Chapter-1

INTRODUCTION
1.1. INTRODUCTION – ANALYTICAL CHEMISTRY

Analytical Chemistry plays a critical role in the development of a compound from its synthesis stage to its marketing stage as a part of a drug formulation and analysis [1]. Before the introduction of chromatographic methods into pharmaceutical analysis in the middle of the 20th century, almost exclusively classical methods such as titrimetry, gravimetry and later on UV spectrophotometry/colorimetry were available for this purpose. It was well known already in those years that, due to the poor specificity of these methods, the value of the percentage figures obtained in such a way for the active ingredient content were of limited value. Nevertheless, due to the lack of specific chromatographic methods these assay methods were considered to be among the most important characteristics of the quality of a bulk drug substance. The purity was checked by means of physical constants, mainly by the melting point and the width of the melting range, limit tests for signal (mainly inorganic) impurities, clarity and colour of the solution of the material, etc.

1.2. THE PRESENT STATE-OF-THE-ART: ASSAY METHODS IN THE CHROMATOGRAPHY ERA

1.2.1. Introduction

The invention and rapid spread of thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) in the 1960s and 1970s [2], respectively, created an entirely new situation in this field. The reasons for this are as follows: (1) both methods enable the detection, separation, identification and quantitative determination of organic impurities which were up to that time not measurable [3]; (2) the selective chromatographic methods were found to be suitable for the reliable determination of also the main component.
1.2.2. Non-specific methods

1.2.2.1. Titrimetric methods

Classical, non-specific methods are still used, especially in the European Pharmacopoeia [4]. Of these, the non-specificity of titrimetric methods is evident: in the majority of cases organic impurities contain the same functional group on which the titration of the drug material is based. Signs of some modernization are the spreading of non-aqueous titration methods expanding the field of application of titrimetric methods to (very) weak acids and bases as well as potentiometric (in the case of nitritometric titrations amperometric) end-point detection improving the precision of the methods. Advantages of these methods are saving time and labour, high precision and the fact that there is no need of using reference standards. However, due to their poor specificity the accuracy of titrimetric methods is also poor in the presence of related impurities.

1.2.2.2. Spectrophotometric/colorimetric methods

Another group of non-specific methods in pharmacopoeias are spectrophotometric methods based on natural UV–VIS absorption and to a lesser extent visual (VIS) spectrophotometric methods based on chemical reactions (colorimetric methods) [5]. The reason for their non-specificity is the same as in the case of titrimetric methods: most of the impurities of drugs contain the same or similar chromophoric groups as those of the drug material. The low time and labour consumption of the methods as well as good precision are advantages in this case also, especially if the method is based on natural absorption. There is no clear picture regarding the necessity of reference standards. In the majority of pharmacopoeial monographs of US Pharmacopoeia [6] the use of reference standards is prescribed, while in the European
Pharmacopoeia the calculation of the content is mainly (but not exclusively) based on specific absorbance values given in the monographs. Although the principles of the validation of the determination of specific absorbance have been set up [7], and this is the less time consuming approach, this can be the source of further analytical error, if not high-level spectrophotometers are used for the assay. It is worth mentioning that (although not too many) startlingly outdated colorimetric methods based on chemical reactions are still in use for the assay of bulk drug materials.

1.2.2.3. Other methods

Although some other non-specific methods (polarimetry, polarography, fluorimetry, etc.) do not play an important role in the assay of bulk drugs, it is to be noted that even the precision of these methods is by no means sufficient for this purpose.

1.2.3. Specific chromatographic methods

1.2.3.1. High-performance liquid chromatography

HPLC methods appeared for the first time for the assay of bulk drug materials in 1980 [8]. This has become the predominant method in USP XXVII [6] and—although to a lesser extent—it is one of the most widely used methods also in Ph. Eur. 4 [4]. The reason for this is that, in contrast to the above discussed non-specific methods the specificity of this method is excellent and at the same time sufficient precision is also attainable. Due to these advantageous features and the disadvantages of the methods discussed so far it can be stated HPLC is certainly one of the methods applicable for the assay of drug materials which can afford accurate results. However, it has to be mentioned that the high specificity, precision and accuracy are attainable only if lengthy system suitability tests are carried out prior to the HPLC assay. For this reason the price to be paid for the high specificity, precision and accuracy is also high:
the HPLC method is by about one order of magnitude more time consuming and labour extensive than the above discussed non-specific methods.

### 1.2.3.2. Gas chromatography (GC)

Due to the insufficient volatility and thermal stability of the majority of drug materials, gas chromatography can be used for their assay in a limited number of cases only. For the specificity, precision and accuracy as well as the time and labour consumption of this method the same considerations apply that are described for HPLC.

### 1.2.3.3. Thin-layer chromatography–UV spectrophotometry

Before the introduction and widespread adoption of HPLC, the high specificity of TLC was often exploited for quantitative analytical purposes using spot elution followed by spectrophotometric measurement and is still prescribed in some cases in USP XXVII [6] inspite of being a labour-intensive and less precise method. The low acquisition, operational and maintenance costs needed to successfully perform the TLC analytical technique are very important because it can provide product quality assessment capability in areas where laboratory facilities for pharmaceutical quality analysis are minimal or do not exist.

Kenyon et al. demonstrated that TLC can be used to provide a semi-quantitative yet versatile and robust testing of pharmaceuticals in a resource limited environment [9]. The ease of deployment with low operational costs of the TLC based analytical techniques has been a key to the vast increase in its use to detect counterfeit/substandard medicines in markets particularly in resource constrained settings [10, 11].

Recent advancements in technology have contributed to a marked improvement of repeatability and reliability of TLC based testing. Automating the TLC sample
application step has markedly improved repeatability of the sample application process, and thereby the overall test procedure. In addition the detection technology has been developed to measure the intensity of a spot of interest on the plate by which comparisons to standards can be related to drug content. With the aid of software, the complex mathematics needed to calculate the drug content from the reflected light can be easily performed. These two key developments have made TLC-Densitometry a reliable method for pharmaceutical drug analysis. The separation media also have been improved by reducing the particle size and uniformity which has evolved into HPTLC. The HPTLC offers all of the advantages of TLC but with improved separation capacity by marked improvement in plate numbers which approach those afforded by the conventional HPLC columns.

The above developments have increased the acquisition costs for HPTLC plates but the new systems have brought improved versatility, throughput and robustness to the TLC technique while retaining the low running and maintenance costs.

1.2.3.4 Ultra Performance Liquid Chromatography (UPLC)

Ultra Performance Liquid Chromatography (UPLC) could be considered to be a new direction of liquid chromatography. UPLC, as its first producer Waters proclaims, means “speed, resolution and sensitivity” [12]. As it is very well known from Van Deemter equations, the efficiency of chromatographic process is proportional to particle size decrease. According to his model describing band broadening, which describes relationship between height equivalent of theoretical plate (HETP) and linear velocity, one of the terms (path dependent term), is dependent on a diameter of particle packed into the analytical column. Smaller particle diameter can significantly reduce HETP which results in higher efficiency and the flatter profile of Van Deemter curve (Figure 1). Consequently, the mobile phase flow-rate increase does not have
negative influence to the efficiency as it could be observed at 10 or 5 μm particles [13-15]. The negative aspect of small particle packed columns used in HPLC is, however, high back-pressure generating.

In conventional HPLC the choice of particle size must be a compromise. The smaller the particle size, the higher the column back-pressure is occurring in the HPLC system. That could be a limitation of the use of such columns in HPLC systems. Small column diameters like 2.1 or 1.0 mm could also cause similar problems and disable their use under the conventional conditions. Throughout the history of HPLC there has been a trend to use smaller particles packing material. Due to the pressure limitation of conventional equipment, shorter columns packed with small particle diameter particles were used.

However, in order to use ultra high pressure chromatography routinely in the laboratory, some practical concerns, such as sample introduction, reproducibility and detection still needed an improvement. Ultra high pressure columns required extremely narrow sample plugs to minimize any sample volume contribution to peak broadening. To overcome these problems, Acquity UPLC system was developed because many of ultra high pressure systems used before needed in-house modification of commercial products by laboratory itself and also the own manufacturing of analytical columns [16-18] often capillary columns, as was stated above.
1.3 VALIDATION

The demonstration of the ability of an analytical method to quantify is of great importance to ensure quality, safety and efficacy of pharmaceuticals. Consequently, before an analytical method can be implemented for routine use, it must first be validated to demonstrate that it is suitable for its intended purpose.

1.4 LIQUID CHROMATOGRAPHIC METHOD

The actual meaning and utilization of the various phases of a validation are exemplified by a survey of published methods on LC analysis of drug substances and dosage forms.

- **Linearity**-

The response function for an analytical procedure is the existing relationship, within a specified range, between the response (signal, e.g. area under the curve, peak height, absorption) and the concentration (quantity) of the analyte in the sample. The
calibration curve should be described preferably by a simple increasing or decreasing) response function that gives reliable measurements, i.e. accurate results. The response function – or standard curve – is widely and frequently confounded with the linearity criterion.

The linearity criterion refers to the relationship between the quantity introduced and the quantity back-calculated from the calibration curve while the response function refers to the relationship between the instrumental response and the concentration.

For an analyst, the “test results” are, without ambiguity, the back-calculated measurements evaluated by the “regression line” that is in fact the calibration curve, established using appropriate statistics methodologies. Another aspect that is very important is the fit-for purpose principle [19]. The central idea is very logical: the purpose of an analytical procedure is to give accurate measurements in the future; so a standard curve must be evaluated on its ability to provide accurate measurements. A significant source of bias and imprecision in analytical measurements can be caused by the inadequate choice of the statistical model for the calibration curve. The statistical criteria such as $R^2$, lack-of-fit or any other statistical test to demonstrated quality of fit of a model are only informative and barely relevant for the objective of the assay [19-23]. For that intend, several authors [24-26] have introduced the use of the accuracy profile based on the tolerance intervals (or prediction intervals) to decide if a calibration model will give quality results.

● Accuracy

In document ICH Q2R1 part 1 [27], accuracy is defined as: “... the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.”
Accuracy is usually established through spiked placebo studies (simulated samples) in which, placebo is fortified with drug at various concentrations above and below the target claim. Frequently 0, 80, 100 and 120% or 0, 75, 100 and 125% of claim are used. These samples are then passed through the processing scheme, assayed and the linearity of recovery is calculated with appropriate statistical analysis.

Under certain circumstances use of the spiked placebo method is impossible such as in academic settings or in government labs, which cannot obtain authentic placebo and its exact composition is unknown. Here the standard addition method (SA) should be used to verify accuracy by beginning with a sample and then adding known amounts of standard to it in order to derive a linearity expression. This method is also commonly practiced in impurity analysis for drug substance in which various levels of impurity are added to the lot of bulk drug showing lowest impurity levels. Linearity of recovery of degradation products likewise can be calculated following their addition to placebo for drug products.

Recovery studies can be performed using different columns or on different days are drug substance recovery studies are also performed which do not relate to method accuracy but only to reproducibility of standard preparation.

**Precision**

The ICH Q2R1 Part 1 definition of precision is: “The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.”

Precision is expressed as standard deviation (s), variance (s^2) or relative standard deviation (RSD) or coefficient of variation (CV). It measures the random error linked to the analytical procedure, i.e. the dispersion of the results around their average.
value. The estimate of precision is independent of the true or specified value and the mean or trueness estimate. For ICH Q2R1 and ISO documents, three levels could be assessed:

(1) Repeatability which “expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.”

(2) Intermediate precision which “expresses within-laboratories variations different days, different analysts, different equipment, etc.”

(3) Reproducibility which “expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).”

The repeatability conditions involve the re-execution of the entire procedure to the selection and preparation of the test portion in the laboratory sample and not only the replicate instrumental determinations on a single prepared test sample. The latter is the instrumental precision which does not include the repetition of the whole analytical procedure.

The document of the FDA, also distinguish “within-run, intra batch precision or repeatability, which assesses precision during a single analytical run”, and “between-run, inter-batch precision or repeatability, which measures precision with time, and may involve different analysts, equipment, reagents, and laboratories”.

As can be seen in the regulatory documents what makes the difference between repeatability and intermediate precision is the concept of series or runs. These series or runs are composed at least of different days with eventually different operators and/or different equipments. A run or series is a period during which analyses are executed under repeatability conditions that remain constant. The rational to select the different factors which will compose the runs/series is to mimic conditions that will be encountered during the routine use of the analytical procedure.
It is evident that the analytical procedure will not be used only 1 day. So including the variability from one day to another of the analytical procedure is mandatory. Then during its routine use, will the analytical procedure be used by only one operator, and/or on only one equipment? Depending on the answers of these questions, different factors representing the procedure that will be used during the routinely performed analysis will be introduced in the validation protocol, leading to a representative estimation of the variability of the analytical procedure.

When the selection of the appropriate factors is made, an experimental design can be made in order to optimize the number of runs or series to account for the main effects of these factors with a cost effective analysis time. For example if the factor selected are days, operators and equipments, each of them at two levels, then a fractional factorial design allows to execute four runs or series in only 2 days. The design is shown in Table 1.1.

**Table 1.1** Experimental design of four runs taking into account days, operators and equipments as sources of variability

<table>
<thead>
<tr>
<th>Run 1</th>
<th>Run 2</th>
<th>Run 3</th>
<th>Run 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>Day 1</td>
<td>Day 2</td>
<td>Day 2</td>
</tr>
<tr>
<td>Operator 1</td>
<td>Operator 2</td>
<td>Operator 1</td>
<td>Operator 2</td>
</tr>
<tr>
<td>Equipment 2</td>
<td>Equipment 1</td>
<td>Equipment 1</td>
<td>Equipment 2</td>
</tr>
</tbody>
</table>

Usually, precision is commonly expressed as the percent Relative Standard Deviation (RSD). The classical formula is:

$$\text{RSD} (%) = 100 \times \frac{\sqrt{\sigma^2}}{\bar{x}}$$

When an RSD precision is expressed, the corresponding variance is used, e.g. repeatability or intermediate precision. The computed RSD is therefore the ratio of two random variables, giving a new parameter with high uncertainty. However, in the
case of validation of analytical procedure, because the true or reference value is known, then the denominator should be replaced by its corresponding true value $\mu_T$. The RSD computed by this way depends only on the estimated precision (estimated variances), regardless of the estimated trueness.

The precision tests include tests for precision of the system which is measured by replicate analysis of a single standard solution, ordinarily run before initiation of sample analysis as part of a system suitability test. This precision measurement should be carried out on each day a particular analysis is performed giving rise to the expression of results for different days. Method precision is shown by replicate analysis of a pooled sample such as the thoroughly mixed contents from 20 capsules, 20 finely ground tablets or five ampoules. Each measured aliquot is carried through the entire sample preparation scheme and assayed. This measurement can be done on more than and also by using more than one column. Precision of recovery is based on multiple measurements made on placebos spiked at one concentration. Precision of linearity of recovery is the measure derived from the linearity of recovery study in which percents recovered at each concentration, possibly in replicate, are analysed to give the RSD. A third term in the context of precision is robustness or ruggedness.

The US Pharmacopeia [28] defines ruggedness as: "The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same sample under a variety of normal test conditions, such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperatures, different days, etc.

In the chemical literature however, a ruggedness test was defined as [27, 29]: "An intra laboratory experimental study in which the influence of small changes in the operating or environmental conditions on measured or calculated responses is
The changes introduced reflect the changes that can occur when a method is transferred between different laboratories, different experimentators, different devices, etc."

As part of a ruggedness test, the method precision is determined by assaying the same set of samples in different labs. Method ruggedness is also indicated by results from tests in which standard mixtures are chromatographed using mobile phase variations of 10-20% (organic/aqueous) and by use of one mobile phase with three to five columns of different age for analysis of a standard mixture. A final precision measure that is determined is a method precision in which different lots of bulk drug are assayed.

**The steps of a ruggedness test**

A ruggedness test requires an experimental design approach. It consists of the following steps:

- Selection and identification of the operational or environmental factors to be investigated;
- Selection of levels for the factors to be examined. In a ruggedness test 2 or 3 levels for each factor are normally considered. The ruggedness for the factors in the intervals between the factor levels is then investigated;
- Selection of the experimental design;
- Carrying out the experiments described in the design. This is the experimental part of the ruggedness test;
- Computation of the effect of the factors on the response(s) of the method, to derive which factors might have experimentally relevant effects;
- Statistical analysis of the results. In this part of the test statistically significant effects are identified;
• Drawing chemically relevant conclusions;

• When necessary giving advice for improvement of the performance of the method and definition of suitability criteria.

Selection of the factors

As a first step one selects a number of factors to examine. The selected factors should be chosen from the description of the analytical procedure or from environmental parameters which are not necessarily specified explicitly in the analytical method. The factors can be quantitative (continuous, numerical) or qualitative (discrete). The factors to be tested should represent those that are most likely to be changed when a method is transferred, for instance, between different laboratories, different devices, or over time, and that potentially could influence the response of the method. However it is not always obvious which factors will influence a response and which will not. This is one of the reasons why screening designs are used. They allow to screen a large number of factors in a relatively small number of experiments.

A list of different factors investigated in different publications is given [30-34]. The list is not exhaustive

Some salient features to be noted include:

• The selection of the factor "type of acid" in a ruggedness test could be accepted when only the pH is specified by the method rather than the acid used to bring the solution or the buffer up to the desired pH. Clearly, however, in such a case the method is poorly defined.

• A group of factors causing problems are HPLC columns. Some articles [31, 35, 36] propose to include the factor "batch of material" or "manufacturer of material" in a two level design and do this by comparing two columns. However, it is far from evident that these two selected columns are extreme levels for the whole
population of batches from one manufacturer or for the population of columns from different manufacturers. The problem could be tackled by examining more than two columns. One possibility is to consider the column factors in the same way as the factors "different laboratories, different analysts, different instruments".

**Selection of the levels of the factors**

In a second step the levels for the chosen factors are selected. For quantitative factors one considers a low and a high extreme level that is respectively smaller and larger than the nominal one. The nominal level is the level for the factor as it is given in the description of the procedure or the one that is most likely to occur in the case it is not specified in the analytical procedure. The levels for the factors are chosen in such a way that they represent the maximum difference in the values of the factors that could be expected to occur when a method is transferred from one laboratory to another without the occurrence of major errors [31].

A common error is to select levels that are too far apart from each other. In a ruggedness test one selects the extreme levels of the factors to be somewhat larger than the changes that would occur for this factor under normally changing conditions (different laboratories, etc.). In a number of published ruggedness tests one finds levels that are quite far from each other, much further than can occur by transferring a method between different laboratories. Since one does not know the effect of the factor in advance one will introduce a large possibility of finding a significant effect which is not relevant for the evaluation of the ruggedness. If in a method description the pH of the mobile phase is 5.0 then one normally should be able to work in an interval between 4.8 and 5.2. This then is the interval proposed to be examined in a ruggedness test and not for example 4.0 and 6.0. Examples of levels of factors that
seem too far from each other and that were tested in different ruggedness tests are given in Table 3.2.

**Table 3.2** Some levels of factors that are tested with large intervals in a ruggedness test (HPLC methods)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Levels as tested in the literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>Nominal ± 1 [31]</td>
</tr>
<tr>
<td></td>
<td>Nominal ± 0.5 [32, 34, 36]</td>
</tr>
<tr>
<td>Flow rate</td>
<td>Nominal ± 0.3 ml/min [32]</td>
</tr>
<tr>
<td></td>
<td>Nominal ± 0.5 ml/min [34, 36]</td>
</tr>
<tr>
<td>Wavelength (UV)</td>
<td>Nominal ± 8 to 12 nm [36]</td>
</tr>
</tbody>
</table>

**Selection of the experimental design**

To examine the ruggedness of the factors that were selected one could test these factors one variable at a time, i.e. change the level of one factor and keep all other factors at nominal level. The result of this experiment is then compared to the result of experiments with all factors at nominal level. The difference between the two types of experiments gives an idea of the effect of the factor in the interval between the two levels. The disadvantage of this method is that a large number of experiments is required when the number of factors is large.

For this reason one prefers to apply an experimental design. In the literature a number of different designs are described, such as saturated fractional factorial designs and Plackett-Burman designs, full and fractional factorial designs, central composite designs and Box-Behnken designs [37].

**Decision rule**

Most of the regulatory documents do not make any recommendation on acceptance limits to help the analyst to decide when an analytical procedure is acceptable. The only exception found concerns the FDA document on bio-analytical methods that
clearly indicates in the pre-study validation part: “The mean value should be within ±15% of the theoretical value, except at LLOQ, where it should not deviate by more than ±20%. The precision around the mean value should not exceed 15% of the CV, except for LLOQ, where it should not exceed 20% of the CV.” Later, when referring to in-study validation, the same document indicates: “Acceptance criteria: At least 67% (4 out of 6) of quality control (QC) samples should be within 15% of their respective nominal value, 33% of the QC samples (not all replicates at the same concentration) may be outside 15% of nominal value. In certain situations, wider acceptance criteria may be justified.”

**Dosing range**

For any quantitative method, it is necessary to determine the range of analyte concentrations or property values over which the method may be applied. ICH Q2R1 part 1 document defines the range of an analytical procedure as “the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity”. The FDA Bio-analytical Method validation definition of the quantification range is “the range of concentration, including ULOQ and LLOQ that can be reliably and reproducibly quantified with accuracy and precision through the use of a concentration–response relationship”, where LLOQ is the lower limit of quantitation and ULOQ is the upper limit of quantitation. Thus, the above mentioned definitions are quite similar because for both of them, the range is correlated with the linearity and the accuracy (trueness + precision). Moreover, both documents specify that the range is dependent on the specific application of the procedure. ICH Q2R1 part 2 states that the specified range is “established by confirming that the analytical procedure provides an acceptable
degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure”. The range should be anticipated in the early stage of the method development and its selection is based on previous information about the sample, in a particular study. The chosen range determines the number of standards used in constructing a calibration curve.

ICH Q2R1 part 2 recommends the minimum specified ranges for different studies:

- for the assay of a drug substance or a finished (drug) product: normally from 80 to 120% of the test concentration;
- for content uniformity, covering a minimum of 70–130% of the test concentration, unless a wider more appropriate range, based on the nature of the dosage form (e.g. metered dose inhalers), is justified;
- for dissolution testing: ±20% over the specified range;
- for the determination of an impurity: from the reporting level of an impurity to 120% of the specification.

Therefore, the dosing range is the concentration or amount interval over which the total error of measurement – or accuracy – is acceptable. It is essential to demonstrate the accuracy of the results over the entire range. Consequently, and in order to fulfill these definitions, the proposition of ICH document to realize six measurements only at the 100% level of the test concentration to assess the precision of the analytical method should be used with precautions to be in accordance with the definition of the range. Accuracy, and therefore trueness and precision should be evaluated experimentally and acceptable over the whole range targeted for the application of the analytical procedure.
Limit of quantitation

ICH considers that “quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products”. ICH Q2R1 part 1 defines the quantitation limit of an individual analytical procedure as “the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy”. Limit of quantitation (or quantitation limit) is often called LOQ. Both terms are used in regulatory documents, the meaning being exactly the same. ICH document defines only one limit of quantitation. But the quantification range of the analytical procedure has two limits: LLOQ and ULOQ.

ICH Q2R1 part 2 proposes exactly the same approaches to estimate the (lower) quantification limit as for the detection limit. A first approach is based on the well known signal-to-noise (s/n) ratio approach. A 10:1 s/n is considered by ICH document to be sufficient to discriminate the analyte from the background noise. The main problem appears when the measured signal is not the signal used to quantify the analyte. For example, in chromatography with spectral detection, the measured signal represents the absorption units, i.e. the signal height but for the quantitation the areas are generally used. Therefore, the quantitation limit is not expressing the lowest level of the analyte, but lowest quantified absorbance.

The other approaches proposed by ICH Q2R1 part 2 documents are based on the “Standard Deviation of the Response and the Slope” and it is similar to the approach used for detection limit computation. The computation ways for detection (DL) and quantitation limit (QL) are similar, the only difference being the multiplier of the standard deviation of the response:

\[ DL = \frac{3.3\sigma}{S}; \quad QL = \frac{10\sigma}{S} \]
where \( \sigma \) is the standard deviation of the response and \( S \) = the slope of the calibration curve [19, 38-55].

**System Suitability**

System suitability tests include resolution factor, precision of standard analysis or precision of impurity analysis and can include such measures as tailing factor or standard linearity. Other parameters measured under system suitability can include capacity factor (\( k' \)), retention time (\( t \)), relative retention (\( a \)), number of theoretical plates (\( N \)) or peak symmetry (\( s \)).

These terms have been adequately described in many reviews and in the USP [56].

**Capacity factor (capacity ratio) \( k' \)**

This value gives an indication of how long each component is retained on the column (ie how many times longer the component is retarded by the stationary phase than it spends in the mobile phase). \( k' \) is used in preference to retention time because it is less sensitive to fluctuations in chromatographic conditions (ie flow rate) and therefore ensures greater reproducibility from run to run. In practice the \( k \) value for the first peak of interest should be >1 to assure that it is separated from the solvent.

\[
k' = \frac{t_R - t_m}{t_m}
\]

\( t_m = \) unretained peak’s retention time

\( t_R = \) retention time of the peak of interest

**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 Separation
factor calculation \( k \) for the later peak is always placed in the numerator to assure a value >1.

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. The separation factor gives no indication of the efficiency of the column.

**Peak Resolution R**

This is not only a measure of the separation between two peaks, but also the efficiency of the column. It is expressed as the ratio of the distance between the two peak maxima, \((A_t)\) to the mean value of the peak width at base, \((W_b)\).

**Specificity**

Specificity studies include the subcategory selectivity. Selectivity implies that the method separates potential process impurities (I), degradation products (D) and structural analogues (A). Specificity, as a broader concept, also includes peak homogeneity. This means that a particular peak corresponds to a single chemical entity rather than several different molecules whether structural, geometrical or configurational isomers or unrelated compounds with overlapping retentions. This property can be indicated by diode-array detection in which spectra taken at various times while a peak is eluting are compared with standard spectra known to be due to a single entity. A second means of showing peak homogeneity is to collect the fraction as the peak elutes and run the sample in an alternate chromatographic system such as TLC or a different mode of LC. Alternatively a non-chromatographic stability indicating method such as capillary electrophoresis or certain electrochemical methods may verify that a collected peak and a standard substance are the same. When a method is shown to be specific for a particular compound, this implies that
the method is stability indicating. Further proof of this implication comes from stress studies in which drug product and/or drug substance are degraded chemically (acid, base, oxygen, air), thermally and photo-chemically. These forced degradations (FD) give rise to reaction products which can be separated from the parent compound and quantified. If this is done as part of a stability study, degradation kinetics (DK) can be established. A stressed placebo study can be included as well to show that no products resulting from possible excipient decomposition will interfere with measurement of components of interest [57].

1.5 CLEANING VALIDATION

In pharmaceutical industry the cleaning procedure is one of the most important tasks to avoid the cross contamination for subsequent batches manufactured in the same equipment. Analytical methods used to determine residuals or contaminants should be specific for the substance or the class of substances to be assayed (e.g., API residue, detergent residue) and be validated prior to cleaning validation [58-60]. Guidelines recommend thin layer chromatography (TLC), UV photometric, total organic carbon analysis (TOC), conductivity, gas chromatography (GC) and conventional high performance liquid chromatography (HPLC) methods for cleaning control or validation [61]. The use of other analytical methods, including capillary gas chromatography [62], over-pressured layer chromatography (OPLC) [63] or micellar electro-kinetic chromatography (MEKC) [64], have also been described. Ion mobility spectrometry (IMS) [65] and TOC [66] have the advantage of speed over the abovementioned methods but TOC is not specific and IMS is usually not available at pharmaceutical manufacturing facilities. Liquid chromatography–mass spectrometry (LC–MS) [67, 68] and ultra performance liquid chromatography–mass spectrometry (UPLC–MS) [69] techniques applied in pharmaceutical cleaning verification have the
advantage of improved sensitivity, selectivity and general applicability even for UV-inactive compounds. However, these techniques are more expensive than the other techniques mentioned above and not widespread yet in cleaning control analysis. Nowadays HPLC–UV is the most commonly applied technique for cleaning control and validation [70-75]. In liquid chromatography, the analysis time can be reduced by using small columns packed with sub-2μm particles. In addition, with sub-2μm particles, due to the higher efficiency and smaller retention volume, sensitivity is also improved, compared to conventional HPLC. However, extra column effects are more significant for scaled down separations, therefore it is essential to minimize extra column dispersion.

A dedicated low dispersion system for ultra-high pressure separation (UPLC) with the particle size of stationary phases reduced down to 1.7μm, small dwell and extra column volume is able to work up to 1000 bar (15,000 psi). In such a way the analysis time could be reduced down to 1–3 min, without the loss of resolution and sensitivity [76-77].

The cleaning procedures for the equipment must be validated according to good manufacture practice (GMP) rules and guidelines [78-79].

During the cleaning validation following factors should be taken into consideration: equipment construction material, sealing part and parts that offers greater risk of contamination. It is important to standardize cleaning procedures and cleaning material, verification of residues chemical products and post-cleaning microbial load. Other factors such as time that the equipment can be considered clean, sampling procedure and analysis of contaminating residues in the equipment should also be considered. The analysis method and selected sampling procedure should be validated.
and presents adequate extraction-recovery to favor the analysis of possible contaminating residues [80].

The acceptable limit for residue in the equipments is not established in the current regulations. However, Food and Drug Administration (FDA) mention that the limit should be based on logical criteria, involving the risk associated to residues of a determining product [81]. The calculation of acceptable residual limit for active products in production equipments should be based on therapeutical doses, pharmacological activity and toxicological index. Several mathematical formulas were proposed that can be used to establish acceptable residual limit [80].

To summarise, GMP/GLP compliance is vital to the success of the pharmaceutical industries. In this regard sensitive and selective methods for checking purity of new drug candidates, monitoring changes during scale up or revision of synthetic procedures, evaluating new formulations, and running control/assurance of the final drug product. Cross contamination with active ingredients is also a matter of real concern. The Code of Federal Regulations (CFR) states that “Equipment and utensils shall be cleaned, maintained, and sanitized at appropriate intervals to prevent malfunctions or contamination that would alter the safety, identity, strength, quality, or purity of the drug product beyond the official, or other established requirements”. Cleaning validation is required in the pharmaceutical field to avoid potential clinically significant synergistic interactions between pharmacologically active chemicals.
1.6 AIM AND OBJECTIVE

Thus the main objectives of our work are

Method development for cleaning validation using Loratadine as a model pharmaceutical

Method development and validation for protein based drugs in the presence of stabilizers used model drugs- erythropoietin, parathyroid Hormone. Sensitive and selective method development for pharmaceutical model drug- ketoconazole.
1.7 References


[12]. Ultra Performance LCTM by design, 2004. Waters Corporation, USA, 720000880EN LL&LW-UL.


[27]. International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use, Topic Q2 (R1): Validation of Analytical Procedures: Text and Methodology, Geneva, 2005


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[52]. The Fitness for Purpose of Analytical Methods, Eurachem, Teddington, 1998.


[54]. F. Satterthwaite, Psychometrika 6 (1941) 309.


