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Review Article

HPLC: Isocratic or Gradient Elution and Assessment of Linearity In Analytical Methods

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ABSTRACT

The aim of pharmaceutical analysis is to obtain the necessary qualitative and quantitative information about the investigated sample. HPLC is the dominant separation technique in modern pharmaceutical and biomedical analysis because it results in highly efficient separations and in most cases provides high detection sensitivity. Chromatographic optimization procedures are becoming more multidisciplinary to obtain more and more information on the separations which may be isocratic or gradient. During validation of analytical methods a correlation coefficient close to unity ($r = 1$) is considered sufficient evidence to conclude that the experimenter has a perfect linear calibration since it is common practice to check the linearity of a calibration curve by inspection of the correlation coefficient. The aim of the presented review is divided into two parts, first is to compare isocratic and gradient elution and to propose suitable statistical procedures to access linearity of the analytical method. We found that many of the previous reasons for avoiding gradient elution (i.e. long re-equilibration times, poor precision and difficult optimization) appear much too pessimistic and linear functional analysis or lack of fitness test as another suitable statistical tool to evaluate linearity of the analytical method. Some other validation parameters are also discussed here in brief.

Keywords: Linearity, Method validation, linear functional analysis, lack of fitness test, High Performance Liquid Chromatography.

1. INTRODUCTION

In recent years, research in analytical chemistry has concerned mainly on the development and application of physical and physicochemical analytical methods, instrumental analysis, which in their speed and sensitivity have far surpassed the classic methods of gravimetric and even volumetric analysis. Most of the major developments in analytical chemistry take place after 1900. During this period instrumental analysis becomes progressively dominant in the field. In particular many of the basic spectroscopic and spectrometric techniques were discovered in the early 20th century and refined in the late 20th century [1].

The separation sciences follow a similar time line of development and also become increasingly transformed into high performance instruments. In the 1970s many of these techniques began to be used together to achieve a complete characterization of samples [2].

2. IMPORTANCE OF ANALYTICAL TESTING

Globalization of the pharmaceutical industry has the potential to rapidly spread poor-quality medicines worldwide before adequate detection and intervention are possible [3].

With the evolution of today's specialized therapy, the role of the compounding pharmacist in the practice of pharmacy is more evident than ever. The art and science of compounding allows the pharmacist to prepare dosage forms that best suit the needs of individual patients. For several years compounding has been on the rebound, influenced by the modernized practice of pharmacy. In 2001, the FDA's Division of Prescription Drug Compliance and Surveillance conducted a limited survey of common drugs and dosage forms compounded by 12 pharmacies located throughout the United States. This survey enabled the FDA to assess quality, purity, and potency of the compounded drug products. Twenty-nine samples were collected and subjected to original and repeated analytical testing during the survey. Ten (34%) of 29 sampled products failed standard quality tests. Nine of the 10 products failed potency testing (less of the active ingredient(s) than declared on the label) with a failure range of 59 to 89%.

In 2006, the FDA conducted a second survey in which it collected both active pharmaceutical ingredients (API) and finished compounded drug products from compounding pharmacies.3 Of the 36 samples analyzed, 12 (33%) failed analytical testing, with potency ranging from 67.5% to 268.4% of the amount declared on the label [4].

Similarly, the Indian government estimates that counterfeit drugs account for 0.34% of the total pharmaceutical market and substandard drugs account for 9.34% [5]. Evidence suggests that a significant proportion of

drugs consumed in the developing world are of poor quality [6].

At the same time drug analysts play a predominant role in assuring the quality of bulk drug materials and drug formulations and this is also closely related to the safety issue [7].

The goal of pharmaceutical manufacturers is to produce drug substance of acceptable quality for formulation into a drug product. An array of tests is used to determine if the material has acceptable quality prior to formulation of the drug product [8]. The purity of a drug product is in turn determined on the basis of the percentage of the labeled amount of API found in it by a suitable analytical method [9].

3. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High-performance liquid chromatography (HPLC) was introduced to pharmaceutical analysis not long after its discovery in the late 1960s. By now it has developed into a generally applicable analytical method providing rapid and versatile separation possibilities that meet the increasing requirements for purity testing of bulk pharmaceuticals and pharmaceutical products. It is also suitable for the determination of drugs in biological and environmental samples. It provides a number of highly selective variants to resolve almost every type of separation problem: on the basis of this, HPLC and related techniques can be regarded as the most important analytical method in contemporary pharmaceutical analysis [10].

HPLC is the prevailing separation technique for nonvolatile organics, drugs, metabolites or toxic residuals and element speciations using isocratic and gradient elution being still in progress. The RP-HPLC, ion-pair HPLC, ion, ion-exchange HPLC or sometimes size exclusion chromatography are currently used and developed in practice. The HPLC based on ion exchange, chelate formation, and ion interactions is highly suitable for the separation and determination of inorganic species or ions [11].

HPLC as an assay method for bulk drug materials (with a share of about 50% in USP [12] and 15% in Ph. Eur. [13] the picture is clear and positive. It is evident that a well-designed, carefully optimised and validated HPLC method has sufficient selectivity to furnish accurate assays.

Assay by HPLC is one of the most common techniques used to measure the quality of the drug substance. The utility of HPLC for this use is impacted by the precision that can be achieved [8]. Görög [7] estimated the precision of compendial HPLC methods in the range of 0.5–1.0%. A literature review of typical intermediate precision values for HPLC assays shows ranges of about 0.2–1.7% with averages between 0.6 and 1.1% [14-16]. *Gradient elution* gave a shorter overall analysis with similar resolution of the critical pair compared to isocratic elution without sacrificing repeatability in retention time, peak area and peak height or linearity of the calibration curve [17].

In terms of separation speed, gradient elution is generally considered to be an inherently slower technique than isocratic elution since a widely accepted rule of thumb indicates that the column should be flushed (i.e. equilibrated) with at least 10 column volumes of initial eluent before reliable retention can be obtained in the next run [11]. Furthermore, many chromatographers have a phobia of "ghost" peaks [18], baseline noise [19] and other disturbances (e.g. eluent mixing) [20] associated with gradient elution that can lead to inaccurate values of peak area and peak height and impede quantitation. Also, gradient elution instrumentation is more complex and requires more regular maintenance compared to isocratic elution [17].

In gradient elution, a modulator is often used in the mobile phase to adjust eluent strength for better results in chromatographic separations. Compared with isocratic elution, the modulator concentration in the mobile phase in gradient elution is increased or decreased continuously with time [21]. Therefore, gradient elution can be used to separate components which have a wide range of retentivity with no loss of resolution. Gradient elution is able to produce high peak heights in a shorter operation cycle compared with isocratic elution. For these reasons, gradient elution has been widely used in high performance liquid chromatography for analytical purposes. Increasingly, gradient elution is applied to preparative- and large-scale chromatography for the separation of various macromolecules, such as proteins. The wide range of retentivity of proteins makes gradient elution indispensable [22]. However, simple extension of analytical instrumentation and procedures may not be sufficient for large-scale separations. For instance, when two or more mobile phases are mixed in gradient elution chromatography, air bubbles are often formed and then captured in the closed mixer, which may lead to distortion of gradient shape. In the laboratory, various methods, including heating, helium and nitrogen gas purging, decompression, ultrasonification and using special degassing devices, are employed for removal of air from the mobile phases. These degassing methods are impractical in large-scale separations [23].

After the end of a previous gradient run, the column must be completely reequilibrated with its initial mobile-phase before the next injection, usually by switching to its initial mobile phase rather than by a reverse gradient. Incomplete equilibrium with the initial mobile phase after the prior run will cause earlier elution and poor separation of the sample compounds in the next run [22].

4. HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

HPTLC allows fast and inexpensive method of analysis in the laboratory and in the field. The modern HPTLC technique, combined with automated sample application and densitometric scanning, is sensitive and completely reliable, suitable for use in qualitative and quantitative analysis. HPTLC is a valuable tool for reliable identification because it can provide chromatographic fingerprints that can be visualized and stored as electronic images [24].

HPTLC does not require expensive instrumentation, when only modest precision and reproducibility are necessary. Under such circumstances HPTLC can be carried out almost anywhere. All the necessary tools, including chemicals can be conveniently carried [25].

High Performance TLC layers are smaller, contain sorbent with smaller, more uniform particle size; are thinner; and are developed for a shorter distance compared to TLC layers. These factors lead to faster separations, reduced zone diffusion, better separation efficiency, lower detection limits, less solvent consumption, and the ability to apply more samples per plate. However, smaller samples, more exact spotting techniques and more reproducible development techniques are required to obtain reproducible results [26].

5. ANALYTICAL METHOD VALIDATION

Validation (evaluation of suitability) of an analytical technique a procedure aimed at obtaining experimentally justified evidence of the ability of this technique to give re-sults characterized by the required *accuracy* and *precision* [27-34].

Analytical method validation is the systematic process of establishing that an analytical method is acceptable for its intended purpose. In general the developer or user of the method generates evidence on specificity, linearity range, accuracy, precision, detection limit, quantitation limit, ruggedness and robustness of the method for regulatory submissions or in-house application. The iterative process of method development and validation has a direct impact on the quality of the above data. Such validated analytical methods for qualitative or quantitative testing of drug molecules assume greater importance when they are employed to generate quality and safety compliance data during development and post-approval of drug products [35].

For an analytical result to be fit for its intended purpose it must be sufficiently reliable that any decision based on it can be taken with confidence. In the light of this, analytical method validation can be considered as the process of defining the analytical requirements, and confirming that the method under consideration has performance capabilities consistent with what the application requires [32]. Method validation procedures and acceptance criteria were for a long time matter of personal prudence until various industrial committees and regulatory agencies developed framework guidelines for performing such validations for methods applicable to drugs and pharmaceuticals [36].

A method has to be validated when it is necessary to verify whether its performance parameters are adequate for use for a particular analytical problem. For example, (a) when a new method is developed for a specific problem; (b) when indications exist that an established method is changing with time; (c) when an established method is revised to incorporate changes/improvements or to extend it for another purpose; (d) when an established method is used in a different laboratory, or with different analysts or different instrumentation; (e) to demonstrate the equivalence between two methods, e.g. a new method and a standard [35].

So many variables exist during methods validation that it makes it very difficult, if not impossible, to give an accurate prediction of the length of the validation project. There is an enduring myth in the pharmaceutical industry that it should *only take six weeks* to validate a method. The formal validation portion itself should only take about six weeks, but the preparation and documentation take significantly more time [37]. Everyday many chromatographers face the need to develop a high performance liquid chromatography (HPLC) separation. Any systemic approach to HPLC method development should be based on this knowledge of the chromatographic process. In most cases, a desired separation can be achieved easily with only a few experiments. In other cases, a considerable amount of experimentation may be needed. A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result.

Ideally, every experiment will contribute to the end result so that there are no wasted run. Usually, this requires that the result of each chromatographic run be assessed before proceeding with the next experiments.

Chemical composition of the sample can provide valuable clues for the best choice of initial conditions for an HPLC separation. Some chromatographers try to match the "chemistry" of the sample to a best choice of initial HPLC conditions. To do this, they rely heavily on their own past experience (i.e., separation of compounds of similar structure) and/or they supplement this information with data from the literature. Other workers proceed directly to initial chromatographic separations, paying little attention to the nature of the sample. These two kinds of HPLC method development might be characterized as theoretical vs empirical. Once an initial separation has been carried out, the choice of ensuing experiments can be made on the basis of similar considerations (theoretical vs empirical).

Either a theoretical or an empirical approach to HPLC method development can be successful, and a "best" strategy is often some blend of these two procedures [38].

6. LINEARITY ASSESSMENT

It is common practice to check the linearity of a calibration curve by inspection of the correlation coefficient *r*. A correlation coefficient close to unity ($r = 1$) is considered sufficient evidence to conclude that the experimenter has a perfect linear calibration. Although the Analytical Methods Committee and some articles on analytical validation discouraged using the correlation coefficient in the context of testing for linearity [39-41], many laboratories around the world base the linearity of their instrumental methods on a so-called (by the author of the present article) "*r*-test". Very few publications seen using lack of fitness test for the assessment of linearity in analytical methods thus, we recommend this as another alternative for the purpose.

6.1. Linear calibration function

Ordinary least-squares (OLS), or weighted least squares (WLS), which consider heteroscedasticity in the response variable, are probably the most widely used regression techniques. However, they are of limited scope because they consider that the *x*-axis is free of error. For this reason, OLS and WLS should not be applied in the cases described above since the uncertainties associated with the results in both axes are habitually of the same order of magnitude [42].

The tests recommended by this procedure were chosen considering the theoretical background and ease of application but there are causes of lack-of-fit other than non-linearity that can arise in calibration curves [43]. This makes sufficient background to elaborate linear functional analysis for the evaluation of linearity in calibration models.

The expression $y = f(x) + \delta$ can be rewritten as

$$\hat{y}i = \Phi xi + \delta \quad (1)$$

where $\hat{y}i$ and xi represent the estimated experimental response and the analytical concentration respectively, both at a concentration level i. The coefficients Φ and δ represent the sensitivity (slope) of the analysis and the intercept respectively [44].

Test is performed in three steps replication, error sum squares and degrees of freedom calculations.

6.2. *Replication*

The experimenter must have a reasonable number of standard solutions and instrumental replicates. Calibration experiments with only one standard per concentration level are a poor calibration strategy and must be avoided unless the standard solutions are effectively error-free. Based on the reported behaviour of the uncertainty as a function of the replication and considering that the minimum number of concentration levels proposed by various guidelines and articles on analytical validation varies between five and six, it is reasonable to measure the linearity of a calibration function by preparing a minimum of five concentration levels in triplicates [29, 36, 41].

6.3. Error sum squares

After selecting a sensible number of concentration levels (*I*) and replicating every concentration level *J*-times in a particular calibration experiment, the summation of three squared differences, namely the residual error sum of squares (*SSr*) pure experimental error sum of squares (*SS*ε) and lack-of-fit error sum of squares (SS_{lof}), must be calculated according to the following equations:

$$SS_r = \sum_{i=1}^{I}\sum_{j=1}^{J_i}(y_{ij} - \hat{y}_i)^2 \quad (2)$$

$$SS_\varepsilon = \sum_{i=1}^{I}\sum_{j=1}^{J_i}(y_{ij} - \bar{y}_i)^2 \quad (3)$$

$$SS_{lof} = SS_r - SS_\varepsilon = \sum_{i=1}^{I}(\bar{y}_i - \hat{y}_i)^2 \quad (4)$$

The term *yij* represents the experimental response, $\hat{y}i$ is the estimated response obtained by using Eq. (1), and $\bar{y}_i$ is the average response at every concentration level.

6.4. Degrees of freedom

The degrees of freedom (*DF*) associated to Eqs. (2)-(4) are respectively:

DFr = (IJ − 2) .....(5)

DFε = (IJ − I) .....(6)

DFlof = (I − 2).....(7)

The bracketed number 2 in Eqs. (5) and (7) and associated with Eqs. (2) and (4) respectively, represents the number of parameters described by Eq. (1) (the Φ slope + the δ intercept =2 parameters). If a model with a different number of parameters to those described by Eq. (1) were studied, for instance:

$$\hat{y}_i = \phi xi - \varphi x^2 i + \delta \quad (8)$$

The degrees of freedom associated with Eqs. (2) and (4) would be (*IJ* − 3) and (*I* − 3) respectively. The bracketed number 3 in this case, represents the three parameters $(\phi + \varphi + \delta)$ of Eq. (8).

6.5. Acceptability of linearity data

Analytical Method Committee suggests using the *F*-test as a reliable approach to check the linearity of any calibration function. The procedure is as follows:

- The purely experimental variance and lack-of-fit variance designated by σ_{ε}^2 and σ_{lof}^2 are estimated by computing the quotients $SS\varepsilon/(IJ-I)$ and $SS_{lof}/(I-2)$ respectively.
- The calculated σ_{ε}^2 and σ_{lof}^2 variance terms are used to calculate the Fisher variance ratio or *F*-test by the expression:

  $F(I-2)/(IJ-I) = \sigma_{lof}^2 / \sigma_{\varepsilon}^2$ .....(9)
- The value of $F(I-2)/(IJ-I)$ calculated experimentally is compared against the critical value of *F* found in statistical tables, generally at the 95% confidence level for $I-2$ and $IJ-J$ degrees of freedom in the numerator and denominator respectively. If the experimental data set describes a genuine linear calibration of the form given by Eq. (1) then the condition $F_{tabulated} > F(I-2)/(IJ-I)$ must be fulfilled [41].

7. ACKNOWLEDGEMENT

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8. CONCLUSION

It is apparent from the present considerations that the goal of fast chromatography can best be approached in liquid chromatography by employing gradient elution. Gradient elution RPLC is a powerful technique required to separate samples that otherwise exhibit the general elution problem under isocratic conditions. Delivering competent analytical judgment on samples in a timely manner is becoming more difficult as the sample load in quality control laboratories continues to increase. Same class of drugs may have almost same functional groups and gradient RPLC will be more useful to separate such complicated mixtures. The main factors that drive chromatographers to use gradient elution are multi-component samples, which span a wide range in retention.

Ordinary least-squares (OLS), or weighted least squares (WLS) and r-test are probably most widely used concept for the evaluation of linearity in calibration curves. Based on presented facts we recommend linear calibration function as another statistical tool to assess the linearity of calibration curves of analytical procedures. Correlation coefficient close to one is also sign of good linear calibration curve. Other parameters of method validation and system suitability parameters are presented under Table 1 and Table 2 respectively. Analyte concentration versus precision within or between days and analyte recovery at different concentrations are presented under Table 3 and 4 respectively.

Table 1: Validation parameters definitions according to various guidelines as per ICH, US FDA, AOAC, USP and IUPAC along with common methods of their measurements

Validation parameters	Definitions	Measurement methods
Specificity	• Ability to assess unequivocally the analyte in the presence of components, which may be expected to be present [28-30]. • Ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample [32]. • Ability of a method to measure only what it is intended to measure [44]. • Ability to assess unequivocally the analyte in the presence of components, which may be expected to be present [34] • The degree to which a method can quantify the analyte accurately in the presence of interferants [46].	Sufficient separation of all compounds. Check peak purity with a diode-array detector and/or a mass selective detector. Run the sample under different chromatographic conditions and/or with different columns [45].
Selectivity	The USP monograph [34] defines 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.	Obtained by choosing optimal columns and setting chromatographic conditions, such as mobile phase composition, column temperature, and detector wavelength [45].

Continued.....

Linearity and range	• Ability (within range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample. Range: Interval between the upper and lower concentration (amounts) of analyte in the sample including the concentrations for which suitable level of accuracy, precision, and linearity has been demonstrated [30]. • Calibration/Standard Curve: Relationship between response and known concentration of analyte [32]. • Defines the ability of the method to obtain test results proportional to the concentration [44]. • Ability (within range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample. Range: Interval between the upper and lower concentration (amounts) of analyte in the sample including the concentrations for which suitable level of accuracy, precision, and linearity has been demonstrated [34].	Inject five standards containing the full working concentrations. Inject each standard three times. Average the peak area. Plot the averaged peak area vs. concentration. Calculate the linear regression [45]. Lack of fitness test [41].
Accuracy	• Expresses the closeness of agreement between the values, which is accepted either as a conventional true value or the value found. (Also referred as trueness) [30]. • Closeness of mean test results obtained by method to the true value (concentration of analyte) [32]. • Closeness of the determined value to the true value [44]. • Closeness of test results obtained by that method to true value [34]. • Trueness is the closeness of agreement between a test result and accepted reference value. Smaller bias means greater trueness [46]	Spike a blank sample with the analyte at three different concentrations. Calculate the deviation of the results obtained with the method to be validated with the true value [45]. Also see Table 4
Precision	• The precision of analytical procedures expresses the closeness of agreement (degree of scatter) between series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions [30]. • Describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of single homogenous volume of biological matrix [32] • Degree of agreement of measurements under specific conditions [44]. • Degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample [34]. • Closeness of agreement between test results obtained under stipulated conditions [46].	• Repeatability is obtained when the analysis is carried out in one laboratory by one operator using one piece of equipment over a relatively short timespan [45]. • Intermediate precision is a term that has been defined by ICH [26] as the long-term variability of the measurement process and is determined by comparing the results of a method run within a single laboratory over a number of weeks. • Reproducibility as defined by ICH [26,27] represents the precision obtained between laboratories Also see Table 3
Limit of detection (LOD)	• Lowest amount of analyte in the sample, which can be detected but not necessarily quantitated under stated experimental conditions [30]. • Lowest content that can be measured with reasonable statistical certainty [44] • Lowest amount of analyte in the sample, which	• Visual definition • Calculation from the signal-to-noise ratio to 3 or 2. Calculation from the standard deviation of the blank • Calculation from the calibration line at low concentrations

Continued……

	can be detected but not necessarily quantitated under stated experimental conditions [34]. • Smallest amount of conc. of analyte in the sample that can be reliably distinguished from zero [44].	DL/QL = F×SD/b Where, F: Factor of 3.3 SD: Standard deviation of the blank, standard deviation of the ordinate intercept, or residual standard deviation of the linear regression *b*: Slope of the regression line [47]
Limit of quantitation (LOQ)	• Lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy [30]. • The lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy also called LLOQ (Lower limit of quantification) [44]. • The limit of quantitation is the lowest amount of analyte in a sample, which can be quantitatively determined with precision and accuracy appropriate to analyte and matrix considered [34]. • Lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy [46].	• Visual definition • Calculation from the signal-to-noise ratio to 3 or 2. Calculation from the standard deviation of the blank • Calculation from the calibration line at low concentrations DL/QL = F×SD/b Where, F: Factor of 10 SD: Standard deviation of the blank, standard deviation of the ordinate intercept, or residual standard deviation of the linear regression *b*: Slope of the regression line [47]

Table 2: System suitability parameters and recommendations

Parameter	Definition	Calculation	Recommendations
Capacity Factor[38]	The migration rate of an analyte on a column	$k' = t_R - t_0 / t_0$ t_R = band retention time t_0 = column dead time	$0.5<k'<20$
Resolution (Rs)	Resolution, the separation between two peaks [45]	$R = \frac{2(t_2 - t_1)}{W_2 - W_1}$ t_1 and t_2 are retention time of two peaks W_1 and W_2 are width of the two peaks [38, 48]	At least 1.5 from all other sample components. If this cannot be achieved the unresolved components at their maximum expected level will not affect the final assay result by more than 0.5% [36]
Tailing Factor (TF)	It is defined as the distance from the front slope of the peak to the back slope divided by twice the distance from the center line of the peak to the front slope, with all measurements made at 5% of the maximum peak height [38].	$TF = W_{0.05} / 2F$ F = Width of the front half of the peak $W_{0.05}$ = Width of peak All measurements are at 5% height of peak. [Please see Figure 1]	0.8-1.5, if the peaks have significantly different heights (e.g., used in the determination of impurities) and/or exhibit tailing, CDER recommends that *Rs* 2.0. It should be noted that this situation is most frequently encountered in practice during the analysis of drugs [27].
Asymmetric Factor (ASF)	It is defined as the distance from the center line of the peak to the back slope divided by the distance from the center line of the peak to the front slope, with all measurements made at 10% of the maximum peak height [49].	AsF = BC/CA BC = distance from the center line of the peak to the back slope CA = distance from the center line of the peak to the front slope All measurements are at 10% height of peak [38] [Please see Figure 1]	<1.5, Good column produce peaks with ASF 0.95-1.1 (exactly symmetrical peaks have an ASF of 1.0) [38]
No. of theoretical Plates [48]	Separate equilibrations of the sample between the stationary and mobile phase occur in these "plates" [Fig. 2]. The analyte moves down the column by transfer of equilibrated mobile phase from one plate to the next.	$N = 5.545\left[\frac{t_R}{W_{1/2}}\right]^2$ N = No. of theoretical plates t_R = Retention time $W_{1/2}$ = Peak width at half height	>2000 [34]

Continued…….

Relative retention time [49]	The ratio of the retention time (RT) of a component relative to that of a standard, obtained under identical conditions	$RRT = t_{RA} / t_{RB}$ RRT = Relative retention time t_{RA} = retention time of component A t_{RB} = Retention time of main component B in the same chromatogram	Not defined, RRT <1 elutes before the main peak, and any peak with an RRT >1 elutes after the main peak [27]

Table 3: Analyte concentration versus precision within or between days [45]

Analyte (%)	Analyte ratio	Unit	RSD(%)
100	1	100%	1.3
10	10-1	10%	1.8
1	10-2	1%	2.7
0.1	10-3	0.1%	3.7
0.01	10-4	100 ppm	5.3
0.001	10-5	10 ppm	7.3
0.0001	10-6	1 ppm	11
0.00001	10-7	100 ppb	15
0.000001	10-8	10 ppb	21
0.0000001	10-9	1 ppb	30

Table 4: Analyte recovery at different concentrations [45]

Active ingredient (%)	Analyte ratio	Unit	Mean recovery
100	1	100%	98-102
10	10-1	10%	98-102
1	10-2	1%	97-103
0.1	10-3	0.1%	95-105
0.01	10-4	100 ppm	90-107
0.001	10-5	10 ppm	80-110
0.0001	10-6	1 ppm	80-110
0.00001	10-7	100 ppb	80-110
0.000001	10-8	10 ppb	60-115
0.0000001	10-9	1 ppb	40-120

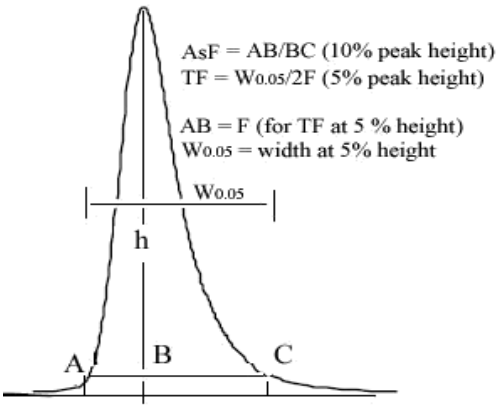

Figure 1: Calculation of tailing and asymmetric factor

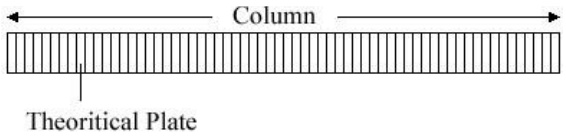

Figure 2: Hypothetical plates of the column showing equilibrium of the sample between the stationary and mobile phase

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