Bioanalytical Method Validation and the FDA

Andy de Jager

May 2001

Guidance for Industry

Bioanalytical Method Validation

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Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Veterinary Medicine (CVM)
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Themed Issue: Bioanalytical Method Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays
Guest Editors - Mario L. Rocci Jr., Vinod P. Shah, Mark J. Rose, Jeffrey M. Sailstad

Workshop/Conference Report — Quantitative Bioanalytical Methods Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays

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INTRODUCTION

Bioanalysis, employed for the quantitative determination of drugs and their metabolites in biological fluids, plays a significant role in the evaluation and interpretation of bioequivalence, pharmacokinetic (PK), and toxicokinetic studies. The quality of these studies, which are often used to support regulatory filings, is directly related to the quality of the underlying bioanalytical data. It is therefore important that guiding principles for the validation of these analytical methods be established and disseminated to the pharmaceutical community.

The first American Association of Pharmaceutical Scientists (AAPS)/Food and Drug Administration (FDA) Bioanalytical Workshop in 1990 focused on key issues relevant to bioanalytical methodology and provided a platform for scientific discussions and deliberations. The workshop and the report[1] raised awareness of the need for validated bioanalytical methods for the regulatory acceptance of bioequivalence and pharmacokinetic data. Although the workshop addressed bioanalysis in general, it acknowledged the differences between chromatographic and ligand binding (non-chromatographic based) methods. The workshop identified the essential parameters for bioanalytical method validation, ie, accuracy, precision, selectivity, sensitivity, reproducibility, limit of detection, and stability. The outcome of the first workshop and its report resulted in improved quality of data submissions to regulatory agencies.

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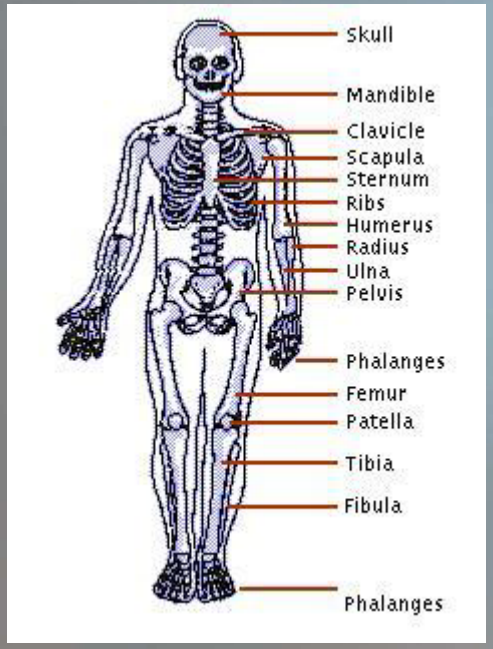
Following the first workshop report[1] and the experience gained at the FDA, the draft Guidance on Bioanalytical Methods Validation was issued by the FDA in January 1999. This draft guidance provided stimulus and opportunity for further discussion at the 2nd AAPS/FDA Bioanalytical Workshop in January 2000. In addition, newer technology, such as chromatography coupled to tandem mass spectrometry (LC-MS/MS), was discussed along with an update on ligand-binding assays. This workshop resulted in a report "Bioanalytical Method Validation—A Revisit with a Decade of Progress"[2] and formed the basis for the FDA Guidance on Bioanalytical Methods Validation in May 2001.[3]

The evolution of divergent analytical technologies for conventional small molecules and macromolecules, and the growth in marketing interest in macromolecular therapies, led to the workshop held in 2000 to specifically discuss bioanalytical methods validation for macromolecules. Because of the complexity of the issues, the workshop failed to achieve a consensus. To address the need for guiding principles for the validation of bioanalytical methods for macromolecules, the AAPS Ligand-Binding Assay Bioanalytical Focus Group developed and published recommendations for the development and validation of ligand-binding assays in 2003.[4]

As bioanalytical tools and techniques have continued to evolve and significant scientific and regulatory experience has been gained, the bioanalytical community has continued its critical review of the scope, applicability, and success of the presently employed bioanalytical guiding principles. The purpose of this 3rd AAPS/FDA Bioanalytical Workshop was to identify, review, and evaluate the existing practices, white papers, and articles and clarify the FDA

- Basic Anatomy of a Validation



- Chemical Assay
- Microbiological/ligand-binding assay

Chemical Assays

- Selectivity
- Accuracy, Precision, Recovery
- Calibration Curves
- Stability
- Principles of method validation and establishment
- Specific recommendations for validation

Selectivity

- Absence of ‘signal’ or ‘response’ in blank matrix
- At least six sources of matrix (not pooled)
- Impact at LLOQ

Mb/L-B assays

Endogenous

Metabolites

Accuracy

- Accuracy defined as deviation of mean measured from nominal
- Minimum n = 5 determinations per conc.
- Test at min n = 3 levels in ‘range of expected conc’s’
- Within ± 15% of nominal (non LLOQ levels)
- Within ± 20% of nominal at LLOQ

Precision

- Precision : reproducibility of measurement at a concentration (CV% = S.Dev/Mean x 100)
- Minimum n = 5 determinations per conc.
- Test at min n = 3 levels in ‘range of expected conc’s’
- Within ± 15% of nominal (non LLOQ levels)
- Within ± 20% of nominal at LLOQ

Recovery

- % Measurand that makes it through the sample preparation process (maximum 100%)
- L, M, H – within assay range
- Prepared samples compared with ‘pure authentic standard)
- No prescribed min. recovery, consistent, precise and reproducible

Pure authentic STD

Precipitations

Matrix effects

Calibration Curve

- Relationship between concentration and detector response

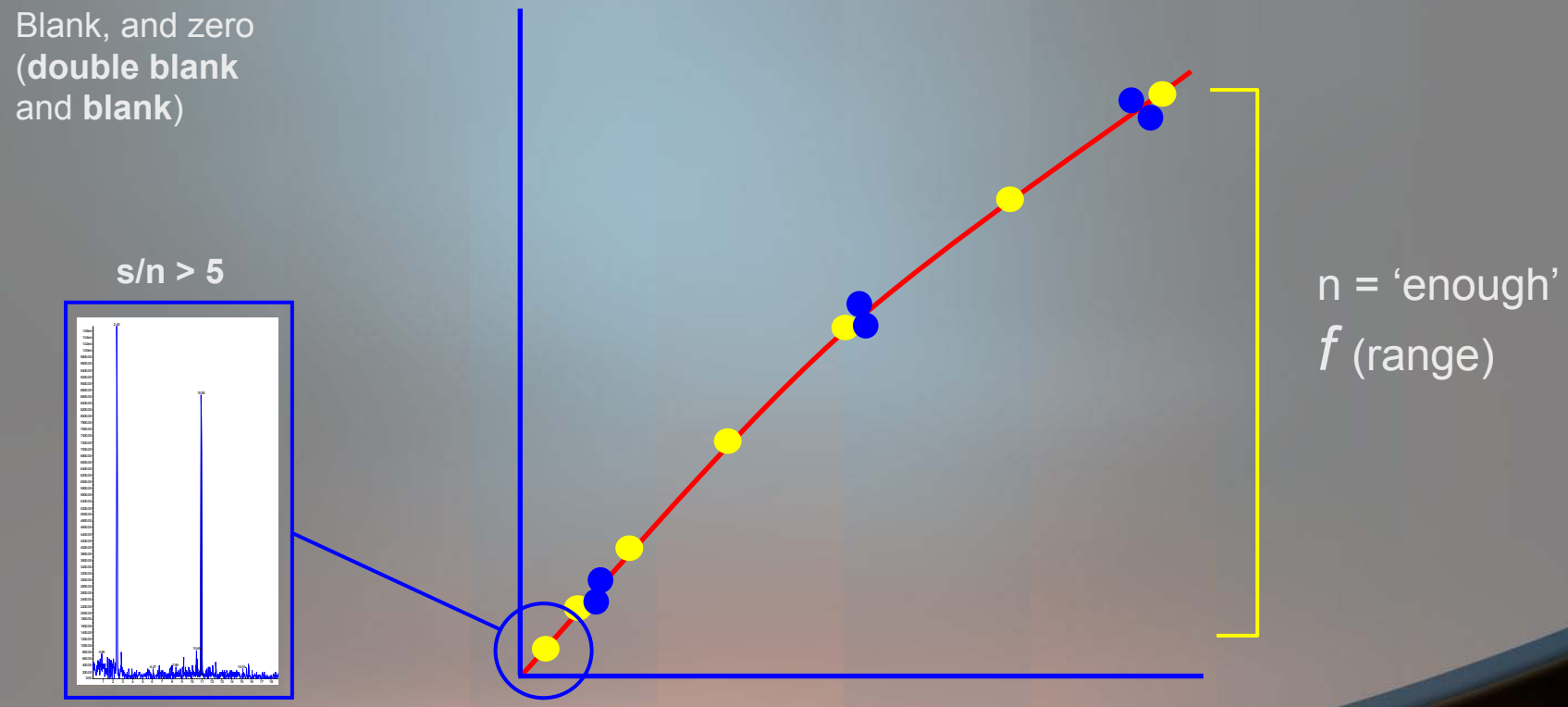
Simplest model

Weighting

Anchoring pts.

Calibration Curve

- Relationship between concentration and detector response



Calibration Curve

- Simplest model
- Justify weighting (paper dealing with calculation of residuals)

Stability Studies

Collection

Process (centr., freeze)

Transfer samples

Thaw and assay

Review

QA, QC final report

FT

- Why – samples FT a number of times (re-assays, etc.)
- How – comparative QC sample FT=0 compared to sample with multiple FT cycles

- Done on QCs

- Thaw, refreeze (12-24 hr), three cycles, assay within single batch

Short-Term Stability

- Why – Samples might degrade while processing
- How – comparative QCs exposed to lab (4-24 hr) with unexposed sample

- Done on QCs
- Decrease only ?

- Assay within single batch

Long-Term Stability

- Why – Samples may degrade over storage life-cycle
- How – comparative QCs which have been stored* compared with freshly prepared QCs

- Stability of QCs demonstrated over interval which exceeds the period over which samples were stored
- Specific to tube-type and storage temperature

Solution Stability

- Calibrators prepared from stocks – investigate stability (IS too)
- How – comparative. ‘Old’ STD solutions compared to freshly prepared STD solutions
- Stability of solutions characterised, and use of appropriate exp. dates
- Specific to solvent, vessel and temperature

Extract Stability

- Final residue, processed on analytical instrument
- How – compare fresh extracts with ‘old’ extracts residing in instrument for extended interval

- Stability of solutions characterised, and use of appropriate exp. dates
- Specific to solvent, vessel and temperature

Specifics

for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

5. Post-Preparative Stability

The stability of processed samples, including the resident time in the autosampler, should be determined. The stability of the drug and the internal standard should be assessed over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards.

Although the traditional approach of comparing analytical results for stored samples with those for freshly prepared samples has been referred to in this guidance, other statistical approaches based on confidence limits for evaluation of an analyte's stability in a biological matrix can be used. SOPs should clearly describe the statistical method and rules used. Additional validation may include investigation of samples from dosed subjects.

E. Principles of Bioanalytical Method Validation and Establishment

- The fundamental parameters to ensure the acceptability of the performance of a bioanalytical method validation are accuracy, precision, selectivity, sensitivity, reproducibility, and stability.
- A specific, detailed description of the bioanalytical method should be written. This can be in the form of a protocol, study plan, report, and/or SOP.
- Each step in the method should be investigated to determine the extent to which environmental, matrix, material, or procedural variables can affect the estimation of analyte in the matrix from the time of collection of the material up to and including the time of analysis.
- It may be important to consider the variability of the matrix due to the physiological nature of the sample. In the case of LC-MS-MS-based procedures, appropriate steps should be taken to ensure the lack of matrix effects throughout the application of the method, especially if the nature of the matrix changes from the matrix used during method validation.
- A bioanalytical method should be validated for the intended use or application. All experiments used to make claims or draw conclusions about the validity of the method should be presented in a report (method validation report).

Fundamentals

Method Document

Integrity, entire lifecycle

Matrix effects

Validation report

- Whenever possible, the same biological matrix as the matrix in the intended samples should be used for validation purposes. (For tissues of limited availability, such as bone marrow, physiologically appropriate proxy matrices can be substituted.)
- The stability of the analyte (drug and/or metabolite) in the matrix during the collection process and the sample storage period should be assessed, preferably prior to sample analysis.
- For compounds with potentially labile metabolites, the stability of analyte in matrix from dosed subjects (or species) should be confirmed.
- The accuracy, precision, reproducibility, response function, and selectivity of the method for endogenous substances, metabolites, and known degradation products should be established for the biological matrix. For selectivity, there should be evidence that the substance being quantified is the intended analyte.
- The concentration range over which the analyte will be determined should be defined in the bioanalytical method, based on evaluation of actual standard samples over the range, including their statistical variation. This defines the *standard curve*.
- A sufficient number of standards should be used to adequately define the relationship between concentration and response. The relationship between response and concentration should be demonstrated to be continuous and reproducible. The number of standards used should be a function of the dynamic range and nature of the concentration-response relationship. In many cases, six to eight concentrations (excluding blank values) can define the standard curve. More standard concentrations may be recommended for nonlinear than for linear relationships.
- The ability to dilute samples originally above the upper limit of the standard curve should be demonstrated by accuracy and precision parameters in the validation.
- In consideration of high throughput analyses, including but not limited to multiplexing, multicolumn, and parallel systems, sufficient QC samples should be used to ensure control of the assay. The number of QC samples to ensure proper control of the assay should be determined based on the run size. The placement of QC samples should be judiciously considered in the run.
- For a bioanalytical method to be considered valid, specific acceptance criteria should be set in advance and achieved for accuracy and precision for the validation of QC samples over the range of the standards.

Same matrix (proxy?)

Stability during collection, prio if possible

Labile metabolites

Still specific when endog's and metab's?

Curve pre-defined, appropriate to samples?

STDs, 6 – 8, blanks, zero

Dilution test

QCs, judicious, H, M, L

STDs, QCs, a-priori criteria

F. **Specific Recommendations for Method Validation**

- The matrix-based standard curve should consist of a minimum of six standard points, excluding blanks, using single or replicate samples. The standard curve should cover the entire range of expected concentrations.

- Standard curve fitting is determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighting and statistical tests for *goodness of fit*.

- LLOQ is the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision. The LLOQ should be established using at least five samples independent of standards and determining the coefficient of variation and/or appropriate confidence interval. The LLOQ should serve as the lowest concentration on the standard curve and should not be confused with the limit of detection and/or the low QC sample. The highest standard will define the upper limit of quantification (ULOQ) of an analytical method.

- For validation of the bioanalytical method, accuracy and precision should be determined using a minimum of five determinations per concentration level (excluding blank samples). The mean value should be within ±15% of the theoretical value, except at LLOQ, where it should not deviate by more than ±20%. The precision around the mean value should not exceed 15% of the CV, except for LLOQ, where it should not exceed 20% of the CV. Other methods of assessing accuracy and precision that meet these limits may be equally acceptable.

- The accuracy and precision with which known concentrations of analyte in biological matrix can be determined should be demonstrated. This can be accomplished by analysis of replicate sets of analyte samples of known concentrations • QC samples • from an equivalent biological matrix. At a minimum, three concentrations representing the entire range of the standard curve should be studied: one within 3x the lower limit of quantification (LLOQ) (low QC sample), one near the center (middle QC), and one near the upper boundary of the standard curve (high QC).

- Reported method validation data and the determination of accuracy and precision should include all outliers; however, calculations of accuracy and precision excluding values that are statistically determined as outliers can also be reported.

- The stability of the analyte in biological matrix at intended storage temperatures should be established. The influence of freeze-thaw cycles (a minimum of three cycles at two concentrations in triplicate) should be studied.

Curve min 6 points (daily)

Simplest model, statistically justify (residuals)

LLOQ, test 5x, top STD = ULOQ

Test 5x, acc and prec ± 15% (LLOQ, ± 25%)

QCs, L, M, H (3x LLOQ, mid, 75% of ULOQ)

All values in calc's, can report without outliers (option)

Matrix stability studies

Stability Studies cont.

- The stability of the analyte in matrix at ambient temperature should be evaluated over a time period equal to the typical sample preparation, sample handling, and analytical run times.
- Reinjection reproducibility should be evaluated to determine if an analytical run could be reanalyzed in the case of instrument failure.
- The specificity of the assay methodology should be established using a minimum of six independent sources of the same matrix. For hyphenated mass spectrometry-based methods, however, testing six independent matrices for interference may not be important. In the case of LC-MS and LC-MS-MS-based procedures, matrix effects should be investigated to ensure that precision, selectivity, and sensitivity will not be compromised. Method selectivity should be evaluated during method development and throughout method validation and can continue throughout application of the method to actual study samples.
- Acceptance/rejection criteria for spiked, matrix-based calibration standards and validation QC samples should be based on the nominal (theoretical) concentration of analytes. Specific criteria can be set up in advance and achieved for accuracy and precision over the range of the standards, if so desired.

Re-inject if inst, stoppage

Matrix effects (n = 6)

A-priori criteria

The AAPS Workshop

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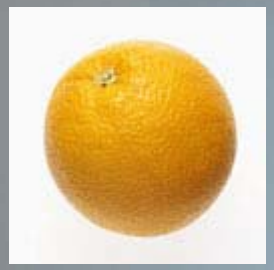
- L-B assays
- Incurred sample re-analysis
- Recognition that "QCs may not adequately mimic study samples"

ncurred samples (Jemal et al)

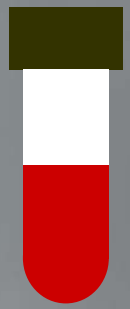


Analyte introduced into plasma

No metabolites

Plasma FT prior to introduction

None of the chemistry associated with red blood cells



Analyte introduced into living system

Metabolites (known and unknown)

Fresh *in vivo* introduction

Enzymes, binding, etc

Revisit

RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2002; 16: 1538–1547
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/rcm.757

RCM

A strategy for a post-method-validation use of incurred biological samples for establishing the acceptability of a liquid chromatography/tandem mass-spectrometric method for quantitation of drugs in biological samples

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Validated liquid chromatography/tandem mass spectrometric (LC/MS/MS) methods are now widely used for quantitation of drugs in post-dose (incurred) biological samples for the assessment of pharmacokinetic parameters, bioavailability and bioequivalence. In accordance with the practice currently accepted within the pharmaceutical industry and the regulatory bodies, validation of a bioanalytical LC/MS/MS method is performed using standards and quality control (QC) samples prepared by spiking the drug (the analyte) into the appropriate blank biological matrix (e.g. human plasma). The method is then declared to be adequately validated for analyzing incurred biological samples. However, unlike QC samples, incurred samples may contain an epimer or another type of isomer of the drug, such as a *Z* or *E* isomer. Such a metabolite will obviously interfere with the selected reaction monitoring (SRM) transition used for the quantitation of the drug. The incurred sample may also contain a non-isomeric metabolite having a molecular mass different from that of the drug (such an acylglucuronide metabolite) that can still contribute to (and hence interfere with) the SRM transition used for the quantitation of the drug. The potential for the SRM interference increases with the use of LC/MS/MS bioanalytical methods with very short run times (e.g. 0.5 min). In addition, a metabolite can potentially undergo degradation or conversion to revert back to the drug during the multiple steps of sample preparation that precede the introduction of the processed sample into the LC/MS/MS system. In this paper, we recommend a set of procedures to undertake with incurred samples, as soon as such samples are available, in order to establish the validity of an LC/MS/MS method for analyzing real-life samples. First, it is recommended that the stability of incurred samples be investigated 'as is' and after sample preparation. Second, it is recommended that potential SRM interference be investigated by analyzing the incurred samples using the same LC/MS/MS method but with the additional incorporation of the SRM transitions attributable to putative metabolites (multi-SRM method). The metabolites monitored will depend on the expected metabolic products of the drug, which are predictable based on the functional groups present in the chemical structure of the drug. Third, it is recommended that potential SRM interference be further investigated by analyzing the incurred samples using the multi-SRM LC/MS/MS method following the modification of chromatographic conditions to enhance chromatographic separation of the drug from any putative metabolites. We will demonstrate the application of the proposed strategy by using a carboxylic acid containing drug candidate and its acylglucuronide as a putative metabolite. Plasma samples from the first-in-man (FIM) study of the drug candidate were used as the incurred samples. Copyright © 2002 John Wiley & Sons, Ltd.

The high-throughput approaches currently adopted in drug discovery and development in pharmaceutical companies have resulted in an increased number of drug candidates. To meet the increased number of samples for fast-turnaround analysis, bioanalytical chemists have adopted the technique of liquid chromatography coupled with atmospheric pressure ionization (API) tandem mass spectrometry (LC/MS/MS) as the technique of choice for quantitating small-molecule drugs, metabolites and endogenous biomolecules in biological matrices (plasma, serum,

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- Putative MS scans
- Method transfer
- FT
- RT
- Extract stability
- LTFSS

Remarks

- No need for wholesale re-think, in most cases, QC mimic is adequate
- CI sample analysis is prudent (revisit FT, RT, etc)
- CI repeat assays likely to appear new guidance
- Tricky in a modular system (clinical and lab facility separate – disconnect)
- Q-Pharm has a fit-for-purpose procedure in place, discuss afterwards if desired

Questions