Bioanalytical method validation

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Abstract

A syntetic discussion on bioanalytical methods validation is presented from the point of view of regulatory documents, scientific articles and books. The validation parameters are described, together with an example of validation methodology applied in the case of chromatographic methods used in bioanalysis, taking in account to the recent Food and Drug Administration (FDA) guidelines and documents of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).

Introduction

The reliability of analytical method is a matter of great importance in analysis and is, of course, a prerequisite for correct interpretation of data.

As it is known, analytical method validation is an experimental procedure which demonstrates that a specific analytical method generates **reliable, accurate and precise** information about a sample. Bioanalytical methods are used for the quantitation of drugs and their metabolites in biological matrices. Should we validate a bioanalytical method is not a question anymore, validation proving the quality of the analyst's work, users of bioanalytical data get confidence in the results and it is required by the regulatory agencies. Before using a bioanalytical method for quantitative determinations of drugs and their metabolites, an applicant laboratory must first demonstrate that the envisaged method fulfills a number of performance criteria after following a **method validation** protocol.

Publications on bioanalytical methods validation

The scientific literature about analytical and bionalytical methods validation is reach and includes different categories: guidelines of the European and US committees, review articles, books, documents published by international conferences or congresses etc.

Since the publications of the European and US committees at the beginning of '90 years, many laboratories have started to redesign their processes by involving analysts and statisticians, in order to define strategies that will allow the fulfillment of the regulatory requirements, while being practicable and scien-

tifically consistent. The Romanian National Agency of Drugs harmonized their requirements under the latest international regulations which provide assistance in developing bioanalytical method validation used in human clinical pharmacology, bioavailability and bioequivalence studies requiring pharmacokinetic evaluation.

Frank T. Peters and Hans H. Maurer[19] made in 2002 an excelent summary of the most important documents published since 1991:

- The review on validation of bioanalytical methods published by Karnes et al. (1991) - intended to provide guidance for bioanalytical chemists[15].
- The Shah et al. report (1992) on the conference on "Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies" held in Washington in 1990[22] - guidance for bioanalysts for the next years; contains the parameters of bioanalytical methods which should be evaluated, and some acceptance criteria were established but no specific recommendations on practical issues like experimental designs or statistical evaluation had been made.
- Hartmann et al. (1994) analyzed the 1990 Conference Report performing statistical experiments on the established acceptance criteria for accuracy and precision[9]. Based on their results they questioned the suitability of these criteria for practical application.
- Hartmann et al. (1998) review on validation of bioanalytical chromatographic methods - theoretical and practical issues were discussed in detail[10].
- The Shah et al. (2000) report on an update conference of the 1990 Washington conference[23] - template for the guidelines (2001) of the U.S. Food and Drug Administration (FDA)[25].
- The documents of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and approved by the regulatory agencies of the European Union, the United States of America and Japan: the first document, approved in 1994, concentrated on the theoretical background and definitions in validation[13], the second, approved in 1996, on methodology and practical issues[14]. The recent book edited by Ermer J. and Miller J.H. (2005)[7] is the latest document about these topics. Despite the fact, that these were focussed on analytical methods for pharmaceutical products rather than bioanalysis, they still contain helpful guidance on some principal questions and definitions in the field of analytical method validation.

Compliance with the 2001 FDA guidance can be considered today a minimum requirement to test the performance of a bioanalytical method. At the beginning of this document the FDA states very clearly that its guidance for bioanalytical method validation represents its current thinking on this topic and that an alternative approach may be used if such an approach satisfies the requirements of applicable statutes and regulations. This statement allows bioanalytical laboratories to adjust or modify the FDA recommendations, depending on the specific type of bioanalytical method used.

In addition to such important documents, different scientific journal published their opinion on these aspects. Journals like Journal of Chromatography B[17] or Clinical Chemistry have established their own criteria for validation. Other perspectives are included in a recent valuable book edited by Chan C.C. et al. (2004)[3].

It is also necessary to present the guide published in 1997 by La Société Française des Sciences et Techniques Pharmaceutiques (SFSTP)[5] that provided the bioanalyst, on the one hand, with a better understanding on the way to proceed and on the other hand, real data for qualifying his own computations that he could perform using a commercial spreadsheet[4, 6, 11]. It should be noted that this guide was published

before the recent FDA's guide and introduces new concepts in three different areas: stages of the validation, test of acceptability of a method and design of experiments to perform. The main authors of SFSTP guide recently published (2003) an article which objectives were to identify and explain the progress permitted by the SFSTP guide, point out some of the limitations and suggest ways to overcome them[2]. An interesting thing is that no references about the recently published FDA guide was made in this article, just the first FDA guide (1992) is cited, even if the FDA document published in 2001 provides more detailed aspect regarding experimental procedures.

Many other scientific articles try to add a practical point of view on bioanalytical and analytical methods validation[1, 8, 12, 16, 18, 20, 24, 26].

Current validation practice on bioanalytical methods validation

In today's drug development environment, highly sensitive and selective methods are required to quantify drugs in matrices such as blood, plasma, serum, or urine. Chromatographic methods are the most commonly used technology for the bioanalysis of small molecules and the general terms presented below take in account to this type of analytical method.

It is well accepted the FDA Guidance for Industry, Bioanalytical Methods Validation (2001) as a reference for current validation practice and a briefly description of it is given here.

a. Glossary

The general concepts could be expressed as follows:

- *Validation* - the process of checking if something satisfies a certain criterion.
- *Analytical method* - a comprehensive description of all procedures used in sample analysis.
- *Analytical method validation* - a procedure employed to demonstrate that an analytical method used for quantification of analytes in a biological matrix quantifies the analyte with a degree of accuracy and precision appropriate to the task.
    - *Full validation:* establishment of all validation parameters to apply to sample analysis for the bioanalytical method for each analyte.
    - *Partial validation:* modification of validated bioanalytical methods that do not necessarily call for full revalidation.
    - *Cross-validation:* comparison of validation parameters of two bioanalytical methods.

In order to understand the validation process it is necessary to define the analytical terms used, including the validation parameters (*Figure 1*):

- ***Specificity/selectivity*** – the ability of the method to measure and differenciate the analyte signal in the presence of components that may be expected to be present. There has been some controversial discussion about the terminology for this validation characteristic. In contrast to the ICH, most other analytical organisations define this as selectivity, whereas specificity is regarded in an absolute sense, as the "ultimate degree of selectivity" (IUPAC). *Selectivity* is the ability of the bioanalytical method to measure and differentiate the analytes in the presence of components that may be expected to be present. *Specificity* is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present. For example, in high-performance liquid chromatography with UV detection (HPLC-UV), a classic chromatographic method, the method is specific if the assigned peak at a given retention time belongs only to one chemical entity; in liquid chromatography with mass spectrometry detection (LC-MS) the detector could measure selective an analyte, even if this

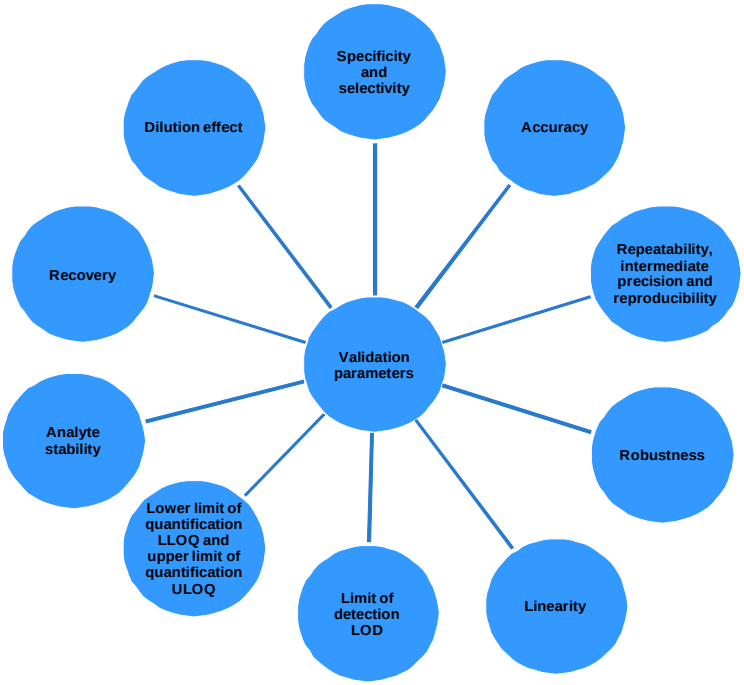

Fig. 1. Validation parameters of a bioanalytical method

is not fully separated from endogenous compounds etc. Despite this controversy, there is a broad agreement that specificity/selectivity is the critical basis of each analytical procedure.

- ***Precision*** - the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. As parameters, the standard deviation, the relative standard deviation (coefficient of variation) should be calculated for each level of precision.
  - *Repeatability* expresses the analytical variability under the same operating conditions over a short interval of time (within-assay, intra-assay).
  - *Intermediate precision* includes the influence of additional random effects within laboratories, according to the intended use of the procedure, for example, different days, analysts or equipment, etc. (between-assay, inter-assay).
  - *Reproducibility*, i.e., the precision between laboratories (collaborative or inter-laboratory studies), is not required for submission, but can be taken into account for standardisation of analytical procedures.
- ***Accuracy:*** the degree of closeness of the determined value to the nominal or known true value under prescribed conditions. This is sometimes termed *trueness*. It is expressed as bias% or relative error%.
- ***Robustness:*** a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage
- ***Limit of detection (LOD)****:* the lowest concentration of an analyte that the bioanalytical procedure can reliably differentiate from background noise.
- ***Lower limit of quantification (LLOQ)****:* the lowest amount of an analyte in a sample that can be determined quantitatively with suitable

precision and accuracy.

- ***Upper limit of quantification (ULOQ)****:* the highest amount of an analyte in a sample that can be determined quantitatively with precision and accuracy.
- ***Standard curve:*** the relationship between the experimental response value and the analytical concentration (also called a *calibration curve*); usually this relationship is linear.
- ***Quantification range:*** The range of concentration, including the LLOQ and ULOQ that can be reliably and reproducibly quantifi ed with suitable accuracy and precision through the use of a concentration response relationship.
- ***Recovery:*** the extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method.
- ***Dilution effect:*** The ability to dilute samples originally above the upper limit of the standard curve should be demonstrated by accuracy and precision parameters in the validation.
- ***System suitability:*** determination of instrument performance (e.g., sensitivity and chromatographic retention) by analysis of a reference standard prior to running the analytical batch.
- ***Reinjection reproducibility:*** It is necessary to be determined if an analytical run has to be reanalyzed in the case of instrument failure.
- ***Stability:*** the chemical stability of an analyte in a given matrix under specific conditions for given time intervals.

Other definitions should be given:

- *Biological matrix:* a discrete material of biological origin that can be sampled and processed in a reproducible manner. Examples are blood, serum, plasma, urine, feces, saliva, sputum, and various discrete tissues.
- *Stock solutions:* the original solutions prepared directly by weighing the reference standard of the analyte and dissolving it in the appropriate solvent. Usually, stock solutions are prepared at a concentration of 1-3 mg/mL in methanol or acetonitrile and kept refrigerated at −20ºC if there are no problems of stability or solubility.
- *Working solutions:* solutions prepared from the stock solution through dilution in the appropriate solvent at the concentration requested for spiking the biological matrix.
- *Calibration standard:* a biological matrix to which a known amount of analyte has been added or *spiked*. Calibration standards are used to construct calibration curves from which the concentrations of analytes in QCs and in unknown study samples are determined.
- *Internal standard:* test compound(s) (e.g., structurally similar analog, stable labeled compound) added to both calibration standards and samples at known and constant concentration to facilitate quantification of the target analyte(s).
- *Sample:* a generic term encompassing:
  - *Blank:* a sample of a biological matrix to which no analytes have been added that is used to assess the specificity of the bioanalytical method.
  - *Quality control sample (QC):* 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. They are also used to calculate the accuracy and precision of the method.
  - *Unknown sample:* a biological sample that is the subject of the analysis.

b. Validation methodology of the chromatographic methods applied for drugs determination in human plasma

Includes two phases:

- pre-study method validation – method developing; method validation; it is performed before the unknown samples analysis
- routine-run method validation – during unknown samples analysis

Pre-study method validation

Specificity

As a first step of method validation, specificity is verified using six different plasma blanks obtained from healthy human volunteers who had not previously taken any medication. A general approach to prove the selectivity (specificity) of the method is to verify that: the response of interfering peaks at the retention time of the analyte is less than 20% of the response of an LLOQ standard, or the response at the LLOQ concentration is at least five times greater than any interference in blanks at the retention time of the analyte; the responses of interfering peaks at the retention time of the internal standard are ≤5% of the response of the concentration of the internal standard used in the studies.

Standard curve. Quantification range

The relationship between the detector response and concentration should be demonstrated to be well defined and reproducible. The calibration curve model is determined usually by the least squares analysis. In general, a polynomial function is considered:

$y = b_0 + b_1x + b_2x^2 + b_3x^3 + ...;$

for the linear model, the terms x^2 and larger are ignored and for the quadratic model, terms larger than x^2 are not considered. Even if the use of the quadratic model is allowed and used extensively by some bioanalytical laboratories, the use of linear regression models should be attempted first. Usually, a deviation from the linear model should be investigated and avoided. Nonlinearity could be due to injection techniques, sample holdup on glassware, cross-talk in MS/MS, interferences, and too wide a concentration range. Weighing functions reduce the influence of values obtained for higher concentrations on slope and intercept but selection of weighing should be justified. From statistical considerations the most common weighing function for LC-MS- and LC-MS/MS-based assays should be $1/x$, due to the fact that variance in y increases in proportion to the concentration. In HPLC-UV-based assays the use of $1/x^2$ weighed linear regression analysis can significantly reduce the LLOQ obtainable, where the standard deviation of y varies with x. A comparison between the weighed least squares procedure and the conventional least squares calibration shows improvements in accuracy at the lower end. The principal advantage in this case is for clinical pharmacology and pharmacokinetic studies when concentration values being measured by the method are near LLOQ.

Calibration is performed using singlicate, duplicate or triplicate (it depends on method precision) calibration standards on five different occasions. The concentration range should cover the expected concentration in biological samples. A calibration curve should consist of a blank sample (matrix sample processed without the IS), a zero standard (matrix sample processed with internal standard), and 6 to 8 nonzero standards. The number of standards can be increased for a complex curve or a curve covering a very large range. The simplest relationship that provides acceptable backcalculated concentrations for the standards should be used first to fit the calibration curve. If a weighting factor is used, it should be defined during validation. Distribution of the residuals (% difference of the back-calculated concentration from the nominal concentration) should be investigated. The calibration model is accepted, if the residuals were within ±20% at the lower limit of quantification (LLOQ) and within ± 15% at all other calibration levels and at least 2/3 of the standards met this criterion, including highest and lowest calibration levels. Calibration standards not meeting the acceptance criteria should be eliminated from the calibration curve calculations.

Lower limit of quantification

The lower limit of quantification is established as the lowest calibration standard with an accuracy and precision less than 20%.

Accuracy and precision

The within- and between-run precision (expressed as coefficient of variation, CV%) and accuracy (expressed as relative difference between obtained and theoretical concentration, Bias%) of the assay procedure is determined by analysis on the same day of five different samples at each of the lower (2 or 3 x LLOQ), medium (30-50% of the ULOQ) and higher (80% of the ULOQ) levels of the considered concentration range and one different sample of each on five different occasions, respectively. Sometimes, the selected concentrations could include values which are relevant in practice.

Recovery

The recoveries at each of the previously three levels of concentration and limit of quantification are measured by comparing the response of the treated plasma standards with the response of standards in solution with the same concentration of analytes as the prepared plasma sample.

Stability

Stock solution stability: The stability of the stock solutions of drug and internal standards should be evaluated at room temperature for at least 6 hours. If the stock solutions are kept refrigerated or frozen over a period of time, the stability over that period should be evaluated by comparing the response of the aged stock solution to that of a freshly prepared stock solution. Stock solution stability should be performed at one concentration in at least duplicate.

The stability of the analytes in human plasma: it is investigated in four ways, in order to characterize each operation during the process of bioequivalence studies: room-temperature stability (RTS), post-preparative stability (PPS) in the autosampler, freeze-thaw stability (FTS) and long-term stability (LTS) (at a freezing temperature at which it is known that the analyte is stable). For all stability studies, plasma standards at low and high concentrations are used. The acceptance criterion: the mean found concentration should be within ±15% and CV% < 15%.

Four plasma standards at each of the two levels are prepared and let at room temperature four hours before processing (RTS study). After that the extracted samples are analyzed with fresh standards.

Other four pairs are prepared, immediately processed and stored in the HPLC autosampler (PPS study). The samples are injected periodically over the expected longest storage times of the samples in autosampler before injection. The extracted samples (ready to inject) kept at autosampler temperature is finally analyzed with fresh standards.

For the freeze-thaw stability (FTS), aliquots at the same low and high concentrations are prepared. These samples are subjected to three cycles of freeze-thaw operations in three consecutive days. After the third cycle the samples are analyzed against calibration curve of the day. The mean concentration calculated for the samples subjected to the cycles and the nominal ones are compared.

For long-term stability (LTS), in the first validation day, there are injected and analyzed four samples at each of low and high concentrations, and values are calculated against calibration curve of the day. Other two sets with the same plasma concentrations were stored in freezer and analyzed together with calibration samples after the expected storage period. The values are calculated against calibration curve of the day and the mean values for the stored samples and nominal concentrations are compared. The requirement for stable analytes is that the difference between mean concentrations of the tested samples in various conditions and nominal concentrations had to be in ±15% range.

Dilution study

The ability to dilute samples with concentrations above the upper limit of quantification is also investigated. Plasma standards (n = 5) with the concentration levels above the

ULOQ are diluted with blank plasma in order to get a concentration within the calibration range, then processed and analyzed, five samples in the same run and one sample on five different occasions. The mean found concentration is compared with the nominal value. The accuracy and precision had to be within ±15% range.

Routine-run method validation

Requirements for the calibration curve of the run:

- Good fitting of the experimental data
- 75% of the calibration points (including LLOQ and ULOQ) has a bias less than 15%, except LLOQ when is acceptable a limit of 20%
- Values falling outside can be discarded if this does not change the established model

The accuracy and precision of the validated method is monitored to ensure that it continued to perform satisfactorily during analysis of unknown plasma samples. To achieve this objective, a number of QC samples prepared in duplicate at the three concentration levels (lower, medium and higher) are analyzed in each assay run together with the calibration standards and unknown samples. At least 67% (four out of six) of the QC samples should be within 15% of their respective nominal values; 33% of the QC samples (not all replicates at the same concentration) can be outside ±15% of the nominal value.

Conclusion

As a conclusion generally accepted by all those involved in this topic, any analytical method validation should be performed considering the facts underlined by Shah V.P. in 2006 (21): „No conference report or guidance can cover „ALL ISSUES", and/or „ALL WHAT IFS". No substitute for common sense. Each issue needs to be evaluated in full light of objectives and aims of analysis, scientific basis and proof for deviation or anomalous observation. No substitute for Good Science. The Workshop Report and/or the Guiance can provide only the guiding principles."

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