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A Detailed Study of Validation Parameters and System Suitability Test in HPLC

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ABSTRACT

All analytical techniques used for the development of drugs and pharmaceuticals and for the determination of their quality characteristics have to be validated. The purpose of this paper is to present the issues to consider when evaluating chromatographic test methods from a regulatory perspective. It has been discussed in this paper the points to note and weaknesses of chromatography so that Center for Drug Evaluation and Research (CDER) reviewers can ensure that the method's performance claims are properly evaluated, and that sufficient information is available for the field chemist to assess the method. This paper presents the discussions about the parameters for Validation of HPLC methods for Drug substance and Drug product and practical recommendations and criteria for finding correct solutions and also presents the importance of the system suitability test to ensure the performance of the HPLC system. With proper validation and tight chromatographic performance (system suitability) criteria, an improvement in the reliability of the data can be obtained.

Key words: HPLC, ICH, USFDA, CGMP

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INTRODUCTION

Validation (evaluation of suitability) of an analytical technique is a procedure aimed at obtaining experimentally justified evidence of the ability of this technique to give results characterized by the required accuracy and precision [1-7]. All analytical techniques used for the development of pharmaceuticals and for the determination of their quality characteristics have to be validated. In the case of using methods stipulated and described in the State Pharmacopoeia, it is not necessary to evaluate their suitability, provided that the analyses are conducted with strict observation of the text of each particular article. In most other cases, especially in cases of modification of the drug composition, the scheme of synthesis, or the analytical procedure, it is necessary to re-evaluate the suitability of the analytical techniques. The purpose of this paper is to present the issues to consider when evaluating chromatographic test methods from a regulatory perspective. The document discusses the points to note and weaknesses of chromatography so that CDER reviewers can ensure that the method's performance claims are properly evaluated, and that sufficient information is available for the field chemist to assess the method. Analytical terms, as defined by the International Conference of Harmonization (ICH), 1993, have been incorporated in this paper.

Chromatographic methods are commonly used for the quantitative and qualitative analysis of raw materials, drug substances, drug products and compounds in biological fluids. The components monitored include chiral or achiral drug, process impurities, residual solvents, excipients such as preservatives, degradation products, extractable and leachables from container and closure or manufacturing process, pesticide in drug product from plant origin, and metabolites. The objective of a test method is to generate reliable and accurate data regardless of whether it is for acceptance, release, stability or pharmacokinetics study. Data are generated for the qualitative and quantitative testing during development and post approval of the drug products. The testing includes the acceptance of raw materials, release of the drug substances and products, in-process testing for quality assurance, and establishment of the expiration dating period. Validation of a method is the process by which a method is tested by the developer or user for reliability, accuracy and preciseness of its intended purpose.

This paper is aimed at (i) critically assessing the main approaches to evaluation of the validation characteristics of HPLC method and providing practical recommendations and criteria for finding correct solutions and (ii) the importance of the system suitability test to ensure the performance of the HPLC system

2. Parameters for Validation of HPLC Methods For Drug Substances and Drug Products

2.1 Introduction

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice.

Analytical methods need to be validated or revalidated

- before their introduction into routine use;
- whenever the conditions change for which the method has been validated (e.g., an instrument with different characteristics or samples with a different matrix); and
- whenever the method is changed and the change is outside the original scope of the method.

In the early days it was a long period of trial and error to establish a document where common users could benefit from. Nowadays the use of computer programmes like Drylab or HIPAC makes it easier to optimize the intended procedure to a practical analysis for other laboratories and schools. This document will try to explain some of the basic jargon and glossary concerning HPLC.

Validation is required for any new or amended method to ensure that it is capable of giving reproducible and reliable results, when used by different operators employing the same equipment in the same or different laboratories. The type of validation programmed required depends entirely on the particular method and its proposed applications.

Method validation has received considerable attention in the literature and from industrial committees and regulatory agencies.

- The U.S. FDA CGMP [8] request in section 211.165 (e) methods to be validated: The accuracy, sensitivity, specificity, and reproducibility of test methods employed by the firm shall be established and documented. Such validation and documentation may be accomplished in accordance with Sec. 211.194(a). These requirements include a statement of each method used in testing the sample to meet proper standards of accuracy and reliability, as applied to the tested product. The U.S. FDA has also proposed an industry guidance for Analytical Procedures and Methods Validation [9].
- ISO/IEC 17025 includes a chapter on the validation of methods [10] with a list of nine validation parameters. The ICH [11] has developed a consensus text on the validation of analytical procedures. The document includes definitions for ten validation characteristics. ICH also developed a guidance with detailed methodology [12].
- The U.S. EPA prepared a guidance for method's development and validation for the Resource Conservation and Recovery Act (RCRA) [13]. The AOAC, the EPA and other scientific organizations provide methods that are validated through multi-laboratory studies.

The USP has published specific guidelines for method validation for compound evaluation [14]. USP defines ten steps for validation:

- Accuracy (2.2)
- Precision (2.3)
- Specificity (2.4)
- Limit of detection (2.5)

- Limit of quantitation(2.6)
- Linearity (2.7)
- Range (2.8)
- Ruggedness (2.9)
- Robustness (2.10)
- Stability (2.11)

2.2 Accuracy

Accuracy is the measure of how close the experimental value is to the true value. Accuracy studies for drug substance and drug product are recommended to be performed at the 80, 100 and 120% levels of label claim as stated in the guideline for submitting samples and analytical Data for Methods Validation. For the drug product, this is performed frequently by the addition of known amounts of drug by weight or volume (dissolved in diluents) to the placebo formulation working in the linear range of detection of the analyte. This would be a true recovery for liquid formulations. For formulations such as tablet, suppository, transdermal patch, this could mean evaluating potential interaction of the active drug with the excipients in the diluent. From a practical standpoint, it is difficult to manufacture a single unit with known amount of active drug to evaluate recovery. This test evaluates the specificity of the method in the presence of the excipients under the chromatographic conditions used for the analysis of the drug product. It will pick up recovery problems that could be encountered during the sample preparation and the chromatographic procedures. However, it does not count the effect of the manufacturing process. At each recommended level studied, replicate samples are evaluated. The RSD of the replicates will provide the analysis variation or how precise the test method is. The mean of the replicates, expressed as %label claim, indicates how accurate the test method is.

Recommendations

Recovery data, at least in triplicate, at each level (80, 100 and 120% of label claim) is recommended. The mean is an estimate of accuracy and the RSD is an estimate of sample analysis precision.

2.3 Precision

Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions. ICH has defined precision to contain three components: repeatability, intermediate precision and reproducibility. Ruggedness as defined in USP XXll <I 225>, 1990 incorporates the concepts described under the terms "intermediate precision", "reproducibility" and "robustness" of this paper.

2.3.1. Repeatability

2.3.1.1. Injection Repeatability

Sensitivity is the ability to detect small changes in the concentration of the analyte in the sample. Sensitivity can be partially controlled by monitoring the specification for injection reproducibility (system suitability testing). The sensitivity or precision as measured by multiple injections of a homogeneous sample (prepared solution) indicates the performance of the HPLC instrument under the chromatographic conditions and day tested.

Recommendations

As part of methods validation, a minimum of 10 injections with an RSD of 11% is recommended. With the methods for release and stability studies, an RSD of 11% RSD for precision of the system suitability tests for at least five injections (n>= 5) for the active drug either in drug substance or drug product is desirable. For low level impurities, higher variations may be acceptable.

2.3.1.2. Analysis Repeatability

Determination, expressed as the RSD, consists of multiple measurements of a sample by the same analyst under the same analytical conditions. For practical purpose, it is often combined with accuracy and carried out as a single study.

2.3.2. Intermediate Precision

Intermediate precision was previously known as part of ruggedness. The attribute evaluates the reliability of the method in a different environment other than that used during development of the method. The objective is to ensure that the method will provide the same results when similar samples are analyzed once the method development phase is over.

Recommendations

As a minimum, data generated as described under section 2.2 Accuracy, for two separate occasions, is recommended to indicate the intermediate precision of the test method.

2.3.3. Reproducibility

As defined by ICH, reproducibility expresses the precision between laboratories as in collaborative studies. Multiple laboratories are desirable but not always attainable because of the size of the firm.

Recommendations

It is not normally expected if intermediate precision is accomplished.

2.4 Selectivity/Specificity

The terms selectivity and specificity are often used interchangeably. A detailed discussion of this term, as defined by different organizations, has been presented by Vessmann [15]. He particularly pointed out the difference between the definitions of specificity given by IUPAC/WELAC and the ICH.

Although it is not consistent with the ICH, the term *specific* generally refers to a method that produces a response for a single analyte only, while the term *selective* refers to a method that provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is said to be *selective*. Since there are very few methods that respond to only one analyte, the term *selectivity* is usually more appropriate. The USP monograph defines the selectivity of an analytical method as its ability to measure accurately an analyte in the presence of interference, such as synthetic precursors, excipients, enantiomers and known (or likely) degradation products that may be expected to be present in the sample matrix. Selectivity in liquid chromatography is obtained by choosing optimal columns and setting chromatographic conditions, such as mobile phase composition, column temperature and detector wavelength. Besides chromatographic separation, the sample preparation step can also be optimized for best selectivity.

It is a difficult task in chromatography to ascertain whether the peaks within a sample chromatogram are pure or consist of more than one compound. Therefore, the analyst should know how many compounds are in the sample or whether procedures for detecting impure peaks should be used.

While in the past chromatographic parameters such as mobile phase composition or the column were modified, now the application of spectroscopic detectors coupled on-line to the chromatograph is being used. UV/visible diode-array detectors and mass spectrometers acquire spectra on-line throughout the entire chromatogram. The spectra acquired during the elution of a peak are normalized and overlaid for graphical presentation. If the normalized spectra are different, the peak consists of at least two compounds.

The principles of diode-array detection in HPLC and their application and limitations with regard to peak purity are described in the literature [16]. Examples of pure and impure HPLC peaks are shown in Figure 1. While the chromatographic signal indicates no impurities in either peak, the spectral evaluation identifies the peak on the left as impure. The level of impurities that can be detected with this method depends on the spectral difference, on the detector's performance and on the software algorithm. Under ideal conditions, peak impurities of 0.05 to 0.1 percent can be detected.

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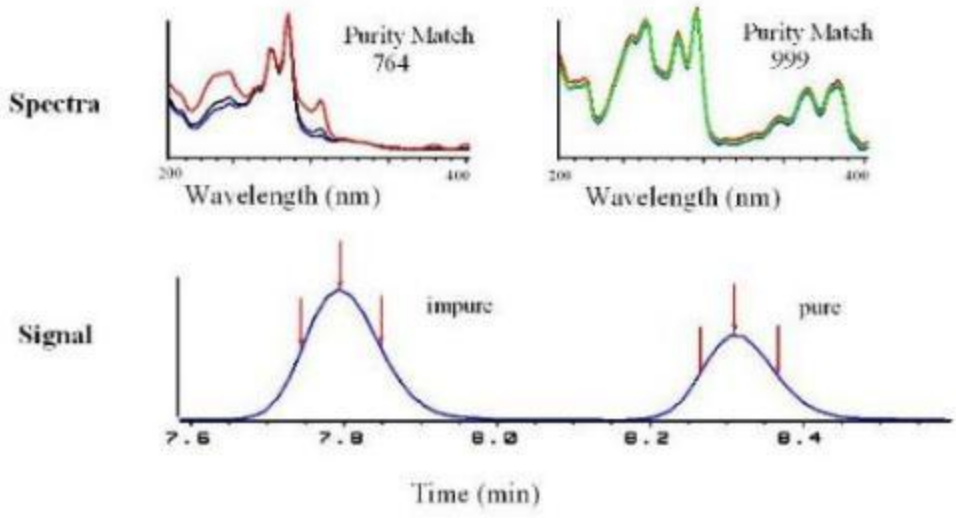

Figure 1. Examples of pure and impure HPLC peaks. The chromatographic signal does not indicate any impurity in either peak. Spectral evaluation, however, identifies the peak on the left as impure.

Selectivity studies should also assess interferences that may be caused by the matrix, e.g., urine, blood, soil, water or food. Optimized sample preparation can eliminate most of the matrix components. The absence of matrix interferences for a quantitative method should be demonstrated by the analysis of at least five independent sources of control matrix.

Recommendations

Representative HPL chromatograms should be submitted for stressed and non-stressed samples that include impurities test method, preservative(s), etc, with the related placebo sample. Representative HPL chromatogram(s) to show selectivity by the addition of known extraneous compounds also should be submitted.

2.5 Limit of Detection

The limit of detection is the point at which a measured value is larger than the uncertainty associated with it. It is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified. The limit of detection is frequently confused with the sensitivity of the method. The sensitivity of an analytical method is the capability of the method to discriminate small differences in concentration or mass of the test analyte. In practical terms, sensitivity is the slope of the calibration curve that is obtained by plotting the response against the analyte concentration or mass.

In chromatography, the detection limit is the injected amount that results in a peak with a height at least two or three times as high as the baseline noise level. Besides this signal/noise method, the ICH describes three more methods:

1. Visual inspection: The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.
2. Standard deviation of the response based on the standard deviation of the blank: Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.
3. Standard deviation of the response based on the slope of the calibration curve: A specific calibration curve is studied using samples containing an analyte in the range of the limit of detection. The residual standard deviation of a regression line, or the standard deviation of y-intercepts of regression lines, may be used as the standard deviation.

Recommendations

1. Analysis repeatability and injection repeatability data at the quantitation limit.
2. Use of an additional reference standard at the quantitation limits level in the test method.

2.6 Limit of Quantitation

The limit of quantitation is the minimum injected amount that produces quantitative measurements in the target matrix with acceptable precision in chromatography, typically requiring peak heights 10 to 20 times higher than the baseline noise.

If the required precision of the method at the limit of quantitation has been specified, the EURACHEM [17] approach can be used. Figure 3 illustrates the Limit of Quantitation by EURACHEM method. A number of samples with decreasing amounts of the analyte are injected six times. The calculated RSD percent of the precision is plotted against the analyte amount. The amount that corresponds to the previously defined required precision is equal to the limit of quantitation. It is important to use not only pure standards for this test but also spiked matrices that closely represent the unknown samples.

For the limit of detection, the ICH [12] recommends, in addition to the procedures as described above, the visual inspection and the standard deviation of the response and the slope of the calibration curve.

Any results of limits of detection and quantitation measurements must be verified by experimental tests with samples containing the analytes at levels across the two regions. It is equally important to assess other method validation parameters, such as precision, reproducibility and accuracy, close to the limits of detection and quantitation. Figure 5 illustrates the limit of quantitation (along with the limit of detection, range and linearity). Figure 2 illustrates both the limit of detection and the limit of quantitation.

2.7 Linearity and Calibration Curve

The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of analytes in samples within a given range or proportional by means of well-defined mathematical transformations. Linearity may be demonstrated directly on the test substance (by dilution of a standard stock solution) and/or by using separate weighings of synthetic mixtures of the test product components, using the proposed procedure.

Linearity is determined by a series of 3 to 6 injections of 5 or more standards whose concentrations span 80–120 percent of the expected concentration range. The response should be directly proportional to the concentrations of the analytes or proportional by means of a well-defined mathematical calculation. A linear regression equation applied to the results should have an intercept not significantly different from 0. If a significant nonzero intercept is obtained, it should be demonstrated that this has no effect on the accuracy of the method.

Frequently, the linearity is evaluated graphically, in addition to or as an alternative to mathematical evaluation. The evaluation is made by visually inspecting a plot of signal height or peak area as a function of analyte concentration. Because deviations from linearity are sometimes difficult to detect, two additional graphical procedures can be used. The first is to plot the deviations from the regression line versus the concentration or versus the logarithm of the concentration, if the concentration range covers several decades. For linear ranges, the deviations should be equally distributed between positive and negative values.

Another approach is to divide signal data by their respective concentrations, yielding the relative responses. A graph is plotted with the relative responses on the y-axis and the corresponding concentrations on the x-axis, on a log scale. The obtained line should be horizontal over the full linear range. At higher concentrations, there will typically be a negative deviation from linearity. Parallel horizontal lines are drawn on the graph corresponding to, for example, 95 percent and 105 percent of the horizontal line. The method is linear up to the point where the plotted relative response line intersects the 95 percent line. ***Figure 4*** shows a comparison of the two graphical evaluations on a sample of caffeine using HPLC.

The ICH recommends, for accuracy reporting, the linearity curve's correlation coefficient, y-intercept, slope of the regression line and residual sum of squares. A plot of the data should be included in the report. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity. Some analytical procedures, such as immunoassays, do not demonstrate linearity after any transformation. In this case, the analytical response should be described by an appropriate function of the concentration (amount) of an analyte in a sample. In order to establish linearity, a minimum of five concentrations is recommended. Other approaches should be justified.

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Plotting the sensitivity (response/amount) gives clear indication of the linear range. Plotting the amount on a logarithmic scale has a significant advantage for wide linear ranges. Rc = Line of constant response.

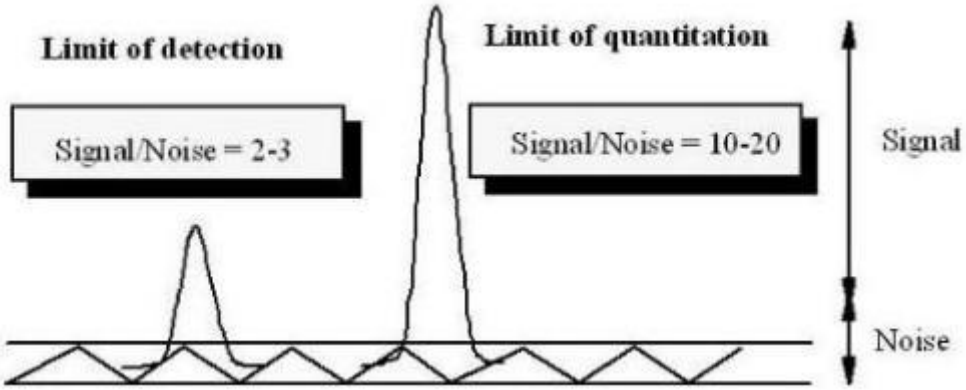

Figure 2. Limit of detection and limit of quantitation via signal to noise

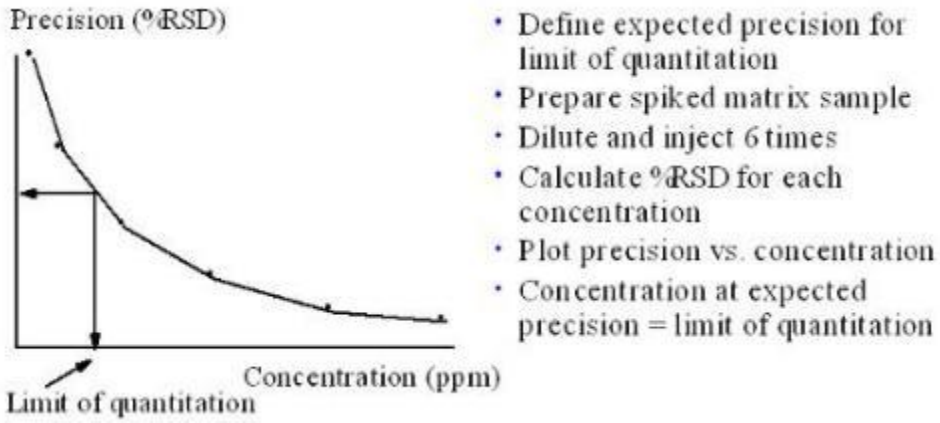

Figure 3. Limit of quantitation with the EURACHEM method.

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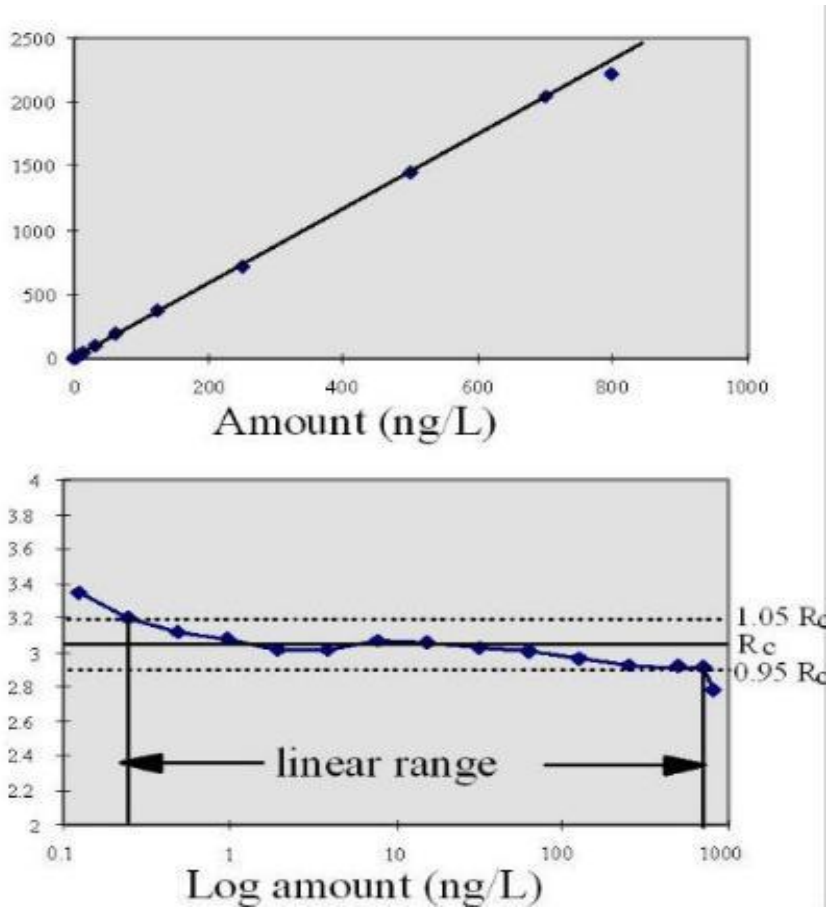

Figure 4. Graphical presentations of linearity plot of a caffeine sample using HPLC.

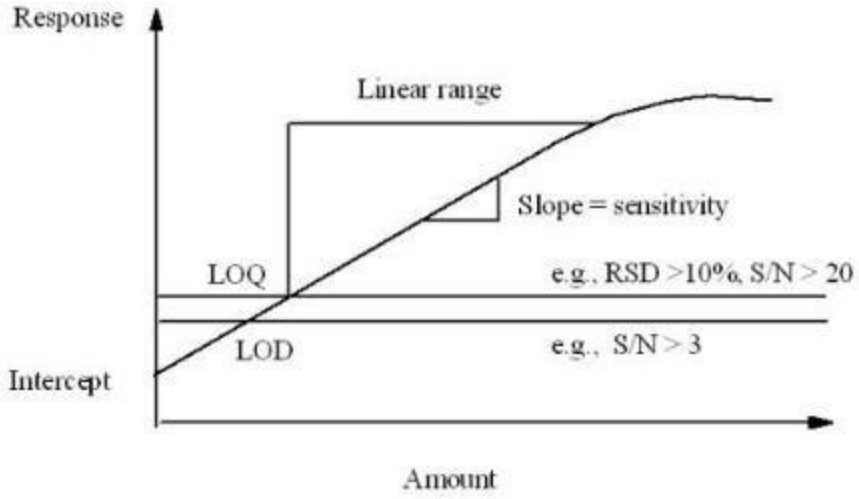

Figure 5. Definitions for linearity, range, LOQ, LOD

Recommendations

The linearity range for examination depends on the purpose of the test method. For example, the recomended range for an assay method for content would be NLT +/- 20% and the range for an assay/impurities combination method based on area % (for impurities) would be +20% of target concentration down to the limit of quantitation of the drug substance or impurity. Under most circumstances, regression coefficient (r) is >=0.999. Intercept and slope should be indicated.

2.8 Range

The range of an analytical method is the interval between the upper and lower levels (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. The range is normally expressed in the same units as the test results (e.g., percentage, parts per million) obtained by the analytical method.

For assay tests, the ICH [12] requires the minimum specified range to be 80 to 120 percent of the test concentration, and for the determination of an impurity, the range to extend from the limit of quantitation, or from 50 percent of the specification of each impurity, whichever is greater, to 120 percent of the specification.

2.9 Ruggedness

The ruggedness of an analytical method is the absence of undue adverse influence on its reliability of performance by minor changes in the laboratory environment [18]. This validation parameter is not recognized by all organizations with testing oversight, as this characteristic is implied by collaborative validation programs. The difference in chromatographic performance between columns of the same designation (i.e., C_{18}) is the most common source of chromatographic variability. To check the column-to-column ruggedness, the specificity (selectivity) of at least three columns from three different batches supplied by one column manufacturer should be checked [19]. A similarly designated column from another manufacturer should also be evaluated. The list of specifications recommended to define a liquid chromatographic column can be obtained from literature [20,21]. Testing procedures have also evolved for the evaluation of gas chromatographic capillary columns [22]. Variability is also caused by the degradation of the chromatographic column. Besides the sorbent stability, consideration should also be given to the stability of the sample solution. The widespread use of automatic sample injectors makes it necessary to determine the length of time that a sample is stable.

2.10 Robustness

Robustness tests examine the effect that operational parameters have on the analysis results. For the determination of a method's robustness, a number of method parameters, for example, pH, flow rate, column temperature, injection volume, detection wavelength or mobile

phase composition, are varied within a realistic range, and the quantitative influence of the variables is determined. If the influence of the parameter is within a previously specified tolerance, the parameter is said to be within the method's robustness range. Obtaining data on these effects helps to assess whether a method needs to be revalidated when one or more parameters are changed, for example, to compensate for column performance over time. In the ICH document [12], it is recommended to consider the evaluation of a method's robustness during the development phase, and any results that are critical for the method should be documented. This is not, however, required as part of a registration.

Recommendations

Data obtained from studies for robustness, though not usually submitted, are recommended to be included as part of method validation.

2.11 Stability

Many solutes readily decompose prior to chromatographic investigations, for example, during the preparation of the sample solutions, extraction, cleanup, phase transfer or storage of prepared vials (in refrigerators or in an automatic sampler). Under these circumstances, method development should investigate the stability of the analytes and standards.

The term system stability has been defined as the stability of the samples being analyzed in a sample solution. It is a measure of the bias in assay results generated during a preselected time interval, for example, every hour up to 46 hours, using a single solution. System stability should be determined by replicate analysis of the sample solution. System stability is considered appropriate when the RSD, calculated on the assay results obtained at different time intervals, does not exceed more than 20 percent of the corresponding value of the system precision. If, on plotting the assay results as a function of time, the value is higher, the maximum duration of the usability of the sample solution can be calculated.

Recommendations

Data to support the sample solution stability under normal laboratory conditions for the duration of the test procedure, e.g., twenty-four hours, should be generated. In exceptional cases where multiple days are needed for sample preparation or solution storage, an appropriate stability time should be selected.

3. SYSTEM SUITABILITY TESTS (SST)

SST is commonly used to verify resolution, column efficiency, and repeatability of a chromatographic system to ensure its adequacy for a particular analysis. According to the United States Pharmacopeias (USP) and the International Conference on Harmonization (ICH), SST is an integral part of many analytical procedures. Although USP and ICH are not regulatory agencies, their guidelines are "bibles" followed closely in the industry because they are

accepted by the FDA. SST is based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as a whole. The chromatographic systems used for most pharmaceutical analyses such as assays of the active ingredients, impurity determinations, and dissolution testing (measuring the dissolution rate for a particular form of dosage) must pass a set of predefined acceptance criteria (SST limits) before sample analysis can commence.

Primary SST parameters are resolution (R), repeatability (RSD—relative standard deviations—of peak response and retention time), column efficiency (N), and tailing factor (T). These parameters are most important as they indicate system specificity, precision, and column stability. Other parameters include capacity factor (k) and signal-to-noise ratio (S/N) for impurity peaks. Most chromatographic data systems can automate the measurement and reporting of these SST parameters. Acceptance criteria or SST limits are predefined in most "official" analytical methods. Limits may vary with different tests and are typically less stringent for biologics and trace impurities.

SST limits should represent the minimum acceptable system performance levels rather than typical or optimal levels. Many analytical methods simply adopted the general limits from the CDER guidance document [23]. While acceptable from the regulatory standpoint, these limits might be too wide to detect emerging system problems. For instance, if historical analysis data show performance of a specific method to be R = 4–6, N = 8000–10,000, and T = 1.0–1.3, then the general CDER limits (R > 2, N > 2000, and T $\leq$2.0) might not reflect the normal performance range and perhaps not truly fulfill the role of determining system suitability.

Several studies [24-26] have suggested the use of statistical analysis (e.g., Plackett and Burman or other fractional factorial designs) on data gathered during method optimization or validation. This is in line with guidance from ICH, which regards SST as one of the method validation steps. Another approach particularly useful during method revisions is to apply the 3-sigma rule to historical performance data (preferably from different laboratories). For instance, suppose the average column efficiency (N) is found to be 8000 plates, with a standard deviation of 1000 plates. The expected range is then the mean ±3 SD, or 5000–11,000 plates. The efficiency criterion can then be set to >5000 plates.

Once a method or system has been validated the task becomes one of routinely checking the suitability of the system to perform within the validated limits. The simplest form of an HPLC system suitability test involves a comparison of the chromatogram trace with a standard trace. This allows a comparison of the peak shape, peak width, baseline resolution. Alternatively these parameters can be calculated experimentally to provide a quantitative system suitability test report: Number of theoretical plates (efficiency) Capacity factor, Separation (relative retention) Resolution, Tailing factor Relative Standard Deviation (Precision). These are measured on a peak or peaks of known retention time and peak width.

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3.1 Plate number or number of theoretical plates (N)

$$N=(t_R/ t_W)^2=L/H$$

where
W_h = peak width at 1/2 peak height
W_b = peak width at base
t = retention time of peak
t_R = retention time of the analyte
t_W = peak width measured at baseline of the extrapolated straight sides to baseline Theoretical plate number is a measure of column efficiency, that is, how many peaks can be located per unit run-time of the chromatogram. N is fairly constant for each peak on a chromatogram with a fixed set of operating conditions. H, or HETP, the height equivalent of a theoretical plate, measures the column efficiency per unit length (L) of the column. Parameters which can affect N or H include peak position, particle size in column, flow-rate of mobile phase, column temperature, viscosity of mobile phase, and molecular weight of the analyte. **Figure 6** represents the Plate Number Calculation.

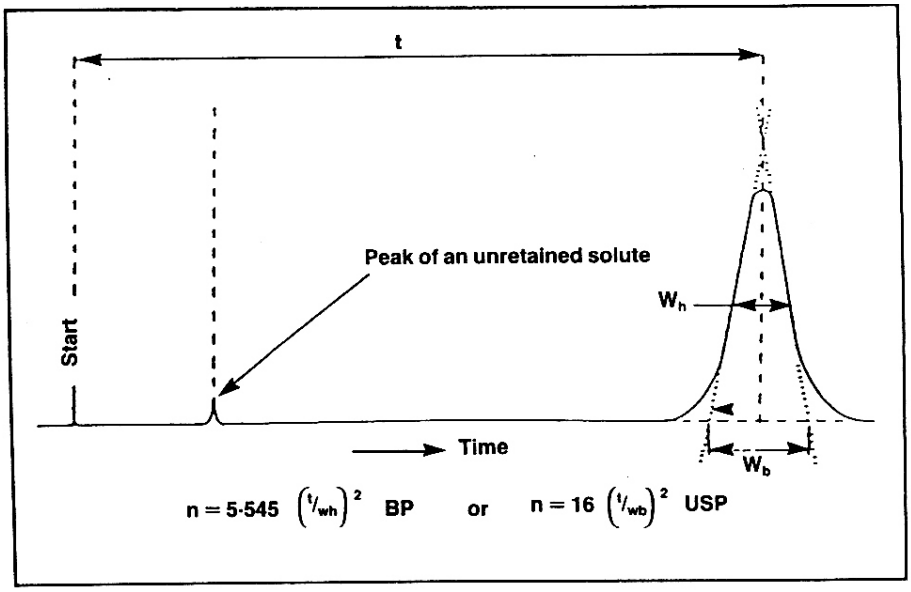

Figure 6 Plate Number Calculation

3.2 CAPACITY FACTOR ($K^{/}$)

The time elapsed between the injection of the sample components into the column and their detection is known as the Retention Time (t_R). The retention time is longer when the solute has higher affinity to the stationary phase due to its chemical nature. For example, in reverse phase chromatography, the more lypophilic compounds are retained longer. Therefore, the retention time is a property of the analyte that can be used for its identification.

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A non-retained substance passes through the column at a time t_0, called the Void Time. The Retention Factor or Capacity Factor k' of an analyte is measured experimentally as shown in Figure 7 and Eqn 1:

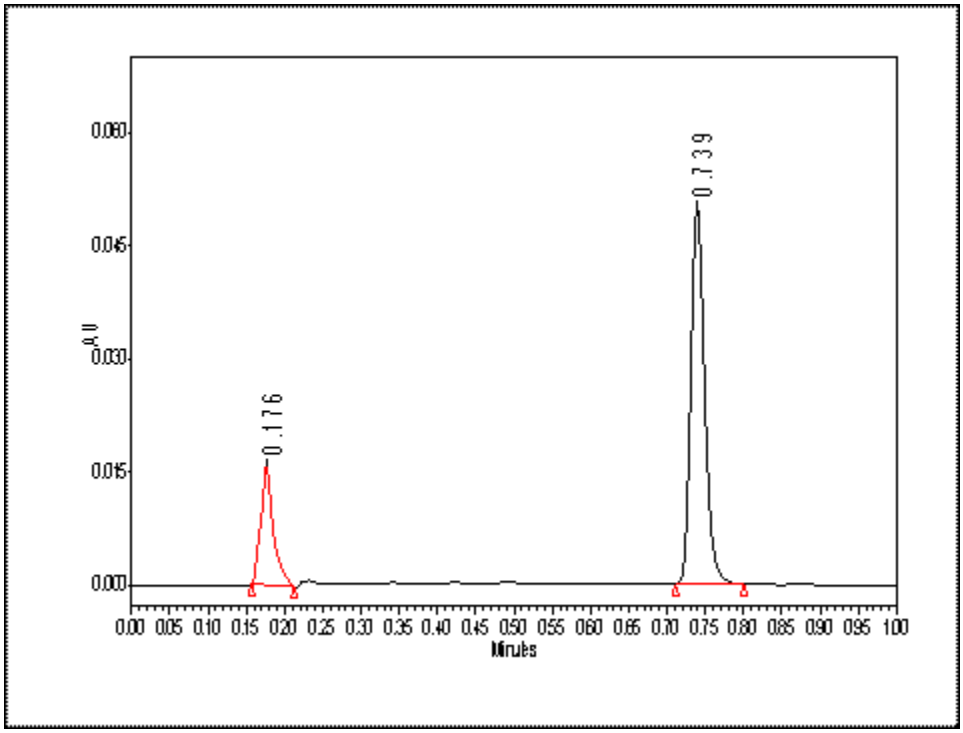

Figure 7 Example of Capacity factor calculation in LC. In this case: t_R = 0.739, t_0 = 0.17, therefore k' = (0.739-0.176)/0.176 = 3.20

$$k' = \frac{t_R - t_0}{t_o} \quad (1)$$

The Capacity Factor describes the thermodynamic basis of the separation and its definition is the ratio of the amounts of the solute at the stationary and mobile phases within the analyte band inside the chromatographic column:

$$k' = \frac{C_s}{C_m}\Phi \quad (2)$$

Where C_s is the concentration of the solute at the stationary phase and C_m is its concentration at the mobile phase and 'Φ' is the ratio of the stationary and mobile phase volumes all within the chromatographic band.

The Retention Factor (Eqn 1) is used to compare the retention of a solute between two chromatographic systems, normalizing it to the column's geometry and system flow rate. The need to determine the void time can be tricky sometimes, due to the instability of the elution

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time of the void time marker, t_0, therefore, when the chromatogram is complex in nature, and one known component is always present at a certain retention time, it can be used as a retention marker for other peaks. In such cases the ratio between the retention time of any peak in the chromatogram and the retention time of the marker is used ($t_{R\ (Peak)}$ / $t_{R\ (Marker)}$) and referred to as the Relative Retention Time (RRT). RRT is also used instead of the capacity ratio for the identification of the analyte as well as to compare its extent of retention in two different chromatographic systems.

Recommendations

The peak should be well-resolved from other peaks and the void volume. Generally the value of k' is > 2.

3.3 Separation Factor (relative retention)

This describes the relative position of two adjacent peaks. Ideally, it is calculated using the capacity factor because the peaks' separation depends on the components' interaction with the stationary phase. Therefore considering peaks A and B in Fig. 8, where separation factor has been calculated. k for the later peak is always placed in the numerator to assure a value >l. If the capacity factor is used then the separation factor should be consistent for a given column, mobile phase, composition and specified temperature, regardless of the instrument used.

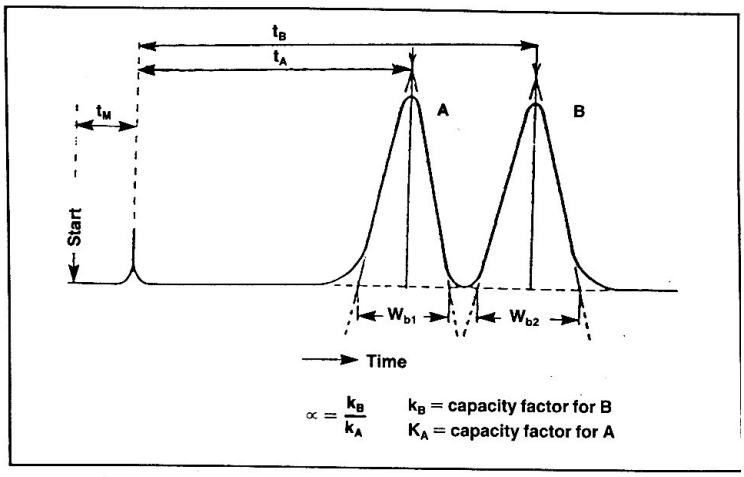

Figure 8 Separation Factor calculation

3.4 Peak Resolution (R)

There are several formulas available for calculating resolution factors. The formula recommended in USP 23 for GC and HPLC is as follows:

$$R= 2(t_2 – t_1)/(W_2 + W_1)$$

Where t_2 and t_1 are the retention times of the two components and W2 and W1 are the corresponding widths at the peak base. The width is obtained by extrapolating the relatively straight sides of the peaks to the baseline. Baseline resolution is achieved when R = 1.5

It is useful to relate the resolution to the number of plates(N) in the column, the selectivity factor($K^{/}$) and the retention factors(α) of the two solutes;

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{1 + k'_B}{k'_B}\right)$$

To obtain high resolution, the three terms must be maximised. An increase in *N*, the number of theoretical plates, by lengthening the column leads to an increase in retention time and increased band broadening - which may not be desirable. Instead, to increase the number of plates, the height equivalent to a theoretical plate can be reduced by reducing the size of the stationary phase particles.

It is often found that by controlling the capacity factor, *k'*, separations can be greatly improved. This can be achieved by changing the temperature (in Gas Chromatography) or the composition of the mobile phase (in Liquid Chromatography).

The selectivity factor, can also be manipulated to improve separations. When Selectivity Factor is close to unity, optimising *k'* and increasing *N* is not sufficient to give good separation in a reasonable time. In these cases, *k'* is optimised first, and then Selectivity Factor is increased by one of the following procedures:

1. Changing mobile phase composition
2. Changing column temperature
3. Changing composition of stationary phase
4. Using special chemical effects (such as incorporating a species which complexes with one of the solutes into the stationary phase)

3.5. Asymmetry Factor (Tailing Factor)

If the peak to be quantified is asymmetric, a calculation of the asymmetry would also be useful in controlling or characterizing the chromatographic system [27]. Peak asymmetry arises from a number of factors. The increase in the peak asymmetry is responsible for a decrease in chromatographic resolution, detection limits, and precision. Measurement of peaks on solvent tails should be avoided. The peak asymmetry factor (tailing factor) can be calculated by several different methods. By the USP,

$$T = W_{0.05}/2f$$

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Where $W_{0.05}$ is the width of the peak at 5% peak height and f is distance at 5% height from the leading edge of the peak to the distance of the peak maximum as measured at the 5% height. The system suitability test for antibiotics and antibiotic drugs recommends measurement at 10% of the peak height from the baseline [28]. Representative values from the USP are presented in Table 1.6. Values vary from 1 to 3. For a symmetrical peak, the factor is unity which increases as tailing becomes more pronounced. A variety of alternative models have been proposed to more accurately characterize peak tailing [29]. Fig. 9 represents the Tailing factor calculation.

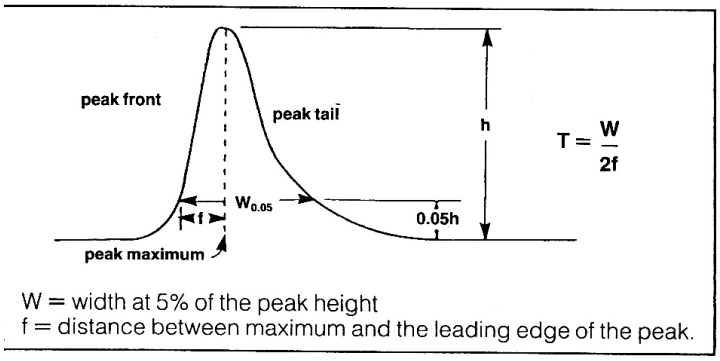

Fig. 9 : Tailing factor calculation

3.6 Relative Standard Deviation or precision

For an HPLC system this would involve the reproducibility of a number of replicate injections (ie 6) of an analytical solution. The USP requires that unless otherwise specified by a method: - if a relative standard deviation of <2% is required then five replicate injections should be used - if a relative standard deviation of >2% is required then six replicate injections should be used

3.7. Column Efficiency

The resolution factor is considered to be a more discriminating measure of system suitability than column efficiency [30]. Yet, column efficiency determinations are required for the assay of antibiotics and antibiotic containing drugs [28]. The reduced plate height (h_r) for the column is determined by first calculating the number of theoretical plates per column:

$$N = 5.545(t/w_{h/2})^2 \quad \text{or } N=16(t/w)^2$$

Where t is the retention time of the analyte and $W_{h/2}$ is the peak width at half-height or W is the width at the base of the peak. The height equivalent to one theoretical plate is calculated by

$$h=L/n$$

Where L is the length of the column. Finally, the reduced plate height is determined by

$h_r=h/d_p$

Where dp is the average diameter of particles in the column. The reduced plate has the advantage of being independent of column length and particle diameter. The resulting number can also be compared to the theoretical limiting value of 2. The calculation of column theoretical plates by the width at half-peak height is insensitive to peak asymmetry. This is because the influence of tailing usually occurs below that measurement location. The consequence will be an overestimate of the theoretical plates for non-Gaussian peaks. Nine different calculation methods for efficiency have been compared for their sensitivity to peak asymmetry [29]. Besides being influenced by the calculation method, column efficiency is sensitive to temperature, packing type, and linear velocity of the mobile phase.

3.8. Column Capacity

The column capacity factor is calculated by

$$\mathbf{K=(t_r-t_m)/t_m}$$

Where the retention time of the solute is t_r and the retention time of solvent or unretained substance is t_m. The corresponding retention volume or distance can also be used, as they are directly proportional to retention time. Retention volumes are sometimes preferred, because t_r varies with flow rate. The factor is then calculated by

$$V=(V_r-V_{m)} / V_m$$

Where Vr is the retention volume of the solute and V_m is the elution volume of an unretained substance. There is no universally accepted method for the accurate measurement of the volume of an unretained substance. Numerous methods have been proposed [54].
For TLC,

$$\mathbf{K^{/} = (1-R_f)/R_f}$$

Where R_f is the distance traveled by the analyte to that of the mobile phase [31]. The factors which influence the reproducibility of retention in HPLC have been studied [32]. The conclusion is that the relative method of recording retention (e.g., relative capacity factors of retention indices) is more robust for reliable inter laboratory comparisons than the use of capacity factors.

General Recommendation for SST

System suitability testing is essential for the assurance of the quality performance of the chromatographic system. The amount of testing required will depend on the purpose of the test method. For dissolution or release profile test methods using an external standard method, k', T and RSD are minimum recommended system suitability tests. For acceptance, release, stability, or impurities/degradation methods using external or internal standards, k', T, R, and

RSD are recommended as minimum system suitability testing parameters. In practice, each method submitted for validation should include an appropriate number of system suitability tests defining the necessary characteristics of that system. Additional tests may be selected at the discretion of the applicant or the reviewer.

CONCLUSION

Reproducible quality HPLC results can only be obtained if attention has been paid to the method development, validation and the system's suitability to carry out the analysis. There are numerous variables to consider in developing an accurate and rugged chromatographic method. The extent depends on the purpose of the test that is, stability-indicating assays are the most demanding, whereas identification tests are the least demanding. Methods should not be validated as a one-time situation, but methods should be validated and designed by the developer or user to ensure ruggedness or robustness throughout the life of the method. The variations due to the drug product manufacturing process, the laboratory sample preparation procedure and the instrument performance contribute to the accuracy of the data obtained from the analysis. With proper validation and tight chromatographic performance (system suitability) criteria, an improvement in the reliability of the data can be obtained. Variations, except from the drug product manufacturing process, will be minimized. Only with good reliable validated methods, can data that are generated for release, stability, and pharmacokinetics be trust-worthy.

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