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Hybrid assays: the next big thing?

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In the near-term, the wide-spread use of hybrid assays appears to be ‘the next big thing’ in bioanalysis as evidenced by its inclusion in recent professional publications (Table 1) and discussions at scientific meetings. Consequently, the US FDA has been prompted to issue guidance/specifications/expectations for hybrid assays; however, regulatory experience with these modalities has been extremely scarce to date. Much of the interest in FDA guidance has been for acceptance criteria around accuracy and precision for hybrid assays. Several authors have privately and publicly promulgated the use of the ligand-binding assay (LBA) criteria (20/25%) because of the use of LBA to enrich the samples [1]. The FDA has not stated a position on this issue yet [2], due to the lack of data in regulatory submissions, but it has indicated that those criteria would be based on the data generated over time. However, review of the recent literature (Table 1) tends to indicate overwhelmingly that chromatographic acceptance criteria are easily met. So, with data like these, chromatographic acceptance criteria would seem to be the more reasonable choice.

The typical validation parameters in and of themselves are not the issues of greatest interest. This combinatorial approach of a hybrid assay, and the inclusion of a digestion step is much more complex than the average assay for either a small-molecule drug, measured by LC–MS or therapeutic protein assayed by LBA. Consequently, the FDA has questions about these ‘new’ steps in the process and how they affect reliability of the assay.

- **What peptide should be selected to monitor?** Injecting the intact protein onto the LC/MS is currently not feasible, and most groups are making decisions about digesting the protein and isolating a particular peptide that represents the intact molecule. In nonclinical studies, a ‘universal’ peptide, which is a segment of the protein that is conserved across animal species, has been used to generate assays and data. But once the studies in humans begin, a new assay based on a signature peptide is needed (because humans do not share these conserved stretches of antibody). How do the nonclinical data compare with the human assays (do they need to)? Is there a bias that has to be accounted for in order to extrapolate nonclinical findings to humans to assess safety evaluations?
- **How reliable is the selection of the signature peptide in human studies?** These are often the segments of the heavy chain of an antibody. Do these reliably indicate the identity of the intact analyte of interest? Or could they be confounded by fragments of the antibody in the circulation or other plasma contaminants? Should we be monitoring two fragments: heavy chain and light chain? would that be sufficient? Some sample preparations might exclude these fragments, in which case, they would not be measured. But should they be monitored? Would a qualitative assessment of the remaining peptides within a digest also be appropriate? If so what percentage of the therapeutic protein should be monitored qualitatively (e.g., 10, 50 or 80%). Furthermore, there are some anecdotal reports that monoclonal antibodies (mAbs) may undergo biotransformations (e.g., oxidation) that may impact biological activity (are we on the verge of exploring/understanding mAb metabolism?); can these fragments be assessed reliably?
- **What instrumentation will be utilized in assay validation and during study runs?** A number of platforms (e.g., Triple Quads, Ion traps, Q-TOFs and Orbi Traps) are available for the quantitation of therapeutic proteins. Early in assay development high-resolution instruments are typically used as they provide the user with more information (spectral counting) and selectivity through higher mass resolution. Conversely, later in assay validation and during study runs, unit resolutions instruments are used as they cost less than high-resolution instruments and are more user friendly. Is switching between platforms an acceptable practice? If so how do we translate data generated across multiple platforms? Should high-resolution instruments be used

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Table 1. Recent articles reporting hybrid assay results.

Study (year)	Analyte	Bias % LLOQ (bold)	Precision % LLOQ (bold)	Internal standard	FDA BMV guidance LC/MS criteria	Ref.
Willrich M <i>et al.</i> (2015)	Infliximab heavy chain; human serum; 1–100 µg/ml	NA	7 to 15	Horse IgG peptide and stable isotope-labeled (SIL) peptide	Yes	[3]
	Infliximab light chain; human serum; 1–100 µg/ml	NA	5 to 13		Yes	
Becher F <i>et al.</i> (2017)	Cetuximab (HT4) heavy chain; human serum; 1–1000 µg/ml	-6.7 to 3	9.5 to 14.7	Proteotypic peptides (SIL)peptides	Yes	[4]
	Cetuximab (LT3) heavy chain; human serum; 1–1000 µg/ml	-11.6 to 7	5.9 to 14.3; 6.4		Yes	
Li H <i>et al.</i> (2013)	mAC; rat plasma; 0.1–15.0 µg/ml	-5.6 to 7.4; 6.5	7.9 to 11.2; 8.1	SIL-αDA that are situated at the similar CDR location	Yes	[5]
	αDA; rat plasma; 0.1–15.0 µg/ml	-8.2 to 5.8; 4.9	2.4 to 6.6; 4.1		Yes	
	αDB; rat plasma; 0.1–15.0 µg/ml	-7.8 to 0.9	5.3 to 9.6		Yes	
	αK; rat plasma; 0.1–15.0 µg/ml	1.5 to 5.9; 2.8	6.7 to 17.7		Yes for accuracy	
Heudi O <i>et al.</i> (2008)	mAb; marmoset serum; 5–750 ug/ml	2.6 to 8.3	6 to 15.9	Synthetic signature peptide	Yes for accuracy	[6]
Xu K <i>et al.</i> (2014)	mAb-A; human serum; 0.1–20 ug/ml	7.66 to 10.5	2.85 to 7.18	6.56/5.85	Yes	[7]
	mAb-B; human serum; 0.1–20 ug/ml	-4.18 to 11.5	3.67 to 11.9	10.6/10.1	Yes	
Ewles M <i>et al.</i> (2016)	Trastuzumab QCs; human serum; 0.25–250 ug/ml	-4.9 to 0.3	1.4 to 5.1	0.7/3.0	Yes	[8]
	Bevacizumab QCs; human plasma; 0.1–100 ug/ml	-7.3 to 4.5	4.7 to 13.6	3.9/3.1	Yes	
Mekhssian K <i>et al.</i> (2014)	Rituximab QCs; human serum 1–150 ug/ml HC	-4.5 to 0.8	3.3 to 10.1	na	Yes	[9]
	Rituximab QCs; human serum 1–150 ug/ml LC	-1.9 to 0.4	4.6 to 12.8	na	Yes	
Furlong MT <i>et al.</i> (2014)	IgG mAb; human serum 5–400 ug/ml	-7.8 to 7.2	3.2 to 7.3	na	Yes	[10]
Kaur S <i>et al.</i> (2016)	mAb; monkey serum; 1 0.1–19 ug/ml	1.96 to 10.9	5.14 to 8.84	na	Yes	[11]
Peng K <i>et al.</i> (2014)	RG7652; human serum 0.1 ug/ml	not reported	4 to 9	na	Yes for precision	[12]

throughout a therapeutic protein's life cycle as they provide more information that can be assessed retrospectively (e.g., biotransformations)?

- **How will sample stability be assessed?** Long-term stability is often conducted with the peptide, but does that reflect what we need to know? Does the intact protein behave the same way? How do you handle changes in peptide solubility? And which species should be monitored on the benchtop? The peptide? The intact molecule? Both because we are handling both?
- **Furthermore, what about protein digestion?** Since the digestion generates the reference standards, is the digestion step complete (or at least linear) across the range of protein concentrations needed? The outcomes would have a profound impact on the accuracy of the assay. So would the selection of the internal standard. Options include stable labelled mAb (SILmAb), SIL labeled peptides and 'winged' peptides. Each of these offers different advantages; each is added at different points in sample processing, and consequently, may not account for all potential issues that might arise during sample preparation.

Other questions that arise include what mass fragments should be monitored. Peptide mass spectra often generate multiple peaks: which ones should be monitored – some representative peaks, or do we need all of them?

These are some of the questions we have to grapple with regarding hybrid assays; and undoubtedly others will crop up later. Traditionally, there is always fear about being 'the first guy' to try out something new in the regulated space. But a lot of work has been done in this field, and the agency has traditionally worked with drug development scientists to employ new technologies and approaches. Hybrid assays are no different in that respect. The best advice that can be offered is to seek regulatory input on the use of hybrid assays early during development (ideally before

first in human studies). The FDA meets with industry representatives around many issues in drug development and this is a good pathway to resolve many of the questions about hybrid assays. The questions posed in this article are good starting point for discussions with the agency. We look forward to the implementation of hybrid assays in future applications.

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