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# Validation of chromatographic methods in biomedical analysis Viewpoint and discussion

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**Abstract**

Bioanalytical method validation is the process used to establish that a quantitative analytical method is suitable for biomedical applications. Reassurances as to the quality of the method and its reliability come from adopting a minimum series of validation experiments and obtaining satisfactory results. Consistent evaluation of the key analytical parameters: recovery, response function, sensitivity, precision, accuracy, selectivity and stability, is discussed with a view to improving scientific standards in manuscripts submitted for publication.

*Keywords:* Validation; Biochemical analysis

## 1. Introduction

At a time when we are recognising the significant contribution made by Dr. Karel Macek in establishing the Journal of Chromatography B: Biomedical Applications as a leading journal, it is appropriate that we continue to strive for improvements which will further enhance the scientific value of the journal's publications.

The application of chromatographic methods to the quantitative analysis of compounds of biomedical interest continues to generate large numbers of published methods. For these methods to be useful, some reassurances as to the quality of the work, particularly the reliability of data generated in biological matrix samples must be provided. In the past this has been largely at the discretion of the authors and reviewers, with the result that bioanalytical methods are published which are difficult to reproduce outside the originating laboratory and much time can be wasted trying to set up methods which turn out to be unreliable when applied to real samples. If a bioanalytical method claims to be for quantitative biomedical application, then it is important to ensure that a minimum package of validation experiments has been conducted and yields satisfactory results.

Bioanalytical method validation in the pharmaceutical industry is influenced by regulations from the US Food and Drug Administration (FDA), the UK Medicines Control Agency (MCA) and similar bodies from Canada, Japan and other countries. A consensus on the requirements for analytical method validation was reached by a panel of experts at the Washington conference on Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies in 1990 and reported by Shah and colleagues, 1992 [1]. This report provides the guidelines for analytical method establishment, validation and application to drug analysis in biological matrices. In the authors opinion, the lead shown by the pharmaceutical industry in providing formal

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validation for quantitative bioanalytical methods, should be incorporated into the criteria that scientific journals apply in their judgement of the suitability of manuscripts covering the biomedical application of quantitative chromatographic techniques for publication.

## 2. Method development

Method development involves evaluation and optimisation of the various stages of sample preparation, chromatographic separation, detection and quantitation. Authors should be encouraged to discuss the rationale behind their method development experiments and state clearly how they arrived at the final method which was selected for validation and application. To merely provide a method menu with no explanation as to the underlying science is unacceptable. With chromatographic methods, an important consideration at the method development stage is whether to use internal or external standardisation. For external standardisation, the response of the analyte is plotted against concentration to generate the calibration line, while for internal standardisation, a structural or isotopic analogue of the analyte is added to standards and samples prior to sample pre-treatment and the ratio of the response of the analyte to that of the internal standard is plotted against concentration. The only way to assess which of these approaches is best practice is to evaluate both approaches [2].

## 3. Method validation

The validation of a bioanalytical method is the process used to establish that the analytical performance parameters are adequate for their intended use. For chromatographic methods used in biomedical applications there is more consistency in validation practice [3] with key analytical parameters including: (1) recovery; (2) response function; (3) sensitivity; (4) precision; (5) accuracy; (6) selectivity and (7) stability.

Analysis of analytes in biological matrices is subject to many variables and as such a bioanalytical method is never truly validated until it has been applied successfully to at least one study. Generation of ongoing performance data is the best way to monitor minor changes to the method, e.g. changing the HPLC column, and assess if the method was performing appropriately in a particular study. Application of validated bioanalytical methods to clinical trials in patients can reveal complications not seen in the validation with spiked drug-free matrices. Thus the potential interferences from metabolites and concomitant medication should also be mentioned and investigated once the method is being used on a regular basis.

### *3.1. Recovery*

The absolute recovery of a bioanalytical method is measured as the response of a processed spiked matrix standard expressed as a percentage of the response of a pure standard, which has not been subjected to sample pre-treatment and indicates whether the method provides a response for the entire amount of analyte that is present in the sample [4]. It is best established by comparing the responses of extracted samples at low, medium and high spiked matrix concentrations in replicates of at least 6 with those of non-extracted standards which represent 100% recovery.

$$\text{Absolute recovery} = \frac{\text{response of analyte spiked into matrix (processed)}}{\text{response of analyte of pure standard (unprocessed)}} \times 100$$

In addition, the effect of co-extracted biological material should be studied by comparing the response of extracted samples spiked before extraction with the response of extracted blank matrix samples to which analyte has been added at the same nominal concentration; just before injection [5]. If an internal standard is used, its recovery should be determined independently at the concentration level used in the method. The recovery of the internal standard should be within 15% of that determined for the analyte.

Although it is desirable to attain recovery as close to 100% as possible in order to maximise the sensitivity of the method, it is unlikely that recoveries of 50% or more will compromise the

integrity of the method. Good precision and accuracy can be obtained from methods with moderate recoveries, provided they have adequate sensitivity. Indeed it may be desirable to intentionally sacrifice high recovery in order to achieve better selectivity with some sample extraction procedures.

### *3.2. Response function*

In chromatographic methods of analysis peak area or peak height may be used as the response function to define the linear relationship with concentration known as the calibration model. It is essential to verify the calibration model selected to ensure that it adequately describes the relationship between response function ($y$) and concentration ($x$). The difference between the observed $y$-values and the fitted $y$-value or residual, should be examined for a minimum of six standard curves each constructed with a minimum of six unique concentrations. A plot of studentised residual (raw residual/standard error) vs. log concentration will then show how well the model describes the data. The most common occurrence is an increase in variance with increase in concentration or heterocedasticity and this is best managed by use of a weighted regression [6]. Weightings of $1/x$, $1/y$ and $1/y^2$ are suitable approximations of this variance and should be selected by examination of residual vs. concentration plots, using each weighting factor [7].

### *3.3. Sensitivity*

The sensitivity of an analytical method is determined from the slope of the calibration line. A method is said to be sensitive if small changes in concentration cause larger changes in the response function [8]. The limits of quantification (LOQ) or working dynamic range of a bioanalytical method are defined as the highest and lowest concentrations which can be determined with acceptable accuracy and precision. It is suggested that this be set at ±15% for both the upper and lower limit of quantification (ULOQ and LLOQ), respectively. The ULOQ and LLOQ determined by these acceptance criteria will become the highest and lowest calibration standards of the method in routine use, respectively. Any sample concentrations that fall outside the calibration range cannot be interpolated from the calibration line and extrapolation of the calibration line is discouraged. If the concentration is over range the sample should be diluted in drug-free matrix and reassayed. If study samples are to be routinely diluted into the calibration range with something other than the matrix being validated, e.g. buffer, the linearity of dilution should be assessed. This is best performed at a minimum of two concentrations, representing the low and high regions of the calibration curve, after appropriate dilution factors, e.g. two and ten-fold. It is suggested that dilution linearity controls are analysed in replicates of six and should back-calculate to within ±15% of their nominal concentration allowing for the dilution factor used. If the concentration is below the LLOQ, a concentration cannot normally be assigned and the result is best expressed as below LLOQ or BLOQ. Occasionally, it may be possible to increase the volume of sample matrix in order to obtain a concentration below the LLOQ. In this case, spiked samples can be prepared in the same way to validate this procedure. Validation is necessary to show that selectivity is not compromised and the predefined criteria for accuracy and precision is met with the larger sample volume. In addition, selectivity should be verified using the increased blank volumes.

### *3.4. Precision*

The precision of a bioanalytical method is a measure of the random error and is defined as the agreement between replicate measurements of the same sample. It is expressed as the percentage coefficient of variation (%C.V.) or relative standard deviation (R.S.D.) of the replicate measurements.

$$\%\text{C.V.} = (\text{standard deviation}/\text{mean}) \times 100$$

Precision can be considered as having a within assay batch component or repeatability which defines the ability to repeat the same methodology with the same analyst, using the same equipment and the same reagents in a short interval of time, e.g. within a day. This is also known as intra-assay precision. The ability to repeat the same methodology under different conditions, e.g. change of analyst, reagents or equipment; or on subsequent occasions, e.g.

across several weeks or months, is covered by the between batch precision or reproducibility, also known as inter-assay precision. The reproducibility of a method is of most interest to the analyst since this will give a better representation of the precision during routine use as it includes the variability from a greater number of sources.

For the validation of a new bioanalytical method for routine use with clinical studies it is suggested that precision be assessed at four unique concentrations in replicates of six, on four separate occasions, i.e. $4\times6\times4$. This approach will allow the data for individual analytes to be analysed by a one-way analysis of variance (ANOVA) which gives estimates of both the intra-assay and the inter-assay precision of the method at each concentration. To be acceptable, both measures should be within $\pm15\%$ at all concentrations.

### 3.5. Accuracy

The accuracy of a bioanalytical method is a measure of the systematic error or bias and is defined as the agreement between the measured value and the true value. Accuracy is best reported as percentage bias which is calculated from the expression:

$$\%\text{Bias} = [(\text{measured value} - \text{true value})/\text{true value}] \times 100$$

Since for real samples the true value is not known, an approximation is obtained based on spiking drug-free matrix to a nominal concentration. The accuracy of a bioanalytical method is then determined at each concentration by assessing the agreement between the measured and nominal concentrations of the analytes in the spiked drug-free matrix samples. For the validation of a new bioanalytical method for use with clinical studies, the measured concentrations will be those obtained during the estimation of precision, i.e. from the $4\times6\times4$ experiment. All results other than those rejected for analytical reasons, i.e. poor chromatography, should be used in the calculation and the accuracy of the method and should be within $\pm15\%$ at all concentrations.

### 3.6. Selectivity

A selective bioanalytical method is one which provides response functions for a number of chemical entities which may or may not be distinguished from each other [9]. If the response function is distinguished from all others, the method can be described as specific. Putting this another way, specificity is defined as the ability of the method to distinguish the analyte from all other substances present in the sample. This may be established by comparing the chromatographic retention time of the analyte in extracted matrix samples, with its retention time in at least one reference solution or by mass spectrometric determination following chromatographic analyte separation. Specificity can also be investigated by analysing at least six independent sources of the target matrix and checking for interferences by endogenous matrix components. Any interference should be less than 20% of the detector response at the LLOQ. Exceptions may be made when analysing rare biological matrices, e.g. aqueous humour, in which case a smaller number of individual matrices should be thoroughly assessed. Any available, known or potential drug metabolites, degradation products, concomitant medication and their major metabolites and common OTC drugs where applicable, should be spiked into test matrix and analysed for potential interference.

### 3.7. Stability

Stability data is required to show that the concentration of analyte in the sample at the time of analysis corresponds to the concentration of analyte at the time of sampling [10]. The stability of the analyte in analytical stock solutions, biological matrix and processed samples (extracts) should be established. The storage stability of the analyte in analytical stock solutions is best investigated during the validation, with samples to define expiry dates prepared immediately before method validation starts, stored at the appropriate temperature and analysed in separate assays after appropriate time intervals, e.g. 15 and 30 days. The stability of the analyte in biological matrix should be conducted at the temperature, e.g. ambient and 4°C, and light levels that will exist over the period needed to process a batch of study samples, and should include the effects of freeze–thaw, with a minimum of three cycles separated by at least 12 h and/or other processes involved in the bioanalytical method e.g. heat inactivation of HIV/HBV. Stability of the

analyte in processed samples, i.e. in an autosampler carousel, is best assessed after 24 and 48 h. This can be achieved by re-injection of extracts within another validation run. Stability should be assessed at a minimum of two concentrations, reflecting the expected concentration of the analyte in study samples, or where this is not known, representing the low and high concentration regions of the calibration curve.

Stability samples must be compared against freshly prepared 100% controls analysed in the same analytical run, preferably in replicates of six. Changes in stability of greater than −10% are likely to compromise the integrity of the data, although variations in stability of up to −20% may be acceptable under certain conditions [11]. When instability is proven, appropriate additives, e.g. buffers, antioxidants or enzyme inhibitors may be essential in order to minimise degradation of the analytes or losses due to adsorption [12].

### *3.8. Method application*

Bioanalytical support to clinical studies usually involves grouping between 50 to 100 clinical samples together with at least six unique calibration standards in duplicate and at least three unique quality control (QC) samples in duplicate. This grouping is known as a batch or run of samples. Monitoring the performance of a validated bioanalytical method is best achieved by establishing batch acceptance criteria [13]. These typically involve system suitability tests (SSTs) such as qualitative assessment of the chromatography obtained from a test mixture, e.g. resolution, peak symmetry and retention time [14], together with quantitative assessments of the calibration standards and QC samples. Monitoring QC results from subsequent batches over time is conveniently performed by some form of control chart, e.g. Levy and Jennings [15], Shewart or CuSum [16]. According to Shah et al. [1], to be acceptable, an analytical batch must contain at least four out of the six QC samples back-calculating to within ±20% of the nominal concentration and contain at least one acceptable QC at each of the three concentrations, (4-6-20 rule). This fixed range acceptance criteria is not sufficiently stringent for chromatographic methods of bioanalysis and many in the field have pushed for changes which range from setting a lower percentage of nominal for the QCs, most commonly ±15%, to replacement of the fixed range approach with inspection by variables [17].

The application of a validated bioanalytical method is incomplete without chromatograms from subjects or animals dosed with the compound (if appropriate) and this author always prefers to see at least one chromatogram at the $C_{max}$ and one at the terminal phase of the elimination. Additionally, a plasma concentration–time curve for each species/matrix together with basic pharmacokinetic parameters including $C_{max}$, $T_{max}$, $t_{1/2}$ and AUC are useful in demonstrating the application of the method to real study samples.

## 4. Conclusion

One of the recurring problems in manuscripts submitted for publication to the Journal of Chromatography B: Biomedical Applications has been inadequate bioanalytical method validation. In an effort to bring about improvements in this area, the essential validation characteristics for bioanalytical methodology have been discussed with a view to improving the standard and consistency of future submissions.

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