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An Updated Review on Bioanalytical Method Development and Method Validation by LC-MS/MS

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ABSTRACT:
The validation of bioanalytical methods for drug quantification and research sample inspection is the main topic of this material. In order to guarantee quality, uniformity, harmonize regional guidelines, and expedite drug development in accordance with the three R (Reduce, Refine and Replace), it provides standards for acceptance and validation criteria. For bioavailability, bio equivalency, and safety, validation is essential.

Keywords: Method Development, Method Validation, Solid Phase Extraction, Liquid Liquid Extraction, Protein Precipitation.

I. INTRODUCTION:

A biological matrix for a chemical substance is obtained, processed, stored, and analysed via a set of steps identified as a bioanalytical method. The process used to determine whether a quantitative analytical method is suitable for biochemical applications is known as bioanalytical method validation, or BMV.

Securing satisfactory results from a minimum series of validation experiments provides reassurances about the method's quality and reliability. An essential part of biological assay validation is the characterization of the stability of analyses in biological samples obtained during clinical studies along with essential assay reagents, such as analyte stock solutions.

All procedures that demonstrate that a particular technique used for quantitative measurement of analyte in a biological matrix, such as blood, plasma, serum, or urine, is dependable and repeatable for the intended use are included in the process of bioanalytical validation of the method.

Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended analytical applications.

The increased number of biological agents used as therapeutics (as recombinant proteins, monoclonal antibodies, vaccines, etc.) has prompted the pharmaceutical industry to review and redefine aspects of the development and validation of bioanalytical methods for the quantification of these therapeutics in biological matrices to support preclinical and clinical studies.

Bioanalytical method validation employed for the quantitative determination of drugs and their metabolites in biological fluids plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic, and toxicokinetic study data. These studies support regulatory filings. The quality of these studies is directly related to the quality of the underlying bioanalytical data.

Therefore, it is important that guiding principles for the validation of these analytical methods be established and disseminated to the pharmaceutical community. Both RP-UHPLC and LCMS-MS can be used for the bioanalysis of drugs in plasma. Each of the instruments has its own merits. RP-UHPLC coupled with UV, PDA, or fluorescence detectors can be used to estimate many compounds.

These chromatographic principles have low detection limits, the capacity to produce structural information, minimal sample treatment requirements, and the ability to cover a broad range of analytes with varying polarity differences. These are their main advantages. Every bioanalytical method validation procedure includes steps that demonstrate the use of a specific method for the quantitative determination of the analyte.

The key performance indicators for this validation are recovery, robustness, stability, range, limit of detection, precision, linearity, robustness, and stability. Validating bioanalytical procedures aims to demonstrate their suitability for the intended use. In the fields of pharmaceutical and medical science, the ICH guidelines Q2 (R1) are

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the most commonly used guidelines for method validation.

Other guidelines are specifically focused on bioanalysis and are much more detailed, requiring more thorough validation and having defined strict limits for most of the determined parameters. The FDA's "Guidance for Industry, Bioanalytical Method Validation" and the EMA's guidelines on bioanalytical method validation are respectively represented.

Recently, it has become necessary to determine additional parameters, such as matrix effects, carryover, and dilution integrity, as part of the validation process. A crucial feature of bioanalytical methods is a thorough examination of the analytes stability under various conditions during method application.

The guidelines support the development of bioanalytical method validation data used in clinical pharmacology, bioavailability, and bioequivalence studies by sponsors of investigational new drug applications (INDs), new drug applications (NDAs), abbreviated new drug applications (ANDAs), and supplements.

1. Method Development

The creation of a process for the identification and measurement of a novel or unknown compound in a matrix is known as bioanalytical method development. A compound can often be measured using a variety of techniques. When selecting an analytical method, considerations include the analytes chemical properties, concentrations, sample matrix, cost of the analysis and its instruments, the speed and duration of the analysis, the measurement's quantitative or qualitative nature, precision, and required equipment. Sample preparation, sampling, separation, detection, results evaluation, and conclusion are all included in the process of developing a method.

2. Sample collection and preparation:

Sample gathering and preparation: The analyte is typically found in living media such as blood, plasma, urine, serum, etc. Typically, a hypodermic needle is used to draw up to 5-7 millilitres of blood from human volunteers or subjects during a vein puncture. Heparin is used along with the anticoagulant EDTA when drawing venous blood into tubes. Plasma is obtained by centrifugation at 4000 rpm for 15 minutes.

Between thirty and fifty percent of the volume is collected. Cleaning the sample in advance of analysis is the goal of sample preparation. The chromatographic column or detector in biological samples may be impacted by endogenous macromolecules, proteins, salts, small molecules, and metabolic byproducts.

Additionally, during sample preparation, the analyte from the biological matrix is transformed into a solvent that can be added to the chromatographic system. Liquid/liquid extraction, solid-phase extraction (SPE), protein precipitation, chromatography, and ligand binding assays (LBA) are among the common techniques used to prepare samples.

3. Bioanalytical method:

Some following bioanalytical methods are used:

- Extraction method
- Protein precipitation chromatography
- Ligand binding assay (LBA).

Extraction method:

Liquid-liquid extraction:

It is predicated on the ideas of analyte molecule partitioning equilibrium and difference solubility between the organic and aqueous phases (the sample). The process of extracting a substance from one liquid phase into another is known as liquid-liquid extraction. These days, more sophisticated and effective techniques like liquid phase micro-extraction, supported membrane extraction, and single-drop liquid phase micro-extraction have replaced liquid extraction.

SPE:

Analyte is bound to a solid support, interferences are removed, and the analyte is selectively eluted from a variety of sorbent options using the selective elution technique (SPE). SPE is an effective method.

The four phases of the solid phase are sample loading, conditioning, washing, and elution.

An organic solvent that solvates the functional groups of the sorbent triggers and serves as a wetting agent on the packing material

1. Preparation The pillar. In order to properly activate the column for adsorption mechanisms, water or an aqueous buffer is added.
2. Importance of the sample Following apH shift, the sample is fed into the column by gravity feed, vacuum aspiration, or pumping.
3. Washing The analyte is kept while interferences from the matrix are eliminated.

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4. Resolving Analyte-sorbent interactions are distributed using an appropriate solvent, with the goal of minimizing residual interferences.

The sorbents used in SPE are made of silica gel with pore diameters of about 60 µm and a diameter of 40 µm. Functional groups are chemically bonded in this silica gel. The most popular format is a syringe barrel with a 20-µm frit with the sorbent material at the bottom and another frit, called packed columns, on top.

Syringe barrels are filled with extraction disks. These disks are made up of 8–12 µm packing material particles that are embedded in an inert matrix. The way that disks are used and conditioned is comparable to that of packed columns.

Easily applied higher flow rates is the main benefit of disks over packed columns. Analytes fall into one of four groups: amphoteric, basic, neutral, or acidic compounds. Amphoteric analytes can act as cations, anions, or zwitterions based on the pH, primarily the pH of 13, because they contain both basic and acid functional groups.

Protein precipitation:

In routine analyses, protein precipitation is a common method for removing proteins. The solubility of the proteins is affected by pH changes or precipitation caused by the addition of an organic modernizer, such as salt. Following centrifugation of the samples, the supernatant can either be added to the HPLC system or evaporated until it is completely dry and then dissolved in an appropriate solvent. After that, the sample's concentration is reached.

When it comes to clean-up techniques, the precipitation method has certain advantages over SPE. Little amounts of organic modifier or other solvents are used, and it takes less time. Nevertheless, there are drawbacks as well; samples frequently contain protein particles, and the method of sample clean-up is non-selective. There's a chance that the reversed-phase HPLC system will restrict endogenous compounds or other medications.

To create a clean extract, SPE is frequently used in conjunction with the protein precipitation technique. Among the organic solvents, methanol is favoured because it can yield a transparent supernatant suitable for being added straight to HPLC. Another substitute for acid organic solvent precipitation is salts. We refer to this process as salt-induced precipitation. Proteins gather and separate from solutions as the concentration of salt in the mixture rises.

4. Validation of bioanalytical methods is necessary:

To produce accurate results that can be appropriately interpreted, bioanalytical methods that are thoroughly validated and well-characterized must be used.

It is acknowledged that bioanalytical approaches and procedures are at the forefront of technology and are always changing and improving.

It is crucial to emphasize that each bioanalytical method has distinct qualities that vary depending on the analyte. For each analyte, specific validation standards will need to be developed.

Furthermore, the purpose of the study may also have an impact on how appropriate the technique is. In order to establish inter-laboratory reliability, when sample analysis for a given study is carried out at multiple sites, it is required to validate the bioanalytical methods at each site and provide relevant validation information for different sites.

- **Partial validation:**

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

- **Cross-validation:**

It is required to show 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) using both methods and laboratories. Bias can be evaluated using Bland-Altman plots, Deming.

Regression or other appropriate methods

5. Additional considerations:

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

• Surrogate matrix
• Surrogate analyte
• The background subtraction
• Typical augmentation

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Evaluation of the recovery (or extraction efficiency) is necessary for procedures that use sample extraction.

In LBA, the Minimum Required Dilution (MRD) can be used to lessen matrix interference or background signal. For every sample, including calibration standards and QCs, the MRD must be the same.

When developing a novel drug, the applicant should validate any commercial or diagnostic kit used to measure chemical or biological drug concentrations to make sure it complies with drug development regulations.

Cross-validation with an existing technology is not necessary when a new or alternative technology is used as the only bioanalytical technology from the beginning of drug development.

Additional sampling approach validation is required for dried matrix methods, including sample homogeneity, extraction of the sample from the dried matrix, and sample collection for ISR.

6. Application of a validated method for routine drug analysis.

Using a tried-and-true technique for regular drug analysis.

Analyte assays for every sample in a biological matrix ought to be finished within the time frame for which stability data is accessible. If the assay method has a satisfactory and acceptable variability as defined by validation data, biological samples can be analysed with a single determination without the need for duplicate or replicate analysis.

This is valid for processes where variability, accuracy, and precision consistently fall within acceptable bounds. Duplicate or even triplicate analyses can be carried out for better analyte estimation in a challenging procedure involving a labile analyte, where it may be challenging to meet high precision and accuracy requirements. When using a bioanalytical method for routine drug analysis, keep the following suggestions in mind.

A minimum of six to nine standard points, excluding blanks (single or duplicate), should make up a matrix-based standard curve that covers the whole range. Response function: The standard curve in the study would normally be fitted using the same curve fitting, weighting, and goodness of fit that were established during pre-study validation. On the basis of the actual standard points during each validation run, the response function is ascertained using the relevant statistical tests. Some issues are indicated by changes in the response function relationship between routine run validation and pre-study validation.

The run must be accepted or rejected using the QC samples. The matrix spreads these QC samples along with the analyte.

7. Bioanalytical Method Validation:

The following should be part of a thorough validation:

- Matrix Effect
- Selectivity and Specificity
- Calibration Curve and Range; Including an Upper and Lower Limit of Quantification (ULOQ)
- Accuracy and Precision (Relative to Nominal Concentration);
- Carry-Over;
- Dilution Integrity;
- Stability
- Reproducibility of Reinjection

Selectivity:

Does the biological matrix contain substances that interfere with the ability to distinguish and quantify analytes?

Analyses of blank biological matrix samples (plasma, urine, or other matrix) from at least six sources should be performed in order to ensure selectivity. Selectivity at the lower limit of quantification (LLOQ) should be guaranteed, and interference should be tested on each blank sample. In the cases of the lipaemic and haemolysed matrix ($n \geq 1$ source each), as well as the blank matrix ($n \geq 6$ sources), there should be no discernible reaction or interference with the analyte and internal standard (IS).

Acceptance Criteria:

Interfering signal $\leq$ 20% of the analyte LLOQ response and $\leq$ 5% of the IS response.

Specificity:

The capacity to identify and distinguish the analyte from other substances—including those that are related to it.

Because other substances are present in the matrix, there shouldn't be any noticeable reaction or interference with the analyte or IS.

Acceptance Criteria:

Interfering signal: $\leq$ 20% of the analyte LLOQ response and $\leq$ 5% of the IS response.

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The back-conversion of the metabolite into the parent during sample processing should be evaluated when relevant.

Matrix effect:

Are elements in the matrix causing interference the reason for the change in the analyte response?

Acceptance Criteria:

Accuracy and precision within 15% using ≥3 low-quality control samples (LQC) and 3 high-quality control samples (HQC) of the matrix (n ≥ 6 sources).

When available, it should also be assessed in pertinent patients or specific populations, such as those with renal or hepatic impairment.

Calibration curve range:

The relationship between nominal analyte concentration and response is demonstrated.

A calibration curve includes blank samples and zero samples.

And at least six calibration standards, including LLOQ and ULOQ.

Acceptance Criteria:

≥ 3 runs over several days

Accuracy ± 15%, except at LLOQ ± 20%

Should meet 75% of the calibration standards with a minimum of 60%

Accuracy/ Precision:

Evaluated using at least four quality control sample (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).

Acceptance Criteria:

Within-run: n ≥ 5 for 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%)

Carry-over:

Is the instrument's residual analyte from a previous sample the cause of the altered measured concentration?

Evaluated by injection of blank samples after injection of a sample at the ULOQ.

Acceptance Criteria:

Analyte response in the blank sample is ≤ 20% of the analyte LLOQ response and ≤ 5% of the IS response.

Dilution integrity:

Is it clear from the evaluation of the sample dilution process that the procedure has no effect on the analytes measured concentration?

Evaluated by dilution with the matrix (or surrogate, if rare, Matrix) of the sample with an analyte concentration > ULOQ.

The dilution factor and concentration(s) applied during the study sample analysis should be within those evaluated during validation.

Acceptance Criteria:

n ≥ 5 per dilution factor; accuracy and precision of the analyte concentration in a diluted sample ≤ 15%.

Stability:

Analyte concentrations should be checked to make sure that none of the procedures used in sample preparation, processing, and analysis—or the storage conditions employed—affect the analytes concentration.

Conditions applied during the evaluations should reflect those used for the study samples.

The freeze-thaw, bench-top, and long-term stability tests should be carried out using the for fixed-dose combination products and specially labelled medication regimens.

The matrix was spiked with the dosed compounds.

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 using the LQC and HQC.

Acceptance Criteria:

n ≥ 3 aliquots of one bulk sample at each QC level; mean accuracy within 15%.

The following stability tests should be performed:

Stability of the analyte in the matrix:

1. Freeze-thaw stability
2. Bench-top (short-term) stability
3. Long-term stability
4. Stability of the analyte in the processed sample
5. Stability of the analyte and IS in the stock/working solutions
6. Stability of the analyte in whole blood, if applicable.

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Reinjection reproducibility:

In order to confirm the viability of the processed samples and to support their storage prior to reinjection, samples that are capable of being reinjected should have their reproducibility during reinjection assessed.

Assessed by injecting a run again that includes calibration standards and, following storage, a minimum of five duplicates of the low, middle, and high QCs.

The viability of the processed samples is determined by the accuracy and precision of the re-injected QCs.

The study samples, QCs, and calibration standards should be processed using the approved analytical procedure.

An analytical run comprising a blank sample, a zero sample, calibration standards at least six concentration levels, and at least three levels of quality control (low, medium, and high) in duplicate should be used to analyse the study samples.

To minimize variability in comparative BA/BE studies, it is recommended to analyse all samples of a given subject in a single analytical run.

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.

At every level of quality control, the overall between-run accuracy and precision should be within 15%; if this is not the case, an explanation must be given.

Calibration range:

The analyte concentrations in the research samples should be sufficiently covered by the calibration curve range. If not, the calibration range needs to be reduced, the current QC concentrations need to be adjusted, or new QCs at different concentrations need to be added.

In the study samples, at least two QC levels ought to lie within the concentration range that was measured.

System suitability:

Based on the analyte and technique, a specific standard operating procedure (or sample) must be identified to ensure the optimum operation of the system.

Any required sample dilutions should use a matrix (e.g., human to human beings), obviating the need to incorporate an actual within-study dilution matrix in QC samples.

Repeat analysis:

It is important to establish an SOP or guidelines for repeat analysis and acceptance criteria. This SOP, or guideline, explains the reasons for repeating sample analysis. Reasons for repeat analyses may include repeat analysis of clinical or preclinical samples for regulatory purposes, inconsistent replicate analysis, samples outside the assay limit, sample processing errors, equipment failure, poor chromatography, and inconsistent PK data. Reassays must be performed in triplicate if the sample volume allows. The basis for repeat analysis and the reporting of repeat analysis should be clearly documented.

Sample data reintegration:

An SOP or guideline for sample data reintegration should be established. This SOP or guideline should explain the reasons for reintegration and how it is to be performed. The rationale for reintegration should be clearly described and documented. Original and reintegration data should be reported.

8. Documentation and Archives:

• The data generated for bioanalytical method validation should be documented and available for data audit and

• The recommended documentation for submission to the regulatory authorities and documentation that should be available at the analytical site of inspection.

• Summary information should be provided, including a summary of the methods used, a summary table of all relevant validation reports, and information on regulatory site inspections with comparative BA/BE studies.

To ensure adequate reproducibility and reliability of bioanalysis, results obtained in analytical method validations and study sample analyses should be documented in a validation report and a study sample analysis report, as described below. Reports should be stored along with relevant records and raw data in an appropriate manner.

All relevant records and raw data should be kept, including those obtained in rejected analytical runs, specifically records of reference materials and blank matrices (receipt/release, use, and storage), records of samples (receipt/release,

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preparation, and storage), records of analyses, instruments (calibration and settings), deviations, communications, and raw data such as analytical data and chromatograms.

Validation report

• Summary of the validation
• Information on reference standards
• Information on blank matrices
• Analytical method
• Validated parameters and acceptance criteria
• Validation results and discussion
• Rejected runs with the reason for rejection.
• Information on reanalysis
• Deviations from the protocol and/or SOP, along with the impact on the study results
• Information on reference studies, protocols, and literature
• Representative chromatograms of the study sample analysis report
• Summary of the study: sample analysis
• Information on reference standards
• Information on blank matrices
• Information on receipt and storage of study samples
• Analytical method
• Parameters, acceptance criteria, and results of the validity evaluation
• Results and discussion of the study sample analysis
• Rejected runs with the reason for rejection.
• Information on reanalysis
• Deviations from the protocol and/or SOP, along with their impact on the study results
• Information on reference studies, protocols, and literature
• Representative chromatograms, as required

II. CONCLUSION:

Drug discovery and development in the pharmaceutical industry heavily relies on bioanalysis and the generation of pharmacokinetic, toxicokinetic, and metabolic data. An effort has been made to comprehend and elucidate the development and validation of bioanalytical methods from the perspective of the quality assurance department. Several techniques and the process of validation were explained in relation to the various scenarios that arose during the research. This article has reported on a sample analysis. We've talked about these different crucial aspects of bioanalytical methodology development and validation in order to raise the bar and increase acceptance in this field of study.

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