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SOP 12: Validation of Bioanalytical Methods

Background and Objectives

A bioanalytical method is a set of procedures involved in the collection, processing, storage, and analysis of a biological matrix for a chemical compound. The availability of selective and sensitive bioanalytical methods is a prerequisite for the generation of reliable data on pharmacokinetics, bioavailability, and bioequivalence of drugs. These methods should allow the quantification of drugs and their metabolites in biological matrices, e.g. plasma, urine, and cerebrospinal fluid, and must be validated with respect to their reliability for the intended use. Bioanalytical method validation comprises all criteria determining data quality, such as selectivity, accuracy, precision, recovery, sensitivity, and stability. This SOP is applicable for the analysis of preclinical as well as clinical samples.
A specific, unique chemical moiety that would be found in a biological matrix is referred to as *analyte*.

Types of Method Validation

Full Validation
A full validation is required
- If the method is developed and implemented for the first time,
- If a new drug entity is analyzed, or
- If metabolites are added to an existing assay for quantification.

Partial Validation
A partial validation can be performed if validated bioanalytical methods have been modified. It can range from the determination of a within-day accuracy and precision to a nearly full validation. Typical situations for a partial validation are
- Method transfers between laboratories and analysts,
- Instrument and/or software platform changes,
- Changes in species within the same matrix,
- Changes in matrix within the same species,
- Change in analytical methodology, and
- Change in sample processing procedures.

Cross Validation
In a cross validation two bioanalytical methods for the same analyte are compared. Cross validations are necessary when two or more bioanalytical methods are used to generate data within the same study. They should be conducted with spiked matrix standards and subject samples. A cross validation should be also considered when
- Sample analyses within a single study are conducted in more than one laboratory or
- Data generated using different analytical techniques in different studies are included in a regulatory submission.

Validation Parameters

In this section the fundamental parameters for the validation of a chemical assay, e.g. gas chromatography (GC), high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE), are described. For immunoassays special considerations have to be taken into account (see below 'Special Considerations for Immunoassays').

Selectivity
Selectivity is the ability of an analytical method to differentiate and quantify in a sample the analyte in the presence of other components. For selectivity, analyses of blank samples of the respective biological matrix should be obtained from at least 6 sources. Each blank sample should be tested for interference which could originate from endogenous matrix components, metabolites, decomposition products and concomitant medication.

Accuracy
The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the nominal value (concentration) of the analyte. Accuracy is

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determined by replicate analysis of samples containing known amounts of analyte. Accuracy should be measured using a minimum of 3 concentrations and 5 determinations per concentration. *The mean value should be within 15% of the nominal value except at lower limit of quantification (LLOQ, see below), where it should not deviate by more than 20%.* The deviation of the mean from the nominal value (relative error) serves as the measure of accuracy.

Precision

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using a minimum of 3 concentrations and 5 determinations per concentration. *The imprecision determined as coefficient of variation (CV) at each concentration level should not exceed 15% except for the LLOQ (see below), where it should not exceed 20%.*

Precision is further subdivided into

- Within-day precision, which assesses precision during a single analytical run, and
- Between-day precision, which measures precision with time, and may involve different analysts, equipment, reagents, and laboratories.

Recovery

The recovery of an analyte is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the nominal concentration of the pure authentic standard. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations with unextracted standards that represent 100% recovery. *Recovery of the analyte need not be 100% but the extent of recovery of an analyte and an internal standard should be consistent, precise, and reproducible.*

Calibration Curve

The relationship between the experimental response value and known concentrations of the analyte is referred to as *calibration curve*. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte. In case that there is not enough blank sample availiable, e.g. from cerebrospinal fluid, an appropriate calibration matrix should be selected, e.g. 0.9% NaCl, and the response obtained from both matrices should be compared.

Concentration of standards should be chosen on the basis of the concentration range expected in a particular study. A calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample with internal standard), and 5–8 non-zero samples covering the expected range, including the LLOQ.

Calibration curve fitting should be performed by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighting and statistical tests for *goodness-of-fit*. The following conditions should be met in developing a calibration curve:

- 15% deviation of standards other than LLOQ from nominal concentration and
- 20% deviation of the LLOQ from nominal concentrations.

At least 4 out of 6 non-zero standards should meet the above criteria, including the LLOQ and the calibration standard at the highest concentration.

Lower Limit of Quantification

The LLOQ is the lowest concentration of an analyte that can be measured with acceptable accuracy and precision. The lowest standard on the calibration curve should be accepted as LLOQ if the following conditions are met:

- The analyte response should be at least 5 times the response compared to blank response.
- The analyte response should be reproducible with an imprecision of maximum 20% and an accuracy of 80–120%.

Stability

The stability of the analyte in the biological matrix at intended storage temperatures should be established. Moreover, the stability should be investigated at ambient temperature over a time period that encompasses the duration of typical sample preparation, sample handling, and analytical run time. The procedure should also include an evaluation of analyte stability in stock solution for at least 6 h. The influence of freeze/thaw cycles should be studied – at least 3 cycles at 2 concentrations in triplicate.

All stability determinations should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate blank, interference-free biological matrix.

Validation Report

A specific, detailed description of the bioanalytical method, the validation experiments and the validation results will be written in form of a report by the laboratory responsible for drug analysis. The validation report covers all validation parameters described in 'Validation Parameters'.

Routine Drug Analysis

This section describes the validation parameters that should be evaluated during routine application of a validated bioanalytical method to a particular study.

Following a successful validation, analysis of biological samples can be done by single determination. The need for duplicate analysis may arise for special cases, e.g. for labile analytes, when precision and accuracy tolerances are difficult to achieve. In this case, a rationale for duplicate analysis should be developed a priori.

System Suitability
Based on the analyte and technique, a specific system suitability test or sample should be identified to assure the optimum operation of the system employed.

Calibration
A matrix-based calibration curve should be generated for each run and should be used for calculating the analyte concentration in the unknown samples assayed with that run.
It is important to use a calibration curve that covers the entire range of concentrations in the unknown samples. Estimation of unknowns by extrapolations below the LLOQ or above the upper range is not acceptable. Instead, the calibration curve should be re-determined or samples should be re-assayed after dilution with the matrix.

Quality Control Samples
Duplicate quality control (QC) samples at a minimum of 3 concentrations (one within 3 × of the LLOQ, one in the midrange, and one approaching the high end of the range) should be incorporated into each run. There should be a minimum of 5% QCs relative to the number of samples in a run or 6 total QC samples, whichever is greater.

Acceptance Criteria
The results of the QC samples provide the basis for accepting or rejecting a run. At least 67% (4 out of 6) of the QC samples must be within 15% of their respective nominal values. 33% of the QC samples (but not all replicates at the same concentration) may be outside the ±15% range of the nominal value.

Repeat Analysis
A guideline for repeat analysis should be established a priori. Anticipated reasons for repeating analysis should be defined, e.g. sample processing errors, equipment failure, poor chromatography. The rationale for the repeat analysis should be clearly documented.

Special Considerations for Immunoassays

Most of the bioanalytical validation parameters and principles mentioned above are also applicable to immunoassays. However, there are some specific differences which are described in this section.

Selectivity
As for chemical assays, immunoassays must be shown to be selective for the analyte. Two types of selectivity have to be considered:
- *'Specific' nonselectivity* (interference from compounds that are physicochemically similar to the analyte), e.g. cross-reactivity of metabolites, concomitant medication or endogenous compounds:
  If possible, the immunoassay should be compared with a validated reference method, e.g. HPLC, using study samples.
- *'Nonspecific' nonselectivity* (interference from matrix components):
  Calibration curves in biological fluids should be compared with those in buffer in order to detect matrix effects.

Accuracy and Precision
Criteria for accuracy and precision of immunoassays should match those of chemical assays. Both upper limit of quantification as well as LLOQ must be defined by acceptable accuracy and precision.

Recovery
If separation is employed prior to assay for study samples but not for standards, it is necessary to assess efficiency and reproducibility of recovery and use recovery in determining results.

Calibration Curve
Calibration curves of immunoassays are essentially nonlinear and in general require more concentration points to define the fit than do chemical assays. A minimum of 6 nonzero calibrator concentrations, run in duplicate, is required.

Routine Drug Analysis
Due to the greater inherent imprecision of immunoassays, QC sample acceptance criteria in routine assay implementation are ±25% for accuracy. Thus, at least 67% of QC samples must be within ±25% of their nominal values, with no more than 2 at the same concentration level exceeding ±25% of nominal values.

Documentation and Archiving of Analytical Data

The data generated for bioanalytical method establishment and the QCs should be documented and available for data audit and inspection. In particular, documentation should comprise
- An operational description of the analytical method,
- Evidence of purity and identity of drug standards, metabolite standards, and internal standards used in validation experiments and routine analysis,

- All available full, partial and cross validation reports (see 'Validation Report'),
- Typical chromatograms, electropherograms or mass spectrograms, if applicable,
- Summary tables containing information on sample processing and storage, including sample identification, collection dates, storage prior to shipment, information on shipment batch, and storage prior to analysis,
- Summary tables of analytical runs of clinical and preclinical samples, including assay run identification, date and time of analysis, assay method, analysts, start and stop times, duration, significant equipment and material changes, and any potential issues or deviation from the established method,
- Summary tables of calibration curve data,
- Summary information on QC data used for accepting the analytical run,
- Data tables from analytical runs of clinical and preclinical samples, including assay run identification, sample identification, and raw data,
- Complete serial chromatograms from 5–20% of subjects with calibration and QC samples from those analytical runs,
- Reasons for missing samples,
- Documentation for repeat analyses, including the initial and repeat analysis results, the reported result, and the reason for the repeat analysis,
- Documentation for re-integrated data, including initial and repeat integration results, the method used for re-integration, the reported result, and the reason for the re-integration.

The documents should be archived for no less than 10 years.

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