CHAPTER 1

INTRODUCTION

Nowadays, impurities or the degradation products of the drugs are at the forefront in pharmaceutical industries, medical and regulatory agencies. This chapter deals with the introductory aspects of impurities and also the importance of appropriate identification and quantification of impurities in regard to the regulatory submissions of manufactured drug products or substances.

In today's scenario, with the increase in number of synthetic and generic drugs in the market, all the regulatory bodies are intended for evaluation of these drugs in terms of impurity determination. Hence, there is an absolute necessity and requirement for development and introduction of efficient and quality designed analytical methods for the unbiased evaluation and quantification of these impurities in pharmaceutical drug substances and their formulations. Impurity profile plays a pivotal role in the accurate determination and the evaluation of impurities to their stringent specification limits.

Various national and international pharmacopoeias like, United States Pharmacopoeia [1], British Pharmacopoeia [2] and European Pharmacopoeia [3] have enforced the stringent necessities for setting the specification and proper evaluation of impurities present in the active drug substances and their products. To provide better awareness and guidelines on impurity profile, International Council for Harmonization (ICH) has provided a vast bank of information in its Quality guidelines [4, 5]. Current requirements for the evaluation and quantification of trace level of potent degradation products cannot be satisfied with the age old classical chemistry methods. Hence, the introduction of highly sophisticated and tandem techniques, which are capable of accurate determination of impurities and degradation products, has become an unavoidable mandate.

In the way of enhancement and upgrdation of the better and accurate quality designed analytical methods, HP/UP-LC (High/Ultra performance liquid chromatography), gas chromatography, mass and nuclear magnetic resonance spectrometry has gained the interest of researchers worldwide.

1.1 CLASSIFICATION OF IMPURITIES AND INSTRUMENTS USED FOR THE IDENTIFICATION AND QUANTIFICATION

Worldwide, regulatory bodies and pharmacopoeias classify the impurities in different ways. In this chapter, the classification based on ICH, has been taken as reference since these guidelines gained worldwide acceptance [4, 5]. As per ICH, impurities can be classified into the following three categories.

1.1.1 Inorganic impurities

Inorganic impurities are result from the manufacturing process of active pharmaceutical ingredient [4, 6]. They are normally known and identified which include:

- Reagents, ligands and catalysts
- Heavy metals or other residual metals
- Inorganic salts
- Other materials (e.g., filter aids, charcoal)

Inorganic impurity determination is a field of major concern to the researchers as these impurities cannot be determined with appropriate sensitivity by utilizing general techniques (Iron content by residue method). With the advance in scientific knowledge and instrumentations, various sophisticated instruments are now available to determine these impurities at trace levels (Part per billion, Part per trillion). Some of the instrumentation techniques widely utilized are Inductive Coupled Plasma technique tandem with Mass Spectrophotometer (ICP-MS) is one of the most successful and sensitive technique to determine trace level inorganic impurities in the compounds. Inductive Coupled Plasma technique Atomic Emission Spectroscopy/ Optical Emission Spectrometry (ICP-AES/OES) and flame photometry techniques also used for trace level determinations of the inorganic impurities [7-11].

1.1.2 Residual solvents

Solvents are inorganic or organic liquids used as vehicles for the preparation of solutions or suspensions during the synthesis of a new drug substance and their products. Since these impurities in new drug substances are toxic in nature, the selection of their controls is necessary. Generally Gas Chromatographic technique (GC) with different types of detectors (FID, TCD, Mass etc) is used for the determination of these residual solvents in the drug products. Gas chromatography equipped with mass detector (GC-MS) provides an excellent platform for determination of volatile compounds with appropriate sensitivity [12-15].

1.1.3 Organic impurities (process and drug related)

Organic impurities may come up in the new drug substances and their products during their manufacturing process and/or storage of drug substances i.e

- Starting materials
- By-products
- Intermediates
- Degradation products

Researchers utilize various techniques and sophisticated instruments to identify and monitor these impurities. But HPLC/UPLC (High/Ultra Performance Liquid Chromatography) is the first choice of ever researcher due to ease of use and availability of the instrumentation worldwide. Liquid Chromatography tandem with mass detector (LC-MS), provides an excellent platform for the determination of trace level genotoxic impurities [4].

1.2 SPECIFICATIONS / LIMITS OF IMPURITIES

With the increasing attention of regulatory bodies (USP, BP, IP and ICH) on the appropriate quantification and control of degradation products in the drugs for human consumption, it was in immediate need to lay down general guidelines of specifications/limits for the impurities. To address these requirements, ICH had made its Q3 quality guidelines [4, 5] [16], which set the limits for impurities in drugs substances and products as mentioned in Table 1.1.

Table: 1.1: Threshold limits for impurities as per ICH

	Maximum Daily Dose	**Threshold**
Reporting Thresholds	≤ 1 g	0.1%
	> 1 g	0.05%
Identification Thresholds	< 1 mg	1.0% or 5 µg TDI, whichever is lower
	1 mg - 10 mg	0.5% or 20 µg TDI, whichever is lower
	>10 mg - 2 g	0.2% or 2 mg TDI, whichever is lower
Qualification Thresholds	< 10 mg	1.0% or 50 µg TDI, whichever is lower
	10 mg - 100 mg	0.5% or 200 µg TDI, whichever is lower
	>100 mg - 2 g	0.2% or 3 mg TDI, whichever is lower
	> 2 g	0.15%

TDI=Total daily intake

The above information, addresses the specification limits for general non-genotoxic impurities properly. But recently, with the emergence of high concern regarding genotoxic impurities evaluation and setting limit for these highly potent impurities, ICH had come up with its new multidisciplinary guideline M7 [17]. The concentrations, at which the degradation products have an adverse effect on human health, should identify and quantify as it has become an object of prime importance for pharmaceutical industries. Hence, highly specific, accurate and sensitive analytical method for impurity profiling has become the need of researchers and medical industries, worldwide.

1.3 ANALYTICAL CHALLENGES

As per the limits mentioned in ICH guidelines [3, 4], presence of impurities in pharmaceuticals should be controlled to lower level. Hence sensitivity and selectivity of the method become more important and that should be addressed followed by other validation parameters like accuracy, precision, linearity and robustness. For this, researchers require analytical techniques ranging from basic classical techniques (titrations and thin layer chromatography etc.) to high end sophisticated analytical techniques (Mass spectrometry, NMR, HP/UP-LC). A specific stability indicating analytical method is required for the evaluation of these impurities and to know the

shelf life of pharmaceutical formulations and bulk drugs. Among all these, HPLC/UPLC techniques are widely accepted by regulating agencies and pharmaceutical companies due to their low cost, high sensitivity, selectivity and easy adaptability. Hence, to fulfill the objectives of the current research work, both HPLC/UPLC techniques are chosen to quantify the impurities in drugs [18-20].

1.4 COMPONENTS OF LIQUID CHROMATOGRAPHY

The important aspects of various components of HPLC/UPLC instruments are discussed in below given sections.

1.4.1 Pump

The pump in liquid chromatography has the capability to provide a wide range of flow rates (0.1 mL/min (UPLC) to 2.5 mL/min (HPLC)) and all the materials used in the pump should be chemically inactive with the solvents. The pump used in liquid chromatography must be allowing the solvents from one reservoir or multiple reservoirs, containing different solvents simultaneously. Different types of pumps are available in the market [21-23], but the most accepted one used today is the reciprocating pump.

1.4.2 Mobile Phase

The retention of molecule depends on its mutual interaction with both the stationary phase and mobile phase. Here if the analysis run with a fixed composition of the mobile phase, it is called as isocratic elution, whereas the mobile phase composition changes with time it is called as gradient elution. In the latter, a mobile phase is chosen which provides adequate separation of both early eluting analytes and the longer-retained analytes over a period of time by changing the solvent strength. The optimum solvent strength is obtained by changing the solvent composition on trial and error method [24-26].

1.4.3 Stationary Phase

This is an important part in HPLC analysis and separation of compounds take place on the stationary phase. The majority of HPLC analysis utilizes reversed-phase modes and widely used columns are C18 and C8, which silica surface bonded with alkyl group. A stainless steel filled with stationary phase used as a column for the analysis. Retention of sample analytes depends on the polarity differences between sample analytes and bonding site on the stationary phase. The elution of compounds in RP-HPLC is given as below [27-29].

Polar Molecules > Weekly polar molecules > Non polar molecules

1.4.4 Detectors

It is a key part of the HPLC as which senses the analytes as they come from column and converts the concentration into electrical signals. Generally, UV-VIS detector is the most widely used detector and its response is specific to the compound, based on its light absorbance. Photodiode array detector (PDA) detector is used to monitor more than one absorbing component at different wavelengths simultaneously. If the analyte compounds are fluorescence, the ideal detector is fluorescence detector and it has more sensitivity than a UV-VIS detector. In addition to the above mentioned detectors, other detectors such as electrochemical detector, conductivity detector, evaporative light scattering detector, mass detector and IR detector are also employed in the HPLC analysis [30-34].

1.5 METHOD DEVELOPMENT

This part highlights the important aspects of method development, along with a complete characteristics evaluation and the flow of the activity to reach a quality designed stability indicating method. As the method development is always a non ending activity, with up gradation and improvement at every step of formulation changes, an appropriate strategy and design for a robust method development is of prime importance. The various steps involved in the method development as follows [31].

1.5.1 Literature collection

Vast and appropriate literature collection for molecules having similar structures in the available journals and domains (like pharmacopeias, journals, patents, and the internet forums etc.) is very important in the method development. Physical and chemical properties available in the literature give the information regarding the characteristics of the analyte molecules. Based on the above information researcher can sketch a proper strategy to initiate method development based on these properties.

1.5.2 Chemical properties

Functional groups (organic moieties attached to the main skeleton of the molecules) give relevant information about the acidity or basicity and pKa of the molecule [30-34]. This information helps the researchers to select the appropriate pH of the mobile phase to initiate the method development activity. The basic nature of molecules types tabulated in Table 1.2.

Table 1.2: The basic nature of the molecules

S. No	Analyte	Chemical property
1	Neutral	No significantly acidic or basic functional groups
2	Weak acid	Has carboxylic acid functional or phenolic
3	Weak base	Aromatic amine
4	Strong base	Non-aromatic amine

1.5.3 Solubility

Solubility of the compound in different mediums and diluents need to be known before proceeding for development activity as it plays a vital role for accurate determination of the active and degradation products in the compound. Solubility aspect needs to be addressed with utmost care for an accurate determination of analyte [30-34].

1.5.4 Mobile phase selection

Selection of the mobile phase and the buffer is among the crucial steps for a quality designed and robust analytical method. Separation of analytes in the column is drastically affected by the mobile phase. Appropriate buffer strength, pH and organic modifiers ratio are of prime importance for a specific method. Commonly used buffers are phosphates, acetates, borates and formats, as these provides a complete range of pH required for an elution of either an acidic, basic or neutral analytes. Organic modifiers generally used in practice are acetonitrile, methanol, isopropanol and ethanol etc [24-26]. Different compositions of these buffers and organic modifiers, either in isocratic or gradient mode used for better resolution of analytes.

In reverse phase HPLC, retention of the analyte depends on its hydrophobicity as given in Figure 1.1. Mobile phase pH should never be same as the pKa of the molecule (should be at least ±1.5), as it will result into a variation of retention time with concurrent runs, resulting into non reproducibility of the method.

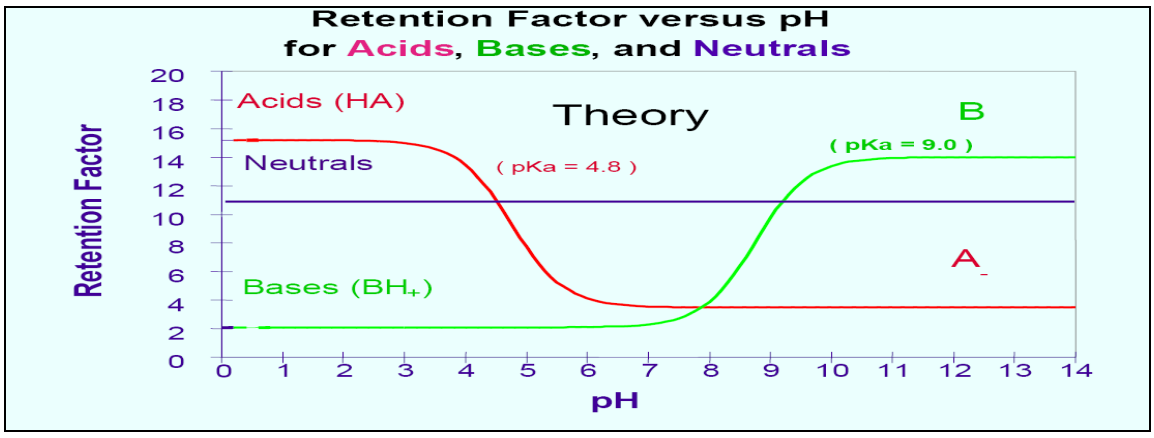

Figure 1.1: Relationship between pH and retention factor of the analyte

1.5.5 Organic Modifiers

First choice of organic modifiers for every researcher is acetonitrile/methanol due to their low UV cutoff (limit the absorption interference of solvents during chromatography) [24-26]. Effect of organic modifier on separation of analytes are shown in Figure 1.2, in which optimum resolution of analytes are found with 20% of MeOH.

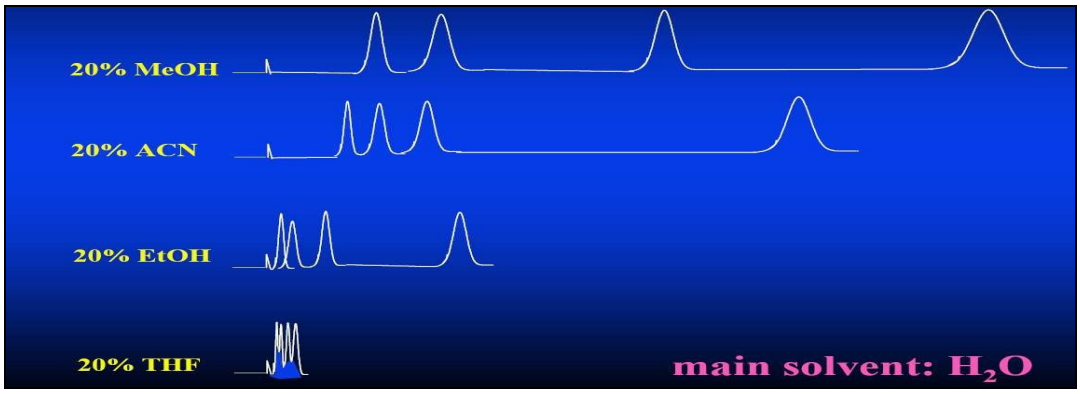

Figure 1.2: Effect of organic modifiers on separation of analytes

1.5.6 Column selection

Column selection is the backbone of method development activity. It is one among the crucial components which determines the robustness and specificity of the method. With increasing scientific knowledge and engineering in the field of column manufacturing, lot of columns are now available in the market for proper method development for the analysis of different analytes. Reverse phase analysis utilizes polar mobile phase and a non-polar stationary phase (column). Examples for the various stationary phases are C4, C8, C18, phenyl, cyano and amino columns etc. United States Pharmacopoeia classifies stationary phases as 59 different categories [1] and [27-29], starting from L1 to L59. Structural view of few stationary phases is shown in the Figure 1.3.

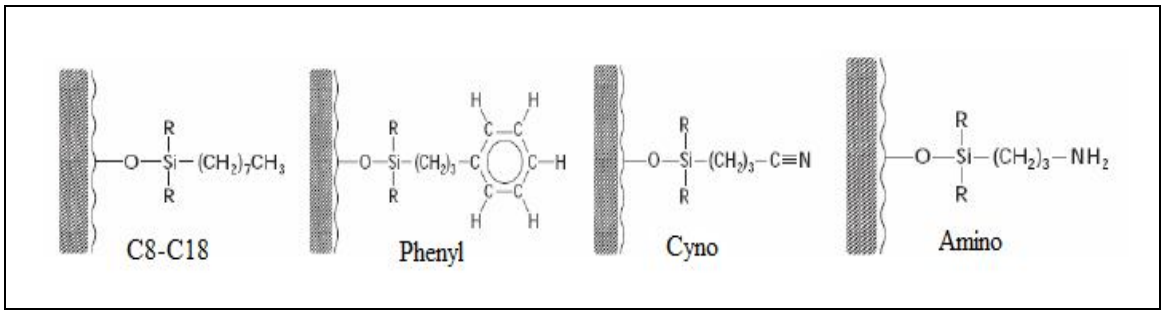

Figure 1.3: Structural view of column common stationary phases**.**

Apart from column stationary phase material, various other parameters like particle shapes (spherical or irregular, Figure 1.4), internal diameter, carbon load, particle surface area, volume and size of pore also need to be considered for the proper analysis of compounds through chromatography. Spherical particle columns are very reproducible and irregular particle are not reproducible.

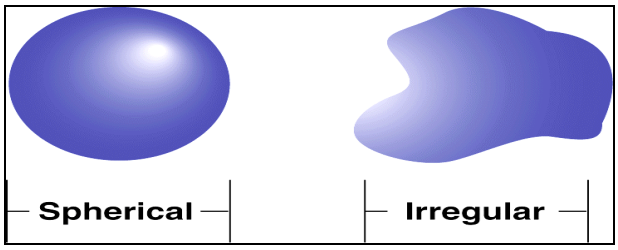

Figure 1.4: Particle shapes of column stationary phase

1.5.7 Particle size

Column particle size varies (Figure 1.5) in the range of 1.6 µm to 10 µm. and based upon the requirement, researcher has to evaluate the efficiency of the method and select the appropriate particle size, required. Smaller particle size improves the resolution between the eluants but also simultaneously lead to increase in column back pressure. Bigger particle size produces low back pressure, long life of the column, but the results in poor resolution of analytes. Similarly, other factors like pore size, type of bonding, pore volumes, surface area of particles, etc., play a vital role in selecting a column for the development of a neat chromatographic method [27-29].

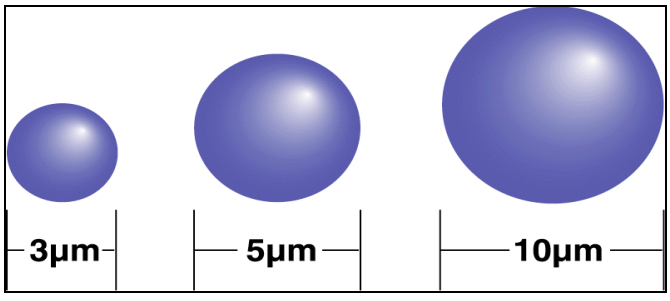

Figure 1.5: Sizes of particle in column stationary phases

1.5.8 Selection of elution mode

The target analytes must be eluted with good resolution without interference of any matrix and which is influenced by proper selection of elution mode. The separation and resolution of analytes depends mainly polarity of the molecules**.** The interaction of target molecules with stationary and mobile phase need to be optimized approximately to make the elution of molecule in a proper and well separated manner**.** Generally, gradient and isocratic modes of operation can be implied for proper separation of the analytes from each other and without any interference from the matrix. Constant composition of the buffer and organic modifiers are used in isocratic mode, whereas in gradient mode, the composition of the buffer and organic modifiers is not constant which varies with the time to modify the interactions between analytes and stationary and mobile phases [35-37].

1.5.9 Detector selection

This section discusses about the selection of detector based on the nature of the analyte molecule. Organic compounds may not always possess attached chromospheres, due to which, its determination on UV and PDA detectors is not easily feasible. Derivatization can be performed for non chromophoric molecules to detect it on UV/PDA detectors. But dervitization itself is not easily reproducible and sensitive technique. To overcome these limitations, researchers worldwide make use of different detectors available, like Refractive Index (RI), Evaporative Light Scattering Detector (ELSD) etc. RI and ELSDs are generally termed as universal detectors due to their compatibility and detectability of all types of molecules [30-34]. In case of UV/PDA detectors a wavelength maximum is of prime importance to achieve sensitivity of the analytical methods. Wavelength selection should be nearby to 1^{st} or 2^{nd} derivative of the maxima, to achieve proper sensitivity of analytical methods.

1.6 METHOD VALIDATION OF ANALYTICAL METHODS

The International Organization for Standardization (ISO) defines the validation as it is the authorization of the method in a systematic way to meet the specific requirements of an intended purpose [38]. This definition describes that a complete examination of developed analytical method, when properly applied, results that are fit for objective as well as it confirms the effectiveness and high accuracy of the analytical method [18-20]. Now the method validation is an important component in any laboratory engaged with the development and establishment of standard analytical methods. Validation of analytical method drawn more attention by cGMP [39], US FDA [40] , ISO/IEC 17025 [41] , ICH [42] , US EPA [43] , USP [44] [45] as they included specific guidelines for validation of the analytical method. Among all Washington conference report with the title of *"Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies***"** became a most comprehensive document. As there are variations in the definitions of various method validation parameters, ICH took forefront step to synchronize the parameters of all these organizations for pharmaceutical application as it is involved various representatives from the industry and regulatory agencies from various countries. The validation parameters of the HPLC/UPLC method are discussed in detail in the below given sections.

1.6.1 Selectivity/Specificity

It is an important step in analytical method validation as it describes the ability of a method to determine the analyte in the presence of all other components. If specificity of the method is not good, remaining validation parameters like linearity, accuracy and precision are not completely addressed. Specificity also assesses interferences that may be caused by the matrix, e.g. placebo components, mobile phase and diluent components. In the assessment of specificity of the method and to prove the method is stability indicating, forced degradation studies has to be performed. Stability indicating methods correctly estimate the changes in the content of drug substance and impurities with respect to time during the storage of drug substance and drug products.

1.6.1.1 Forced degradation studies

Forced degradation studies of the active compound in formulations are essential to understanding their stability and identify the possible degradation products. These studies are carried out in extreme conditions, i.e. acidic, basic, photolytic, oxidative and thermal conditions at various intervals. Generally, the mass balance is applied to compare the unstressed and stressed drug samples and to evaluate the stability data. Calculation of the drug substance and impurities up to 100% of the initial value, including analytical error is called as mass balance.

Purpose of mass balance

- To demonstrate that the method is suitable for the determination of all significant degradation products.
- To establish that the method is sensitive enough to the content changes with the time.
- It is useful to understand the degradation pathway of the active drug compound/drug product.

Procedure for mass balance determination

Steps involved in mass balance are

1. Conducting an assay study for main drug substance and content of impurities in undegraded sample.
2. Performing the assay study for main drug substance and other degraded impurities in degraded sample.
    a. If known impurities, use its response factor and then, correct for its molecular weight.
    b. If unknown impurities, consider the same response factor of active substance and adopt same molecular weight.
    c. Express the assay of undegraded sample in terms of *%w/w* of initial sample concentration (Level/Label claim).
    d. Express the impurities in terms of *% w/w* of initial degraded sample (concentration/level/label claim) as active substances or as % area, depending upon the method.
    e. Justification for the analytical variability of results followed by mass balance calculations.

1.6.2 Precision and reproducibility

Precision is the degree of agreements among the results of individual tests when the same the procedure is applied repeatedly to homogeneous samples of multiple samplings [45]. As per the ICH, which can be categorized into 3 classes i.e repeatability, intermediate precision and reproducibility [46]. Repeatability is attained by performing the analysis under the same operating conditions in a laboratory by an analyst. This can be measured either through instrument precision by injecting the same preparation for six times and determination of average and the relative standard deviation (%RSD) or by analysis of samples at different preparations by the same analyst under the similar conditions. Intermediate precision includes analysis of the sample under above similar conditions on different days, different analyst or instrument within the lab. Reproducibility examines the precision between different laboratories and often determined in collaborative studies or method transfer methods.

1.6.3 Linearity and range of calibration curve

The linearity is the ability of a proposed liquid chromatography method to produce test results that the concentrations of analytes are directly proportional to the chromatogram peak responses within a given range (LOQ to 150% of specification level of impurities) This can be demonstrated by injecting the sample at different analyte concentrations (LOQ to 150%) followed by calculation of slope, intercept and correlation coefficient through least square regression analysis. A linear regression equation applied to the test results should have an intercept significantly from zero. If a significant non-zero intercept is obtained, it should be addressed that this has no effect on the accuracy of the method. A linearity correlation coefficient (r) of above 0.99 is acceptable for the most analytical methods.

The range of an analytical method can be defined as the lower and upper concentrations for which the method has adequate accuracy, precision and linearity. For related substances tests, the ICH requires the minimum specified range as LOQ to 150 percent of the test concentration and for the determination of an impurity.

1.6.4 Limit of detection and limit of quantitation

Sensitivity of the method is expressed in terms of the limit of detection (LOD) and limit of quantification (LOQ). Limit of detection is the lowest concentration that can be detected without accurate quantification. Limit of quantification is smallest concentration that can be quantified accurately. In chromatography, LOD is measured on signal to noise ratio, typically at least 3:1 LOQ measured on signal to noise ratio, typically 10:1. Figure 1.6 shows the limit of detection and limit of quantitation of HPLC method through signal to noise.

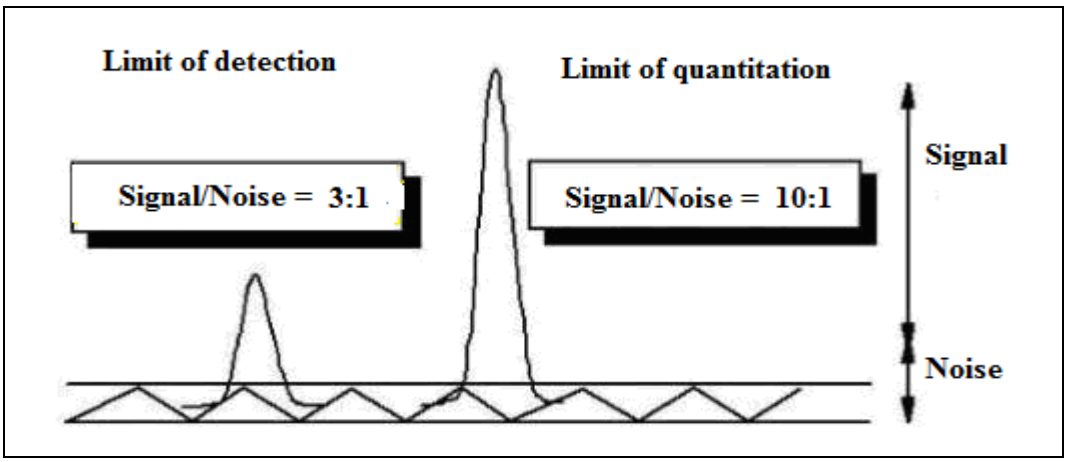

Figure 1.6: Limit of detection and limit of quantitation through S/N ratio

1.6.5 Ruggedness

This can be defined as the reproducibility of results when the method is performed under actual conditions, which includes different labs, columns, analysts, instruments, reagents, solvents and chemicals. Ruggedness is not presented in the ICH guidelines. As per the United States Pharmacopeia, it is replaced by reproducibility, which has the same meaning as ruggedness, as the degree of reproducibility of results obtained under a variety of conditions as mentioned above.

1.6.6 Robustness

Robustness is ability of a proposed analytical method to remain constant by changing the method parameters to a small extent. Robustness of HPLC method determined by varying the method parameters, for example, pH, flow rate, column temperature, injection volume, sample temperature, wavelength or mobile phase composition. If the influence of the parameter is within a previously specified limit, the parameter is said to be within the method's robustness range. In the ICH document, it is recommended to consider the evaluation of a method's robustness during the development phase, and any variations that are critical for the method should be documented. This is not, however, required as a part of the registration.

Aim: The aim of the present work is to develop and validate the stability indicating liquid chromatography methods for the quantification of impurities in drug substances and drug products.

Objectives: The objective of the present research work

- Development and validation of stability indicating RP-HPLC methods for determination of related substances in anti-cancer drugs
- Development and validation of a stability indicating HPLC method for the quantification of impurities in fampridine dosage forms
- Development and validation of a stability indicating HPLC method for the quantification of eleven impurities in lamivudine and tenofovir disoproxil fumarate
- RP-UPLC method for the simultaneous quantification of related substances in emtricitabine, tenofovir disoproxil fumarate and efavirenz pharmaceutical dosage forms.