.

8. Biolayer Interferometry using the Octet platform (22) was compared with the more traditional bridging ELISA and a homogenous electrochemiluminescence assay (MSD) for detection of ADA. Biotinylated mAb drug was immobilized onto streptavidin-coated biosensor tips. Serum samples containing drug and ADA were incubated with the tips. Sensitivity for the ADA

in presence of drug was 10 $\times$ greater in Octet vs. the electrochemiluminescence immunoassay (ECLIA) and 100 $\times$ greater than the bridging ELISA. The superiority of the Octet method to detect ADA in the presence of the drug was explained by the fact that for the bridging ELISA and ECLIA, two binding events were required for a signal to be generated while for the Octet a single binding event was required and the chance of the latter occurring in the assay milieu was higher. The assay can measure total ADA.

9. Immuno-PCR was also used to measure a simulated ADA response in standardized human serum using as a model drug, goat anti-mouse IgG (23). This assay utilized high sample dilution to shift equilibrium of ADA-drug complexes to a more free form of ADA, and then measure the ADA with the high sensitivity inherent in the immuno-PCR platform. At high dilution, the sensitivity of the immuno-PCR method was not affected by virtue of the exponential amplification inherent in PCR. On the other hand, matrix dilution limited the sensitivity of ELISA.
10. Neubert *et al.* (8) used magnetic bead-based immunoprecipitation, enzymatic digestion, liquid chromatography, and MALDI-MS to indirectly measure ADA in a serum samples containing human growth hormone analog. Quantitation was based on stable isotope-labeled peptides of the therapeutic protein as internal standards. In this method, instead of the therapeutic protein being a liability in ADA measurement, it was used as an asset. Excess therapeutic protein was used to saturate all specific IgG and the complex was isolated by immunoprecipitation. The therapeutic protein measured was used to infer the amount of ADA present in the sample.
11. A homogeneous biotin-digoxigenin-based bridging ELISA was developed to replace an electrochemiluminescence ADA assay on the BioVeris ECL platform. An assay with comparable sensitivity and drug tolerance was established (24). The low background afforded by biotin-digoxigenin assays could also be explored in drug assays.
12. Using LC-MS/MS to quantify therapeutic monoclonal antibodies, the authors employed a uniformly heavy-isotope-labeled common whole mAb internal standard and a common immunocapture for sample processing (25). The sample processing had the added benefit of disrupting any potential immune complexes. This facilitates the detection of the drug. The use of the common mAb internal standard obviates the need for a new internal standard every time a new assay is to be developed. This strategy will likely prove useful as LCMS/MS for detecting mAb becomes more routine.

Unless there is a known or expected interference in the method, there is generally limited effort made early in the development of the method to mitigate the impact of ADA. However, since these disruption strategies are investigative tools, it may be appropriate to test them in a method development phase if the appropriate surrogate ADA is available. Once developed, there is no need to further validate the process unless variable PK profiles in the study suggest a suspicion of ADA presence. In that case, some degree of validation would be necessary in order to re-analyze the samples to obtain better PK parameter estimates.

Table I. Methods Used to Disrupt ADA–Drug Complex to Increase the Sensitivity of ADA Detection

Chemical disruption methods	Author	Method description to detect ADA in citation	Theoretical reverse approach to detect drug
Acid dissociation	Patton <i>et al.</i> (3)	300 mM acetic acid was added to serum sample containing ADA for 1 h; 1 M Tris neutralization; dissociated ADA was detected in an ELISA <i>in situ</i> without separation of the drug antibody and the anti-drug antibody	After the (1) neutralization step, (2) capture free drug with target or anti-idiotypic antibody for (3) quantification
Affinity capture elution	Bourdage <i>et al.</i> (13)	300 mM acetic acid added to the serum in the presence of drug bound to 96-well ELISA plate; the freed Ab could bind to the immobilized drug and the free was washed away. The bound ADA was eluted and added to fresh immobilized drug. Detection occurred using biotinylated antigen, SA-HRP and substrate. Claim to be usable in the presence of 1,000-fold excess drug	(1) Coat plates with target or anti-idiotypic Ab. (2) Add acidified samples to buffered plate. (3) Wash off dislodged ADA. (4) Elute drug with acid and (5) transfer to fresh buffered plates. (6) Allow drug to coat well. (7) Add detection reagents for quantification
SPE with acid dissociation	Smith <i>et al.</i> (14)	Solid phase extraction of ADA–drug complex from ADA; extract treated with 300 mM acetic acid and neutralized with 1 M Tris to measure liberated ADA by ELISA	(1) Add biotinylated anti-human IgG and sample to SA plate. (2) Wash. (3) Acidify. (4) Transfer to fresh well to neutralize. (5) Measure drug by ELISA
pH shift anti-ID	Schouwenburg <i>et al.</i> (15)	0.1 M glycine-HCl (pH2.5) for 30 min; F(ab) of anti-ID added; pH neutralized with 1 M Tris; ADA measured by binding test. ADA–drug complex dissociated by pretreatment of serum at low pH. Addition of anti-Id F(ab) prevented reformation of the complex	(1) Dissociate complex in presence of labeled F(ab) to quantitate drug. (2) Use appropriate reagents to quantify drug in the ELISA
Acid dissociation with bridging ECL on MSD	Zhong <i>et al.</i> (16)	300 mM acetic acid for 2 h; 50 μ L aliquot neutralized with 1 M Tris (pH9.5); biotinylated and ruthenium- labeled drug were incubated with the acid treated samples before transferring to MSD for detection	(1) After acid dissociation and neutralization, (2) incubate with biotinylated and ruthenylated target (or antibody) (3) to drug to quantify drug
Acid dissociation with Biacore	Sickert <i>et al.</i> (17)	Acidification of both control rabbit ADA-complex and human ADA complex to pH 2 and 3 respectively with 1 M HCl; Neutralization to pH 6.0 and 6.5 respectively and applied to Biacore analysis	(1) Acidify. (2) Neutralize sample (3). Flow the sample over target-bound (or id-Ab) chip to quantify drug
Extreme pH dissociation/ denaturation	Salimi-Moosavi <i>et al.</i> (18)	High pH denatured therapeutic mAbs to soluble targets which were able to survive the treatment. With mAbs denatured the total target concentration in a sample was measured by ELISA	(1) Dissociate non-mAb drug/ADA complexes with high or low pH. (2) Measure non-denatured drug in LBA for a total drug assay

It is conceivable that modification of these methods would facilitate the detection of the drug

In some cases, a drug under development will be very similar to a marketed drug (or one further along in development) which has a history of ADA with an impact on PK. It would not be unexpected then, that the new drug would experience similar ADA generation, with similar effect on PK. In such cases, early efforts to minimize potential ADA impact on assay development may be beneficial.

Some toxicology studies only require evidence of exposure at certain time points. When ADA interference is not absolute, *i.e.*, some drug can be quantified, the ADA impact might not be a serious issue. In contrast, for a critical PK or TK study that experiences a high level of ADA, it may be prudent to consider re-developing the method for improved recovery of drug in the presence of ADA and re-analyzing the study samples to get more meaningful PK data.

Consideration should also be given to when ADA-tolerant PK methods are important to draw study conclusions. For example, when the ADA incidence is under 10%

(only 10% of the subjects demonstrate an immune response in some or all of their samples) the PK parameters could be calculated from the remaining 90% of the subjects. Since most biologics usually do not have a 100% ADA rate, this strategy will serve most PK studies well. However, with the introduction of complex therapeutics, like fusion proteins, that possess possible antigenic components (*e.g.*, bridging sequences, known immunogenic cytokines, and toxins) a strategy of early investigation into the possibility of ADA interference in the PK assay may be prudent.

CASE STUDIES

The ADA's potential impact on drug exposure has been reported in the literature, and a summary on this topic is provided in the previous section as well as in Table I. As pointed out by Ponce *et al.* (26), ADA could have very different effects on PK. Some ADA show no effect while

Table II. Platform Methods that May Facilitate the Detection of the Drug in the Presence of the ADA

Platform	Author	Method description to detect ADA in citation
Biolayer interferometry	Li <i>et al.</i> (22)	Octet platform; most sensitive when compared with both ELISA and MSD platforms; immobilized biotinylated drug coated onto streptavidin biosensors; samples with ADA complex added
Immuno-PCR	Spengler <i>et al.</i> (23)	Measure simulated ADA response; high sample dilution to shift equilibrium from ADA–drug complex to a more free form of ADA; measure ADA with high sensitivity inherent in immuno-PCR technology
LC-MALDI-MS coupled with IP/digestion	Neubert <i>et al.</i> (8)	Magnetic bead Immunoprecipitation, enzymatic digestion; LC-MALDI-MS to indirectly measure ADA in sample; quantification based on stable isotope-labeled peptide internal standards
Biotin-digoxigenin based bridging ELISA	Qiu <i>et al.</i> (24)	Using a homogeneous biotin-digoxigenin-based bridging ELISA, a highly sensitive and drug-tolerant assay was developed for immunogenicity profiling
LC-MS/MS	Li <i>et al.</i> (25)	This approach employed a uniformly heavy-isotope-labeled common whole mAb IS and a common immunocapture for sample processing. The sample processing had the added benefit of disrupting any potential immune complexes

others can alter the PK of a biotherapeutic, making it appear to have a faster/slower clearance as compared with samples that have no detectable ADA. Case studies described below demonstrate this dichotomy. The first three cases are examples from the authors' internal investigations, in which the application of disruption methods/alternate technologies may have been beneficial to the accurate quantitation of total drug. The fourth case identifies an example in the literature on how these methods were applied to measure free and total drug.

The first case study describes a free/partially free ligand-binding assay to support PK. The PK results were impacted by ADA development with some correlation between the level of impact and ADA reactivity in the samples. In this example, a therapeutic recombinant human receptor-Fc fusion protein (drug A) was evaluated in cynomolgus

monkeys with weekly subcutaneous and IV dosing up to 100 mg/kg. Drug A concentrations were determined by a ligand-binding assay in which drug was captured by a recombinant version of human ligand (drug target) coated on MSD multi-array plates. Bound drug A was detected with a biotinylated goat anti-human IgG monkey cross-absorbed antibody followed by interaction with MSD Sulfo TAG Streptavidin reagent. Assay plates were read using MSD Sector Imager 6000. Unknown sample concentrations were determined by interpolation from a standard curve that was fit using a 4-PL logistic equation (see Table III).

Reduced drug A levels and faster drug clearance were observed in the 2-mg/kg dose group and correlated with a detection of anti-drug antibodies with a possible correlation between the ADA titer and the level of impact on the drug A PK (Fig. 5). Presence of ADA reactivity was determined in the

Table III. Drug A Serum Concentrations (in µg/mL) in Individual Monkeys After 2 and 100 mg/kg Subcutaneous Administration

Time post-drug administration (h)	2 mg/kg dose group			100 mg/kg dose group	
	Animal 1	Animal 2	Animal 3	Animal 4	Animal 5
	Drug A concentrations (data (µg/mL))				
0.083	BQL	BQL	BQL	BQL	BQL
1	0.4	0.5	2.4	15.7	29.3
24	4.8	4.7	10.3	875	723
48	5.8	9.5	12.9	833	784
96	12.0	11.6	13.2	584	691
168	11.2	10.6	10.4	495	522
240	8.0	8.3	8.3	367	428
336	9.5	BQL	11.7	360	483
456	9.5	BQL	1.2	281	239
504	8.3	BQL	BQL	250	315
672	0.1	BQL	BQL	179	197
840	BQL	BQL	BQL	151	196
1008	BQL	BQL	BQL	129	117
1176	BQL	BQL	BQL	57.1	41.6
1344	BQL	BQL	BQL	22.2	23.3

BQL below quantifiable level for the assay

Fig. 5. Drug A concentration profiles after a single 2-mg/kg dose subcutaneous administration in cynomolgus monkeys

samples collected at the first post-dose time point, 19 days post-drug A administration (see Table IV). Animals remained positive throughout the sample collection, up to day 56, with increasing ADA reactivity levels. In contrast, there were no abnormalities in PK profiles observed in the 100-mg/kg drug A dose group where all animals scored negative for ADA induction. However, high circulating drug A concentrations were measured in this dose group even at the last post-dose collection point (56 days). It is possible that the negative ADA sample scoring was due to the drug interference in the ADA assay. In this case study, a free or partially free quantitative assay was used to monitor PK of a receptor-Fc fusion compound. Impact on the drug PK had a possible correlation with the level of ADA response observed.

A second study describes an antibody-like recombinant protein (drug B) with a dual specificity for two separate ligands (L1 and L2). In the method developed to support PK, drug B is captured on an ELISA plate *via* immobilized L1 and detected by biotin-L2 conjugate. The assay therefore could detect only the free version of drug B. A qualified version of the assay was used to quantitate drug B in a multi-dose cynomolgus monkey study. Anti-drug B antibody development was evaluated using a “sandwich”-type LBA protocol employing biotin- and ruthenium-labeled drug B reagents. Drug B was evaluated in a four-cycle cynomolgus study in which drug B was administered weekly *via* the IV route. PK profiles were evaluated after the first drug B dose (day 1) and 3rd drug B dose (day 22). Table V shows serum concentrations of drug B in individual serum samples

collected during days 1 (first dose) and 22 (fourth dose) of the study. In the low-dose (10 mg/kg) group, one of five animals developed specific anti-drug B antibody response detected on day 29 (log titer 2.81) while other animals remained ADA negative. While the drug B PK profile was unaffected in any of the animals after the day 1 dose, analysis of the day 22 PK data demonstrated that the one ADA-positive animal had an elevated rate of drug B clearance as compared with other four unaffected animals (Fig. 6). One of the ADA-negative animals also showed an increased rate of drug B clearance while no ADA reactivity was detected in the animal at all evaluated time points. In this case, a free quantitative assay was used to monitor PK of a recombinant protein with dual specificity. Impact on the drug PK was observed in ADA-positive as well as ADA test-negative animals. It is possible that ADA could have impacted either drug clearance or the PK assay ability to correctly report drug concentration or both. By applying an ADA-drug complex disruption technique, one could clarify and determine the actual impact of the ADA development on the drug PK profile.

The next example describes an antibody-like recombinant protein (drug C) with specificity to ligand X. In the PK assay, drug C is captured on an ELISA plate *via* immobilized ligand X and detected by specific noncompetitive assay with the ligand X-binding site anti-drug C rabbit antibody reagent. The assay therefore is designed to detect the free and partially free versions of the drug. The presence of anti-drug C antibody was detected using a bridge-based ligand-binding assay protocol using biotin- and ruthenium-labeled drug C reagents. Presence of neutralizing anti-drug C activity (NAb assay) was evaluated in ADA-positive samples using a competitive LBA-based protocol. In the NAb assay, samples were spiked with a constant amount of biotinylated ligand X and allowed to incubate with plate immobilized drug C. A reduction of the assay signal signified the presence of neutralizing anti-drug C antibody. Drug C was evaluated in a multi-dose clinical study where treatment emergent development of ADA and NAb were determined. Only a low proportion of ADA-positive patients developed NAb reactivity. The presence of ADA activity in the patients did not appear to impact drug C clearance rates. The investigation of PK profiles of the subjects with positive NAb reactivity indicated that while these individuals tended to have a faster elimination of the drug C, the impact appeared to precede the occurrence of positive NAb onset and therefore complicated causal link of NAb development and impacted PK. Overall, the limited number of subjects with a positive NAb response

Table IV. Anti-drug Antibody Status for the Cynomolgus Monkey Samples After 2 mg/kg Subcutaneous Administration of Drug A

Sample time (days)	ADA score/log ADA assay signal				
	Animal 1	Animal 2	Animal 3	Animal 4	Animal 5
0	Negative/<1.40	Negative/<1.40	Negative/<1.40	Negative/<1.40	Negative/<1.40
19	Positive/2.70	Positive/5.19	Positive/2.82	Negative/<1.40	Negative/<1.40
28	Positive/3.47	Positive/5.80	Positive/5.23	Negative/<1.40	Negative/<1.40
42	Positive/5.03	Positive/5.87	Positive/5.13	Negative/<1.40	Negative/<1.40
56	Positive/5.25	Positive/92	Positive/4.89	Negative/<1.40	Negative/<1.40

Assay results are presented as sample log titer values

Table V. Serum Concentration (drug B ($\mu\text{g/mL}$)) in Individual Animals After Intravenous Administration

Time (h)	Animal number									
	1	2	3 ^a	4	5	1	2	3 ^a	4	5
	Study day 1 (1st dose)					Study day 22 (4th dose)				
0.08	366	382	363	290	353	674	686	382	756	701
1	314	385	276	263	356	567	631	414	828	689
3	271	410	281	302	364	564	580	359	629	590
7	242	301	287	268	383	580	522	346	562	699
24	192	237	189	195	246	446	445	232	486	573
72	143	165	138	147	172	287	443	119	426	442
168	117	129	99.4	107	120	100	329	0.443	355	345

Data shown for the 10 mg/kg dose group, study days 1 (1st dose) and 22 (4th dose)

^a Animal number 3 was ADA positive on day 29; ADA log titer, 2.81; minimum log titer in the assay, 1.40

made it challenging to draw significant conclusions regarding whether the presence of NAb antibody may have affected the PK profiles of drug C. Actual impact of the NAb antibody development could be evaluated by applying an appropriate ADA–drug disruption technique allowing to measure accurate drug concentration in NAb containing samples.

The last case study is a historical example since the presence of anti-drug antibodies has long been recognized to impact the measurement of the drug in the case of insulin. Anti-insulin antibodies (AIA) are generated in a large percentage of patients receiving exogenous insulin (27). The impact of these antibodies is often unpredictable. In some cases, the binding of insulin by AIA may prevent its detection, causing an underestimation of analyte concentration. However, in the traditionally used competitive radioimmunoassay, binding of the insulin tracer by AIA may cause an overestimation of the amount of analyte present. For these reasons, the utilization of “free” insulin methods has been practiced for years.

The assay for free insulin usually involves pretreating the sample with polyethylene glycol at sufficient percentage to cause precipitation of total IgG from the sample.

Fig. 6. Drug B concentration profiles after intravenous administration at 10-mg/kg dose. Study days 1 (1st dose) and 22 (4th dose) are shown

Centrifugation then removes IgG, including the AIA, as well as any bound insulin. Assay of the supernatant thus yields free insulin. Pitfalls of this approach were described by Arnqvist, *et al.* (27). PEG precipitation has also been combined with acid dissociation to differentiate free, total, and antibody-bound insulin (28,29). The utility of this method is illustrated when Gennaro *et al.* found that in nonfasting normal subjects, the free and total insulin levels were comparable (mean, 27 UIU/ML vs. 25 UIU/ML, respectively). In insulin-treated diabetic subjects, by contrast, free and total insulin were widely different (mean, 47 UIU/ML vs. 2,676 UIU/ML), which is explainable by the presence of insulin-binding antibodies in these subjects. Therefore, correct evaluation of the total insulin PK required application of this ADA–drug complex disruption protocol.

CONCLUSIONS

Immunogenicity responses to biotherapeutic compounds that result in development of anti-drug antibodies may lead to potential implications for the drug PK assays. Various approaches to mitigate ADA impact on PK assays are discussed in the present report. Modifications to the assay format allowing detection of various analyte types, e.g., free vs. total drug, are suggested and reviewed. It should be also understood that the impact on various PK assays may depend on the binding characteristics, such as specificity and affinity of the ADA developed.

As previously stated, ADA can impact drug PK/PD by altering clearance of the drug, by neutralizing bioactivity or through interference in PK assays that detect primarily free drug. Assays that allow detection of total drug in the presence of ADA could be used, in conjunction with free drug assays and biomarker measurements, to fully characterize the effect of ADA on drug disposition. LC-MS-based total drug assays have been shown to be unaffected by the presence of ADA (30). Other total PK assay methodologies are possible where drug is measured after complexes of drug with binding matrix components, including ADA, have been dissociated. Because the nature of the ADA found in each sample could vary, the recovery following sample pretreatment steps should be carefully evaluated.

Currently there is no guidance or industry consensus regarding the evaluation of ADA impact on PK assays during assay validation, or when to institute PK methods that are ADA tolerant. Some investigators previously conducted such studies for all PK assay validations, but later changed the practice due to limited value the studies provide. This change in practice is primarily due to the lack of relevance of surrogate ADA used to that in real samples. However, as drug development becomes more complex, the relative value of investigating ADA impact on PK assays during assay validation is being reconsidered. Such assessment hinges on the availability of relevant or most appropriate type of ADA reagent. Based on the project type and the requirements, questions should be reviewed as related to the most appropriate surrogate ADA with relevant binding characteristics to be used during PK assay validation.

In conclusion, understanding what the reported data means in terms of free or total available drug is relevant and early assessments using some manipulation may help the kineticists to more accurately evaluate the results. The choice of the PK assay format, the type of measured analyte and the need of the evaluation of potential ADA impact on the PK assay should be considered and carefully reviewed especially during assay development, but may be performed in validation, as well. Final decisions are based on the project needs, expectations of compound immunogenicity and the overall value of the assessment.

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