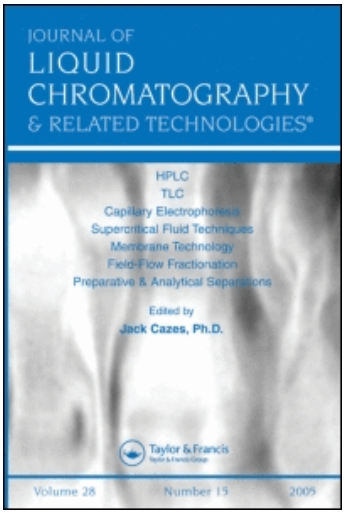

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

BIOANALYTICAL LIQUID CHROMATOGRAPHIC METHOD VALIDATION. A REVIEW OF CURRENT PRACTICES AND PROCEDURES

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Online publication date: 18 January 2000

To cite this Article Rosing, H. , Man, W. Y. , Doyle, E. , Bult, A. and Beijnen, J. H.(2000) 'BIOANALYTICAL LIQUID CHROMATOGRAPHIC METHOD VALIDATION. A REVIEW OF CURRENT PRACTICES AND PROCEDURES', Journal of Liquid Chromatography & Related Technologies, 23: 3, 329 — 354
To link to this Article: DOI: 10.1081/JLC-100101455
URL: http://dx.doi.org/10.1081/JLC-100101455

J. LIQ. CHROM. & REL. TECHNOL., 23(3), 329–354 (2000)

BIOANALYTICAL LIQUID CHROMATOGRAPHIC METHOD VALIDATION. A REVIEW OF CURRENT PRACTICES AND PROCEDURES

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ABSTRACT

Validation of analytical methodologies is widely accepted as pivotal before they are put into routine use. Within the guidelines issued by Regulatory Authorities, there still exists scope for individual interpretation with respect to their conduct and acceptance criteria. The intention of this paper is to review the performances used 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 studies. A survey of recent literature on liquid chromatographic procedures used in the bioanalysis of anticancer drugs revealed that very variable standards were employed for validation.

INTRODUCTION

Before an analytical method can be implemented for routine use, it is widely recognized that it must first be validated to demonstrate that it is suitable for its intended purpose. While the need to validate analytical methods is clear, the procedures of performing a rigorous validation programme are not generally defined. Questions of interest include (i) which validation parameters should be established, (ii) what specific procedure should be followed to evaluate a particular parameter, and (iii) what is the appropriate acceptance criterion for a certain parameter?

Two factors complicate the definition of an effective validation protocol. Firstly, the applications of chromatographic methods are broad and diverse. Secondly, existing guidelines for general classes of applications allow scope for individual interpretation. The following approaches can be utilized to determine which operational parameters should be included in a formal validation protocol:

> . . . guidelines published by organizations/bodies with recognized authority like the United States Pharmacopeia (USP), Committee for Proprietary Medicinal Products (CPMP) and the Center for Drug Evaluation and Research (CDER) of the US Food and Drug Administration,[1-4] or chemical literature.[5-33]

Guidelines appropriate for every specific analytical situation do not exist. USP and CPMP guidelines are meant for pharmaceutical analysis like identification tests, quantitative tests for impurities, quantitative tests in the drug product, etc., but not for drug analysis in biological matrices. Validation of bioanalytical methods employed to generate data for bioavailability, bioequivalence, and pharmacokinetic studies can be approached by a variety of techniques and is subject to many different methods of interpretation. Meaningful pragmatic guidance with respect to establishing appropriate validation parameters in bioanalytical chromatographic methods, however, can be received from the Washington Conference Report.[5]

In order to gain more insight into the present state of thinking on the general topic of analytical method validation and to put various bioanalytical validation procedures into perspective, a literature review was performed where existing guidelines from several governmental bodies were included. Parameters that should be considered during the validation of bioanalytical chromatographic methods are discussed.

The purpose of this review is to summarize views and to provide some guidance on how to handle the various validation parameters. To answer the question of how validation is put into practice, 37 publications dealing with liquid chromatographic procedures used in the analysis of anticancer drugs were critically reviewed.

PRACTICES AND PROCEDURES

Before validation can commence, an analytical reference standard must be available to prepare stock solutions of known concentrations. This standard should be fully chemically characterized with known purity and stability.[3,4,6,7] The correction for the purity of the reference standard should be included in the calculation of the concentrations. Spiked control samples and calibration standards in the biological matrix should be prepared independently from different stock/working solutions (separate weighings of the reference standard).[8] The volume added to the blank matrix should be less than 2-5% of the matrix volume to simulate a real sample as much as possible.[6,9] Dilution of the biological matrix with working solutions (>1:20) must be compensated by adding an equal volume of the blank working solution to the study samples. Due to poor solubility characteristics of reference standards in water, the use of organic solvents may be inevitable. In these cases the excess of solvent can be evaporated to dryness and the analyte may then be reconstituted into the biological matrix. Ideally, the analyte is dissolved in the blank matrix and then diluted with blank matrix. For each biological matrix, including the same matrix from different species, a validation program should be completed.

An efficient design for the experimental set-up of a bioanalytical method validation has been described recently.[10] It is advisable to establish that selectivity, short term stability, and robustness are acceptable prior to the estimation of parameters which require analysis of a large number of samples, for example accuracy and precision.[10,11] System Suitability Tests (SSTs)[12] to confirm that the method maintains suitability, are not discussed here because these tests should be run in the application phase of the method. The following validation parameters are considered essential:

Specificity and Selectivity

Definition

A method is specific if it produces a response for only one single analyte.[13,14] Since it is almost impossible to develop a chromatographic assay for a drug in a biological matrix that will respond to only the compound of interest, the term selectivity is more appropriate. The selectivity of a method is its ability to produce a response for the target analyte which is distinguishable from all other responses (e.g. endogenous compounds).[7,13,15,16]

Procedures and Acceptance Criteria

Several batches of blank matrix need to be analyzed to determine whether endogenous substances interfere with the assay. Endogenous substances include compounds normally occurring in the biological fluids or tissues (e.g. hormones, proteins, carbohydrates, lipids, dietary substances, etc.).[11,13] The number of

batches that should be analyzed is six.[5,6,10,11,13,14] In practice 10-20 sources will be necessary to obtain meaningful results and to get insight into inter-batch variability.[13] Usually some tested blanks will contain minor interferences. Although the criteria for interferences are often set arbitrarily, they can affect the determination of analyte around the lower limit of quantitation (LLQ) substantially. To avoid this, pre-defined precision and accuracy criteria for the quantitation at the LLQ should be set and respected.[11,13] Frequently deviations and variations up to 20% from the nominal concentrations are tolerated.[5]

To investigate possible interferences by co-administered drugs, formulation vehicles, analyte precursors, metabolite(s), and/or degradation products, amounts of these compounds must be added at the maximum expected concentration in the samples, and analyzed to assure the assumed selectivity of the method.[6] When analyte metabolites or degradation products are not available to serve as reference material, analysis of biological samples from treated subjects is the next best option. Other ways to obtain the respective metabolite(s) and degradation product(s) to establish the selectivity are *in vitro* incubations of the parent compound with liver homogenate or microsomal fractions[11,13] and by stress testing (acid/base hydrolysis, heat, photolysis, oxidation).[3,4]

If the analyte concentration is high enough and the possible interferences have different chromophores, thus different UV spectra, peak purity can be assessed by the use of diode array detector.[4,6,9,11,13,14] Specificity of a method can be enhanced by changing to mass spectrometric detection methods.[6,9,11,14] Mass spectrometric detection provides information on the identity and the purity of the analyte. A good alternative for mass spectrometry to detect any potential interferences, is the use of a second chromatographic method based on different chromatographic mechanisms and which has previously been validated.[9,14,16]

The influences of exogenous interferences on the selectivity of the method can be easily assessed by processing "reagent blanks" in the absence of biological matrix (replacing the biological matrix with an equivalent amount of pure water). Exogenous substances include: reagents and their impurities used in sample work-up, substances used in the manufacture of labware (e.g. plasticizers) or resulting from incomplete washing of labware, apparatus.[7,11,13] The same procedures should be followed, using the same equipment, reagents, etc. If exogenous interferences are found, the source has to be determined and eliminated.[13]

Limit of Detection

Definition

The limit of detection (LOD) is the lowest concentration of analyte in the sample that can be detected but not quantified under the stated experimental con-

ditions.[1,2,4,7,14] The LOD is also defined as the lowest concentration that can be distinguished from the background noise with a certain degree of confidence.[5,6,9,11,14,16,17] There is an overall agreement that the LOD should represent the smallest detectable amount or concentration of the analyte of interest.

Procedures and Acceptance Criteria

The LOD is largely dependent on background noise, which is measured as the difference between the maximum and minimum value of the blank-signal, ("peak-to-peak noise"). These fluctuations are due to signals from endogenous substances in the matrix and electronic signals from the equipment. The LOD should therefore be determined by injecting the blank biological sample rather than the reference solution.[7]

For chromatographic analysis the noise level should be determined by measuring the variation in the baseline noise in the region of a blank chromatogram where the analyte peak is expected.[12,17] This region is often set at 20 times the width of the analyte peak (either at half height of the peak[12] or the peak width at the baseline[17]).

The noise is measured as the largest peak-to-peak height fluctuation and its standard deviation is the largest peak-to-peak height divided by 5.[17] Once the noise value has been determined the LOD can be obtained by the following approaches:

Based on the signal-to-noise approach. Blank samples are measured together with samples with concentrations of the analyte at the expected LOD. The signals of the blank and the analyte samples are compared and expressed as a signal-to-noise ratio. The found LOD is considered to be acceptable when the signal-to-noise is equal or larger than the predefined ratio. Different ratios have been considered to be acceptable varying between 2 and 5.[1,3,7,12,16]

Based on the standard deviation of the noise. The LOD is defined as 2-3 times the standard deviation of the background noise of an appropriate number of representative blank samples.[1,3,18]

Based on the standard deviation of the noise and slope of the calibration curve. The LOD is calculated as 3.0 or 3.3 times the standard deviation of the noise divided by the slope of the calibration curve.[3,15-17] The standard deviation can be estimated based on an appropriate number of blank sample signals or can be extracted from the calibration curve. The residual standard deviation of a regression line (in the range of the LOD) or the standard deviation of the y-intercept of the regression line may used as the standard deviation of the noise.[3]

The LOD is not a very stable characteristic because of its susceptibility to minor changes in the conditions of the analytical method, like temperature, purity of reagents, sample matrices, and instrumental system changes.[16] For this reason the LOD concentration level should not be included in the calibration curve. Although the LOD is not of great importance for the analysis of drugs in biological samples it has been used to estimate the lower limit of quantitation (LLQ).[7,16] The LOD as well as the LLQ, (though the latter less frequently) are often reported as being synonymous to the sensitivity of the analytical method.[6,9,10,15,16,19] This is, however, incorrect because these terms are defined in different ways. The sensitivity of the method is obtained by plotting the measured signals against the sample concentrations. The calculated slope of the linear calibration curve is the sensitivity of the method.[6,15,16]

Lower Limit of Quantitation

Definition

The lower limit of quantitation (LLQ) is the lowest concentration of the analyte in the sample that can be measured with acceptable accuracy and precision under the stated experimental conditions.[1,2,4,6,7,14,16,17] The LLQ-value is determined by the presence of background signal (accuracy) and the reproducibility of the analytical method (precision). The LLQ is the lowest concentration point in the calibration curve.[5,6]

Procedures and Acceptance Criteria

The LLQ can be determined in numerous ways. The most common procedures are given here:

Based on samples spiked with analyte. The LLQ can be assessed by analyzing at least five replicates of the control samples together with calibration samples, in separate runs. The analyte samples should be prepared independently from the calibration samples (different stock solutions with separate weighings of the reference compound) in the biological matrix at known concentrations around the expected LLQ. At the LLQ the deviation from the nominal concentration as well as the relative standard deviation within each run should not exceed the required precision and accuracy level of 20%.[5,11,13]

Based on the signal-to-noise approach. The signals of the blank samples are compared with the signals from samples which contain known low concentrations of the analyte. Next, a signal to noise-ratio at which the analyte can be reliable quantified is determined. Typical ratios of 3, 5, and 10 have been used to define the LLQ.[3,13,14,16]

Based on the standard deviation of the noise. The LLQ is defined as 10 times the standard deviation of the background noise of an appropriate number of representative blank samples.[1,3,16]

Based on the standard deviation of the noise and the slope of the calibration curve. By this approach the LLQ is expressed as 10 times the standard deviation of the noise divided by the slope of the calibration curve.[3,14,15,17] The standard deviation of the noise can be estimated as described in the section "Limit of detection".

Based on the calculated confidence intervals around the calibration curve. Calibration data sets are fitted (by unweighted linear regression), after which the confidence intervals are calculated.[20] The LLQ is then defined as the concentration for which the interval at the 95% (or any other) probability level does not overlap with the confidence level of the blank matrix standard.

This method exhibits some weaknesses. For instance, the LLQ is derived from data that uses one single matrix source. Another drawback is that there is a 2.5% possibility for a false positive value for the LLQ at a 95% probability level, in case there is an overlap of the confidence level of the LLQ with the confidence level of the blank standard.[13]

Based on background interferences and reproducibility of the response. The responses from the estimated LLQ and blank matrix samples are determined in four analytical runs. The means of the obtained responses are tested statistically (with the *t*-test).

If the difference is significant, the variability of the response of the estimated LLQ sample is evaluated by comparing the mean response to its standard deviation. If the mean response is equal or greater than 3 standard deviations, the concentration is accepted as the LLQ.[21]

Based on the confidence intervals. The LLQ has also been defined as the concentration at which the lower confidence level of the mean response is at least four times greater than the upper confidence level for the mean blank response of approximately 10 independent matrix sources.[13]

The measured concentration of the proposed LLQ should lie between 80 and 120% of its theoretical concentration and the relative standard deviation should be less than 20%.[5,11,13,22] These criteria are generally accepted.

LLQ values which do not meet the precision and accuracy criteria may lead to unreliable results. To avoid this the LLQ value should be increased to that concentration where the criteria are met.

Linearity, Range of the Calibration Curve

Definition

Linearity means that the assay provides test results which are proportional to the concentration of the analyte in the sample within a given range, either directly or via a mathematical transformation.[6,42,49,51] The range of an analytical procedure is the interval between the upper and lower limits of quantitation (ULQ and LLQ) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity.[1,2,6,7,14]

The concentration range of the calibration curve should at least span those concentrations expected to be measured in the study samples. Usually the dynamic range of the analyte in bioanalytical methods span 3 or more orders of magnitude.[7] If the total range cannot be described by a single calibration curve, two calibration ranges can be validated.[6] If the available study sample volume is less than the validated sample volume, a partial volume can be used for analysis, provided that it is supplemented with blank matrix to the validated volume. As an alternative to the extension of a calibration curve for the measurement of concentrations which are above the highest calibration standard, the sample can be re-analyzed after dilution with blank matrix.[7,11] These procedures need to be validated. To prevent this situation the range is often chosen wider than the expected concentration range in the study samples. Nevertheless it should be kept in mind that the accuracy and precision of the method will be negatively affected at the extremes of the range by extensively expanding the range beyond necessity.[6]

Procedures and Acceptance Criteria

The calibration curve should consist of at least 4-8 non-blank calibration standards analyzed in duplicate,[5,6,15,21,22] or 5-10 non-blank calibration standards in singular.[5,6,9,11] When using more than 7-8 non-blank standards a maximum of two non-blank standards may be rejected from the calibration curve if they are not adjacent, leaving a minimum of 5 non-blank standards remaining valid.[6,10,11] Inclusion or exclusion of standards in the calibration curve should be established *a priori*.[11] The concentrations of the calibration standards should be equally distributed over the total concentration range and the same matrix as for the study samples should be used. For the construction of the calibration curve the blank matrix should not be included, although a blank may be used to check the intercept.

Many calibration curves are calculated by least-squares linear regression, which assumes that variance is independent of the analyte concentration. This assumption, however, is not justified for many analytical methods, particularly when the calibration range expands more than a few orders of magnitude. When the standard deviation is not constant, the calibration data ought to be weighted to increase the accuracy of the lower concentrations.[9,15,23]

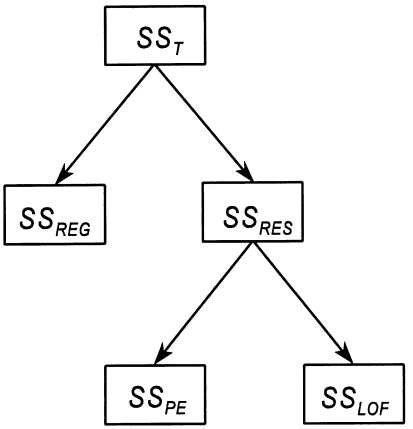

Figure 1. Breakdown of the total sum of squares(SS) when applying a lack-of-fit test.

In order to establish the best weighting factor, back-calculated calibration concentrations are determined. The model with the lowest total bias and the most constant bias across the concentration range of interest is considered to be the best fit.[6,24] Calibration curves should never be forced through zero, as this ignores a potential bias.[6] It is not an absolute requirement to work with a linear model, however, straight-line data are less variable than data from non-linear calibrations and thus preferred.[19] To prove whether the proposed linear model is correct, a linearity test should be applied. There are several tests in use to test the linearity of the method:

Lack-of-fit test.[10,19,21,24,25] This statistical test also provides the information of whether a non-linear model may be more appropriate in some cases. The test requires replicate determinations ($N \geq 2$) at each calibration concentration. When applying the test (see Figure 1) the total sum of squares (SS_T) is broken up into the sum of squares due to regression (SS_{REG}) and the residuals (SS_{RES}) which encompasses the remaining variability of the regression line. The SS_{RES} from the regression is decomposed in a pure error term (SS_{PE}) and a lack-of-fit component (SS_{LOF}). The performance of the lack-of-fit test using ANOVA is accurately described by Lang and Bolton.[21] Wieling et al. also use a lack-of-fit test to evaluate linearity.[10] However, the ANOVA was not weighted, although the calibration curves were calculated by weighted linear regression (weighting factor: X^{-1}, the reciprocal of the analyte concentration). To illustrate how to perform a lack-of-fit test using weighted ANOVA, an example is given in Table 1.

Table 1

Lack-of-Fit Test with Weighted ANOVA, Using Dummies (d1-d4) to Determine the Sum of Squares of the Pure Error (SS_{PE})

Concentration (ng/mL)	Response	Weighting Factor (1/x)	d1	d2	d3	d4
10	0.0147	0.100	0	0	0	0
50	0.0659	0.020	1	0	0	0
50	0.0644	0.020	1	0	0	0
100	0.1491	0.010	0	1	0	0
100	0.1449	0.010	0	1	0	0
500	0.6801	0.002	0	0	1	0
500	0.6467	0.002	0	0	1	0
1000	1.3192	0.001	0	0	0	1
1000	1.3422	0.001	0	0	0	1

Analysis of Variance*

	DF	Sum of Squares (SS)	Mean Squares (MS)	F
Total (T)	8	5.010999×10^{-3}	6.263749×10^{-4}	----
Regression (REG)	1	5.006103×10^{-3}	5.006103×10^{-3}	----
Residual (RES)	7	4.896000×10^{-6}	6.994286×10^{-7}	----
Pure Error (PE)	4	1.491000×10^{-6}	3.728500×10^{-7}	----
Lack of Fit (LOF)	3	3.405000×10^{-6}	1.135000×10^{-6}	3.04
$F_{0.05(3,4)} = 6.59$		$F < F_{0.05(3,4)}$	No lack of fit	

* DF: degrees of freedom; $F_{0.05(3,4)}$: critical value for F with a significance level of 5% and 3,4 degrees of freedom.

To determine SS_{RES}, linear regression with a weighting factor of 1/x is performed with the response as the dependent and the concentration as the independent parameter. An additional analysis of variance is executed to determine the SS_{PE} with the response as the dependent, the dummy the independent parameter, and a weighting factor of 1/x. The difference between SS_{RES} and SS_{PE} is SS_{LOF} (Figure 1). F is determined by calculating the ratio of the mean squares (MS_{LOF}/MS_{PE}). If F is less than or equal to the critical value of F with a level of significance of 5% (α=0.05), linearity can be accepted. The weakness of a lack-of-fit analysis is that the more precise the data, the less the likelihood of passing the test.[10,15]

Applying linear regression analysis to the logarithm of the concentration versus detector response, a linear graph is obtained with a slope not significantly different from 1, in case of linearity.[15,24]

In the polynomial regression approach, linearity is determined when the coefficient on the concentration squared term is not significantly different from zero.[24] This method requires no replicate determinations at each concentration. However, in bioanalytical practice this test has only benefited when the quadratic model is statistically valid.

A high correlation coefficient (>0.99 or 0.999) of the calibration curve is often used to state linearity.[4,6,22,24] Although good linearity always provides a correlation coefficient close to 1, a high correlation coefficient does not necessarily imply linearity. The correlation coefficient merely gives an indication of the absence or presence of a response-concentration relationship.[17,19] Therefore, by assessing an acceptable high correlation coefficient alone the linearity is not guaranteed and further tests on linearity will still be necessary.[26] The slope of the calibration line and its variance do not provide a measure of linearity as stated in the USP.[1] These data are a measure of reproducibility of the detector signal in time.

Accuracy

Definition

Accuracy is defined as the closeness of test results to the nominal value (in house standard) or to the accepted reference value (international standard e.g. pharmacopoeial standard).[1,2,4-7,14,16,17,27,29] Accuracy, together with precision, determines the error of the analysis and is, therefore an important criterion in the evaluation of an analytical method.

Procedures and Acceptance Criteria

The two most commonly used ways to determine the accuracy or method bias of an analytical method, are (i) analyzing control samples spiked with analyte[12,15,25] and (ii) by comparison of the analytical method with a reference method.[15,25]

The accuracy of the method can be determined by analyzing spiked control samples with analyte concentrations around the LLQ, 2-5 times the LLQ, 0.5 times the ULQ, and the ULQ. An additional quality control concentration above the ULQ should be measured after appropriate dilution of the sample to the validated concentration range.[10,11] Each control sample should be analyzed in a minimum of 5 replicates together with a calibration curve, independently prepared from the control samples, in at least 3 analytical runs. The accuracy is usually determined in percent of the nominal concentration or in percent difference

between the mean concentration and the nominal concentration.[6,10,14,27] This is also referred to as the within-run accuracy. The average accuracy is calculated by dividing the average of the accuracy values for each run, and the number of validation runs. This is referred to as the between-run accuracy.

Another approach to asses the accuracy is to perform a two-sided t-test to reveal any significant difference between the mean of the data and the nominal value with a 95% level of confidence.[21,27] However as the t-test approach proved to be a more stringent test for accuracy, the question is not whether there is any statistical difference but whether there is an acceptable difference between the measured and nominal concentrations. As accuracy criterion it is generally accepted that the mean value should not exceed ±15% of the nominal value, except for the mean value round the LLQ, where it should not deviate more than ±20%.[5,6,25] These fixed range criteria are somewhat subjective and the researcher should be guided by what kind of decisions are made upon the acquired data.

The method to be validated can also be compared with an independent, widely accepted reference method. Both methods are applied on at least 6 different samples in duplicate by the same analyst. Paired observations are achieved in this way. A t-test is applied on the differences between the paired observations. The magnitude of a significant difference is a measure for accuracy. The validated method is considered accurate when no significant difference is found.[7] The disadvantage of this method is that the applied reference method is assumed to be free of systematic errors.

The analytical results can also be compared in a graph in which the determined values are plotted against the known concentrations. Linear regression analysis should be performed on the results according to the least-squares method. The blank matrix must be excluded from the calculations. The values of the slope and the intercept can be tested from the nominal values (1 and 0, respectively) by applying t-tests.[9,26] When the intercept is not equal to 0, a constant systematical error has been detected. Proportional systematic errors are concentration dependent and can be demonstrated when the slope significantly differs from 1.

Precision

Definition

Precision is defined as the closeness of the analytical results obtained from a series of measurements of the same homogeneous sample under prescribed assay conditions.[1,2,4-7,14,27] Precision is an indication for random error, the degree of scatter. The precision can be further subdivided into the method's repeatability and reproducibility. Repeatability is the precision of the analytical method in which the variability in experimental conditions is kept within a narrow range and over a short time interval (same analytical run), whereas reproducibility is the

precision determined under a maximum variety of conditions over a longer time interval.[27] The duration of these time intervals are not defined. Within/intra-day, -assay, -run and -batch are commonly used to express the repeatability. Expressions for reproducibility of the analytical method are between/inter-day, -assay, -run and -batch. The expressions intra/within-day and inter/between- day precision are not preferred, because a set of measurements could take longer than 24 hours or multiple sets could be analyzed within the same day.[15]

Procedures and Acceptance Criteria

There are several methods available for the determination of the precision. The most commonly applied method is the measurement of calibration samples together with a set of independently prepared quality control samples to determine the within- and between run precisions. For the determination of the within-run precision a minimum of 3 different concentrations covering the low, medium and high range of the calibration curve are needed. The concentrations should be selected around the LLQ, 2-5 times the LLQ, 0.5 times the ULQ, and the ULQ. A minimum of 5 replicates per concentration level must be analyzed. The standard calibration samples should be independently prepared from the quality control samples but measured during the same analytical run. This procedure is to be repeated preferably on at least 3 occasions.[7-10] To obtain meaningful data, the stability of the samples, reagents or stock solutions must be adequate during the period of the study.

In general the coefficient of variation is used to report the precision.[1,3-7,19,27] However, analysis of variance (ANOVA) is a statistically better approach to determine the within- and between-run precisions.[9,10,21,27] The following formulas are to be used to calculate the precisions:

$$\text{Within - run precision} = \frac{\sqrt{MS_{WG}}}{GM} \times 100\%$$

$$\text{Betw. - run precision} = \frac{\sqrt{\frac{(MS_{BG} - MS_{WG})}{n}}}{GM} \times 100\%$$

where, MS_{WG} is the mean square of the within groups/runs, MS_{BG} the mean square of the between groups/runs, GM the grand mean of the measured control concentration, and n the number of determinations per group/run. When MS_{WG} is greater than MS_{BG}, there is no significant additional variation due to the performance of the assay in different runs. Some authors overestimate the between-run precision by assuming that the expectation of MS_{BG} is the between-run error variance.[10,27]

However, if at least two of the population means (mean concentrations per run) are not equal, the expectation of MS_{BG} ($E(MS_{BG})$) is[28]:

$$E(MS_{BG}) = MS_{WG} + n \times \text{betw. - run variance}$$

The general acceptance criteria are the same for the within-run and between-run precision. For the LLQ it should be less or equal to 20%. For the other higher concentrations it should be less or equal to 15%.[5,6] Separate criteria for accuracy and precision, as proposed in the Washington Conference Report,[8] leads to the acceptance of a relative high total measurement error.[15,29] For example to ensure that 95% of the test results fall within the interval of ± 15% an accuracy and precision of ≤8% (N=5) are required.[29]

Recovery

Definition

Absolute recovery is the amount of analyte extracted from a spiked matrix standard expressed as a percentage of the compound which has not been subjected to sample pretreatment.[15] In most cases biological samples cannot be assayed directly, but require a pretreatment to obtain a suitable sample for analysis. The preparation usually includes isolation steps, which may cause some loss of analyte. If an internal standard is used in the analytical method, the recovery of this compound should be determined independently at the concentration level used in the analytical method.

Procedures and Acceptance Criteria

The recovery is determined by comparing the responses from processed (e.g. biological) and unprocessed (e.g. non-biological) samples.[7,8] If a radioactive analyte is available, every fraction can be traced back easily to ascertain where losses occur.[7] Mean responses of the processed samples at low and high concentrations are compared with the mean signals from the unprocessed standards in one single run or in several runs.[6,8,10,11] Since the recovery can be concentration dependent, it should be determined whether the results are constant throughout the concentration range of interest.[7,16] Another approach to estimate the recovery is by dividing the slope of the calibration curve of the processed samples by the slope of the unprocessed calibration curve. The recovery can then be calculated over the entire range.[15,16]

The recovery should be as high as possible, but it is even more important to achieve consistent recoveries throughout the range of the calibration curve[7,8] to ensure the reproducibility[6,11,16] and selectivity[11] of the analytical method. The recovery should be expressed as a percentage of the direct sample analysis

(unprocessed samples), which is defined as 100%. Although a recovery of 100% is most desirable, in practice analyte and internal standard recoveries higher than 70% with a variation of 15% are accepted.[6,15]

Stability

Definition

An analyte is stable under certain conditions during a certain period of time when the responses compared to freshly prepared samples do not change significantly over time. It is of great importance for the reliability of the assay that the analyte is stable in the matrix for the duration of the test. The aim of a stability test is to detect any degradation of the analyte(s) of interest, during the entire period of sample collection, processing, storing, preparing, and analysis.[16] All but long term stability studies can be performed during the validation of the analytical method. Long term stability studies might not be complete for several years after clinical trials begin. The conditions under which the stability is determined is largely dependent on the nature of the analyte, the biological matrix, and the anticipated time period of storage (before analysis).

Procedures and Acceptance Criteria

Commonly, spiked blank samples are used to conduct stability tests, after which the results are extrapolated to the study samples. This is considered adequate if metabolites do not revert back to the parent drug or metabolism continues to occur in the sample after it has been withdrawn. As it is usually not possible to rule out this possibility it is advisable to incorporate real study samples to assess the stability.[6,7,11,13] Stability data are acquired at two or three different concentrations (low, medium and high) at different time intervals after storing, performing replicate (2-10) analysis.[9,11,13,30] For the quantification, fresh calibration samples must be prepared from stock solutions using the same substance batch and same blank biological matrix as used for the stored samples.[30] A t-test can be applied to assess analyte stability. The analyte is considered stable, when no significant concentration difference is found between the two sets of samples. Alternative approaches to assess analyte stability are (i) the concentrations estimated from the regression line are higher than a pre-determined percentage (90-95%) of the initial concentration[6,10] or (ii) the 95% lower confidence limit is higher than 90% of the initial concentration[6] or (iii) the 90% confidence interval of the calculated difference in response is less than -10%.[30] The following types of stability evaluations are required:

> *Stability of the stock solutions and dilutions*. The stability of the standard solutions of the analyte and internal standard should be evaluated to cover the time interval from preparation until use. The stabilities of the solutions should be determined under the same conditions of light or dark, at the same temperature(s), in the same solvent and container as used during the

study. If no stability data are available, all solutions must be prepared fresh on each day of analysis until enough evidence on their stability is available.[7,13]

In-process sample stability. Stability of the analyte is studied in the biological matrix at ambient temperatures[6,11] over the time interval needed to process the samples, usually a time period of 6 or 24 h[6,7,11] for whole blood and/or plasma suffices. If the samples are urine or faeces the observation time might be adjusted to 24 hours or more, since it takes longer to collect these samples.[6,7,11] Analyte stability, stability of the analyte under these conditions, needs to be demonstrated.[11] Analyte stability in each matrix encountered during sample processing, including partially processed samples or dried residues which are stored, should be evaluated.[11,13]

Processed sample stability. The processed sample stability investigation should at least cover the maximum time required from the completion of the sample work-up until completion of the measurements, allowing extra time to cover possible delay.[13] The conditions of light and temperature at which the investigation are conducted are the same as those at which the samples will be held prior to data collecting (e.g. room temperature or at the temperature of the auto-injector).[11,13] The stability of the analyte in the processed sample can be easily assessed by reinjecting the samples in replicates of six in consecutive analytical runs. The suggested time intervals during which the stability should be demonstrated are after 24, 36, and/or 48 hours.[4,11] These time intervals are chosen, so that in case of incomplete assay due to e.g. instrumental failure, re-analysis can still be conducted the next day. The information obtained from these tests provides guidelines as to the size of analytical run and the maximum time allowed between extraction and (re-) analysis.[6,7,11]

Freeze-thaw stability. Freeze-thaw stability studies are conducted to investigate the influence of repeatedly freezing and thawing of the samples on the stability of the analyte of interest. Freezing samples may cause loss of analyte by several causes e.g. adsorption of the analyte to precipitated plasma proteins, crystallisation from urine. Freeze-thaw studies are an essential part of method validation. Information obtained from these studies is required because almost all samples will undergo a freeze/thaw cycle before assay.[6] After one cycle, samples are completely thawed at room temperature in the absence of any heat source and refrozen separated by a minimum of 24 hours.[6] After three freeze-thaw cycles the results are compared to the initial amount of the fresh unfrozen samples[7,13] or are compared to freshly prepared control samples analyzed in the same analytical run.[6] When instability is demonstrated, re-analysis after each cycle is required in order to determine when instability becomes significant.[11,13]

Long-term stability. Long term stability is investigated to examine whether (i) the analyte is stable in the biological matrix and (ii) whether any matrix degradation occurs that may interfere with the analytical method performance.[13]

For most analytes (in plasma) storage at -20°C is sufficient to guarantee stability. If at -20°C instability is observed precautions have to be taken to avoid these problems like storing the samples under -40°C or -80°C or by pretreating the samples to enhance the stability (e.g. alteration of pH, addition of stabilizers, freeze-drying).[7] Long-term stability tests should at least cover the maximum period of sample storage.[11] The same conditions as in the (clinical) study should be employed concerning the storage temperature, the type of container and the biological matrix.[11,13]

In general, there are two ways to conduct prolonged storage stability tests. The first most widely applied method uses samples which are spiked with the analyte at two[7] or three[6] concentrations and stored at the predefined conditions. At fixed time points replicate samples are analyzed together with freshly prepared calibration samples until instability occurs or the storage period of interest is reached.[11] Another applied procedure is to divide a batch of samples into two sub-batches: one batch is used as study samples and the other batch serves as the control samples. The study samples are stored at a temperature of -20°C and the control samples in liquid nitrogen at -196°C, or in another suitable freezer of at least -130°C. At temperatures this low even normally unstable drugs become stable. Studies have shown that the latter method has a significant higher accuracy and precision, because errors associated with additional processing steps such as the preparation of different batches at each validation time point are eliminated.[11,13]

Ruggedness and Robustness

Definition

Ruggedness establishes a method's ability to perform effectively in the face of variation which can reasonably be expected to occur when the method is implemented.[12,16,31] More specifically, ruggedness is the degree of reproducibility of test results obtained by the analysis of samples under a variety of normal test conditions.[1] Thus, ruggedness addresses unintentional variation in the method introduced by its application, at different times by different analysts at different locations using different instrumentation and materials.[31] Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provide an indication of its reliability during normal usage.[1,2,31]

Procedures and Acceptance Criteria

To evaluate the ruggedness/robustness of the analytical method, variations (within certain limits) are introduced in the method's conditions and their effects on the measurements are determined.[3] If analytical results are susceptible to these variations, then the variables should be subjected to more suitable conditions to maintain reliable results.[3,30] These (changed) conditions should be described and well controlled. A precautionary statement should be included in the procedure(s) if a condition is critical.[3] To enhance the ruggedness/robustness in chromatographic methods the effects of variations of certain variables should be investigated, for example:

columns; a minimum of two columns containing two different lots of the identical packing material should be tested during validation,[3,4,11,16,31]
flow-rate,[3,11,12]
column temperature,[3,4,11,12,31]
mobile phase composition,[3,4,11,16,31]
injection volume,[31]
detection wavelength,[31]
extraction solutions, using a different ratio or a different composition of solutions,[3,4]
time of certain procedures, e.g. extraction time,[3,12,15]
analysts,[11,15]
instrumentation.[11,15]

In order to get more insight into the method's ruggedness/robustness, method reproducibility obtained throughout the changes tested, should be compared to the precision of the assay under 'normal' conditions: the reproducibility thus obtained should not be significantly different from the method's precision obtained under normal operating conditions. Although ruggedness and robustness may be tested by varying each individual factor at a time and keeping the other factors fixed, it is more efficient to use a multifactorial designed experiment.[15,31,32] Besides interactions between variables can be estimated. Another way to test the ruggedness of the method is to follow quality control results from the application phase continuously to determine whether the results have changed with time and/or environment.[15]

TREND ANALYSIS: VALIDATION DATA REPORTED

To answer the question of how validation is performed in practice, 37 articles describing liquid chromatographic procedures used in the analysis of anticancer drugs were selected (HPLC/antitumor/anticancer), published in the past five years in the Journal of Liquid Chromatography and Related Technologies, Journal of Chromatography Biomedical Applications and Journal of Pharmaceutical & Biomedical Analysis.[43-70] Frequency of validation parameters mentioned in these references are presented in Figure 2.

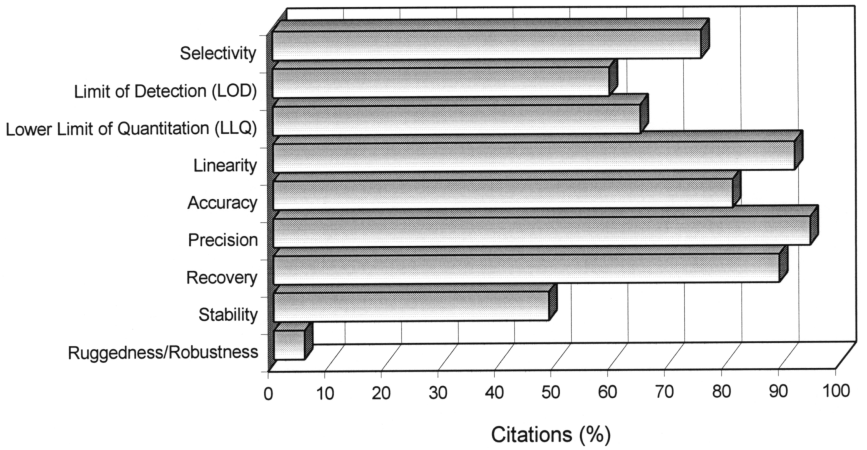

Figure 2. Summary statistics, frequency of validation parameters mentioned in 37 references of liquid chromatographic procedures used in the analysis of anticancer drugs selected from the Journal of Liquid Chromatography and Related Technologies, Journal of Chromatography Biomedical Applications and Journal of Pharmaceutical & Biomedical Analysis.

Certain validation parameters (e.g., selectivity, linearity, accuracy, precision, and recovery) have been evaluated in almost every analytical application. The LOD, LLQ, stability, and ruggedness/robustness have been less investigated frequently. The LOD is not a very stable characteristic and therefore it should not be included in the calibration curve. The LLQ on the other hand must be determined. Stability data was only mentioned in about 50% of the citations. It is obvious that enough stability data should be established during validation, to assure good routine operation of the method. Ruggedness/robustness tests were hardly performed. Although these experiments are time consuming, we feel that at least a minimum of two columns containing two different lots of the identical packing material should be tested.

The procedures to establish method validation vary significantly among laboratories. In Table 2 the validation approaches reported in all 37 references are tabulated. Correlation coefficients were most widely used to test linearity. Although the correlation coefficient is of benefit for demonstrating a high degree of relationship between concentration-response data, it is of little value in establishing linearity. Therefore, by assessing an acceptable high correlation coefficient alone the linearity is not guaranteed and further tests on linearity are necessary, for example a Lack-of-Fit test. Generally the coefficient of variation was used to report the precision and ANOVA was applied in only a few references.

Table 2

Type of Validation Data Reported in 37 Published Descriptions of Liquid Chromatographic Procedures Used in the Analysis of Anticancer Drugs*

Validated Parameter	Approach[a]	Occurance Number	Occurance Percentage
Selectivity/ specificity	Endogenous substances	23	62
	Degradation products	2	5
	Metabolites/precursor	7	19
	Co-administered drugs/ formulation vehicles	9	24
	Second method/purity check diode array detection	2	5
Limit of detection (LOD)	Signal-to-noise	20	54
	Standard deviation blank response/slope calibration curve	2	5
Limit of quantitation (LLQ)	Spiked samples with required accuracy/precision level	20	54
	Signal-to-noise	2	5
	Standard deviation blank response	1	3
	Standard dev. blank response/slope calibration curve	1	3
Linearity	Lack-of-fit test	3	8
	Logarithmic transformation	1	3
	Back-calculated concentrations	4	11
	Correlation coefficient	30	81
	Visual inspection	1	3
Accuracy	Spiked samples: within-run accuracy	21	57
	Spiked samples: between-run accuracy	18	49
	Recovery analysis	1	3
Precision	Within-run precision, Coefficient of Variation	28	76
	Within-run precision, ANOVA	3	8
	Between-run precision, Coefficient of Variation	26	70
	Between-run precision, ANOVA	4	11
Recovery	Spiked samples	33	89
	Radioactive analyte	1	3
Stability	Stock/working solutions	8	22
	Freeze-thaw cycles	5	14
	In process stability	11	30
	Processed stability	12	32
	Long term stability	14	38
Ruggedness/ Robustness		2	5

*Selected from the Journal of Liquid Chromatography and Related Technologies, Journal of Chromatography Biomedical Applications and Journal of Pharmaceutical & Biomedical Analysis (1993-1997). [a] For details, see text.

CONCLUDING REMARKS

In drug development, important decisions are taken which are based on data obtained from bioanalytical methods. Therefore, it is generally accepted that method validation is required to demonstrate the performance of the method and the reliability of the analytical results. During validation, the measured performance and pre-determined acceptance criteria are systematically compared. The acceptance criteria should be clearly established in a validation plan, prior to the initiation of the validation study.

In the last 10 years method validation has been rigorously extended and guidelines have been published by recognized authorities. Although these guidelines provide indications for method validation, they do not include much information on how to conduct these studies or when results should be considered acceptable.

Acceptance criteria can be very dependent on the nature of the sample, the type of analytical methodology, and the purpose of carrying out the analysis. Fixed criteria are subjective and eventually the researcher has to decide what criteria are considered acceptable and relevant.

For bioanalytical liquid chromatographic methods, the essential performance parameters have been discussed here in detail to provide guidance to bioanalytical chemists. A survey of recent literature on liquid chromatographic methods used in the bioanalysis of anticancer drugs revealed that validation was performed but was often very brief and deficient.

The aim of this review, with practical approaches to validation, has been to contribute to a further optimization of bioanalytical method validation.

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Received March 22, 1999
Accepted April 26, 1999
Manuscript 5039