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DRAFT

GUIDELINE ON VALIDATION OF BIOANALYTICAL METHODS

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GUIDELINE ON VALIDATION OF BIOANALYTICAL METHODS

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EXECUTIVE SUMMARY

This guideline defines key elements and provides recommendations for the validation of bioanalytical methods. The guideline focuses on the validation of the analytical methods used for pharmacokinetic sample analysis. In addition, guidance will be provided with regard to the actual analysis of study samples.

1. INTRODUCTION (background)

Measurement of drug concentrations in biological matrices is an important aspect of medicinal product development for those products containing new active substances as well as for line extensions and generic products. Such data may be required to support new applications as well as variations to authorised drug products. The results of toxicokinetic, pharmacokinetic and bioequivalence studies are used to make critical decisions supporting the safety and efficacy of a medicinal drug substance or product. It is therefore paramount that the applied bioanalytical methods used are well characterised, fully validated and documented to a satisfactory standard in order to yield reliable results.

Acceptance criteria wider than those defined in this guideline may need to be used in special situations, such as analysis of complex matrices (e.g. solid tissues), when usual acceptance criteria cannot be met. This should be justified and prospectively defined.

2. SCOPE

This guideline provides requirements for the validation of bioanalytical methods.

In addition, specific aspects of the bioanalytical method itself will be addressed, e.g. the actual analysis of samples from toxicokinetic studies and clinical trials.

Furthermore, this guideline will describe when partial validation or cross validation may represent an appropriate alternative approach to the complete validation of an analytical method.

Some special techniques such as radio-labelled analysis methods using ^{14}C labelled drugs, are not covered here, but even in such cases efforts should be made to apply to the principles of this guideline.

3. LEGAL BASIS

This guideline has to be read in conjunction with the introduction and general principles (4) and Part I and II of the Annex I to Directive 2001/83 as amended. It applies to Marketing Authorisation Applications for human medicinal products submitted in accordance with the Directive 2001/83/EC as amended, and Regulation (EC) No. 726/2004, in which the analysis of drug concentrations in a biological matrix is part of the application.

The validation of bioanalytical methods and the analysis of study samples should be performed in accordance with the principles of Good Laboratory Practice (GLP). However, as human bioanalytical studies fall outside of the scope of GLP, as defined in Directive 2004/10/EC, the sites conducting the human studies are not required to be monitored as part of a national GLP compliance programme. In addition, for clinical trials in humans the principles of Good Clinical Practice (GCP) should be followed.

Furthermore, reference is made to the following EMEA guidelines:

- Note for guidance on good clinical practices (CPMP/ICH/135/95).
- Note for guidance on validation of analytical procedures: text and methodology (CPMP/ICH/381/95).

4. METHOD VALIDATION

4.1 Complete validation of an analytical method

A complete method validation should be performed for any analytical method whether new or based upon literature.

The main objective of method validation is to demonstrate the reliability of a particular method for the determination of an analyte concentration in a specific biological matrix, such as blood, plasma, urine, saliva or tissue. Moreover, validation should be performed using the same anticoagulant as for the study samples. A full validation should be performed for each species concerned.

In some cases, it may be problematic for validation purposes to obtain an identical matrix compared to the matrix of the study samples. A suitable alternative matrix may be used, e.g. synthetically prepared cerebrospinal fluid, if justified.

The main characteristics of a bioanalytical method that are essential to ensure the acceptability of the performance and the reliability of analytical results are: selectivity, lower limit of quantitation, the response function (calibration curve performance), accuracy, precision, matrix effects, stability of the analyte(s) and any internal standard in the biological matrix and the stock and working solutions under the entire period of storage and processing conditions.

Usually one analyte or drug has to be determined, but on occasions it may be appropriate to measure more than one analyte. This may involve two different drugs, but can also involve a parent drug with its metabolites, or the enantiomers or isomers of a drug. In these cases the principles of validation and analysis apply to all analytes of interest.

Reference standards

During method validation, a blank biological matrix will be spiked with the analyte of interest using solutions of reference standard. In addition, an internal standard (IS) is normally used in chromatographic methods.

It is important that the quality of the reference standard and IS is ensured, as the quality (purity) may affect the outcome of the analysis, and therefore the outcome of the study data. Therefore the reference standards used for the analytical validation and analysis should be obtained from an authentic and traceable source. Suitable reference standards, include certified standards such as compendial standards (EPCRS, USP, WHO), commercial available standards, or fully characterised standards prepared in-house or by an external non-commercial organisation. Suitability of the reference standard should be scientifically justified. The use of certified standards is not needed for IS, as long as the suitability for use is demonstrated, e.g. lack of interference is shown for the substance itself or any impurities thereof.

Whoever the supplier, a certificate of analysis is required to ensure quality, stability, storage conditions, expiration date, batch number and purity of the reference standards.

When MS detection is used in the bioanalytical method, a stable isotope-labelled IS is recommended to be used whenever possible. However, it is essential that the labelled standard is of the highest isotope purity and that no isotope exchange reaction occurs. The presence of any unlabelled analyte would otherwise introduce a bias in the results.

4.1.1 Selectivity

The analytical method should be able to differentiate the analyte(s) of interest and IS from endogenous components in the matrix (i.e. blood, plasma, urine) or other components in the sample. Selectivity should be proven by using at least 6 sources of the appropriate blank matrix, which are individually

analysed and evaluated for interference. Absence of interfering components is accepted where the response is less than 20% of the lower limit of quantitation for the analyte.

It may also be necessary to investigate the extent of any interference caused by metabolites of the drug(s), interference from degradation products formed during sample preparation, and interference from possible co-administered medications. Co-medications normally used in the subject population studied should be taken into account.

The possibility of back-conversion of a metabolite into parent analyte during the successive steps of the analysis (including extraction procedures) should also be evaluated, when relevant (e.g. acidic metabolites to ester, unstable N-oxides or glucuronide metabolites, lactone-ring structures). Preferably, blank matrix (and/or samples spiked with analyte at a concentration not higher than 3 times the lower limit of quantitation) should be spiked with concentrations of the metabolite of interest, representing the actual highest in vivo metabolite concentrations, the sample should be processed, and the chromatogram should be evaluated for the formation of the parent analyte. The extent of back-conversion should be established and the impact on the study results discussed. It is acknowledged that this evaluation will not be possible early during drug development of a new chemical entity when the metabolism is not yet evaluated. However, it is expected that this issue is taken into account and the analytical method is revalidated as further knowledge regarding metabolism of the active substance is gained during drug development.

4.1.2 Carry-over

Carry-over should be addressed and minimised during method development. Carry-over may not affect accuracy and precision (see section 4.1.5 and 4.1.6). During validation carry-over should be assessed by injecting blank samples after a high concentration sample or calibration standard. If it appears that carry-over is unavoidable, specific measures should be considered, tested during the validation and applied during the analysis of the study samples. This could include the injection of blank samples after samples with an expected high concentration, before the analysis of the next study sample.

Randomisation of samples should be avoided, as this may interfere with the detection and assessment of carryover problems.

4.1.3 Lower limit of quantitation

The lower limit of quantitation (LLOQ) is the lowest amount of analyte in a sample which can be quantified reliably, with an acceptable accuracy and precision (see Accuracy and Precision). The LLOQ should be adapted to expected concentrations and to the aim of the study.

4.1.4 Calibration curve

The response of the instrument with regard to the analyte should be known, and should be evaluated over a specified concentration range. The concentrations to be analysed (calibration standards) should be prepared in the same matrix as the matrix of the intended study samples by spiking the blank matrix with known concentrations of the analyte (and IS). There should be one calibration curve for each analyte studied in the method validation and for each analytical run.

Before carrying out the validation of the analytical method it should be known what concentration range is expected. This range should be covered by the calibration curve range, defined by the LLOQ being the lowest calibration standard and the upper limit of quantitation (ULOQ), being the highest calibration standard. The range should be justified based on scientific information.

A minimum of six calibration concentration levels should be used, excluding the blank sample (processed matrix sample without analyte and without IS) and a zero sample (processed matrix with IS).

A relationship which can simply and adequately describe the response of the instrument with regard to the analyte should be applied. The blank and zero samples should not be taken into consideration to calculate the calibration curve parameters.

The calibration curve parameters should be submitted (slope and intercept in case of linear fit). In addition, the back calculated concentrations of the calibration standards should be presented together with the calculated mean accuracy values (see definition of Accuracy below). At least 3 calibration curves should be evaluated.

The back calculated concentrations of the calibration standards should be within ±15% of the nominal value, except for the LLOQ for which it should be within ±20%. At least 75% of the calibration standards with a minimum of six, must fulfil this criterion. In case a calibration standard does not comply with these criteria, this calibration standard sample should be rejected, and the calibration curve without this calibration standard should be re-evaluated, including regression analysis.

Although it may be clear from stability data that the analyte is sufficiently stable in the matrix of interest, it is recommended that freshly prepared calibration curves are used during validation of the bioanalytical method.

4.1.5 Accuracy

The accuracy of an analytical method describes the closeness of the determined value obtained by the method to the true concentration of the analyte (expressed in percentage). Accuracy should be assessed on samples spiked with known amounts of the analyte, the quality control samples (QC samples).

During method validation accuracy should be determined by replicate analysis using a minimum of 5 determinations at a minimum of 4 concentration levels which are covering the calibration curve range: the LLOQ, within three times the LLOQ (low QC), around 50% of the calibration curve range (medium QC), and at about 75% of the upper calibration curve range (high QC).

The QC samples are analysed against the calibration curve, and the obtained concentrations are compared with the nominal value. The accuracy should be reported as percent of the nominal value. Accuracy should be evaluated for the values of the QC samples obtained within a single run (the within run accuracy) and in different runs (the between-run accuracy). The latter will support the accuracy over time.

To enable evaluation of any trends over time within one run, it is recommended to demonstrate accuracy of QC samples over at least one of the runs with a size equivalent to a prospective analytical run.

Within-run accuracy

For the validation of the within-run accuracy, there should be a minimum of five samples per concentration level at LLOQ, low, medium and high QC samples in a single run. The mean accuracy value should be within 15% of the nominal values for the QC samples, except for the LLOQ which should be within 20% of the nominal value.

Between –run accuracy

For the validation of the between-run accuracy at least five determinations per concentration per run at LLOQ, low, medium and high QC samples from three runs analysed on at least two different days should be evaluated. The mean accuracy value should be within 15% of the nominal values for the QC samples, except for the LLOQ which should be within 20% of the nominal value.

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 should additionally be reported.

4.1.6 Precision

The precision of the analytical method describes the closeness of repeated individual measures of analyte. Precision is expressed as the coefficient of variation (CV). The statistical method for estimation of the precision should be predefined and calculated according standard practise. Precision should be demonstrated for the LLOQ, low, medium and high QC samples, within a single run and between different runs, i.e. using the results generated for demonstration of accuracy.

Within-run precision

The within-run CV value should not exceed 15% for the QC samples, except for the LLOQ which should not exceed 20%.

Between –run precision

The between-run CV value should not exceed 15% for the QC samples, except for the LLOQ which should not exceed 20%.

4.1.7 Dilution integrity

Dilution of samples should not interfere with the accuracy and precision. Dilution integrity should be demonstrated by spiking the matrix with an analyte concentration above the ULOQ and dilution of this sample with blank matrix (at least five determinations per dilution factor). Accuracy and precision should be within the set criteria, i.e. within ±15%. Dilution integrity should cover the applied dilution of the study samples.

4.1.8 Matrix effect

Matrix effects should be investigated when using mass spectrometric methods, using at least 6 lots of matrix including haemolysed, hyperlipidaemic and if applicable, sample matrix from special populations, such as renally or hepatically impaired populations.

For each analyte and the internal standard, the matrix factor (MF) should be calculated in each lot of matrix, by calculating the ratio of the peak area in the presence of matrix (measured by analysing blank matrix spiked with analyte at a concentration of maximum 3 times the LLOQ after extraction), to the peak area in absence of matrix (pure solution of the analyte). The IS normalised MF should also be calculated by dividing the MF of the analyte by the MF of the IS.

The CV of the IS-normalised MF calculated from the 6 batches of matrix should not be greater than 15 %.

If this approach cannot be used, for instance in the case of on-line sample preparation, the variability of the response from batch to batch should be assessed by analysing at least 6 batches of matrix in triplicate, spiked at a concentration of a maximum of 3 times the LLOQ. The validation report should include the peak areas of the analyte and of the IS and the calculated concentration for each individual sample. The overall CV calculated for the concentration should not be greater than 15 %. The mean concentration should be within 15 % of the nominal concentration. The mean concentration should also be reported for each individual batch of matrix; a deviation of this mean from the nominal concentration of more than 20 % in any individual batch of matrix should lead to additional investigations.

If the matrix is difficult to obtain, less than 6 different batches of matrix may be used, but this should be justified. However, matrix effects should still be investigated.

If a formulation for injection to be administered to the subjects or animals contains excipients known to be responsible for matrix effects, for instance polyethylene glycol or polysorbate, matrix effects should be studied with matrix containing these excipients, in addition to blank matrix. The matrix used for this evaluation should be obtained from subjects or animals administered the excipient, unless it has been demonstrated that the excipient is not metabolised or transformed *in-vivo*.

4.1.9 Stability

Evaluation of the stability should be carried out to ensure that every step taken during sample preparation and sample analysis, as well as the storage conditions used do not affect the concentration of the analyte. Any deviation from the initial concentration that does occur must be within acceptable limits.

Stability should be ensured for every step in the analytical method, meaning that the conditions applied to the stability tests, such as sample matrix, materials storage and analytical conditions should be similar to those used for the actual study samples. Stability cannot be proven by literature data.

Stability of the analyte and IS in the studied matrix is evaluated using at least triplicates samples of the low and high QC samples which are analysed immediately after preparation and after the applied storage conditions that are to be evaluated. The QC samples are analysed against a calibration curve, obtained from freshly prepared calibration standards, and the obtained concentrations are compared to the nominal concentrations. The deviation should be within ±15%.

Stability of the stock and working solutions should be tested with an appropriate dilution, taking into consideration the linearity and measuring range of the detector.

Stability studies should investigate different storage conditions over time periods that equal or exceed those applied to the actual study samples.

Normally, as an example, the following stability tests should be evaluated:

- stock solution and working solution stability,
- freeze and thaw stability of the analyte in the matrix from freezer storage conditions to room temperature,
- stability of the analyte in matrix stored in the refrigerator, if applicable,
- bench top stability of the analyte in matrix at room temperature,
- long term stability of the analyte in matrix stored in the freezer at the same storage temperature as the study samples,
- bench top stability of the processed sample at room temperature or under the storage conditions to be used during the study (dry extract or in the injection phase), if applicable,
- on-instrument/ autosampler stability of the processed sample at injector or autosampler temperature.

Regarding the freeze and thaw stability: The QC samples are stored and frozen in the freezer at the intended temperature and thereafter thawed at room temperature. After thawing, samples are refrozen again applying the same conditions. At each cycle, samples should be frozen for at least 12 hours

before they are thawed. The number of cycles in the freeze-thaw stability should equal or exceed that of the freeze/thaw cycles of study samples.

Regarding long term stability of the analyte in matrix stored in the freezer: The QC samples should be stored in the freezer under the same storage conditions and at least for the same duration as the study samples. For the evaluation of the long term stability it is not acceptable to use study samples, as the nominal concentration is unknown, and can therefore not be used as reference. It is recommended that evaluation of long term stability is carried out before the start of the actual study.

Sufficient attention should be paid to the stability of the analyte in the sampled matrix directly after blood sampling of subjects and further preparation before storage, to ensure that the obtained concentrations by the analytical method reflect the concentrations of the analyte in the subject at the moment of sampling..

4.2 Partial validation

In situation where minor changes are made to an analytical method that has already been validated, a full validation may not be necessary, depending onto the nature of the applied changes. Changes for which a partial validation may be needed include transfer of the bioanalytical method to another laboratory, change in equipment, calibration concentration range, storage conditions etc. All modifications should be reported and the scope of revalidation or partial validation justified.

In most cases, provision of additional accuracy and precision data or relevant additional stability data on the modified issue may be sufficient.

4.3 Cross validation

Where data are obtained from different study sites, comparison of those data is needed, and a cross validation of the applied analytical methods should be carried out. Differences in sample preparation or the use of another analytical method may result in different outcomes between the study sites. Cross validation should be performed in advance of study samples being analysed if possible. For the cross validation, the same set of QC samples should be analysed by both analytical methods. The outcome of the cross validation is critical in determining whether the obtained data are reliable and whether they can be compared and used. The difference between the two measurements should not exceed 15%.

4.4 Ligand-binding assays

Ligand-binding assays or immunoassays are nowadays especially used for macromolecules. In these assays, quantification is based on macromolecular interactions between the macromolecule and antibody.

The validation principles and the issues with regard to analysis of study samples as indicated before, should also be applied in general for ligand-binding assays. However, the following issues need special attention.

One of the issues is the fact that macromolecules tend to be heterogeneous (small differences in for instance glycosylation, or phosphorylation). Therefore validation must cover issues such as batch-to-batch differences in glycosylation and metabolic differences in phosphorylation. In addition, macromolecules may be structurally comparable to endogeneous compounds. Therefore, specificity of the antibody and selectivity of the assay are critical parameters for ligand-binding assays.

Specificity of an antibody refers to its ability to mainly bind the antigen of interest. Ideally the antibody should be specific such that no cross-reactivity with structural related compounds occurs.

Specificity is validated by using at least 10 sources of sample matrix, spiked at or near the LLOQ. The presence of endogenous antibodies to the analyte may interfere with the analysis and should be taken into account. As interference may be concentration dependent, it would be helpful to estimate the concentration below which interference occurs. Due to interference of endogenous compounds, the blank response may exceed 20% of the LLOQ, however this may be acceptable, as long as it does not affect accuracy. If a surrogate matrix is used for validation, the comparability with regard to study sample matrix should be demonstrated, because of the disease state of a subject, the study matrix may contain different components that may interfere. It is important that the sample matrix and the standard matrix are equivalent. If not, it must be shown that the dose-response relation (e.g. the slope and asymptotes of a four-parameter curve) is unaffected by the matrix.

Reference standards should be selected in such a manner that specificity is ensured, and binding characteristics are durable and stable regarding antibody/antigen complex formation. A change in reference standard during analysis may affect this, and suitability of the new reference standard should thus be demonstrated.

With regard to the calibration curve, the response is measured indirectly and as a result the response function is in most cases nonlinear. Moreover, generally the calibration curve range is limited (<2 orders of magnitude). In case anchor calibration standards are used, the additive value should be clearly documented. Accuracy of the back calculated concentrations should be within ±20%, except for LLOQ and ULOQ which should not exceed 25%.

At least three QC samples should be included (low, medium and high) in each analytical run. The within-run and the between-run accuracy and precision should be within ±20%. Furthermore, the total error (sum of the absolute value of mean accuracy and precision should be less than 30% (40% at the LLOQ and ULOQ). The results of all runs should be included for validation, except in cases where errors are obvious and documented.

It is recommended that the size of the run during validation is comparable to that of a run during analysis of study samples (or visa versa).

It should be demonstrated that characteristics of the analyte are not affected by the methods of sample preparation, additives (e.g. anticoagulants), and stability during the whole process. Evaluation should not only address chemical and physical properties, but also biological integrity (i.e. maintenance of antibody binding affinity). In addition, this also accounts for changes after validation, e.g. a change in used diluents or reagents during analysis of study samples.

If for analysis of study samples microtiter plate-based assays are used, a batch may comprise several individual plates, but each plate should contain an individual set of calibration standards and QC samples, to compensate for difference in plate performance.

Commercial kits

Commercial kits may have been developed for other use or purposes than the intended use, e.g. analysis of samples obtained in bioequivalence studies. Therefore, commercial kits need to be re-validated, meaning that the LOQ and the QC samples in the actual concentration range perform accurately and precisely. The principles of validation listed before apply.

5. ANALYSIS OF STUDY SAMPLES

After complete validation of the analytical method, analysis of study or subject samples may be carried out. Depending on the time period between validation and the analysis of the study samples it may be necessary to verify the performance of the method before start of the analysis of study samples.

The study samples should be processed in accordance with the validated analytical method, and together with the QC samples and the calibration curve to ensure the acceptability of the analytical run.

5.1 Analytical run

An analytical run consists of the blank sample (processed matrix sample without analyte and without IS) and a zero sample (processed matrix with IS), a set of calibration standards at a minimum of 6 concentration levels, at least 3 levels of QC samples (low, medium and high) in duplicate (or at least 5 % of the number of study samples, whichever is higher), and study samples to be analysed. All samples (calibration standards, QC, and study samples) should be processed and extracted as one single batch of samples. Analysing as a single analytical run samples prepared separately as several batches should be avoided. If such an approach cannot be avoided, for instance due to bench-top stability limitations, each batch of samples should preferably include low, medium and high QC samples. Acceptance criteria should be pre-established in a Standard Operating Procedure (SOP) or in the study plan and should be defined both globally for the full analytical run, and for each individual batch of samples.

It is advised to analyse all samples of one subject together in one analytical run to reduce the variability in outcome. Moreover, it is considered acceptable to analyse study samples of more than one subject in one analytical run. The QC samples should be divided over the run i.e. at the beginning, middle and at the end of the run. It is also acceptable to place one of each low, medium and high QC samples at the beginning of the analytical run followed by the study samples and at the end of the run again one of each low, medium and high QC samples.

5.2 Acceptance criteria of an analytical run

Criteria for acceptance or rejection of an analytical run should be defined in the analytical study protocol, in the study plan or in a SOP. The following acceptance criteria should apply:

Accuracy:

The back calculated concentrations of the calibration standards should be within ±15% of the nominal value, except for the LLOQ for which it should be within ±20%. At least 75% of the calibration standards with a minimum of six, must fulfil this criterion. If one of the calibration standards does not meet these criteria, this calibration standard sample should be rejected and the calibration curve without this calibration standard should be re-evaluated, and regression analysis performed. Criteria for decision to exclude calibration standards or not should be pre-defined in a SOP, and should be independent from the results of the QC samples.

If the rejected calibration standard is the LLOQ, it should be realised that the LLOQ for this analytical run is the next acceptable higher calibration standard concentration of the calibration curve. If the highest calibration standard is rejected, the ULOQ, for this analytical run is the concentration of the next acceptable lower calibration standard of the calibration curve. The revised calibration range must cover all QC samples (low, medium and high).

The accuracy values of the QC samples should be within ±15% of the nominal values. At least 4 out of 6 (67%) QC samples and at least 50% at each concentration level should comply with this criterion. In case these criteria are not fulfilled the analytical run should be rejected, and the study samples reanalysed.

The between-run (mean) accuracy of the QC samples should be within 15% of the nominal value.

In the case of the simultaneous determination of several analytes, if an analytical run is acceptable for one analyte but has to be rejected for another analyte, the data for the accepted analyte can be used, but the samples should be re-analysed for determination of the rejected analyte.

Precision:

The between-run precision should not exceed 15%.

Ligand-binding assays:

The back calculated concentrations of the calibration standards should be within ±20% of the nominal value, except for the LLOQ and the ULOQ for which it should be within ±25%. At least 75% of the calibration standards with a minimum of six, must fulfil this criterion. This requirement does not apply to 'anchor' calibrators.

The accuracy values of the QC samples should be within ±20% of the nominal values. At least 4 out of 6 (67%) QC samples and at least 50% at each concentration level should apply to this criterion. Exceptions to this criterion should be justified.

5.3 Calibration range

If a large number of the analyte concentrations of the study samples appear to be above the ULOQ, the calibration curve range should be extended, if possible. The same applies if it appears that the calibration curve range is too wide, such that most analyte concentrations fall in the lower part of the calibration curve range. The calibration curve range should then be narrowed, or an additional QC sample level should be included so that at least 2 QC sample levels fall within the range of concentrations measured in study samples. If the calibration curve range is extended, the analytical method should be revalidated to ensure accuracy and precision.

5.4 Reanalysis of study samples

Reanalysis of study samples should be predefined in the study protocol or SOP, before the actual start of the analysis of the samples. The number of samples (and percentage of total number of samples) that have been reanalysed should be discussed in the study report. The reanalysed samples should be identified and the initial value, the reason for reanalysis, the values obtained in the reanalyses, the finally accepted value and a justification for the acceptance should be provided.

The following reasons can be identified to reanalyse study samples:

- rejection of an analytical run because the run did not fulfil the acceptance criteria with regard to accuracy and precision of the calibration standards and/or QC samples,
- internal standard response significantly different from the response for the calibration standard and QC samples, if such criteria have been pre-defined in a SOP,
- improper sample injection or malfunction of equipment,
- the obtained concentration is above the ULOQ or below the run's LLOQ, in runs where the lowest standard sample has been rejected from a calibration curve, resulting in a higher LLOQ compared with other run's,
- identification of sample analyte in pre-dose samples or placebo sample,
- poor chromatography.

Normally reanalysis of study samples because of a pharmacokinetic reason is not acceptable. This is especially important for bioequivalence studies, as this may affect and bias the outcome of such a study. However reanalysis might be considered as part of laboratory investigations, to identify possible reasons for results considered as abnormal and to prevent the recurrence of similar problems in the future.

Re-injection of samples can be made in case of instrument failure if reinjection reproducibility and on-injector stability have been demonstrated during validation. Re-injection of a full analytical run or of individual calibration standard samples or QC samples, simply because the calibration or QCs failed, without any identified analytical cause, is not acceptable.

5.5 Integration

Chromatogram integration should be described in a SOP. Any deviation from this SOP should be discussed in the analytical report.

6. INCURRED SAMPLES REANALYSIS

The use of calibration standards and QC samples during validation may not mimic the actual study samples. Differences for instance in protein binding, back-conversion of known and unknown metabolites, sample inhomogeneity or concomitant medications, may affect the accuracy and precision of the analyte in such samples during processing and storage. It is therefore recommended to evaluate accuracy of incurred samples by reanalysis of study samples over a certain time period. The extent of testing depends on the analyte and the study samples, and should be based upon in-depth understanding of the analytical method and analyte. It should at least provide sufficient confidence that the concentration being reported is accurate. In case incurred sample analysis showed deviating results, this should be investigated, and adequate steps should be taken to minimize inaccuracy (and imprecision).

If pharmacokinetic parameters represent the primary endpoints of a study, incurred sample analysis is recommended. In toxico-kinetic studies it is sufficient to address this issue once per species. For (human) study samples evaluation of incurred samples should be carried out for every subject or patient population, unless otherwise justified. For bioequivalence studies analysis of incurred samples should always be carried out. Incurred sample analysis should be evaluated as early as possible. It is recommended that study samples are obtained from several subjects close to the expected maximal concentration and in the elimination phase. Samples should not be pooled, as pooling may limit anomalous findings. The difference between the two values obtained should be within 20% of the mean (30% for ligand-binding assays) for at least 67% of the repeats.

7. STUDY REPORT

The study director should sign and date the final report to indicate acceptance of responsibility for the validity of the data and to indicate the extent to which the study is complies with the principles of good laboratory practice.

The validation report should include complete documentation of the protocol, conduct and evaluation of the analysis, in accordance with the principles of GLP-rules and in compliance with EU and ICH guidelines. Information regarding conducted audits/inspection should be included in the report.

SOP’s for relevant analysis specific procedures should be appended to the study report.

All individual data should be available in electronic format to be provided upon request.

Any deviation from the analytical protocol should also be discussed in the analytical report.

The validation report should include at least the following information:

- summary of the validation report in a table format,
- summary of the applied analytical method and where appropriate, the source of the analytical method (references from literature and/or modifications in the procedure),

- summary of the assay procedure (analyte, IS, details of the sample pre-treatment, extraction and analysis),
- reference standards (origin, batch, certificate of analysis, stability),
- calibration standards and QC samples (matrix including anti-coagulant, preparation, preparation dates, and storage conditions),
- run acceptance criteria,
- sample tracking (conditions and duration of storage),
- analysis:
    - table of all analytical runs with analysis dates,
    - table of calibration results of all analytical runs, including calibration range, response function, back-calculated concentrations, within- and between-run precision and accuracy,
    - table of QC results of all analytical runs (within- and between-run precision and accuracy),
    - stability data of stock solution, working solution, QC, covering the applied storage conditions,
    - data on selectivity, LLOQ, carry-over, matrix effect, dilution integrity;
- deviating results obtained during validation with full justification of the action taken,
- deviations from method and/or SOPs (description of deviations, impact on study, supportive data).

All measurements with the individual calculated concentrations have to be presented in the report.

Furthermore a specific detailed description of the analysis of the study samples should be written as a separate report and should include at least:

- reference standards (origin, batch, certificate of analysis, stability, storage conditions)
- calibration standards and QC samples (storage conditions)
- run acceptance criteria (short description, method reference)
- assay procedure (short description)
- sample tracking (dates of receipt and contents, sample conditions on receipt, storage location and conditions, if applicable)
- analysis:
    - set up of the analytical run,
    - table of all analytical runs with analysis dates and results, indicating which samples have been analysed in which analytical run,
    - table of calibration results of all (passed) analytical runs; values outside acceptance criteria should be clearly marked,
    - table of QC results of all (passed) analytical runs; values outside acceptance criteria should be clearly marked;
- failed analytical runs (identity, assay date, reason for failure),
- deviations from method and/or SOPs (description of deviations, impact on study, supportive data),
- re-assay (table of sample identification, reason for re-assay, original and re-assay values),
- incurred sample analysis,

- all chromatograms of 5 – 20% of the subjects, including the corresponding QC samples and calibration standards; for bioequivalence studies at least of 20% of the subjects.

DEFINITIONS

1. Anchor calibrators

Anchor calibrators are standards points outside of the range of quantitation, used to assist in fitting the non linear regression of the standard curve in ligand-binding assays.

2. Accuracy

The accuracy of an analytical procedure expresses the closeness of the determined value to the value which is accepted either as a conventional true value or an accepted reference value.

3. Analytical Procedure

The analytical procedure refers to the way of performing the analysis. It should describe in detail the steps necessary to perform each analysis.

4. Calibration range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure meets requirements for precision, accuracy and response function.

5. Carry over

Carry-over is the appearance of an analyte signal in blank sample peaks after the analysis of samples with a high analyte concentration.

6. Incurred samples

Study samples from dosed subjects or animals.

7. Incurred sample reanalysis

The analysis of a portion of the incurred samples to determine whether the original analytical results are reproducible.

8. Lower limit of quantitation (LLOQ)

The lower quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with pre-defined precision and accuracy.

9. Matrix effect

The direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample.

10. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained under the prescribed conditions.

11. Quality control (QC) sample

A spiked sample used to monitor the performance of a bioanalytical method and to assess the integrity and validity of the results of the unknown samples analyzed in an individual batch.

12. Response function

The response function of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

13. Selectivity

Selectivity is the ability of the bioanalytical method to measure and differentiate the analyte in the presence of components which may be expected to be present.

14. Specificity

For ligand binding assays, specificity is the ability to measure the analyte unequivocally in the presence of other compounds, either exogenous or endogenous, in the matrix.

15. Upper limit of quantitation (ULOQ)

The upper quantitation limit of an individual analytical procedure is the highest amount of analyte in a sample which can be quantitatively determined with pre-defined precision and accuracy.