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M10: Bioanalytical Method Validation and Study Sample Analysis

Step 4

Step 4 document – to be implemented

24 May 2022

International Council for Harmonisation of Technical Requirements
for Pharmaceuticals for Human Use

Legal Notice

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Background

- **This document has been signed off as a *Step 4* document (24 May 2022) to be implemented by the ICH Regulatory Members**
- **This document was developed based on a Concept Paper (7 October 2016) and Business Plan (7 October 2016)**

Key Principles

- **This multidisciplinary guideline addresses the validation of bioanalytical methods for chemical and biological drug quantification and their application in the analysis of study samples.**
- **This guideline provides recommendations and criteria to be applied in the validation of bioanalytical methods.**
- **In addition, the guideline provides recommendations and criteria to be applied in the analysis of study samples.**

Key Principles (cont.)

- **The guideline is applicable to chromatographic methods and ligand binding assays (LBA).**
- **Bioanalysis of biomarkers and bioanalytical methods used for the assessment of immunogenicity are not within the scope of this guideline.**

Guideline Objectives

- **This guideline**
  - **provides recommendations for the validation of bioanalytical methods and the analysis of study samples.**
  - **ensures the quality and consistency of the bioanalytical data.**
  - **harmonises current regional guidances and intends to facilitate drug development with the principles of 3Rs (Reduce, Refine and Replace).**

Table of main Guideline Contents

Table of main Guideline Contents (cont.)

- **4. Ligand binding assays**
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Outline

- Objectives and scope of the guideline
- Chromatography
  - validation and study sample analysis
- Ligand binding assays
  - validation and study sample analysis
- Incurred sample reanalysis
- Partial and cross validation
- Additional considerations
- Documentation

Scope

- **Description of the validation of bioanalytical methods and study sample analysis.**
- **Applicable to bioanalytical methods used to measure concentrations of chemical and biological drugs in biological samples from:**
  - **nonclinical toxicokinetic studies conducted according to the principles of Good Laboratory Practice.**
  - **nonclinical pharmacokinetic studies conducted as surrogates for clinical studies.**
  - **all phases of clinical trials, including comparative bioavailability/bioequivalence (BA/BE) studies.**

Scope (cont.)

- **Applicable to the quantitative analysis by chromatographic methods and ligand binding assays.**
- **For studies that are subject to Good Laboratory Practice or Good Clinical Practice, the bioanalysis of study samples should conform to their requirements.**
- **Bioanalysis of biomarkers and bioanalytical methods used for the assessment of immunogenicity are not within the scope of this guideline.**

Scope (cont.)

- **Full method validation is expected for the primary matrix intended to support regulatory submissions. Additional matrices should be validated, as necessary.**
- **For studies not submitted for regulatory approval or not considered for regulatory decisions regarding safety, efficacy or labelling, applicants may decide on the level of qualification.**

Chromatography; validation

- **A full validation should include:**

  - selectivity/specificity

  - matrix effect

  - calibration curve and range, including lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ)

  - accuracy (vs. nominal concentration) and precision

  - carry-over

  - dilution integrity

  - stability

  - reinjection reproducibility

Chromatography; validation (cont.)

- **Selectivity:**
  - **is the ability to differentiate and measure the analyte in the presence of interfering substances in the biological matrix.**
  - **no significant response/interference with the analyte and internal standard (IS) should be observed in blank matrix (n ≥ 6 sources), and lipaemic and haemolysed matrix (n ≥ 1 source each).**
  - **criteria: interfering signal ≤ 20% of the analyte LLOQ response and ≤ 5% of the IS response.**

Chromatography; validation (cont.)

- **Specificity:**
  - **is the ability to detect and differentiate the analyte from other substances, including its related substances.**
  - **no significant response/interference with the analyte and IS should be observed due to other substances in the matrix.**
  - **criteria: interfering signal ≤ 20% of analyte LLOQ response and ≤ 5% of IS response.**
  - **the possible back-conversion of metabolite into parent during sample processing should be evaluated, when relevant.**

Chromatography; validation (cont.)

- **Matrix effect:**
  - **is the alteration of the analyte response due to interfering components in the matrix.**
  - **criteria: accuracy and precision within 15% using ≥3 low quality control samples (LQC) and 3 high quality control samples (HQC) of matrix (n ≥ 6 sources).**
  - **should also be evaluated in relevant patient or special populations, e.g. renally or hepatically impaired patients, when available.**

Chromatography; validation (cont.)

- **Calibration curve range:**
  - **demonstration of relationship between nominal analyte concentration and response.**
  - **a calibration curve includes blank samples, a zero sample and at least 6 calibration standards, including LLOQ and ULOQ.**
  - **criteria: ≥ 3 runs, over several days**

    accuracy ± 15%, except at LLOQ ± 20%

    should be met for 75% of the calibration standards with a minimum of 6.

Chromatography; validation (cont.)

- **Accuracy/precision:**
  - **evaluated using at least 4 quality control samples (QC) concentration levels, i.e. at the LLOQ, ≤ 3 x LLOQ (=LQC), around 30 – 50% of the calibration curve range (=median QC (MQC)) and ≥ 75% of the ULOQ (=HQC).**
  - **criteria:**
    - **within-run: n ≥ 5 each QC level within each run**
    - **between-run: n ≥ 3 runs over 2 or more days**
    - **accuracy and precision within 15%, except at LLOQ (within 20%)**

Chromatography; validation (cont.)

- **Carry-over:**
  - **is the alteration of a measured concentration due to residual analyte from a preceding sample in the instrument.**
  - **evaluated by injection of blank samples after injection of a sample at the ULOQ.**
  - **criteria: analyte response in blank sample ≤ 20% of analyte LLOQ response and ≤ 5% of IS response.**

Chromatography; validation (cont.)

- **Dilution integrity:**
  - **is the assessment of the sample dilution procedure to confirm that the procedure does not impact the measured concentration of the analyte.**
  - **evaluated by dilution with matrix (or surrogate if rare matrix) of sample with an analyte concentration > ULOQ.**
  - **dilution factor and concentration(s) applied during study sample analysis should be within those evaluated during validation.**
  - **criteria: n ≥ 5 per dilution factor; accuracy and precision of analyte concentration in diluted sample ≤ 15%.**

Chromatography; validation (cont.)

- **Stability:**
  - **evaluations should be carried out to ensure that every step taken during sample preparation, processing and analysis as well as the storage conditions used do not affect the concentration of the analyte.**
  - **conditions applied during the evaluations should reflect those used for the study samples.**
  - **for fixed dose combination products and specifically labelled drug regimens, the freeze-thaw, bench-top and long-term stability tests should be conducted with the matrix spiked with all of the dosed compounds.**

Chromatography; validation (cont.)

- **Stability (continued):**
  - **for chemical drugs, if stability is demonstrated at one temperature (e.g. -20°C) it can be extrapolated to a lower temperature (e.g. -70°C).**
  - **is evaluated by use of the LQC and HQC.**
  - **criteria: n ≥ 3 aliquots of one bulk at each QC level; mean accuracy within 15%.**

Chromatography; validation (cont.)

- **Stability (continued):**

  The following stability tests should be evaluated:

  - stability of the analyte in matrix:

  - freeze-thaw stability

  - bench-top (short-term) stability

  - long-term stability

  - stability of the analyte in processed sample

  - stability of the analyte and IS in stock/working solutions

  - stability of the analyte in whole blood, if applicable

Chromatography; validation (cont.)

- **Reinjection reproducibility:**

  - if samples could be reinjected, reinjection reproducibility should be evaluated to establish the viability of the processed samples and to support their storage prior to reinjection

  - evaluated by reinjecting a run that is comprised of calibration standards and a minimum of 5 replicates of the low, middle and high QCs after storage.

  The precision and accuracy of the reinjected QCs establish the viability of the processed samples.

Chromatography; study sample analysis

- **The study samples, QCs and calibration standards should be processed in accordance with the validated analytical method.**
- **The study samples should be analysed in an analytical run consisting of a blank sample, a zero sample, calibration standards at a minimum of 6 concentration levels, and at least 3 levels of QCs (low, medium and high) in duplicate.**
- **For comparative BA/BE studies, it is advisable to analyse all samples of one subject together in one analytical run to reduce variability.**

Chromatography; study sample analysis (cont.)

- **Acceptance criteria analytical run:**
  - **calibration standards: accuracy ± 15%, at LLOQ ± 20%, should be met for 75% of the calibration standards with a minimum of 6.**
  - **at least 2/3 of the total QCs and at least 50% at each concentration level should be within ±15% of the nominal values.**
- **Overall, between-run accuracy and precision at each QC level should be within 15%; in case this is not met a justification should be given.**

Chromatography; study sample analysis (cont.)

- **Calibration range:**
  - **the calibration curve range should cover adequately the analyte concentrations in the study samples. If this is not the case, either the calibration range should be narrowed, existing QC concentrations revised, or QCs at additional concentrations added.**
  - **at least 2 QC levels should fall within the range of concentrations measured in study samples.**

Chromatography; study sample analysis (cont.)

- **Reanalysis of study samples:**
  - **possible reasons for reanalysis of study samples, the number of replicates and the decision criteria to select the value to be reported should be predefined in the protocol, study plan or SOP.**
  - **for comparative BA/BE studies, reanalysis of study samples for a pharmacokinetic (PK) reason (e.g., a sample concentration does not fit with the expected profile) is not acceptable, as it may bias the study result.**

Chromatography; study sample analysis (cont.)

- **Reinjection of processed samples:**
  - **in the case of equipment failure if reinjection reproducibility has been demonstrated, reinjection can be applied.**
  - **reinjection of a full analytical run or of individual calibration standards or QCs simply because the calibration standards or QCs failed, without any identified analytical cause, is not acceptable.**

Chromatography; study sample analysis (cont.)

- **Chromatogram integration and reintegration:**
  - **procedures should be described in a study plan, protocol or Standard Operating Procedure (SOP).**
  - **The reasons for reintegration should be provided.**

Ligand binding assays; validation

- **A full validation should include:**
  - **specificity/selectivity**
  - **calibration curve and range, including LLOQ and ULOQ**
  - **accuracy (vs. nominal concentration) and precision**
  - **carry-over**
  - **dilution linearity and hook effect**
  - **stability**

Ligand binding assays; validation (cont.)

- **Specificity:**
  - **in LBA this is related to cross-reactivity.**
  - **evaluated by spiking blank matrix and LLOQ and ULOQ samples with related molecules at the maximum concentration anticipated.**
  - **criteria: response in spiked blank matrix < LLOQ. accuracy of analyte in LLOQ and ULOQ within 25% of nominal value.**

Ligand binding assays; validation (cont.)

- **Selectivity:**
  - **no significant response/interference with the analyte should be observed in blank matrix (n ≥ 10 sources), and lipaemic and haemolysed matrix (n ≥ 1 source each).**
  - **where applicable, should be evaluated in relevant patient population(s)**
  - **criteria: response in blank ≤ LLOQ in at least 80% of the individual sources.**

    accuracy of analyte concentration within 25% at LLOQ and within 20% at HQC in at least 80% of the individual sources.

Ligand binding assay; validation (cont.)

- **Calibration curve range:**
  - **demonstration of relationship between nominal analyte concentration and response.**
  - **a calibration curve includes blank sample, and at least 6 calibration standards, including LLOQ and ULOQ.**
  - **anchor point samples may be used to improve curve fitting.**
  - **criteria: ≥ 6 runs, over several days**

    accuracy/precision ± 20%, except at LLOQ and ULOQ ± 25%

    should be met for 75% of the calibration standards with a minimum of 6.

Ligand binding assays; validation (cont.)

- **Accuracy/precision:**
  - **evaluated using at least 5 QC concentration levels, i.e. at the LLOQ, ≤ 3 x LLOQ (=LQC), around the geometric mean of the calibration curve range (=MQC), ≥ 75% of the ULOQ (=HQC) and at the ULOQ.**
  - **criteria:**
    - **within-run: n ≥ 3 each QC level within each run**
    - **between-run: n ≥ 6 runs over 2 or more days**

Ligand binding assays; validation (cont.)

- **Accuracy/precision:**
  - **criteria (continued):**
    - **accuracy and precision within 20%, except at LLOQ and ULOQ (within 25%)**
    - **total error ≤ 30%, except at LLOQ and ULOQ (≤ 40%)**

Ligand binding assays; validation (cont.)

- **Carry-over:**
  - **normally not an issue, unless the platform is prone to carry-over.**
  - **evaluated by placing blank samples after the ULOQ sample.**
  - **criteria: analyte response in blank sample should be below the LLOQ response.**

Ligand binding assays; validation (cont.)

- **Dilution linearity and hook effect:**
  - **dilution needed in order to achieve analyte concentrations within the range of the assay.**
  - **accuracy and precision should not be affected by the dilution and samples above the ULOQ should not be impacted by the hook effect.**
  - **dilution factor(s) applied during sample analysis should be within those evaluated during validation.**
  - **criteria: n ≥ 3 dilution factors and independent dilution series; mean accuracy and precision at each dilution factor ≤ 20%.**

Ligand binding assays; validation (cont.)

- **Stability:**
  - **evaluations should be carried out to ensure that every step taken during sample preparation, processing and analysis as well as the storage conditions used do not affect the concentration of the analyte.**
  - **conditions applied during the evaluations should reflect those used for the study samples.**
  - **evaluated by use of the LQC and HQC.**
  - **criteria: n ≥ 3 aliquots of one bulk at each QC level; mean accuracy within 20%.**

Ligand binding assays; validation (cont.)

- **Stability (continued):**

  The following stability tests should be evaluated:

  - stability of the analyte in matrix:

  - freeze-thaw stability

  - bench-top (short-term) stability

  - long-term stability

Ligand binding assays; validation (cont.)

- **Stability (continued):**
  - **for fixed dose combination products and specifically labelled drug regimens, the freeze-thaw, bench-top and long-term stability tests should be conducted with the matrix spiked with all of the dosed compounds, on a case-by-case basis.**
  - **for biological drugs, stability demonstrated at a high and low temperature (e.g. at -20°C and at -70°C) can be interpolated to a temperature in between (e.g. -40°C).**

Ligand binding assays; study sample analysis

- **The study samples, QCs and calibration standards should be processed in accordance with the validated analytical method.**
- **The study samples should be analysed in an analytical run consisting of a blank sample, calibration standards at a minimum of 6 concentration levels, and at least 3 levels of QCs (low, medium and high) applied as two sets, at least.**
- **If multiple microtiter plates are used and each plate contains a set of calibrators and QCs, then each plate should be assessed on its own. If not possible, calibrators should be on the first and the last plate, and QCs should be placed on every single plate.**

Ligand binding assays; study sample analysis (cont.)

- **Acceptance criteria for an analytical run:**
  - **calibration standards: accuracy ± 20%, at LLOQ and ULOQ ± 25%, should be met for 75% of the calibration standards with a minimum of 6.**
  - **at least 2/3 of the total QCs and at least 50% at each concentration level should be within ±20% of the nominal values.**
  - **overall, between-run accuracy and precision at each QC level should be within 20%; in case this is not met a justification should be given.**

Ligand binding assays; study sample analysis (cont.)

- **The calibration curve range should cover adequately the analyte concentrations in the study samples.**
- **If clustering of study samples at one end of the calibration curve range is encountered, additional QC concentration levels should be added, or the calibration curve range should be narrowed, and QC samples concentrations revised.**
- **At least 2 QC levels should fall within the range of concentrations measured in study samples.**

Ligand binding assays; study sample analysis (cont.)

- **Reanalysis of study samples:**
  - **possible reasons for reanalysis of study samples, the number of replicates and the decision criteria to select the value to be reported should be predefined in the protocol, study plan or SOP.**
  - **for comparative BA/BE studies, reanalysis of study samples for a PK reason (e.g., a sample concentration does not fit with the expected profile) is not acceptable, as it may bias the study result.**

Incurred sample reanalysis

- **Incurred sample reanalysis (ISR) is intended to verify the reliability of the reported sample analyte concentrations.**
- **ISR should be performed at least in the following situations:**
  - **For nonclinical studies within the scope of this guideline, at least once per species.**
  - **All pivotal comparative BA/BE studies.**
  - **First clinical trial in subjects.**
  - **Pivotal early patient trial(s), once per patient population.**
  - **First or pivotal trial in patients with impaired hepatic and/or renal function.**

Incurred sample reanalysis (cont.)

- **Samples for ISR should be chosen around the maximum drug concentration (Cmax) and in the elimination phase.**

  - criteria:

    - number of samples: 10% of first 1000 study samples, and 5% of the rest.

    - calculation:

$$\% \text{ difference} = \frac{\text{repeat value} - \text{initial value}}{\text{mean value}} \times 100$$

    - for chromatographic methods: % difference within 20% for 2/3 of repeats; for LBA, within 30% for 2/3 of repeats.

Partial validation:

- **Is required to support a modification to a validated analytical method. Partial validation can range from as little as one within-run accuracy and precision determination, to a nearly full validation.**

Cross validation:

- **Is required to demonstrate how the reported data are related when multiple bioanalytical methods and/or multiple bioanalytical laboratories are involved.**
- **Assessed by measuring a set of QCs and study samples (if available) with both methods/laboratories. Bias can be evaluated by use of Bland-Altman plots, Deming regression, or other appropriate methods.**

Additional considerations:

- **For the analysis of an endogenous molecule, biological matrix to prepare calibration standards and QCs should be the same as the study samples, but free of matrix effect and interference. If this is not available, different approaches can be used to calculate the concentrations of the analyte in study samples, i.e.:**

  - the surrogate matrix approach

  - the surrogate analyte approach

  - the background subtraction approach

  - the standard addition approach.

Additional considerations (cont.):

- **Parallelism in LBA should be evaluated on a case-by-case basis, e.g., where interference is caused by a matrix component (presence of endogenous binding protein) is suspected during study sample analysis.**
- **For methods that employ sample extraction, the recovery (extraction efficiency) should be evaluated.**
- **Minimum Required Dilution (MRD) in LBA may be applied to reduce background signal or matrix interference. The MRD should be identical for all samples, including calibration standards and QCs.**

Additional considerations (cont.):

- **In case a commercial or diagnostics kit is used to measure chemical or biological drug concentrations during the development of a novel drug, the applicant should assess the kit validation to ensure that it conforms to the drug development standards described in this guideline.**
- **In case a new or alternative technology is used as the sole bioanalytical technology from the onset of drug development, cross validation with an existing technology is not required.**

Additional considerations (cont.):

- **For dried matrix methods, additional validation of the sampling approach is needed, such as haematocrit, sample homogeneity, extraction of the sample from the dried matrix and sample collection for ISR.**

Documentation:

- **The data generated for bioanalytical method validation should be documented and available for data audit and inspection.**
- **Table 1 in this guideline describes the recommended documentation for submission to the regulatory authorities and documentation that should be available at the analytical site at times of inspection.**
- **Summary information should be provided, including e.g., a summary of the methods used, a summary table of all the relevant validation reports, information on regulatory site inspections in case of comparative BA/BE studies.**

Conclusions

- **This harmonized guidance on the requirements for validation of bioanalytical methods and analysis of study samples will result in applying the same scientific regulatory requirements in different regions, avoiding unnecessary duplicative testing and support streamlined global drug development.**

Contact

- **For any questions please contact the ICH Secretariat:**

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