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A Review on Bioanalytical Method Development and Validation by Liquid Chromatography–Tandem Mass Spectrometry

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Abstract
The development of sound bioanalytical method(s) is of paramount importance during the process of drug discovery and development, culminating in a marketing approval. The objective of this paper is to review the sample preparation of drug in biological matrix and to provide practical approaches for determining selectivity, specificity, limit of detection, lower limit of quantitation, linearity, range, accuracy, precision, recovery, stability, ruggedness, and robustness of liquid chromatographic methods to support pharmacokinetic (PK), toxicokinetic, bioavailability, and bioequivalence studies. Bioanalysis, employed for the quantitative determination of drugs and their metabolites in biological fluids, plays a significant role in the evaluation and interpretation of bioequivalence, PK, and toxicokinetic studies. Selective and sensitive analytical methods for quantitative evaluation of drugs and their metabolites are critical for the successful conduct of pre-clinical and/or biopharmaceutics and clinical pharmacology studies.

Keywords: Bionalytical Method Development, Validation Parameters, Limit of Detection, bioavailability.

Introduction

The reliability of analytical findings is a matter of great importance in forensic and clinical toxicology, as it is of course a prerequisite for correct interpretation of toxicological findings. Unreliable results might not only be contested in court, but could also lead to unjustified legal consequences for the defendant or to wrong treatment of the patient. The importance of validation, at least of routine analytical methods, can therefore hardly be overestimated. This is especially true in the context of quality management and accreditation, which have become matters of increasing importance in analytical toxicology in the recent years. This is also reflected in the increasing requirements of peer-reviewed scientific journals concerning method validation. Therefore, this topic should extensively be discussed on an international level to reach a consensus on the extent of validation experiments and on acceptance criteria for validation parameters of bioanalytical methods in forensic (and clinical) toxicology. In the last decade, similar discussions have been going on in the closely related field of pharmacokinetic (PK) studies for registration of pharmaceuticals. This is reflected by a number of publications on this topic in the last decade, of which the most important are discussed here.[1]

Need of Bionalytical Method Validation:

It is essential to employ well-characterized and fully validated bioanalytical methods to yield reliable results that can be satisfactorily interpreted. It is recognized that bioanalytical methods and techniques are constantly undergoing changes and improvements, and in many instances, they are at the cutting edge of the technology. It is also important to emphasize that each bioanalytical technique has its own characteristics, which will vary from analyte to analyte. In these instances, specific validation criteria may need to be developed for each analyte. Moreover, the appropriateness of the technique may also be influenced by the ultimate objective of the study. When sample analysis for a given study is conducted at more than one site, it

is necessary to validate the bioanalytical method(s) at each site and provide appropriate validation information for different sites to establish interlaboratory reliability.[2]

Bionalytical Method Development and Validation:

The process by which a specific bioanalytical method is developed, validated, and used in routine sample analysis can be divided into

- Reference standard preparation,
- Bioanalytical method development and establishment of assay procedure and
- Application of validated bioanalytical method to routine drug analysis and acceptance criteria for the analytical run and/or batch.

Validation Parameters:

Linearity

Linearity assesses the ability of the method to obtain test results that are directly proportional to the concentration of the analyte in the sample. The US Food and Drug Administration (FDA) guidelines for bioanalytical method validation. ICH guidelines recommend evaluating a minimum of five concentrations to assess linearity. The five concentration levels should bracket the upper and lower concentration levels evaluated during the accuracy study [3]. ICH guidelines recommend the following concentration ranges be evaluated during method validation.

Selectivity (Specificity):

For every phase of product development, the analytical method must demonstrate specificity. The method must have the ability to unambiguously assess the analyte of interest while in the presence of all expected components, which may consist of degradants, excipients/sample matrix, and sample blank peaks [4]. The sample blank peaks may be attributed to things such as reagents or filters used during the sample preparation.

For identification tests, discrimination of the method should be demonstrated by obtaining positive results for samples containing the analyte and negative results for samples not containing the analyte. The method must be able to differentiate between the analyte of interest and compounds with a similar chemical structure that may be present. For a high performance liquid chromatography (HPLC) [5] identification test, peak purity evaluation should be used to assess the homogeneity of the peak corresponding to the analyte of interest.

For assay/related substances methods, the active peak should be adequately resolved from all impurity/degradant peaks, placebo peaks, and sample blank peaks. Resolution from impurity peaks could be assessed by analyzing a spiked solution with all known available impurities present or by injecting individual impurities and comparing retention to that of the active. Placebo and sample matrix components should be analyzed without the active present in order to identify possible interferences.

If syringe filters are to be used to clarify sample solutions, an aliquot of filtered sample diluent should be analyzed for potential interferences. If the impurities/degradants are unknown or unavailable, forced degradation studies should be performed. Forced degradation studies of the active pharmaceutical ingredient (API) and finished product, using either peak purity analysis or a mass spectral evaluation, should be performed to assess resolution from potential degradant products. [6]

The forced degradation studies should consist of exposing the API and finished product to acid, base, peroxide, heat, and light conditions, until adequate degradation of the active has been achieved. An acceptable range of degradation may be 10–30% but may vary based on the active being degraded. Over degradation of the active should be avoided to prevent the formation of secondary degradants. If placebo material is available, it should be stressed under the same conditions and for the same duration as the API or finished product. The degraded placebo samples should be evaluated to ensure that any generated degradants are resolved from the analyte peak(s) of interest.

Evaluation of the forced degraded solutions by peak purity analysis using a photodiode array detector or mass spectral evaluation must confirm that the active peak does not co-elute with any degradation products generated as a result of the forced degradation. Another, more conservative, approach for assay/related substances methods is to perform peak purity analysis or mass spectral evaluation on all generated degradation peaks and verify that co-elution does not occur for those degradant peaks as well as the active peak.

Whereas the selectivity experiments for the first approach can be performed during a pre-validation phase (no need for quantification), those for the second approach are usually performed together with the precision and accuracy experiments during the main validation phase. At this point it must be mentioned that the term specificity is used interchangeably with selectivity, although in a strict sense specificity refers to methods, which produce a response for a single analyte, whereas selectivity refers to methods that produce responses for a number of chemical entities, which may or may not be distinguished. Selective multianalyte methods (e.g., for different drugs of abuse in blood) should of course be able to differentiate all interesting analytes from each other and from the matrix.[7]

Calibration model:

The choice of an appropriate calibration model is necessary for reliable quantification. Therefore, the relationship between the concentration of analyte in the sample and the corresponding detector response must be investigated. This can be done by analyzing spiked calibration samples and plotting the resulting responses versus the corresponding concentrations. The resulting standard curves can then be further evaluated by graphical or mathematical methods, the latter also allowing statistical evaluation of the response functions. Whereas there is a general agreement that calibration samples should be prepared in blank matrix and that their concentrations must cover the whole calibration range, recommendations on how many concentration levels should be studied with how many replicates per concentration level differ significantly. In the Conference Report II, "a sufficient number of standards to define adequately the relationship between concentration and response" was demanded. Furthermore, it was stated that at least five to eight concentration levels should be studied for linear relationships and it may be more for nonlinear relationships.

However, no information was given on how many replicates should be analyzed at each level. The guidelines established by the ICH and those of the *Journal of Chromatography B* also required at least five concentration levels, but again no specific requirements for the number of replicate set at each level were given. Causon recommended six replicates at each of the six concentration levels, whereas Wieling *et al*. used eight concentration levels in triplicate. This approach allows not only a reliable detection of outliers but also a better evaluation of the behavior of variance across the calibration range. The latter is important for choosing the right statistical model for the evaluation of the calibration curve. The often used ordinary least squares model for linear regression is only applicable for homoscedastic data sets (constant variance over the whole range), whereas in case of heteroskedasticity (significant difference between variances at lowest and highest concentration levels), the data should mathematically be transformed or a weighted least squares model should be applied. Usually, linear models are preferable, but, if necessary, the use of nonlinear models is not only acceptable but also recommended. However, more concentration levels are needed for the evaluation of nonlinear models than for linear models.[8]

After outliers have been purged from the data and a model has been evaluated visually and/or by, for example, residual plots, the model fit should also be tested by appropriate statistical methods. The fit of unweighted regression models (homoscedastic data) can be tested by the analysis of variance (ANOVA) lack-of-fit test. The widespread practice to evaluate a calibration model via its coefficients of correlation or determination is not acceptable from a statistical point of view.

However, one important point should be kept in mind when statistically testing the model fit: The higher the precision of a method, the higher the probability to detect a statistically significant deviation from the assumed calibration model. Therefore, the relevance of the deviation from the assumed model must also be taken into account. If the accuracy data (bias and precision) are within the required acceptance limits and an alternative calibration model is not applicable, slight deviations from the assumed model may be neglected. Once a calibration model has been established, the calibration curves for other validation experiments (precision, bias, stability, etc.) and for routine analysis can be prepared with fewer concentration levels and fewer or no replicates

Accuracy:

Accuracy should be performed at a minimum of three concentration levels. For drug substance, accuracy can be inferred from generating acceptable results for precision, linearity, and specificity. For assay methods, the

spiked placebo samples should be prepared in triplicate at 80, 100, and 120%. If placebo is not available and cannot be formulated in the laboratory, the weight of drug product may be varied in the sample preparation step of the analytical method to prepare samples at the three levels listed above. In this case, the accuracy study can be combined with method precision, where six sample preparations are prepared at the 100% level, while both the 80 and 120% levels are prepared in triplicate. For impurity/related substances methods, it is ideal if standard material is available for the individual impurities. These impurities are spiked directly into sample matrix at known concentrations, bracketing the specification level for each impurity. This approach can also be applied to accuracy studies for residual solvent methods where the specific residual solvents of interest are spiked into the product matrix.

If individual impurities are not available, placebo can be spiked with drug substance or reference standard of the active at impurity levels, and accuracy for the impurities can be inferred by obtaining acceptable accuracy results from the active spiked placebo samples. Accuracy should be performed as part of late Phase 2 and Phase 3 method validations. For early phase method qualifications, accuracy can be inferred from obtaining acceptable data for precision, linearity, and specificity.[9] Stability of the compound(s) of interest should be evaluated in sample and standard solutions at typical storage conditions, which may include room temperature and refrigerated conditions. The content of the stored solutions is evaluated at appropriate intervals against freshly prepared standard solutions. For assay methods, the change in active content must be controlled tightly to establish sample stability. If impurities are to be monitored in the method sample, solutions can be analyzed on multiple days and the change in impurity profiles can be monitored. Generally, absolute changes in the impurity profiles can be used to establish stability. If an impurity is not present in the initial sample (day 0) but appears at a level above the impurity specification during the course of the stability evaluation, then this indicates that the sample is not stable for that period of storage. In addition, impurities that are initially present and then disappear, or impurities that are initially present and grow greater than 0.1% absolute, are also indications of solution instability.

During phase 3 validation, solution stability, along with sample preparation and chromatographic robustness, should also be evaluated. For both sample preparation and chromatographic robustness evaluations, the use of experimental design could prove advantageous in identifying any sample preparation parameters or chromatographic parameters that may need to be tightly controlled in the method. For chromatographic robustness, all compounds of interest, including placebo-related and sample blank components, should be present when evaluating the effect of modifying chromatographic parameters. For an HPLC impurity method, this may include a sample preparation spiked with available known impurities at their specification level or, alternatively, a forced degraded sample solution can be utilized. The analytical method should be updated to include defined stability of solutions at evaluated storage conditions and any information regarding sample preparation and chromatographic parameters, which need to be tightly controlled. Sample preparation and chromatographic robustness may also be evaluated during method development. In this case, the evaluations do not require repeating during the actual method validation.[10]

Establishment of an appropriate qualification/validation protocol requires assessment of many factors, including phase of product development, purpose of the method, type of analytical method, and availability of supplies, among others. There are many approaches that can be taken to perform the testing required for various validation elements, and the experimental approach selected is dependent on the factors listed above. As with any analytical method, the defined system suitability criteria of the method should be monitored throughout both method qualification and method validation, ensuring that the criteria set for the suitability is appropriate and that the method is behaving as anticipated. The accuracy of a method is affected by systematic (bias) as well as random (precision) error components. This fact has been taken into account in the definition of accuracy as established by the International Organization for Standardization (ISO). However, it must be mentioned that accuracy is often used to describe only the systematic error component, that is, in the sense of bias. In the following, the term accuracy will be used in the sense of bias, which will be indicated in brackets.

Bias:
According to ISO, bias is the difference between the expectation of test results and an accepted reference value. It may consist of more than one systematic error component. Bias can be measured as a percent deviation from the accepted reference value. The term trueness expresses the deviation of the mean value of a large series of measurements from the accepted reference value. It can be expressed in terms of bias. Due to the high workload of analyzing such large series, trueness is usually not determined during method validation, but rather from the results of a great number of quality control samples (QC samples) during routine application.[11]

Precision and repeatability:
Repeatability reflects the closeness of agreement of a series of measurements under the same operating conditions over a short interval of time. For a chromatographic method, repeatability can be evaluated by performing a minimum of six replicate injections of a single sample solution prepared at the 100% test concentration. Alternatively, repeatability can be determined by evaluating the precision from a minimum of nine determinations that encompass the specified range of the method. The nine determinations may be composed of triplicate determinations at each of three different concentration levels, one of which would represent the 100% test concentration. Intermediate precision reflects within-laboratory variations such as different days, different analysts, and different equipments. Intermediate precision testing can consist of two different analysts, each preparing a total of six sample preparations, as per the analytical method. The analysts execute their testing on different days using separate instruments and analytical columns.[12]

The use of experimental design for this study could be advantageous because statistical evaluation of the resulting data could identify testing parameters (i.e., brand of HPLC system) that would need to be tightly controlled or specifically addressed in the analytical method. Results from each analyst should be evaluated to ensure a level of agreement between the two sets of data. Acceptance criteria for intermediate precision are dependent on the type of testing being performed. Typically, for assay methods, the relative standard deviation (RSD) between the two sets of data must be $\leq$2.0%, while the acceptance criteria for impurities is dependent on the level of impurity and the sensitivity of the method. Intermediate precision may be delayed until full ICH validation, which is typically performed during late Phase 2 or Phase 3 of drug development. However, precision testing should be conducted by one analyst for early phase method qualification.

Reproducibility reflects the precision between analytical testing sites. Each testing site can prepare a total of six sample preparations, as per the analytical method. Results are evaluated to ensure statistical equivalence among various testing sites. Acceptance criteria similar to those applied to intermediate precision also apply to reproducibility. Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision. Repeatability is sometimes also termed within-run or within-day precision.

Intermediate precision:
Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipments, etc.[13] The ISO definition used the term "M-factor different intermediate precision", where the M-factor expresses the number of factors (operator, equipment, or time) that differ between successive determinations. Intermediate precision is sometimes also called between-run, between-day, or inter-assay precision.

Reproducibility:
Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology). Reproducibility only has to be studied, if a method is supposed to be used in different laboratories. Unfortunately, some authors also used the term reproducibility for within-laboratory studies at the level of intermediate precision. This should, however, be avoided in order to prevent confusion.[14] As already mentioned above, precision and bias can be estimated from the analysis of QC samples under specified conditions. As both precision and bias can vary substantially over the calibration range, it is necessary to evaluate these parameters at least at three concentration levels (low, medium, high). In the Conference Report II, it was further defined that the low QC sample must be within three times LLOQ. The *Journal of Chromatography B* requirement is to study precision and bias at two

concentration levels (low and high), whereas in the experimental design proposed by Wieling *et al*., four concentration levels (LLOQ, low, medium, high) were studied.[15]

Causon also suggested estimating precision at four concentration levels. Several authors have specified acceptance limits for precision and/or accuracy (bias). The Conference Reports required precision to be within 15% RSD except at the LLOQ where 20% RSD is accepted. Bias is required to be within ±15% of the accepted true value, except at the LLOQ where ±20% is accepted.[16] These requirements have been subject to criticism in the analysis of the Conference Report by Hartmann *et al*. They concluded from statistical considerations that it is not realistic to apply the same acceptance criteria at different levels of precision (repeatability, reproducibility) as RSD under reproducibility conditions is usually considerably greater than under repeatability conditions. Furthermore, if precision and bias estimates are close to the acceptance limits, the probability to reject an actually acceptable method (b-error) is quite high. Causon proposed the same acceptance limits of 15% RSD for precision and ±15% for accuracy (bias) for all concentration levels. The guidelines established by the Journal of Chromatography B required precision to be within 10% RSD for the high QC samples and within 20% RSD for the low QC sample. Acceptance criteria for accuracy (bias) were not specified there.

Again, the proposals on how many replicates at each concentration levels should be analyzed vary considerably.[17] The Conference Reports and *Journal of Chromatography B* guidelines required at least five replicates at each concentration level. However, one would assume that these requirements apply to repeatability studies; at least no specific recommendations are given for studies of intermediate precision or reproducibility. Some more practical approaches to this problem have been described by Wieling *et al*., Causon, and Hartmann *et al*. In their experimental design, Wieling *et al*. analyzed three replicates at each of four concentration levels on each of 5 days.[18] Similar approaches were suggested by Causon (six replicates at each of four concentrations on each of four occasions) and Hartmann *et al*. (two replicates at each concentration level on each of 8 days). All three used one-way ANOVA to estimate within-run precision (repeatability) and between-run precision (intermediate precision).

In the design proposed by Hartmann *et al*., the degrees of freedom for both estimations are most balanced, namely, eight for within-run precision and seven for between-run precision. In the information for authors of the *Clinical Chemistry* journal, an experimental design with two replicates per run, two runs per day over 20 days for each concentration level is recommended. This allows estimation of not only within-run and between-run standard deviations but also within-day, between-day, and total standard deviations, which are in fact all estimations of precision at different levels. However, it seems questionable if the additional information provided by this approach can justify the high workload and costs, compared to the other experimental designs. Daily variations of the calibration curve can influence bias estimation.[19] Therefore, bias estimation should be based on data calculated from several calibration curves. In the experimental design of Wieling *et al*., the results for QC samples were calculated via daily calibration curves. Therefore, the overall means from these results at the different concentration levels reliably reflect the average bias of the method at the corresponding concentration level. Alternatively, as described in the same paper, the bias can be estimated using confidence limits around the calculated mean values at each concentration. If the calculated confidence interval includes the accepted true value, one can assume the method to be free of bias at a given level of statistical significance. Another way to test the significance of the calculated bias is to perform a *t*-test against the accepted true value. However, even methods exhibiting a statistically significant bias can still be acceptable, if the calculated bias lies within previously established acceptance limits.[20]

Lower limit of quantification:

The LLOQ is the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy (bias). There are different approaches to the determination of LLOQ.[21]

LLOQ based on precision and accuracy (bias) data: This is probably the most practical approach and defines the LLOQ as the lowest concentration of a sample that can still be quantified with acceptable precision and accuracy (bias). In the Conference Reports, the acceptance criteria for these two parameters at LLOQ are 20% RSD for precision and ±20% for bias. Only Causon suggested 15% RSD for precision and ±15% for

bias. It should be pointed out, however, that these parameters must be determined using an LLOQ sample independent from the calibration curve. The advantage of this approach is the fact that the estimation of LLOQ is based on the same quantification procedure used for real samples.[22]

LLOQ based on signal to noise ratio (S/N): This approach can only be applied if there is baseline noise, for example, to chromatographic methods. Signal and noise can then be defined as the height of the analyte peak (signal) and the amplitude between the highest and lowest point of the baseline (noise) in a certain area around the analyte peak. For LLOQ, *S/N* is usually required to be equal to or greater than 10. The estimation of baseline noise can be quite difficult for bioanalytical methods, if matrix peaks elute close to the analyte peak.

Upper limit of quantification:
The upper limit of quantification (ULOQ) is the maximum analyte concentration of a sample that can be quantified with acceptable precision and accuracy (bias). In general, the ULOQ is identical with the concentration of the highest calibration standard. [23]

Limit of detection:
Quantification below LLOQ is by definition not acceptable. Therefore, below this value a method can only produce semi-quantitative or qualitative data. However, it can still be important to know the LOD of the method. According to ICH, it is the lowest concentration of an analyte in a sample which can be detected but not necessarily quantified as an exact value. According to Conference Report II, it is the lowest concentration of an analyte in a sample that the bioanalytical procedure can reliably differentiate from background noise.

Stability:
The definition according to Conference Report II was as follows: The chemical stability of an analyte in a given matrix under specific conditions for given time intervals. Stability of the analyte during the whole analytical procedure is a prerequisite for reliable quantification. Therefore, full validation of a method must include stability experiments for the various stages of analysis, including storage prior to analysis.[24]

Long-term stability:
The stability in the sample matrix should be established under storage conditions, that is, in the same vessels, at the same temperature and over a period at least as long as the one expected for authentic samples.

Freeze/thaw stability:
As samples are often frozen and thawed, for example, for reanalyis, the stability of analyte during several freeze/thaw cycles should also be evaluated. The Conference Reports require a minimum of three cycles at two concentrations in triplicate, which has also been accepted by other authors.

In-process stability:
The stability of analyte under the conditions of sample preparation (e.g., ambient temperature over time needed for sample preparation) is evaluated here. There is a general agreement that this type of stability should be evaluated to find out if preservatives have to be added to prevent degradation of analyte during sample preparation.[25–27]

Processed sample stability:
Instability can occur not only in the sample matrix but also in prepared samples. It is therefore important to also test the stability of an analyte in the prepared samples under conditions of analysis (e.g., autosampler conditions for the expected maximum time of an analytical run). One should also test the stability in prepared samples under storage conditions, for example, refrigerator, in case prepared samples have to be stored prior to analysis.

Recovery:
As already mentioned above, recovery is not among the validation parameters regarded as essential by the Conference Reports. Most authors agree that the value for recovery is not important as long as the data for LLOQ, LOD, precision and accuracy (bias) are acceptable. It can be calculated by comparison of the analyte response after sample workup with the response of a solution containing the analyte at the theoretical maximum concentration. Therefore, absolute recoveries can usually not be determined if the sample workup includes a derivatization step, as the derivatives are usually not available as reference substances. Nevertheless, the guidelines of the *Journal of Chromatography B* require the determination of the recovery for analyte and internal standard at high and low concentrations.[28–31]

Ruggedness (Robustness):
Ruggedness is a measure for the susceptibility of a method to small changes that might occur during routine analysis like small changes of pH values, mobile phase composition, temperature, etc. Full validation must not necessarily include ruggedness testing; it can, however, be very helpful during the method development/prevalidation phase, as problems that may occur during validation are often detected in advance. Ruggedness should be tested if a method is supposed to be transferred to another laboratory.

Specific Recommendation For Bioanalytical Method Validation:

- The matrix-based standard curve should consist of a minimum of six standard points, excluding blanks, using single or replicate samples. The standard curve should cover the entire range of expected concentrations. Standard curve fitting is determined by applying the simplest model that adequately describes the concentration–response relationship using appropriate weighting and statistical tests for goodness of fit.[32]
- LLOQ is the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision. The LLOQ should be established using at least five samples independent of standards and determining the coefficient of variation (CV) and/or appropriate confidence interval. The LLOQ should serve as the lowest concentration on the standard curve and should not be confused with the LOD and/or the low QC sample. The highest standard will define the ULOQ of an analytical method.
- For validation of the bioanalytical method, accuracy and precision should be determined using a minimum of five determinations per concentration level (excluding blank samples). The mean value should be within 15% of the theoretical value, except at LLOQ, where it should not deviate by more than 20%. The precision around the mean value should not exceed 15% of the CV, except for LLOQ, where it should not exceed 20% of the CV. Other methods of assessing accuracy and precision that meet these limits may be equally acceptable.[33]
- The accuracy and precision with which known concentrations of analyte in biological matrix can be determined should be demonstrated. This can be accomplished by analysis of replicate sets of analyte samples of known concentration QC samples from an equivalent biological matrix. At a minimum, three concentrations representing the entire range of the standard curve should be studied: one within 3× the LLOQ (low QC sample), one near the center (middle QC), and one near the upper boundary of the standard curve (high QC).
- 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 can also be reported.
- The stability of the analyte in biological matrix at the intended storage temperatures should be established. The influence of freeze–thaw cycles (a minimum of three cycles at two concentrations in triplicate) should be studied.
- The stability of the analyte in matrix at ambient temperature should be evaluated over a time period equal to the typical sample preparation, sample handling, and analytical run times.
- Reinjection reproducibility should be evaluated to determine if an analytical run could be reanalyzed in the case of instrument failure.[34]

- The specificity of the assay methodology should be established using a minimum of six independent sources of the same matrix. For hyphenated mass spectrometry based methods, however, testing six independent matrices for interference may not be important. In the case of LC-MS and LC-MS-MS based procedures, matrix effects should be investigated to ensure that precision, selectivity, and sensitivity will not be compromised. Method selectivity should be evaluated during method development and throughout method validation and can continue throughout application of the method to actual study samples.
- Acceptance/rejection criteria for spiked, matrix-based calibration standards and validation QC samples should be based on the nominal (theoretical) concentration of analytes. Specific criteria can be set up in advance and achieved for accuracy and precision over the range of the standards, if so desired.

Method Validation		
1.	Selectivity and Specificity of Analyte and ISTD in Blank Matrix	09 out of 09 plasma lots were passed which consisting of 06 K3EDTA human plasma, 01 haemolyzed, 01 Lipidemic and 01 Sodium Heparin human plasma lot
2.	Recovery of Analyte	% Overall recovery of Analyte and ISTD was found to be 82.3 & 85.6 % respectively
3.	Matrix Factor	% CV of ISTD normalized matrix factor at LQC and HQC level was found to be 3.40 & 2.55 respectively
4.	Linearity	Linearity range was found to be 0.100 - 30.000 ng/ml
5.	Intra-Run Precision	% CV for HQC, MQC 1, MQC 2, and LQC was found to be < 15 % and for LLOQ QC it was found to be < 20 %.
6.	Intra-Run Accuracy	Intra-run accuracy for all high, middle and low quality control samples was found to be within ± 15% and for LLOQ QC ± 20 %.
7.	Inter-Run Precision	% CV for HQC, MQC 1, MQC 2, and LQC was found to be < 15 % and for LLOQ QC it was found to be < 20 %.
8.	Inter-Run Accuracy	Inter - run accuracy for all high, middle and low quality control samples was found to be within ± 15% and for LLOQ QC it was found to be within ± 20 %.
9.	Dilution Integrity	% Mean accuracy of dilution quality control was found to be 98.45 % and % CV of dilution quality control was found to be 2.43 %.
10.	Short Term Stock Solution Stability (At Ambient Temperature for 06 hours)	% Mean stability of drug stock and ISTD stock was found to be 99.99 % and 99.92 % respectively
11.	Short Term Working Solution Stability (At Ambient Temperature for 06 hours)	% Mean stability ULOQ, LLOQ and ISTD dilution was found to be 102.75 %, 99.51 % and 100.38 % respectively
12.	Long Term Stock Solution Stability(At -20 ± 5 °C temperature for 7 days)	% Mean stability of drug stock and ISTD stock was found to be 100.95 % and 99.60 % respectively

13.	Long Term Working Solution Stability (At -20 ± 5 °C temperature for 7 days)	% Mean stability of ULOQ, LLOQ and ISTD dilution was found to 90.96 %, 101.80 % and 99.68 % respectively
14.	Bench Top Stability At Ambient Temperature (6 hrs)	% Mean stability at LQC and HQC level was found to be 96.01 % and 92.04 % respectively
15.	Stability of Analyte in Blood (At Ambient Temperature)	% Mean stability at HQC and LQC level for 30 min was found to be 92.08 % and 96.64 % respectively % Mean stability at HQC and LQC level for 02 hours was found to be 91.78 % and 93.70 % respectively
16.	Stability of Analyte in Blood (At Wet Ice Bath < 10°C and normal temp.)	% Mean stability at HQC and LQC level for 30 min was found to be 91.17 % and 93.45 % respectively % Mean stability at HQC and LQC level for 02 hours was found to be 90.15 % and 93.19 % respectively
17.	Freeze Thaw Stability (At -20 ± 5 °C)	% Mean stability at LQC and HQC level was found to be 92.17 % and 91.46 % respectively
18.	Freeze Thaw Stability (At -78 ± 8 °C)	% Mean stability at LQC and HQC level was found to be 97.70 % and 91.67 % respectively

Conclusion:

The importance of validation, at least of routine analytical methods, can therefore hardly be overestimated. This is especially true in the context of quality management and accreditation, which have become matters of increasing importance in analytical toxicology in the recent years. The US Food and Drug Administration (FDA) guidelines for bioanalytical method validation. ICH guidelines recommend evaluating a minimum of five concentrations to assess linearity. For every phase of product development, the analytical method must demonstrate specificity. Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology). Reproducibility only has to be studied, if a method is supposed to be used in different laboratories In the design proposed by Hartmann *et al*., the degrees of freedom for both estimations are most balanced, It is recognized that bioanalytical methods and techniques are constantly undergoing changes and improvements, and in many instances, they are at the cutting edge of the technology. It is also important to emphasize that each bioanalytical technique has its own characteristics, which will vary from analyte to analyte. In these instances, specific validation criteria may need to be developed for each analyte.

References

1. Thompson M, Ellison SLR, Wood R. Harmonised Guidelines for Single Laboratory Validation of Method of Analysis. *Pure Appl Chem.* 2008;74:835–55.
2. Wood R. How to Validate Analytical Methods. *Trends Analyt Chem.* 2005;18:624–132.
3. McDowall RD. The Role of Laboratory Information Management Systems LIMS in Analytical Method Validation. *Anal Chim Acta.* 2007;54:149–58.
4. Vander HY, Nijhuis A, Verbeke JS, Vandeginste BG, Massart DL. Guidance for rubustness/ruggedness test in method validation. *J Pharm Biomed Anal.* 2009;24:723–53.
5. Puluido A, Ruusanches I, Boque R, Rius FX. Uncertainty of results in routine Qualitative Analysis in Analytical Chemistry. *J Pharm Biomed Anal.* 2005;22:647–54.

6. Kallner A. Quality specification based on the uncertainty of measurement. *Scand J Lab Invest.* 2005;59:513–6.
7. Jhanf J, Chang CC, Fink DJ, Kroll MH. Evaluation of linearity in clinical Laboratory. *Arch Pathol Lab Med.* 2004;128:44–8.
8. Mark H. Application of improved procedure for testing linearity of analytical method to pharmaceutical analsysis. *J Pharm Biomed Anal.* 2003;33:7–20. Trullols E, Ruisanchez I, Rius FX. Trends in Analytical Chemistry. *J Lab Invest.* 2003;23:137–45. Valcarcel M, Cardenas S, Gallego M. Sample Screening system in analytical chemistry. *Trends Analyt Chem.* 1999;23:137–45.
9. Ye C, Liu J, Ren F, Okafo N. Design of Experimental Date Analysis By JMP (SAS Institute) in Analytical Method Validation. *J Pharm Biomed Anal.* 2000;23:581–9.
10. Nowatzke W, Woolf E. Best Practices during Bioanalytical Method Validation for the Characterization of Assay Reagents and the Evaluation of Analyte Stability in Assay Standards, Quality Controls, and Study Samples. *AAPS J.* 2007;9:E117–22. [PMC free article]
11. Braggio S, Barnaby RJ, Grosi P, Cugola A strategy for validation of bioanalytical methods. *J Pharm Biomed Anal.* 1996;14:375–88. James CA, Breda M, Frigerio E. Bioanalytical method validation: A risk-based approach. *J Pharm Biomed Anal.* 2004;35:887–9.
12. Nakashima K. High-Performance Liquid Chromatography of drug of abuse in biological samples. *J Health Sci.* 2009;51:272–7.
13. Boulanger B, Chiap P, Dewe W, Crommen J, Hubert P. An analysis of the SFSTP guide on validation of chromatographic bioanalytical methods: progresses and limitations. *J Pharm Biomed Anal.* 2005;32:753–65.
14. Causon R. Validation of chromatographic methods in biomedical analysis viewpoint and discussion. *J Chromatogr.* 1998;689:175–80.
15. Hartmann C, Smeyers VJ, Massart DL, McDowall RD. Validation of bioanalytical chromatographic methods. *J Pharm Biomed Anal.* 1998;17:193–218.
16. Hubert P, Chiap P, Crommena J, Boulanger B, Chapuzet EN, Laurentie M, et al. The SFSTP guide on the validation of chromatographic methods for drug bioanalysis: from the Washington Conference to the laboratory. *Anal Chim Acta.* 2002;391:135–48.
17. Hartmann C, Smeyers VJ, Massart DL. Validation of bioanalytical chromatographic methods. *J Pharm Biomed Anal.* 1999;17:193–218.
18. Zhoua S, Songb Q, Tangb Y, Weng N. Critical Review of Development, Validation, and Transfer for High Throughput Bioanalytical LC-MS/MS Methods. *Curr Pharm Anal.* 2005;55:3–14.
19. Kelley M, DeSilva B. Key Elements of Bioanalytical Method Validation for Macromolecules. *AAP J.* 2007;9:E156–63. [PMC free article] Mohammad AT, Leung HF. Reversed-phase high-performance liquid chromatography method for the analysis of nitro-arginine in rat plasma and urine. *J Chromatogr.* 1998;235:7–12.
20. Bmscheck T, Meyer H, Wellhrner HH. A High-performance liquid chromatographic assay for the measurement of azathioprine in human serum samples. *J Chromatogr.* 1996;212:287–94.
21. Kees F, Jehnich D, Grobecker H. Simultaneous determination of acetylsalicylic acid and salicylic acid in human plasma by high-performance liquid chromatography. *J Chromatogr.* 1996;677:172–7.
22. Raymond NX, Fan LR, Matthew J, Tawakol A. Recent advances in high-throughput quantitative bioanalysis by LC–MS/MS. *J Pharm Biomed Anal.* 2007;44:342–55.
23. Lau Y, Hanson GD, Carel BJ. Determination of rifampin in human plasma by HPLC with ultraviolet detection. *J Chromatogr.* 1998;676:147–52. Compagnon P, Thiberville, Moore N, Thudlez C, Lacroix C. Simple high-performance liquid chromatographic method for the quantitation of 5-fluorouracil in human plasma. *J Chromatogr.* 2003;677:380–3.
24. Bressolle F, Bromet PM, Audran M. Validation of liquid chromatographic and gas chromatographic methods. Applications to pharmacokinetics. *J. Chromatogr.* 2000;686:3–10.
25. Dadgar D, Burnett PE. Issues in evaluation of bioanalytical method selectivity and drug stability. *J Pharm Biomed Anal.* 2004;14:23–31.
26. Dadgar D, Burnett PE, Choc MG, Gallicano K, Hooper JW. Application issues in bioanalyticalmethod validation, sample analysis and data reporting. *J Pharm Biomed Anal.* 1995;13:89–97.

27. Hartmann C, Massart D, McDowall RD. An analysis of the Washington Conference Report on bioanalytical method validation. *J Pharm Biomed Anal.* 2005;12:1337–43.
28. Karnes HT, Shiu G, Shah VP. Validation of bioanalytical methods. *Pharm Res.* 2001;8:421–6.
29. Lindner W, Wainer IW. Requirements for initial assay validation and publication in J ChromatographyB. *J Chromatogr.* 2006;707:1–2.
30. Shah VP, Midha KK, Dighe S, McGilveray IJ, Skelly JP, Yacobi A, et al. Analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies. Conference report. *Pharm Res.* 2009;9:588–92.