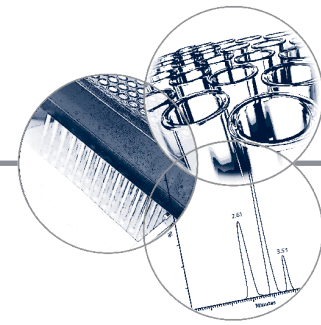


Bioanalytical interferences in immunoassays for antibody biotherapeutics

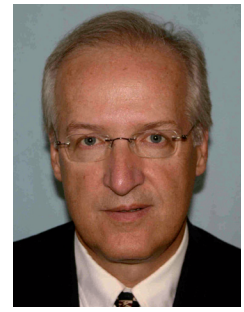
"Assays rarely fully reflect their intended use; knowing the performance boundaries of assays early represents an important approach to effectively managing their use during drug development."

Keywords: ADA ■ antidrug antibody ■ bioanalysis ■ immunoassays ■ interference ■ interferent ■ ligand-binding assays ■ monoclonal antibody ■ PK ■ serum concentration

The measurement of large biological analytes such as antibodies typically requires the use of ligand-binding assays, usually in immunoassay formats such as ELISA, radio-immunoprecipitation, electrochemiluminescence or surface-plasmon resonance. These employ at least one antibody or nonantibody protein (ligand-binding protein or ligand receptor), binding reagents that possess some degree of specificity for the analyte within the sample. For details on immunoassay formats for pharmacokinetic (PK) serum concentration measurements or antidrug antibody (ADA) detection, the reader is directed to other publications [1,2]. Immunoassays do not typically utilize extraction; hence they must be able to bind the biological analyte in the presence of multiple specific, cross-reactive, and/or nonspecific biological binding entities, akin to 'looking for a needle in a haystack'. In addition, in monoclonal antibody (mAb) drug development, the detection of ADAs poses a unique challenge of detecting host antibodies specifically reactive with the mAb amongst a sea of endogenous immunoglobulin, the mAb drug itself and the aforementioned multiple other binding entities, likening it to the challenge of 'looking for a specific straw in a haystack'. The complexities and caveats of immunoassay-based bioanalysis, and the apparency of the resulting concentration values (proximity to the absolute truth), cannot be overstated.

Immunoassay development in complex matrices

The development of immunoassays for the quantification of analyte in a buffer solution has its own challenges, but quantification of the same analyte in a biological matrix (usually serum or plasma) bears additional complexities. The background assay signal changes, biological variability (between matrix samples) exceeds analytical imprecision and recovery of the spiked reference standard can be challenging. Apart from the desired analyte, other binding entities may be present in matrix samples that are able to bind the mAb drug or their soluble natural receptors in preference to assay reagent antibodies. Indeed, drug-target interference in assays is the basis for the development of PK assays, which measure the drug that is not bound to its target ('free' drug) versus that which is partially to fully bound ('total' drug) [3]. Similarly, because ADA assays for monoclonal drugs are generally of the double-antigen bridging format, using drug as both capture and detection reagents, monomeric- or multimeric-drug targets can interfere and lead to false-negative or false-positive results, respectively. The common theme in both types of immunoassays is that one or more cross-reactive species may exist in a test sample that interferes with accurate quantification of the intended analyte. Under such conditions, the reported concentrations of the desired analyte may be overestimated, underestimated or even undetected. As a result, the immunoassay method developer must meticulously take a number of potential interferents into account (among other assay variables) depending upon the known mechanism of action of the drug and the target indication, develop the most optimal method possible, and also consider the method's limitations while interpreting study results. Besides, bioanalytical scientists, being alert but not omniscient, sometimes also discover new assay interferences amidst studies that can cause delays, requiring elucidation of the interference and assay redevelopment before reinitiating bioanalysis.



Charles Pendley
Biologics Clinical Pharmacology Department, Centocor R&D, a division of Johnson & Johnson Pharmaceutical Research & Development

Gopi Shankar
†Author for correspondence:
Biologics Clinical Pharmacology Department, Centocor R&D, a division of Johnson & Johnson Pharmaceutical Research & Development, LLC, 145 King of Prussia Road, Radnor, PA 19087, USA
Tel.: +1 610 407 8922
Fax: +1 610 993 7859
E-mail: gshanka3@its.jnj.com

FUTURE SCIENCE part of fsg

Specific & nonspecific interferences

The extent to which constituents of the sample could specifically interfere or compete with the capture reagents is a complex function of assay format, biotherapeutic drug and reagent binding specificities at the concentrations used in the assay. Specific binding is frequently of high affinity and often has a biologically relevant origin. This may include binding proteins, receptors of drug targets (soluble or cell surface), drug itself or ADA. Whether on- or off-target binding by other specific matrix-sample components can be termed assay 'interference' is contextual and may depend upon the intended goal of the assay. For example, the capture reagent antibodies or binding proteins in a PK immunoassay intended to quantify free mAb drug that is not bound to its target, should recognize the binding/active site of the target molecule, whereas a PK assay for total drug should be able to quantify drug bound with its target.

Nonspecific interference in immunoassays is typically of a lower affinity and may arise from any number of sources of either a biological origin or from assay reagents or hardware. For the purposes of this article, we assume that assay conditions were optimized to eliminate nonspecific binding related to buffers, plates, hardware and so on. The contextual nature of nonspecific binding is also evident in ADA immunoassays, wherein a statistical assay 'cut-point' is carefully established to separate assay results that are specific to an antidrug response from those responses that are nonspecific. The impact of nonspecific binding in PK assays has most impact under conditions that demand high assay sensitivity and depends on the extent of specific binding relative to nonspecific binding as the signal/noise ratio.

Evaluating the interferences in method developement & validation

In mAb drug development, immunoassays are challenged with being specific and sensitive in spite of selectivity; that is, to detect low amounts of the analyte singularly amongst a sea of endogenous immunoglobulin and other interferents. Therefore, specific and nonspecific interferences should be thoroughly investigated during method development and optimization; in subsequent prestudy method validation, those interfering entities should be characterized to identify a concentration at which assay interference is not detected.

In our experience the characterization of specificity is often inadequate and often confused with selectivity. Selectivity is the ability of the assay to measure the analyte of interest in the presence of other constituents of the sample (including concomitantly administered medicines), whereas specificity is the ability to measure ligand unequivocally, despite the presence of structurally similar molecules. For mAb drugs the distinction between specificity and selectivity is obscured by the fact that test matrices always contain the structurally similar endogenous molecules, the immunoglobulins. However, immunoassay validations often differentiate between selectivity and specificity as interference by endogenously extant versus exogenously introduced molecules, respectively.

The selectivity criteria recommended in the White Paper for large-molecule PK method validation is that acceptable recovery of drug must be achieved in 80% of individual matrices tested [4]. Thus, as many as 20% of study subjects could show interference in a validated method. The selection of subject matrix for evaluation of selectivity therefore represents an important consideration during method validation. If the range of potential label indications for the biotherapeutic is known early in development, representative disease matrices should be included in selectivity testing as their constituents might pose unique assay interference issues that have to be dealt with. This testing should include sufficient numbers of samples to fully assess the possible impact of cross-reactivity when samples containing interferents occur at a low frequency. For ADA immunoassays, the validation of cut-point requires an assessment of a statistically significant number of individual matrix samples from the indicated disease states [5,6]. At the time of prestudy validation it can be challenging to obtain sufficient disease matrix samples that reflect the patient population expected in clinical studies or to obtain representative samples that have experienced the conditions and periods of storage comparable with the actual study samples.

Evaluation of specificity should at least reflect the full range of concentrations expected for the interfering substance (the use of supraphysiological concentrations is also recommended) and when inhibition is observed, should include a determination of levels that can be 'tolerated' in the assay. mAb drugs are inherently highly specific for their target ligand, so nontarget binding tends to be associated with preexisting known biological binding partners

or treatment-emergent ADA, or with regions of the antibody outside of the hypervariable region as in the case of nonfully human mAbs. Inhibition by ADA in PK immunoassays is most effectively assessed with incurred study samples that are known to contain ADA and, thus, can be subsequent additions to the prestudy method validation. ADA are polyclonal, so the samples chosen for specificity testing in PK assays should be representative of the titer, and avidity when possible, from study samples. However, it should be noted that failure to detect ADA interference with incurred samples may still be insufficient; interference may only be evident in time–concentration profiles as an accelerated clearance with time. Other portions of the drug molecule outside of hypervariable region may be subject to cross-reactivity by prevalent xenoreactive or autoreactive antibodies. Serum from rheumatoid arthritis patients, in particular, can specifically affect assay performance due to endogenous rheumatoid factor. Rheumatoid factor is an autoantibody against the Fc portion of IgG that readily cross-reacts with assay reagents. Heterophilic cross-reactive antibodies sometimes present in patient populations and healthy individuals may also interfere [7]. However, until the method developer has access to authentic samples representing subject matrix and treatment condition, the ability to evaluate cross-reactivity may be somewhat limited. Validation samples are static representations of potential blood-proteome interferences, but actual study samples better reflect the dynamic changes; compensatory increases target production, treatment induced ADA and so on. Depending on the assay, the selection of samples used for assay characterization should include both early and late time-point samples that represent a range of drug-target ratios, a range of matured IgG type ADA response and the extent of change in drug target production after treatment. Likewise, drug in circulation for months may also experience chemical or metabolic changes to the antibody, whose effects on the assay should be taken into consideration, and tested when possible [8].

Resolving the interferences

The most effective strategy to limit specific and nonspecific interference in immunoassays is to identify and address it early during assay development. For PK assays the choice of assay format remains the single most important decision; monoclonal versus polyclonal, anti-idiotype versus target/binding-protein capture, active versus passive antibody absorption, sandwich versus competition, detection with anti-idiotype versus species specific, detection label and so on. Although there is no universal format that ensures optimal specificity and selectivity there may be other advantages that strike an acceptable balance of performance and development ease/consistency in use. Our preferred strategy is to establish consistency with a single method development and then seek to characterize the assay's behavior through validation and subsequent study-phase execution. At our first clinical or nonclinical study we routinely employ a pair of anti-idiotype noncross-reactive antibody reagent proteins in our PK immunoassays. In the case of mAb drugs, these reagents are specific for the hypervariable region responsible for binding ligand so this type of assay is more likely to detect free drug. When possible, we also seek to employ the same reagents for studies in man. This is done by initiating antibody assay reagents identification in parallel with other development efforts once the new molecular entity has been designated for development. This may also require communication with development teams to manage timeline expectations. Monoclonal anti-idiotype-based assays have other advantages – they are reproducible lot-to-lot, can provide for improved specificity and can be utilized as positive controls in ADA assays.

For ADA assays, either acid-dissociation that enables a shift in immune complex reformation kinetics enabling detection of ADA, pretreatment steps that remove the interferent, or specificity tests that eliminate non-ADA responses, are common approaches. Other assay design factors employed for managing cross-reactivity include seeking the highest affinity monoclonal-reagent antibodies and controlling the time allowed for the ligand capture out of the sample matrix. Monoclonal anti-idiotypic antibodies are very useful as positive controls in ADA assays, but we caution against the selection of the highest affinity antibodies as they often do not represent ADA in study samples, while providing a false sense of confidence in the high assay sensitivity derived through their application. Instead, the use of moderate affinity (for example when the dissociation constant, Kd $\geq 10^{-9}$ M) positive controls, or a range of antibodies when available, is preferred. The use of receptors or another mAb that possess higher affinity for the target/ligand than the mAb drug can be a very useful reagent for the removal of the target/ligand from the matrix before ADA measurement.

Conclusion

The quantification of biotherapeutic drugs with immunoassays, although simple to conduct, can be complex to develop and interpret. This complexity is more substantial in the case of mAb drugs for a several reasons: high drug potencies often result in nonlinear pharmacokinetics for membrane-associated targets, long drug half-lives with multiple exposures that allow the opportunity to development ADA responses, and binding specificities/stoichiometries that challenge a simple concentration response interpretation of target engagement.

The first means of reducing complexity should be the utilization of consistent strategies in development and validation that provide a consistent basis for interpretation and use. Contemporary bioanalytical strategies often employ various assay formats, or even suboptimal assays, to support the different phases of drug development [3]. We advocate investing resources in an inversely proportional manner with regard to the progressive phases of drug development; that is, spending more effort on bioanalytical research in the very early phases of drug development when unexpected assay method impediments, such as interferences, pose a lower risk to product development and offer the opportunity to gain an understanding of the assay and explore enhancement options. This sets the stage for smoother bioanalytical operations in the late phase, large pivotal studies. Taking a contrarian approach, common in resource-restricted environments, can be risky and bear a high opportunity cost (for example, long delays of your drug product's launch due to a health agency's objections to your bioanalytical method and the validity of its results or the comparability of results across studies). Assays rarely fully reflect their intended use; knowing the performance boundaries of assays early represents an important approach to effectively managing their use during drug development.

Financial & competing interests disclosure

The authors are employees of Centocor Research and Development, a division of Johnson & Johnson Pharmaceutical Research & Development, LLC and own stock in Johnson & Johnson, Inc. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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

Bibliography

1 Wu JT. *Quantitative Immunoassay: A Practical Guide for Assay Establishment, Troubleshooting, and Clinical Application*. AACC Press, Washington, DC, USA 255 (2000).

2 Wadhwa M, Bird C, Dilger P, Gaines-Das R, Thorpe R. Strategies for detection, measurement and characterization of unwanted antibodies induced by therapeutic biological. *J. Immunol. Meth.* 278, 1–17 (2003).

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

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

5 Shankar G, Devanarayan V, Amaravadi L *et al.* Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. *J. Pharm. Biomed. Anal.* 48, 1267–1281 (2008).

6 US FDA, Center For Drug Evaluation and Research (CDER), Center For Biologics Evaluation and Research (CBER). Guidance for Industry: Assay Development for Immunogenicity Testing of Therapeutic Proteins (2009).

7 Boscato L, Stuart M. Heterophilic antibodies: a problem for all immunoassays. *Clin. Chem.* 34, 27–343 (1988).

8 Jefferis R. Glycosylation as a strategy to improve antibody-based therapeutics. *Nat. Rev. Drug Discov.* 8, 226–234 (2009).