Review

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Bioanalysis

Bioanalytical challenges and unique considerations to support pharmacokinetic characterization of bispecific biotherapeutics

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Compared with conventional (monospecific) therapeutics, bispecific protein therapeutics present unique challenges for pharmacokinetic (PK) characterization – namely, the characterization of multiple functional domains as well as the consideration of biotransformation or interference by the formation of antitherapeutic antibodies against each functional domain. PK characterization is essential to the success of the overall drug development plan and for molecules with multiple binding domains; multiple bioanalytical methods may be needed to answer critical questions for each phase of drug development. The number of bispecific protein therapeutics entering drug development continues to increase, and therefore, a bioanalytical strategy for the PK characterization of bispecific molecules and study of their *in vivo* structure–function relationship is needed. This review presents case studies and a regulatory perspective.

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Recent advances in protein engineering and manufacturing have enabled the development of increasingly complex protein molecules that engage multiple therapeutic targets with the objective of improving efficacy and/or reducing dosing (to reduce toxicity, minimize safety risks, and improve medication convenience and adherence). Bispecific therapeutics are defined as antibody- or scaffold-based proteins that contain more than one engineered functional domain that bind two or more independent antigenic targets [1,2]. Some of these molecules contain a third functional domain as part of the antibody backbone, such as Fc domain, which may or may not be needed for effector function. This article focuses on bispecific therapeutics with two unique functional domains that bind to proteins integral to disease onset and disease progression regardless of the function of the antibody backbone.

The field of bispecifics has flourished by building on the framework of traditional antibody-based immunotherapies with single molecular targets and by targeting synergistic molecular pathways for more effective treatment of disease [3]. Two bispecific antibodies, blinatumomab (Blincyto) and catumaxomab (Removab), have been approved for oncology indications in the US or EU, demonstrating the therapeutic applications of these molecules [4,5], and more than 60 new bispecific biotherapeutics are currently in clinical development for cancer [6] and other [7] diseases. Bispecific molecules can be presented in a variety of different structures, capitalizing on the availability of various binding domains of antibodies, as discussed in a review by Carter *et al.* [8]. The domains include the variable heavy chain (VH), variable light chain (VL), single-chain molecule (ScFv) and/or Fc regions of antibodies that can be combined to engage more than one molecular target. Large immunoglobulin molecules with multiple Fab binding

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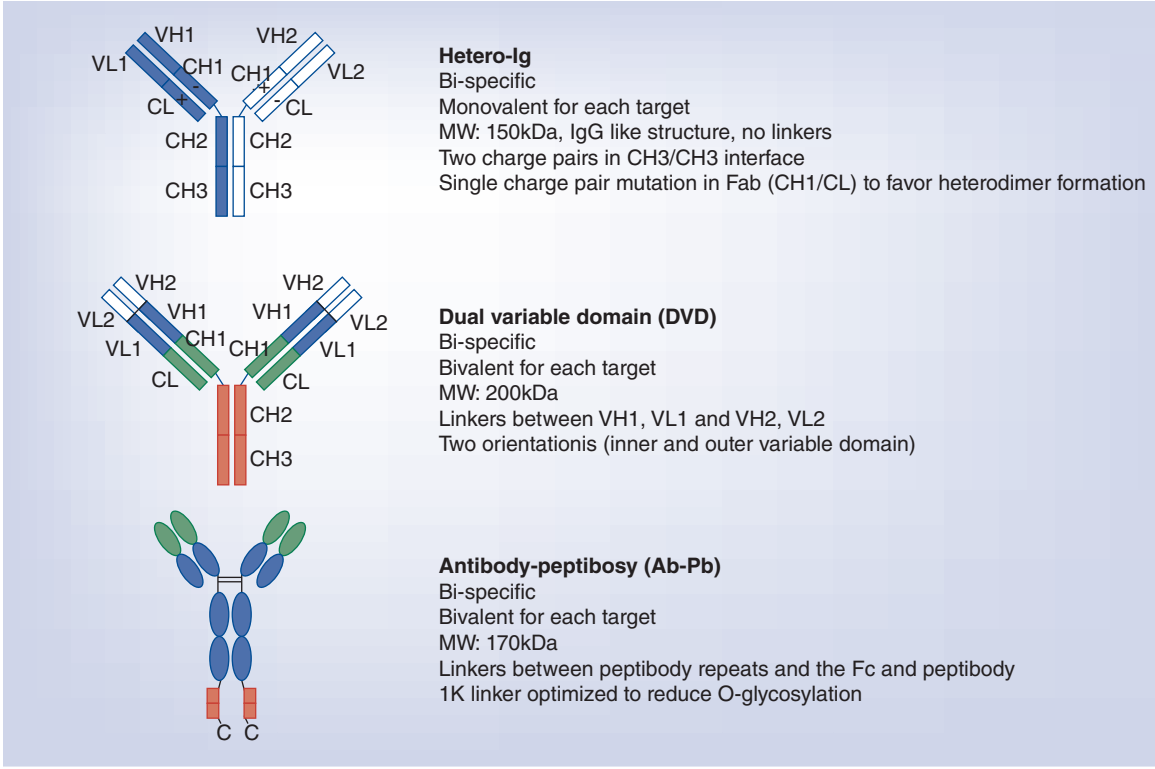

Figure 1. Construct of bispecific molecules. Shown are three examples of bispecific antibodies (hetero-Ig, dual variable domain and antibody–peptibody) illustrating differences in size, structure and complexity.

domains dual-variable domain immunoglobulin (DVD-Ig) and smaller tandem ScFv domains (diabody) represent two extremes in size and structure that can be produced using various protein engineering techniques (Figure 1). Current technology allows for a multiplicity of combinations of protein binding domains and antibody scaffolding, leading to an array of unique modalities. Several functional categories of bispecifics include molecules that bind to soluble targets to inhibit or stimulate function, to cellular targets to antagonize or agonize function, and to cellular targets that recruit immune cells to facilitate antibody-dependent cell-mediated cytotoxicity (ADCC) function.

The unique structures, as well as the mechanism of action (MOA), of bispecific molecules have posed challenges for pharmacokinetic and pharmacodynamic (PK/PD) assessments, for the determination of *in vivo* structure–function relationship, and for bioanalytical support needed during preclinical and clinical drug development phases [9]. Bispecific molecules add a level of complexity to PK modeling efforts, for example, the characterization of target-mediated drug disposition requires consideration of the expression of both targets, which are present in different frequencies on different cell types. And determination of 'free' versus 'total' drug concentration, which is essential for PK/PD modeling particularly if soluble targets are involved, can be complicated due to different expression patterns of the targeted proteins *in vivo* [10].

For the vast majority of clinical studies, drug concentrations have been measured in the so-called 'central compartment' or the systemic circulation (either as plasma or serum samples) largely due to accessibility of easy sampling from dosed subjects. Informed by cumulative scientific data over several decades, systemic drug concentrations are generally considered to be reflective of drug concentrations at the targeted site of action. An exception is in hematological diseases, but even these often have solid tissue components. The relationship between systemic and site-of-action drug concentrations will differ depending on the site, but the appropriate animal model data usually provide a reliable model for humans. Due to the dependency of pharmacological or pharmacodynamic responses on systemic drug concentration, a sound understanding of the PK–PD relationship is extremely useful in projecting the dose and exposure that will achieve the desired response. It is equally important to identify any potential *in vivo* biotransformation that may occur for each unique bispecific molecule, and to thoroughly characterize the different drug variants in terms of exposure and activity [11]. Collectively this level of characterization

aids in understanding the exposure–response relationship, providing a path to optimal design of dosing regimens that are based on pharmacologically active drug variants.

PK assessment is an absolute requirement for nonclinical toxicology studies and clinical development, and also plays an important role in nonclinical disease model evaluation in which dose–response characteristics are studied for lead candidate selection. Systemic exposure data in nonclinical toxicology studies are used to identify a safe starting dose for the first-in-human (FIH) study based on the exposure margin determined from such safety studies. The exposure margin is determined from the ratio of exposure in animals at the no observable adverse effect level (NOAEL) to the projected exposure at the selected human dose, commonly set at 10× lower than the projected human equivalent dose (HED) of the NOAEL. As bispecific molecules may have more potent pharmacological and/or toxicological effect due to the targeting of multiple physiological or pathological pathways, regulatory agencies may require using the 'minimum-anticipated biological effect level' (MABEL) model for selection of the FIH dose, which means a much lower starting dose compared with the NOAEL-based projection. The lower starting dose in clinical studies may well require improved PK method sensitivity, and this can be challenging given the complexity of these molecules.

To demonstrate the reliability and validity of the PK data for a drug's intended use, regulatory agencies expect sponsors to use validated methods to measure drug concentrations for all Good Laboratory Practice (GLP) studies and pivotal clinical trials. Health authorities have provided specific guidelines for bioanalytical assay validations which are generally consistent worldwide across regulatory agencies [12–14]. These guidance documents clearly articulate the regulatory expectations with respect to the performance of assay parameters and their acceptable values, and the integrity of handling study samples during collection, storage and testing. Discussions about the assay reagents, specifically the choice of capture and detection reagents, assay format and technology platform, as well as the relevant analytes to monitor for the PK characterization and for PK/PD correlation analyses, can be found in the scientific literature. For those drugs that have circulating targets that can form drug–target complexes in serum or plasma samples, the most relevant form of the drug – excluding biotransformants – that should be measured remains in dispute: 'total' (bound plus not bound to target) or 'free' or 'active' (not bound to target). This question of total versus free drug concentration is best resolved on a case-by-case basis depending on the known biology of the target, the drug's MOA and characteristics, the opinions of the project team, technical feasibilities, and regulatory feedback if that is sought. Several recent publications have addressed this question [15–20].

The choice of which the bioanalytical strategy to adopt can vary depending upon the specific developmental stage. For example, for a human protein drug in a nonclinical species, a generic PK assay that employs reagents specific to human proteins that do not cross-react with nonhuman species, and in particular nonhuman primate homologs (e.g., recombinant humanized monoclonal antibody [rhuMabs]), can be a fast and cost-effective approach for total drug measurement suitable for supporting overall toxicological assessment. However, this approach will not work in the clinic, where more drug-specific reagents (e.g., target antibodies and anti-idiotypic antibodies) are more likely necessary since: cross-reactivity with endogenous molecules using generic reagents will interfere with assessment of the protein drug concentrations and measurement of free drug is more relevant to establish PK–PD relationship. Another question for nonclinical and clinical bioanalytical strategies is whether measuring the intact bispecific using reagents specific to each target binding site is the most informative, such as a combination of target and/or anti-idiotypic antibody for each target binding moiety. In this regard, the drug's MOA will be important to consider in selecting the appropriate methods, especially in cases where the therapeutic activity depends on engagement of both targets by the bispecific. An example of this is seen with anti-CD3/anti-tumor target bispecifics where measuring the intact active bispecific is the best strategy to fully characterize the molecule. Conversely, if therapeutic activity is dependent upon engagement of one target whose therapeutic activity is then enhanced by engagement of the second target, then it may be that measuring intact drug is less important, and in this case using only one drug-specific reagent would be a reasonable strategy (e.g., 1-arm target, 1-arm for prolonging PK or exposure). An example of yet another approach is the one used for evaluating PK of catumaxomab: the PK assay took advantage of the mouse/rat chimeric structure of the therapeutic antibody and used specific antispecies immunoglobulin capture/detection reagents. A mixed generic assay format was used with the specificity for the drug being directed toward its chimeric structure, with no involvement of the target-engaging complementarity determining regions (CDRs) in the assay [21]. This approach is based on two assumptions: first, monoclonal antibody (mAb) therapeutics are generally very stable *in vivo*. Second, there is no detectable soluble forms of the target proteins. Therefore, this PK method will measure free catumaxomab in the circulation, which is appropriate for its intended use. In general, the default bioanalytical strategy is to utilize the dual specificity of the bispecific,

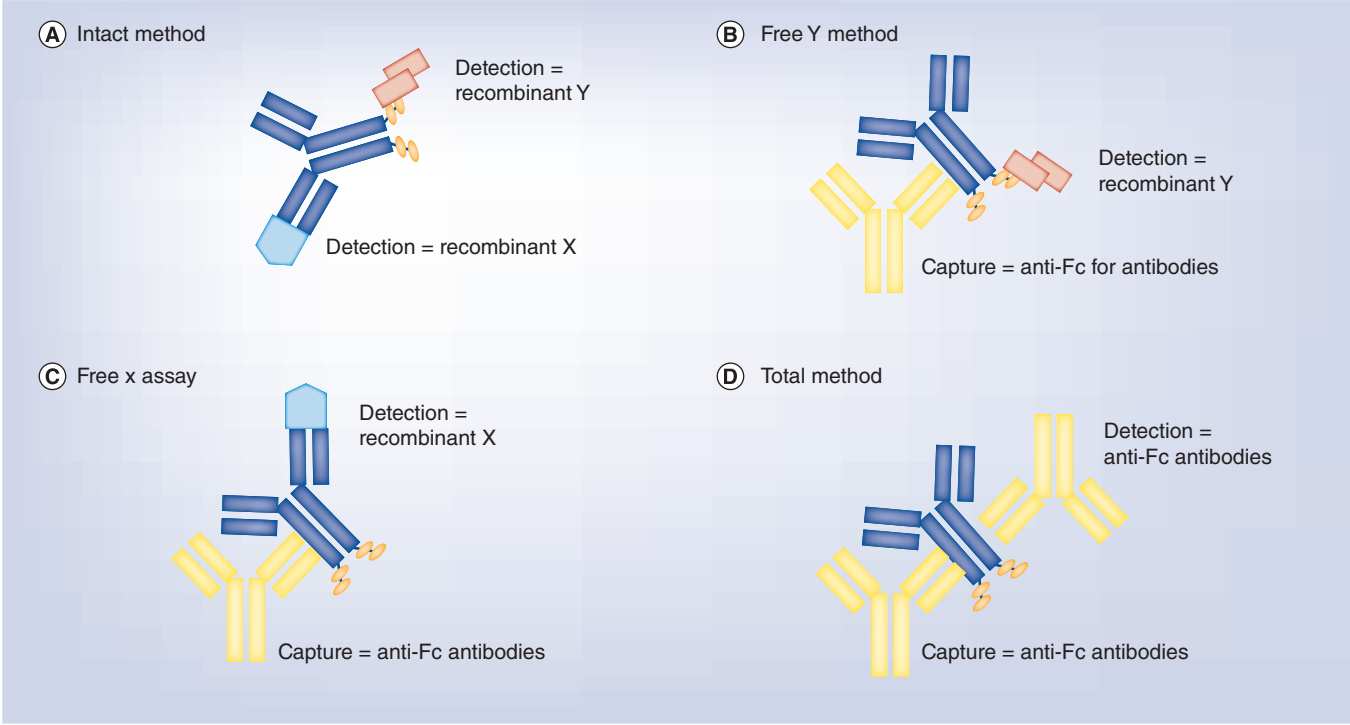

Figure 2. Different assay formats and reagents for quantification bispecific molecules. (A) An intact assay measures the concentration of the intact bispecific molecules by employing both target X protein and target Y protein as assay reagents. **(B)** A functional domain assay measures the concentration of functional domain Y by employing anti-Fc antibodies and target Y protein as assay reagents. **(C)** A functional domain assay measures the concentration of functional domain X by employing anti-Fc antibodies and target X protein as assay reagents. **(D)** A total assay measures all soluble forms of a bispecific molecule by employing two anti-Fc antibodies as assay reagents.

recognizing that reagents for such an assay are time-consuming and challenging to develop and may not be available during drug discovery or even nonclinical studies. The various options are considered in more detail below.

PK method design

Intact bispecific assay

The purpose of this assay type is to measure the intact bispecific molecules in biological matrices. The assay should only measure active bispecific molecules that do not have any functional domain clipped off or blocked by antidrug antibody (ADA) or circulating target. The intact assay typically requires the use of target and/or anti-idiotypic antibodies in order to measure molecules that contain both functional domains that will bind to both the capture and detection reagents. The intact assay is recommended when assessing *ex vivo* or *in vivo* stability (assuming the reagents are available). The assay format for intact measurement is shown in Figure 2A. This format employs two target molecules or two fully neutralizing anti-idiotypic antibodies or a combination of the two. Recently, LC–MS has been used in characterizing intact molecules from biological matrices coupled with affinity pulldown (using target or anti-idiotypic antibodies) for sample isolation. It is a sequential approach, as the free form of the bispecific drug shall be pulled down by anti-idotypic antibody, then the quantification was made via LC–MS method. By using this format, the free concentration of bispecific molecules can be determined. However, during the method development several key factors need to be considered:

- The assay has to be designed to avoid potential steric hindrance effect. Some bispecific molecules are designed to target one soluble molecule and one cell-bound molecule (cell surface receptor). Such bispecific molecules are clearly designed to interact with both targets in order to exert their intended pharmacologic activity. However, the bispecific molecule and assay reagents may interact differently in an assay environment, in other words, on the plastic surface. Therefore, the steric hindrance effects need to be understood and managed vigilantly. Data gathered from such hindrance investigation, whereby domain binding interferences are examined on an Octet

platform, for example, may point to choosing a particular order of capture and detection to avoid unwanted steric hindrance;

- Some bispecific molecules are highly pharmacologically potent through synergistic effects, which can lead to very low clinical doses. Therefore, assay sensitivity will be a significant consideration based on known dose–response data and dose modelling;
- Biotransformation or *in vivo* instability has the potential to occur with bispecific molecules, compared with traditional monospecific antibodies. Biotransformation has the potential to impact not only the activity, but also reliability of measurement of bispecific molecules. Thus, fully neutralizing anti-idiotypic antibody or target molecules (Figure 2A) might allow for detection of potentially consequential biotransformation;
- Similar to monospecific biotherapeutic molecules, such as standard monoclonal antibodies, ADA can interfere with the measurement of bispecific molecules by blocking the capture or detection reagent from binding to the drug (not to be confused with altered drug clearance). Additionally, one domain may be immunodominant, and depending on the assay format, potentially can skew the PK assay results. Furthermore, novel, non-native structures inherent in bispecific molecules, except perhaps for endogenous IgG4 molecules [22], might make bispecifics more prone to ADA responses than monospecific molecules, a consideration for immunogenicity risk assessments.

Functional domain assay

A functional domain assay measures the concentration of one functional domain. This format can be used to demonstrate the concentration of single functional domain of interest. These assays are particularly relevant when the MOA of the bispecific is such that a partial molecule retains partial activity (e.g., soluble targets, fusion proteins or peptide agonist). The goal of functional domain assays is to determine which functional domain is active, and therefore, can explain loss of function in conjunction with the formation of ADA or biotransformation. The assay format for single domain measurement can be designed as shown in Figures 2B and C. The assay intentionally employs the therapeutic target or inhibitory anti-idiotypic antibody to measure the free domain of the bispecific, coupled with anti-Fc or anti-framework antibody as capture or detection reagent. These assays can be used at any development stage. Occasionally a cell-based assay can be used for measuring a single functional domain, such as in the case of blinatumomab.

Total assay

The total assay is used to measure all soluble forms of a bispecific molecule. This format can be used to demonstrate the presence of a bispecific molecule regardless of any structural or functional alternations. The assay is typically designed to measure the backbone of the protein such as an Fc domain. This may not be appropriate for all constructs and depending on the nature of the construct may not be useful for all species, for example, a rhuMab bispecific in clinical studies. The total assay should be tolerant to ADA and target binding. A depiction of a total assay is shown in Figure 2D in which two antihuman Fc antibodies binding to distinct epitopes can be used as capture and detection reagents. Similarly, a single polyclonal antihuman IgG reagent can be used as both capture and detection. This approach is only useful in nonclinical studies. LC–MS is very useful for measuring total drug concentration, especially when no specific reagent is available. The total assay can be used in conjunction with either 'intact' or 'active', or both assays, to assess *ex vivo* and *in vivo* stability and the impact of biotransformation on structure–function relationship, such as certain deamidation or oxidation reactions that may abolish the properties of target engagement [11].

The complexity of the biological milieu in PK samples adds another layer of challenges for consideration of which assay to use. The application of the bioanalytical methods is expected to begin from animal studies during discovery and preclinical safety assessment, continuing through clinical development which extends into a post-marketing setting for life-cycle management (i.e., additional indications and/or combinations). As pointed out earlier, different assay strategies may be used at different development stages, or a single method may be developed and used throughout the course of drug discovery and development when the *in vivo* structure–function relationship is well established. Special considerations of the life-cycle management are needed to enable the integration of PK/PD knowledge from multiple species and various target patient populations for the pursued indications [23].

Method validation & qualification for bispecific molecules

As with other large molecules, method validation for bispecifics includes precision and accuracy, linearity of dilution, selectivity, interference and specificity, and stability testing to ensure the robustness and integrity of the data. However, due to the nature of bispecific molecules, specificity and assay interference evaluation is necessary for both domains when developing an intact assay, as described above.

Case studies

The following case studies are presented to illustrate the bioanalytical challenges and unique considerations that apply to the design and execution of bioanalytical assays and the PK assessments required during different phases of drug development.

Case Study 1: Multiple assays measuring total and intact bispecific drug concentrations with assessment of ADA impact on PK in nonclinical studies

Compound X is a scaffold-based bispecific antibody that targets two cytokines. Preliminary data demonstrated that this particular scaffold product platform can induce a high frequency of immunogenicity in nonclinical species. In order to better interpret the toxicology findings in the presence of ADAs, the bioanalytical team decided to develop two different PK methods to measure total and intact drug in animal serum.

The total PK method was an immunoassay using two antibodies against the framework of scaffold. The intact PK method used one targeted cytokine as capture reagent and the other as detection reagent. It was observed that the concentration versus time profiles generated from the same samples analyzed by the two PK methods were almost identical, except at very low concentrations at later time points when the intact drug concentration was slightly lower than the total drug concentration. The discrepancy observed on low concentrations at later time points only happened to few subjects in the study. Therefore, the ADA and biotransformation were suspected as most likely root causes. An investigation was conducted to identify the root causes of this discrepancy, such as biotransformation-induced instability, target interference and ADA interference. To verify whether the *in vivo* biotransformation (e.g., proteolysis) of this bispecific molecule reveals structural liabilities that impact stability, two ligand-binding mass spectrometry (LBMS) methods were developed using antihuman Fc immunoaffinity capture and target capture followed by tiered mass spectrometric interrogation. Additional higher resolution analysis by nanoscale liquid chromatography interfaced with ESI–MS was used for identification of heterogeneous metabolites. The data showed negative results for biotransformation. An ADA assay was also performed, which demonstrated a higher frequency of ADA in the bispecific molecule than its parent compounds. Despite subsequent confirmation that the majority of the ADAs are not neutralizing ADAs with the characterization of immunogenicity, the ADAs were found to contribute to the faster clearance of the intact drug. The data could also be interpreted to indicate that the drug concentrations were low enough that endogenous cytokines occupied the specific capture/detection sites, in other words, target interference. It was concluded that the structure of the drug is stable *in vivo*. The PK data discrepancy was not related to instability of intact drug, but rather most likely due to high levels of ADAs resulting in faster drug clearance.

Case Study 2: PK analysis of a F(ab')2 that biotransforms *in vivo* into two active F(ab) monomers

This case study involves a bispecific F(ab')2 comprised of an anti-VEGF arm and an anti-Ang2 arm intended for intravitreal administration to treat retinal degenerative diseases such as wet age-related macular degeneration and diabetic macular edema. It has been reported that pre-existing endogenous antibodies (PEA) directed against the hinge region of F(ab')2 antibodies are present in a large percentage of people [24]. This has been confirmed in our laboratory for both cynomolgus monkey and human drug-naive serum samples [25]. Removing the hinge epitopes for these pre-existing endogenous antibodies results in the retention of a single disulfide bond holding the two Fabs together. As a consequence, upon injection into the vitreous, which contains glutathione as part of its defense against oxidation products and to maintain cyrstalline lens integrity [26], the molecule biotransforms into individual Fabs at a clearance rate generally faster ($t_{1/2}$ <1 day) than either Fab ($t_{1/2}$ ~3 days) or F(ab')2 ($t_{1/2}$ ~3 days) clearance rates from rabbit vitreous [Unpublished Observations]. Thus, both intact F(ab')2 and individual Fab fragments are present in both ocular compartments (vitreous and aqueous) and systemically. It was shown using rodent choroidal neovascularization models that the individual Fabs retained biological activity, as might be expected. Therefore, in order to completely characterize the active drug exposure, it was necessary to quantify all three forms of the therapeutic and thus three separate PK assays were developed and validated. The use of a total

PK assay that would measure all three forms with a single assay was considered, but it was decided that since this was a novel construct with which we had limited experience, understanding the kinetics of biotransformation was important for characterizing the *in vivo* behavior of the molecule.

The ELISA assay format for measuring the intact portion of the analyte consisted of capturing the molecule with immobilized recombinant Ang2 protein, followed by biotin-VEGF and finally streptavidin-horseradish peroxidase (HRP) in a sequential sandwich format. Spike recovery experiments demonstrated the specificity of this format for the F(ab')2 form only; neither Fab molecule was detectable. The two Fab assays followed this similar basic format, using the respective target – Ang2 or VEGF – to capture the Fabs from samples. Detection was then accomplished using biotin-sheep antihuman IgG heave and light chain (H&L), followed by streptavidin HRP. Note that with the Fab formats, the intact molecule could also be detected. To our surprise, we found that the intact F(ab')2 was quantitatively detected in the Fab assays despite using the Fabs as standards and controls. Furthermore, using mixtures of F(ab')2 and either Fabs, we could quantify the Fabs by subtracting the value obtained from the intact assay, which is very specific, from the Fab assays. All these assays were validated for use in nonclinical studies for rabbit serum samples, and qualified for use in cynomolgus monkey for serum, aqueous, vitreous and retinal extracts. An additional challenge for the serum assay was the required sensitivity of low to subnanogram/ml concentrations. This is a consistent feature of intravitreally administered biotherapeutics given the small amount typically administered (often <1 of mg/eye), which is diluted many-fold upon reaching the systemic circulation.

Case Study 3: Total and active drug measurement to monitor the biotransformation of a bispecifics from *in vivo* nonclinical studies.

A bispecific mAb was constructed to target two cell surface antigens for oncology indications and the pharmacological effect depends on the intactness of both binding arms of the mAb. However, a post-translational modification (PTM) liability was identified in one of the binding arms during *in vitro* biophysical characterization. This PTM liability cannot be engineered out without significantly compromising the bioactivity because the point mutation at the liable amino acid completely abolishes binding to the target. Furthermore the accumulation of the drug variant with only second arm being functional can potentially mask the second target from the binding by active drug variant and could potentially induce unwanted toxicity with multiple dosing. It was imperative to characterize the *in vivo* biotransformation (e.g., kinetics and extent) of the molecule to provide guidance on further development of the molecule. A total PK method was developed as a bridging format by using assay reagent binding specifically to the Fc portion of the molecule, complemented by an active PK method that employed the target protein as the capture reagent for the PTM-liable functional domain and antihuman Fc reagent as detection reagent. By using a mixture ratio of wild-type drug and mutant drug with the point mutation, the active PK method can differentiate and accurately measure percentage of active drug form in the presence of total drug. A cynomolgus monkey study was conducted to evaluate PK characteristics of the molecule. The total and active PK methods were employed to measure total versus active drug concentrations in the study samples. It was confirmed that the PTM occurred in *in vivo* samples and the percentage of active drug concentration was reduced with time. However, the kinetics of reduction of active drug concentration can be mitigated by increasing dose intervals in clinical studies based on modeling and simulation. The nonclinical study results also provided the path forward to use an active PK method as the primary method to support clinical studies while a total PK method will also be employed to further characterize the potential accumulation of inactive drug variants and its impact on PK/PD and toxicity in FIH study.

Conclusion

To address the bioanalytical support needed during bispecific molecule development, we have discussed some of the unique considerations encountered when developing a PK assay strategy for these molecules. The strategies and approaches presented can apply to bispecific molecules as well as other multidomain large molecule therapeutics, and require a thorough understanding of the particular molecule, its MOA and the underlying target biologies along with an understanding of the data requirements for the PK/PD evaluation. The actual approach to PK assay design and development will be unique to each construct and will require a thorough fit-for-purpose approach and confirmation. Developing a reliable, robust and reproducible PK assay for a bispecific molecule can be very challenging as multiple factors can impact accurate and meaningful measurements. Future discussions and input from industry and regulatory agencies will be valuable for providing guidance on best practices to be used for these highly complex therapeutic proteins.

Future perspective

As the specificity, selectivity and sensitivity of bioanalytical assays continue to improve, more accurate and precise measurement of bispecific antibody concentrations and assessment of their immunogenicity in biologic samples will be achievable. It is expected that the number and variety of bi- and multispecific biotherapeutics will continue to expand, which will lead to the development of new bioanalytical methods to accommodate these molecules' structural complexity and MOAs. Of course, current methods will be adapted to quantify new proteins. Although the ligand-binding assay is the current bioanalytical method-of choice and will continue to be for the foreseeable future, the use of LC–MS/MS may become more widespread to support PK assessments of bispecific biotherapeutics. LC–MS/MS has advantages over the ligand-binding assay including less assay variability and the availability of critical reagents. The use of laboratory automation is anticipated to expand into all phases of bioanalysis reducing manual errors and improving data quality.

Executive summary

- Bispecific antibody therapeutics (antibody- or scaffold-based proteins) have unique structures with multiple binding domains, multiple therapeutic targets and mechanisms of action (MOA), and diverse formats (e.g., small molecules composed solely of the antigen-binding sites of two antibodies and large complex molecules composed of different antigen-binding moieties).
- The unique structure of bispecific protein molecules pose new challenges for assay design, pharmacokinetic (PK) characterization, pharmacokinetic and pharmacodynamic (PK/PD) assessment, bioanalytical preclinical and clinical support, and add complexity to PK modeling.
- The choice of an assay method – intact bispecific assay, functional domain assay and total assay – to measure a bispecific antibody can be different at different stages of drug development.
- Method validation for bispecific molecules includes precision and accuracy, linearity, selectivity, specificity and assay interference evaluation for both binding domains.
- Considerations in the design of bioanalytical assays for bispecific molecules include the complexity of the biological samples, biotransformation or *in vivo* instability, and steric hindrance effects.

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References

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

1 Schlegel P, Lang P, Zugmaier G *et al.* Pediatric posttransplant relapsed/refractory B-precursor acute lymphoblastic leukemia shows durable remission by therapy with the T-cell engaging bispecific antibody blinatumomab. *Haematologica* 99(7), 1212–1219 (2014).

2 Carter PJ, Lazar GA. Next generation of antibody drugs; pursuit of the "high-hanging fruit". *Nat. Rev. Drug Discov.* 17(3), 197–223 (2018).

• **Provides a review of considerable interest in current bispecific development.**

3 Strohl WR. Current progress in innovative engineered antibodies. *Protein Cell* 9(1), 86–120 (2018).

4 Topp MS, Gokbuget N, Stein AS *et al.* Safety and activity of blinatumomab for adult patients with relapsed or refractory B-precursor acute lymphoblastic leukaemia: a multicentre, single-arm, Phase II study. *Lancet Oncol.* 16(1), 57–66 (2015).

5 Seimetz D. Novel monoclonal antibodies for cancer treatment: the trifunctional antibody catumaxomab (removab). *J. Cancer* 2, 309–316 (2011).

6 Zhang X, Yang Y, Zhang L *et al.* Mesenchymal stromal cells as vehicles of tetravalent bispecific Tandab (CD3/CD19) for the treatment of B cell lymphoma combined with IDO pathway inhibitor D-1-methyl-tryptophan. *J. Hematol. Oncol.* 10(1), 56 (2017).

7 Mullard A. Bispecific antibody pipeline moves beyond oncology. *Nat. Rev. Drug Discov.* 16(11), 666–668 (2017).

8 Dillon M, Yin Y, Zhou J *et al.* Efficient production of bispecific IgG of different isotypes and species of origin in single mammalian cells. *MAbs* 9(2), 213–230 (2017).

9 Diao L, Meibohm B. Tools for predicting the PK/PD of therapeutic proteins. *Expert Opin. Drug Metab. Toxicol.* 11(7), 1115–1125 (2015).

10 Trivedi A, Stienen S, Zhu M *et al.* Clinical pharmacology and translational aspects of bispecific antibodies. *Clin. Transl. Sci.* 10(3), 147–162 (2017).

11 Ezan E, Becher F, Fenaille F. Assessment of the metabolism of therapeutic proteins and antibodies. *Expert Opin. Drug Metab. Toxicol.* 10(8), 1079–1091 (2014).

12 FDA. Bioanalytical method validation. Guidance for Industry (2001). https://www.fda.gov/downloads/drugs/guidances/ucm070107.pdf

13 EMA. Guideline on bioanalytical method validation (2011). https://www.ema.europa.eu/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf

14 Japanese bioanalytical method validation guideline (2009). http://www.nihs.go.jp/drug/BMV/BMV_draft_130415_E.pdf

15 Krishnamurthy A, Jimeno A. Bispecific antibodies for cancer therapy: a review. *Pharmacol. Ther.* 185, 122–134 (2018).

16 Poulin P. Drug distribution to human tissues: prediction and examination of the basic assumption in *in vivo* pharmacokinetics–pharmacodynamic (PK/PD) research. *J. Pharm. Sci.* 104(6), 2110–2118 (2015).

17 Viswanathan CT, Bansal S, Booth B *et al.* Quantitative bioanalytical methods validation and implementation: best practices for chromatographic and ligand binding assays. *Pharm. Res.* 24(10), 1962–1973 (2007).

18 O'Hara DM, Theobald V, Egan AC *et al.* Ligand binding assays in the 21st century laboratory: recommendations for characterization and supply of critical reagents. *AAPS J.* 14(2), 316–328 (2012).

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

- **Offers comprehensive understanding of free and total measurement of biological analytes in drug development.**

20 Fischer SK, Yang J, Anand B *et al.* The assay design used for measurement of therapeutic antibody concentrations can affect pharmacokinetic parameters. Case studies. *MAbs* 4(5), 623–631 (2012).

21 Ruf P, Kluge M, Jager M *et al.* Pharmacokinetics, immunogenicity and bioactivity of the therapeutic antibody catumaxomab intraperitoneally administered to cancer patients. *Br. J. Clin. Pharmacol.* 69(6), 617–625 (2010).

22 Aalberse RC, Stapel SO, Schuurman J, Rispens T. Immunoglobulin G4: an odd antibody. *Clin. Exp. Allergy* 39, 469–477 (2009).

23 Samineni D, Girish S, Li C. Impact of shed/soluble targets on the PK/PD of approved therapeutic monoclonal antibodies. *Exp. Rev. Clin. Pharm.* 9(12), 1557–1569 (2016).

24 Villegas VM, Aranguren LA, Kovach JL, Schwartz SG, Flynn HW. Current advances in the treatment of neovascular age-related macular degeneration. *Expert Opin. Drug Deliv.* 14(2), 273–282 (2017).

25 Ruppel J, Brady A, Elliott R *et al.* Preexisting antibodies to an F(ab')2 antibody therapeutic and novel method for immunogenicity assessment. *J. Immunol. Res.* 2016, 1–8 (2016).

26 Fan X, Monnier VM, Whitson J. Lens glutathione homeostasis: discrepancies and gaps in knowledge standing in the way of novel therapeutic approaches. *Exp. Eye Res.* 156, 103–111 (2017).