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Best Practices for Validation of Immunoassays for use in Quantitative Bioanalytical Methods

Emma Waite, Biopharmaceutical Laboratory Manager for Tepnel Research Products and Services reviews the progress and evolution of the guidelines on the validation of immunoassays and discusses the major issues related to their validation and how they differ from chromatographic assays.

Introduction

Bioanalysis can be both qualitative and quantitative in the determination of drugs and metabolites in biological fluids and used to support bioequivalence, pharmacokinetic and toxicokinetic studies. Traditionally this has been carried out using chromatographic techniques for the analysis of small molecules. However, with the ever increasing number of macromolecular therapeutics coming onto the market, bioanalytical methods have diverged and many ligand binding assays, e.g. immunoassays are being used. It is essential that high quality data are produced in these studies since the results will be used in support of regulatory submissions. Several Bioanalytical Method Validation conferences have been held to address the quality of data submitted to regulatory authorities. This article will review the progress and evolution of the guidelines on validation of immunoassays and briefly discuss the major issues related to their validation and how they differ from chromatographic assays.

History of Bioanalytical method validation workshops

The first workshop on Bioanalytical method validation was a meeting between the American Association of Pharmaceutical Scientists (AAPS) and the US Food and Drug Administration (FDA) in 1990[(1)]. This workshop resulted in the draft Guidance on Bioanalytical Method Validation which was issued by the FDA in 1999. A section on microbiological and ligand based assays was included with the recommendations and described only selectivity and quantification issues. The second AAPS workshop was held in 2000[(2)] and was instrumental in finalising the FDA Bioanalytical Guidance which was issued in 2001[(3)]. However, due to the evolution of new therapeutics and advances in technologies (including LC-MS/MS and ligand-binding assays) the 2000 workshop found it difficult to agree on recommendations. As a consequence of this, a guidance document making recommendations for ligand-binding assays to support pharmacokinetic assessments of macromolecules was published in 2003[(4)]. This document gave more detailed recommendations on ligand binding assays and proposed that the sum of the

interbatch precision and absolute value of the mean bias (% RE) be less than or equal to 30% and a 4-6-30 run acceptance rule (i.e. 67% of quality control or validation samples should have a %RE of less than 30%) be adopted.

Previous recommendations for ligand binding assays had been for %RE to be ≤25% and a 4-6-25 rule. It was generally agreed that a separate guidance document with similar aims to the small molecule guidance should be generated but containing criteria more specific for immunoassay of macromolecules.

The summary of the 2003 workshop which discussed bioanalytical method validation for macromolecules was published in 2005[5]. The most recent workshop held in 2006[6], sought to review the existing guidelines, achieve clarification and make recommendations in an effort to improve the uniformity and quality of bioanalytical data produced by different practitioners on different types of molecules. The FDA guidelines of 2001 are still referenced today, though they probably require an update or an addendum since they are more applicable to chromatographic assays than immunoassays.

The assay life-cycle - Development, pre-study and study-phase validation.

Immunoassay method development should initially comprise a selection of the following:

a. Assay reagents (for suitable specificity, selectivity and stability),
b. Assay format (e.g. direct, indirect, sandwich or competition) and
c. Batch size. A batch should include a set of standards, validation samples and an appropriate number of QC samples (to equal 5% of the total number of study samples on each microtitre plate).

Method development is also concerned with establishing the following parameters, which are then confirmed during pre-study validation and applied during the assay validation phase.

Calibration (standard) model/curve and Limits of Quantitation

The calibration model to be used during method development is established by:

- Using well characterised standard (reference) material spiked into the appropriate matrix
- Running at least 10 standard concentration points run in duplicate at least 3 times.

In undertaking this experimental design it must be understood that calibration curves for immunoassays:

- tend to be non-linear (usually following a 4- or 5-parameter logistic regression) and

- require more concentration points (a minimum of 6 non-zero points), spaced evenly on a log scale.
- may also contain anchoring points, to improve curve fitting which may be greater than the Upper Limit of Quantitation (ULOQ) and less than the Lower Limit of Quantitation (LLOQ).
- require a range of calibration that is usually much smaller (e.g.only 1 to 2 orders of magnitude).

Acceptance criteria:

- Standards should have observed concentrations <±20% of the nominal concentrations (except at the LOQs where the deviation can be up to 25%).
- At least 4 out of the 6 non-zero standards should meet the above criteria

Definition of the limits of quantitation:

- Concentration which the % deviation from the nominal value is within ±25%.

Note that the recommendations of immunoassay workshops differ to the FDA guidelines on this criterion where standards can deviate 15% from the nominal value and LOQs deviate by 20%. (4-6-15/20). At the 3rd AAPS/FDA workshop delegates were surveyed for their use of this rule. Only 23% follow the 15/20 rule with most (76%) using a 20/25, 30/30 rule or “other criteria” determined during assay development.

Selectivity / Specificity

Since immunoassays often do not utilise a pre-assay extraction step (unlike small molecule assays) it is essential to establish that the assay detects the analyte of interest without interference from other substances. Specificity for the analyte should be confirmed when it is likely that variant forms or structurally similar forms of the analyte exist and there is potential for cross-reactivity with the test antibodies. Interference from other substances in the matrix unrelated to the analyte (matrix effects) are usually evaluated by comparing recovery of spiked samples in matrix from at least 6 different sources. All spiked samples should be prepared in the same matrix as the samples and the minimum required dilution should be established. This is the minimum magnitude of dilution to which a sample must be subjected to optimize accuracy and precision (overcoming signal to noise and any background signal from the matrix).

Accuracy and Precision

Immunoassays involve antigen-antibody reactions so they can be inherently less selective and consequently less precise than chromatographic assays. Inter-batch precision (%CV) and accuracy expressed as % absolute mean bias should be ±20% or ±25% at the LOQs. Sometimes different limits for accuracy and precision may be determined from method development work.

Different limits can be used so long as there is sufficient justification for the limit based on experimental data and sound scientific and statistical principles and this is agreed with the sponsor or client.

Accuracy and Precision can be evaluated using calibrators (and determining %CV and %RE) at the method development stage. For pre-study validation it is recommended that samples are spiked at 5 or more concentrations and that at least 2 independent determinations per run (replicates) are made for each sample in a minimum of 6 runs. During the study, precision and accuracy is assessed using QC samples spiked at low, medium and high concentrations, with the 4-6-25 or 4-6-30 acceptance rule applying.

Sample stability

Demonstration of the stability of the analyte in the sample matrix should be performed. The conditions should mimic those under which samples will be collected, stored, transported and processed. These can include short and long-term storage, bench-top and freeze-thaw cycles. Stability of stock solutions of standard under relevant storage conditions should also be evaluated.

Dilutional linearity

Immunoassays usually have a narrow range of quantification so it is necessary to show that the analyte, if present at a concentration above the ULOQ, can be diluted into the validated range for analysis. This should be done during method development by spiking the analyte into matrix at a concentration 100 to 1000 –fold higher than the ULOQ diluted to levels above the ULOQ, the upper, middle and lower parts of the curve. The back-calculated values should be within 20% of the nominal value.

Parallelism

This is similar to dilutional linearity except that it is assessed with multiple dilutions of actual study samples or samples which represent the matrix and analyte combination present in the samples. Parallelism should be evaluated either at pre-study phase if samples are available, or during the study.

Robustness / Ruggedness

Assay Robustness is demonstrated by deliberate changes in assay conditions such as incubation times and temperature, light exposure and type of sample matrix. Ruggedness is a measure of assay consistency when routine changes are made e.g. different analysts, instruments, batch sizes, day and time which should not greatly impact on the performance of the assay. These

parameters should be assessed during method development, and evaluation of the major potential variations in method (e.g. operator, incubation time tolerances) can be evaluated during pre-study validation. If an assay is robust and rugged, the inter-assay precision data obtained should be within the limits of the general precision acceptance criteria.

Conclusion

This article has summarised the recommendations for validation of immunoassays and discussed some of the key differences between the acceptance criteria for small molecule and macromolecule analysis.

References

1. Shah VP, Midha KK and Dighe SV. Analytical methods validation: Bioavailability, bioequivalence and Pharmacokinetic Studies (Conference Report), Pharmaceutical Research 9: pp 588-592, 1992
2. Shah VP, Midha KK, Findlay JWA, Hill H, Hulse JD, McGilveray IJ, McKay G, Patnaik RN, Powell ML, Tonelli A, Viswanathan CT and Yacobi A. Bioanalytical Methods Validatio – A Revisit with a Decade of Progress. Pharmaceutical Research 17: pp 1551-1557, 2000.
3. US Food and Drug Administration. Guidance for Industry, Bioanalytical Method Validation, US department of Health and Human Services, FDA, Center for Drug Evaluation and Research, 2001.
4. DeSilva B, Smith W, Weiner R, Kelly M, Smolec J, Lee B, Khan M, Tacey R, Hill H and Celniker A. Recommendations for the Bioanalytical Method Validation of Ligand-Binding Assays to Support Pharmacokinetic Assessments of macromolecules. Pharmaceutical Research 20: pp 1885-1900, 2003.
5. Smolec J, DeSilva B, Smith W, Weiner R, Kelly M, Lee B, Khan M, Tacey R, Hill H and Celniker A. Bioanalytical Method Validation in Support of Pharmacokinetic Studies. Pharmaceutical Research 22: pp 1425-1431, 2005.
6. Viswanathan CT, Bansal S, Booth B, DeStefano AJ, Rose MJ, Sailstad J, Shah VP, Skelly JP, Swann PG and Weiner R. Workshop/Conference Report – Quantitative Bioanalytical Methods Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays. The AAPS Journal 9: pp E30-E42, 2007.