Journal of Pharmaceutical & Biomedical Analysis
Vol. 8, Nos 8–12, pp. 629–637, 1990
Printed in Great Britain

0731-7085/90 $3.00 + 0.00

Method validation in the bioanalytical laboratory*

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Abstract: Bioanalytical methods, based on a variety of physico-chemical and biological techniques such as chromatography, immunoassay and mass spectrometry, must be validated prior to and during use to engender confidence in the results generated. The fundamental criteria for assessing the reliability and overall performance of a bioanalytical method are: the evaluation of drug and analyte stability, selectivity, limits of quantification and detection, accuracy, precision, linearity and recovery. The extent to which a method is validated is dependent on its prospective use, the number of samples to be assayed and the use to which the data are put.

Specific analytical techniques may require additional validation such as antibody-binding characteristics, peak purity determination, evaluation of matrix effects or structural confirmation of the analyte. Ideally each assay should be cross-validated with a method utilizing a highly specific detector such as a mass spectrometer. Once in use, the performance of the method should be monitored using quality control standards. If a method is set up in another laboratory, the performance of that assay should be monitored with quality control standards sent from the originating laboratory.

Keywords: *Bioanalytical method validation; immunoassay; chromatography; mass spectrometry; precision; accuracy; selectivity.*

Introduction

Why validate bioanalytical methods?

The reason for validating a bioanalytical procedure is to demonstrate the performance and reliability of a method and hence the confidence that can be placed on the results. In addition, Shah has stated that all bioanalytical methods must be validated if the results are used to support registration of a new drug or the reformulation of an existing one [1]. It should be noted that the initial validation is only a beginning, as a method should be monitored continually during its application to ensure that it performs as originally validated.

Aims and objectives

The aim of this paper is to discuss method validation and to provide the basis for a comprehensive framework for validating bioanalytical methods. There are very few articles in the literature that address this topic [1–5] and there is little guidance on method validation for the submission of bioanalytical methods to journals; this can result in a variable quality of such manuscripts. There is no standard plan to follow for the validation of a bioanalytical method and the work that is undertaken is dependent on the analyst's experience and judgement. Validation of bioanalytical methods will be discussed mainly in the context of the drug discovery and development process within the pharmaceutical industry; however the principles outlined are, in the opinion of the authors, applicable to all bioanalytical methods.

The responsibilities of the bioanalyst

The rôle of the bioanalyst is a very important one; should analytical data be incorrect, then the efforts of scientists or physicians could be in vain. Dubious analytical data could waste valuable resources, cost money through delays in registration of new compounds or even worse be a cause for wrong diagnosis and treatment. Therefore, the bioanalyst has a vital contribution to make. It is against this background of responsibility that a bioanalyst validates a method to demonstrate to himself, as well as to other scientists, that the data produced are reliable.

International GLP requirements

Directives for bioanalytical method validation are not clearly addressed in the Good Laboratory Practice (GLP) regulations [6–9];

*Presented at the "Second International Symposium on Pharmaceutical and Biomedical Analysis", April 1990, York, UK.

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however, comments that have been made during inspections show that validation of analytical methods is audited, especially by the Food and Drug Administration (FDA). Moreover, a publication by Shah [1] stated the viewpoint that certain criteria must be defined and tested prior to a method being used to support regulatory studies. All GLP guidelines rely on the use of standard operating procedures (SOP) related to the tests conducted during the course of a study to ensure the quality and integrity of the data generated, and a SOP on validation of an analytical method is clearly required and therefore inspected.

A Quality Assurance Group (QAG) working party [10] made the important point that different groups of analysts will have different opinions about which criteria to select for validation but it is important that a system for validating methods is agreed and defined in a SOP.

Differences between pharmaceutical analysis and bioanalysis

It is appropriate first to discuss the differences between pharmaceutical analysis and bioanalysis to show the problems facing a bioanalyst when considering validating a method. Pharmaceutical analysis will be defined as the detection or measurement of an analyte in a pharmaceutical formulation, whilst bioanalysis is the detection or quantification of an analyte in a biological matrix. There are the following differences to consider:

Concentration. In pharmaceutical analysis the analyte concentration is fixed within known manufacturing limits. In bioanalysis, the concentration of the analyte is dynamic, being determined by the absorption, distribution, metabolism, excretion and sometimes chemical degradation of the compound. Other factors that can affect analyte concentration are the dose and its route of administration, drug formulation, whether food was taken prior to ingestion of a dose, species and sex.

Concentration range. The above effects may require the bioanalytical method to have a dynamic range of three or more orders of magnitude in order to monitor the analyte concentrations effectively. This means that analytical equipment, particularly a chromatographic detector, is often used at the lower limit of its specification to measure small concentrations and requires a large dynamic range. In contrast, immunoassays often require sample dilution in order to cope with such dynamic ranges of analyte concentrations.

Matrix effects. There are many different biological matrices that can be encountered in bioanalysis, a selection is presented in Table 1.

Table 1
Some biological matrices encountered in bioanalysis

Fluids	Tissues
Plasma/serum	Liver
Whole blood	Brain
Urine	Lung
Bile	Spleen
Stomach content	Kidney
Cerebrospinal fluid (CSF)	Skin
Milk	Faeces
Saliva	Adipose tissue
Lachrymal fluid	Heart
Vesicle fluid	Muscle
Broncoscopy fluid	
Semen	

Each presents a slightly different challenge to the analyst as each biological matrix can contain many components which can influence or interfere with the method.

Cellular material or debris — varying from erythrocytes in whole blood to disrupted cells in tissue homogenates. These can release enzymes capable of metabolizing an analyte or can sequester analytes with similar physicochemical properties within membranes present in the matrix.

Macromolecules — such as enzymes or other proteins, can bind to or degrade the analyte or interfere with binding in immunoassays. This may require special treatment to the matrix to overcome or inhibit these phenomena.

Metabolites or precursors — of the analyte or compounds in the matrix which can interfere with the detection of the analyte may have to be removed or separated.

Concomitant drug therapy — whereby co-administered drugs or their metabolites can interfere with the determination of an analyte.

Species differences. Different species and sexes can exhibit differences in absorption, metabolism and elimination of the analyte. In

addition, there are differences in the composition of the same matrix from different species which leads to the problem that an assay for a compound in human plasma may not be valid for measuring the same analyte in dog plasma.

Biomedical analysis places special emphasis on parts of the analytical method such as the sample preparation and detection stages to ensure the specificity of an assay.

Validation Parameters

What analytical parameters could be tested and monitored? Ideally, all bioanalytical methods should be evaluated against the parameters listed in Table 2 and defined below.

Analytical reference standard

Before validation can be contemplated, an authenticated analytical reference standard must be available to prepare solutions of known concentrations. This standard should be of a known form, e.g. free base or salt and of known purity, if used over a period of time, should be monitored to ensure no decomposition or contamination has taken place.

Precision

This describes the closeness of replicate determinations of an analyte by an assay. Precision can be further sub-divided into *within-day precision*, *intra-assay precision or repeatability* (which is an assessment of precision during a single analytical run) and *between-day precision*, *inter-assay precision or reproducibility* (which shows the variation of precision with time and may also include different analytical staff, equipment, and reagents).

Accuracy

This is the closeness of the determined concentration to the true value. Accuracy is measured as *within-day or intra-assay* (assessment of a single assay) and *between-day or inter-assay* (over several analytical runs) values; the latter figure is usually more representative and should be quoted. Accuracy is an absolute measurement and an accurate method depends on several factors such as specificity and precision.

Limit of detection (LOD)

The lowest amount of analyte that can be detected but not quantified. The calculation of the LOD is open to misinterpretation as some bioanalytical laboratories just measure the lowest amount of a reference solution that can be detected and others the lowest concentration that can be detected in the biological sample. The latter method is more meaningful as there may be a signal due to the matrix itself. This parameter is dependent on the background signal, whether it be due to endogenous substances or electronic noise, at the time of measurement and a better approach may be to use the limit of quantification.

Table 2
Bioanalytical method parameters that ideally should be examined for every assay and every analyte and every matrix

Calibration curve:	Number of standards, concentrations, replicates and line fit
Precision:	Intra-assay (within-day, repeatability)
	Inter-assay (between-day, reproducibility)
Accuracy:	Intra-assay (within-day bias)
	Inter-assay (between-day bias)
Limit of detection (LOD)	
Limit of quantification (LOQ)	
Working concentration range of the assay	What to do if the measured concentration is outside of this range: extrapolate curve or dilute sample
Linearity	
Selectivity (specificity)	
Matrix effects	
Recovery	
Stability:	Drug solutions
	In the biological matrix under storage and room temperatures
	Investigate stability in the matrix between sampling and storage
	Extracts pending analysis
Cross validation with another analytical technique	

This list assumes the availability of an authenticated reference standard with known purity and confirmed structure.

Limit of quantification (LOQ)

This is defined as the lowest concentration of analyte that can be measured with acceptable precision and accuracy by the method. The definition of *acceptable* is dependent on the aims and objectives of the work which the method is supporting and should be decided by the analyst.

Range and linearity

The *range* of a bioanalytical assay is the concentration interval over which an analyte can be measured with acceptable precision and accuracy. *Linearity* of a method is where the determined response is directly proportional to the analyte concentration. All bioanalytical methods should have a good working range but not necessarily be linear.

Stability

The stability of the analyte must be investigated under various conditions: in the standard solutions used to prepare calibration curves, in any biological matrix stored at −20°C and at room temperature prior to analysis and also in the final extract awaiting analysis. There may also be the need to investigate the stability of the analyte between the sample being taken and stored: some compounds are metabolized by esterases in the blood and have very short half-lives, therefore to stabilize the compound an inhibitor should be added, the effectiveness of which will need to be assessed and validated.

Selectivity

This is the power of the bioanalytical method to distinguish between the analyte and its metabolites, endogenous compounds and artifacts introduced by the analytical method. Initial experiments must be set up to demonstrate selectivity but it is an on-going exercise, throughout the application of the method.

Experimental Plans for the Initial Validation of a Bioanalytical Method

This section will cover the experimental plans, which in the opinion of the authors, should ideally be applied to the validation of every bioanalytical method. The expected results and error resolution of the validation tests are dependent on the nature of work supported by the method. An example of this is shown in Table 3 which outlines the requirements of an assay for the various stages of drug discovery and development. However, there is no universally accepted plan for validation, the testing will depend on the judgement of the individual scientist.

Table 3
Requirements for bioanalytical methods used in drug discovery and development

Development stage	Analytical objectives	Practical validation
Discovery	Comparative assessment of a number of related compounds for absorption, metabolism and elimination	Precision and accuracy to within ±20% or less Basic assessment of stability, and selectivity LOQ relatively high Preliminary recovery data
Preclinical	To support safety and metabolism studies in various animal species	Precision and accuracy to within ±10% or less Selectivity: the method must be revalidated for each animal species and matrix requiring analysis plus on-going evaluation Recovery calculated Stability investigated: on-going assessment LOQ: good sensitivity Range and linearity defined
Clinical	Method sensitive and specific for the determination of human pharmacokinetics Therapeutic monitoring High sample throughput	Precision and accuracy to within ±10% or less Selectivity: cope with the lower concentrations measured in man, known metabolites and concomitant medication. Method validated for each matrix to be analysed Ideal stability: minimum 3 months in samples at known storage conditions LOQ: at least 10% of the minimum effective concentration Cross-validated against another analytical technique Range and linearity defined for each matrix

Validate the method for each matrix

Each biological matrix from each species should be validated. A method that was developed for plasma from one species may not be applicable to urine from the same species or plasma from another species due to the presence of different endogenous compounds or analyte related material. Therefore additional sample preparation or modification of the analytical conditions may be necessary [4].

An area of increasing concern, which may require thought when validating bioanalytical methods in the future, is the supply of control matrix. A relatively large volume is required for standards, quality controls and blank samples during validation and routine operation. Ethical problems may be raised, and methods may need to be cross-validated with aqueous or artificial standard solutions for routine use, possibly with the use of correction factors.

Preparation of spiked samples. The samples used for the validation experiments are usually prepared from a control matrix with known additions of analyte. The preparation of spiked aqueous or semi-aqueous matrices such as plasma, serum, urine and cerebrospinal fluid are relatively easy, as the analyte is normally readily miscible in them. Ideally, the analyte should be added to the matrix to make a stock solution which is then diluted with the control matrix. However, many methods use the direct addition of suitable analyte concentrations to individual aliquots of the matrix. Inappropriate choice of solvent or spiking volume can cause protein precipitation and/or variation in extraction recoveries. If consistency of solvent additions is not maintained between standards and unknown samples then experimental bias can occur.

Matrices become more difficult to spike with analyte the less fluid they are and consequently the analytical results may have more error associated with them. Solid matrices may cause even greater problems [11].

When measuring endogenous compounds in biological samples, how does one obtain an analyte-free control sample? This may be achieved by either chemical degradation or enzymic conversion, e.g. histamine can be removed from plasma by using diamine oxidase, followed by deactivation of the enzyme. If this is impossible, the analysis can be performed using pooled material and the method of standard additions [12].

Analyte stability

As part of validation, analyte stability must be investigated, this is a continuing process that involves a number of components.

Standard solution stability. The analyst needs to know how long and under what conditions standard solutions of the analyte(s) can be kept. In the absence of any information, these solutions should be freshly prepared until evidence of stability is available. Failure to do this could result in standards that do not represent the true concentration of analyte, leading to erroneous determination of unknown samples.

Stability in the biological matrix. Analyte stability should be determined in each matrix and species likely to be studied.

(1) *How long can the sample be stored at both ambient temperatures and deep frozen before the analyte decomposes beyond acceptable limits?* This information is essential as it will determine the time that a sample can be left between sampling and freezing and the time samples can be stored and the time undiluted sample can be left in autosamplers.

To investigate ambient temperature stability, an experiment should cover 6 or 24 h for whole blood or plasma where replicate samples are taken and assayed at hourly or 2-hourly time points after spiking. Urine and faecal samples are usually collected over longer time periods (24 h or more) and stability tests should reflect this. If instability is found, the analyst should investigate what precautions should be taken to prevent degradation or artifact formation. This may include the addition of antioxidants or enzyme inhibitors to sample collection vessels or processing immediately after sampling.

Once the sample has been deep frozen, storage stability is investigated; this is a continuous process and may cover a long and indeterminate time period. Samples are spiked with the analyte at two concentrations, separate aliquots are prepared, and replicate samples taken for analysis at increasing time points until signs of instability are observed. The length of a stability study is usually determined by the initial storage requirements,

although it is always useful to know likely maximum sample storage times. Once instability is observed further experiments could be conducted to determine the actual time limits of storage.

The normal storage temperature for the majority of biological samples is −20°C. Plasma is normally frozen solid at these temperatures but around macromolecules, such as glycoproteins, water still has sufficient potential energy to be mobile at temperatures below −20°C. Urine, depending on the salt content, may still contain micro droplets of water at temperatures as low as −25°C which may influence stability of the analyte. Ideally laboratories should have facilities for storing samples at −40°C and −80°C which may overcome any instability problems observed at −20°C. Some instability problems may be overcome by various methods of sample pretreatment such as heat inactivation, alteration of pH or freeze-drying.

(2) *The effects of freeze–thaw cycles must be investigated.* This can be undertaken by spiking a control matrix with known concentrations of the analyte(s) and subjecting aliquots to freezing and thawing cycles. This approach will determine the stability of the analytes to freezing and thawing, but unless the stability of all metabolites is known, extrapolation of the findings to *in vivo* samples may be erroneous as unknown compounds (e.g. labile glucuronides) may break down to one of the analytes.

(3) *The final area to investigate is the stability of sample extracts.* This information could determine the size of the batch run and the maximum time between extraction and reanalysis. This area concerns mainly chromatographic analysis. In contrast, the final stage of a radioimmunoassay involves labelled material which is stable over several days.

Recovery

The recovery of an analyte from sample pretreatment prior to analysis is important as it is a determining factor in the limit of quantification of the whole analytical procedure. It can be used to improve extraction efficiencies and is an indicator of the robustness. Recovery can be calculated in one of two ways: either comparison of extracted and unextracted standards or using radioactive analyte. The former method can be applied to virtually all bioanalytical procedures provided that a pure reference standard exists. During initial method development, a preliminary indication of recovery can be obtained very quickly by comparing the responses of extracted and unextracted standards at the mid-point of the calibration curve. For a formal validation of a method, two calibration curves prepared in this way should be compared over the whole range of the assay to determine if recovery varies with concentration. The latter approach is limited by the availability of radio-labelled analyte and the facilities to use and measure it. Labelled analyte is added to the control matrix at physiological concentrations and then taken through the method. The advantage is that every fraction from the extraction scheme can be rapidly measured to ascertain where any losses occur.

Linearity of detector response

The linearity of chromatographic detector response should be established. This is important when an assay is required to quantify an analyte over a large concentration range. Solutions of pure analyte are injected in duplicate into the chromatograph and the response recorded. The increments between successive analyte concentrations should not exceed an order of magnitude in order to determine the concentration at which the detector becomes non-linear. This is not usually done if the dynamic range required of the method is one or two orders of magnitude but is encouraged as the bioanalyst can never guarantee when the range of an assay needs to be extended.

Range and linearity of the assay

During the validation, procedures should be developed to cope when a result is outside of the calibration range of the assay. There are a number of options to consider:

— How far can a calibration line be extrapolated outside of the concentration range of the standards?
— Should a sample be repeated using a smaller or larger aliquot to ensure that the result is within the working range of the assay? If so, what is the effect of the matrix when a larger sample volume is assayed? If a smaller volume is used, should it be made up to the nominal volume used in the assay by using the control matrix or a buffer?
— Should the analysis be repeated after dilution of the sample? What are the effects

of diluting the sample with either a buffer or control biological fluid?

The option chosen will depend on the analytical technique used, e.g. immunoassay or chromatography, and the linear range of the detector used. Regardless of the approach, the analyst should have this information available before it is required rather than having to investigate retrospectively.

Precision and accuracy experiments

Initial validation of a method should give an indication of the intra-assay and inter-assay variation and accuracy; therefore two experiments should be set up. Each will have six replicates at three concentrations covering the top, middle and bottom of the calibration curve. After comparison with calibration standards, the analyte concentration in the samples can be measured and then the precision and accuracy of the assay can be calculated. Readers may question the approach of using separate samples for this work, but it is the authors' opinion that the samples for precision and accuracy should be determined against a normal set of calibration standards. For more detailed validation, a third experiment should be performed to give more confidence in the robustness of the assay.

Precision is expressed either as relative standard deviation (RSD) or coefficient of variation (CV); the smaller the values the better the assay performance. As precision can vary over the range of an assay, it is important to determine it at the top, mid point and bottom of the range as a minimum requirement. Usually the precision of an assay is constant over most of the range of an assay but it may decrease at the extremes of the calibration curve, hence the importance of testing at the top and bottom of the range.

The accuracy of analytical methods is expressed as percentage bias or error which can be a positive or negative figure; like precision the lower the value, the better the assay performance. Accuracy can be measured against an in-house, an inter-laboratory or an international standard. In some respects accuracy is a difficult parameter to measure for two reasons. Firstly, accuracy is an absolute measurement and most analytical methods used in a bioanalytical laboratory are comparative, e.g. chromatography and immunoassay. Secondly, how closely does a spiked sample reflect the *in vivo* situation? Comparison with another more selective method, where this is possible, can provide more information about the accuracy of the method.

The limit of quantification (LOQ) should be assessed by analysing replicate samples of low analyte concentrations to determine at what concentration acceptable limits of precision and accuracy have been exceeded. The limit of detection (LOD) is often taken as two or three times the background response which may be due to electrical noise and/or minor chromatographic peaks but should be determined in samples derived from the biological matrix. Estimates of concentrations that fall between the LOD and LOQ may be useful in some cases for pharmacokinetic interpretation, but in this instance an indication of the precision attainable at these concentrations is highly desirable lest too much weight be put on such results.

Selectivity

Assay selectivity is an important part of bioanalytical method validation as the main techniques used for analysis are comparative and not absolute. This means that standards are used to calculate the analyte concentration in the samples. There are two sources of interference in bioanalytical methods: *endogenous* substances that are present in the biological matrix and *exogenous* material that arises from the apparatus (e.g. plasticizer additives) and reagents used in the method. In addition, binding of the analyte to the glass or plastic vessels used in the analysis is a common phenomenon [11].

The impact of interference on an assay can be variable. This can range from a small but constant bias at very low concentrations to intermittent problems caused by interference arising from the use of different batches of reagent or materials, and such factors can make an assay impracticable. Therefore the specificity must be monitored continually to ensure that there is no interference.

The nature of the analytical techniques used means that the basic assumptions of each must be investigated, for example peak purity in chromatography and antibody-binding in immunoassay. Therefore work must be undertaken to test these assumptions continually during the use of the assay.

The initial application of a chromatographic assay in a drug's discovery may involve the use of predose samples only with reagent and

matrix blanks included with each analytical run to monitor assay specificity. However in drug development, specificity investigations may also include the use of diode array or mass spectroscopic detectors to improve confidence in the method. Immunoassay methods require an investigation of cross-reactivity with similar structural compounds, non-specific binding and matrix effects as a means of ensuring specificity. As more information about the compound becomes known then any metabolites, co-administered drugs and their metabolites should be tested for either co-elution or cross-reactivity as appropriate.

Often there are instances when one analytical method will be replaced by another; during the change-over period the results generated by the two methods on the same samples should be compared.

Additional validation options

Other factors to consider when validating a method may be turn-around time, cost per analysis and data management costs. Turn-around time may be a required option depending on the context of the result; with accident and emergency cases the physician may require to know if a patient has ingested a poison and request measurement of its concentration in body fluids. Time in this context may be life-saving hence the need to validate the correctness of the analytical result when the analyst is operating under pressure. Again, rapid turn-around and high throughput may be required to support early studies on new drugs in man when little pharmacokinetic information may be available. Assay performance needs to be assessed critically in these circumstances because of the possibility of unknown factors affecting results.

In a cost conscious business environment, it may be essential to assess the method against operating costs such as those for instrument time, disposable apparatus required and analyst's time, and against the efficiency of the data management procedures.

Routine Operation of a Bioanalytical Method

Once validated, a method should be monitored by quality control procedures to show how it performs routinely.

In-house monitoring of assay performance

Once the initial stability of the analyte, as outlined earlier, has been determined, then analysing quality control (QC) samples is the most popular method for monitoring assay performance. The assay precision, defined during validation, sets the acceptable limits of performance, and the results can be displayed on a Shewart plot [12]. Alternatively, the same QC results can be plotted as a cumulative summation (cusum) [12].

Brooks and Weinfeld [2] used two concentrations of QC samples near the ends of the calibration curve to monitor assay performance. This is a good approach but the acceptance or rejection criteria of any QC results that fall outside the predefined limits should be established. Such criteria may be dependent on how much sample remains to repeat the analysis, how far outside the limits the results were and the speed at which the results are required. In the end it is the responsibility of a bioanalyst to judge whether to accept or reject the results of an analytical run.

Method modification and revalidation

If changes are made to a validated method, then it is the bioanalyst who must judge how much revalidation should be undertaken. This can range from a change in the grade or supplier of a chemical to a major modification of the method: the greater the modification, the greater the need to revalidate the method. In this latter context, precision and accuracy, LOQ and LOD are the minimum parameters that should be redetermined. Changes in stability, apart from that in different sample extracts should not occur.

Acknowledgements — The authors wish to thank Dr P.D. Whiteman, Department of Bioanalytical Sciences, Wellcome Research, and Dr B.V. Fisher, Wellcome Biotech for discussions and comments during the preparation of this article.

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[*Received for review 5 April 1990; revised manuscript received 1 May 1990*]