INTRODUCTION

Pharmaceutical industry plays an important role in today’s society by developing, manufacturing and marketing the pharmaceutical products for human use (McGuire et al, 2007). The pharmaceutical products are developed in order to meet the increasing demand for medicines with good quality, efficacy and safety. Also, drug development is required as and when a new disease or health ailment is discovered. The main challenge of drug development process is in determining suitable dosage form and the dose.

In order to have good efficacy and safety, the product has to be of optimum quality. Hence the pharmaceutical product from its development stage and till it reaches the customer, the quality will be checked by using different analytical techniques. In modern chemistry, all these analytical techniques are classified under analytical chemistry.

Analytical chemistry is a branch of chemistry dealing with different analytical techniques used for quantification of analytes in a given sample matrix and to check their quality. In pharmaceutical industry, Analytical chemistry plays a key role in establishing the quality in products by accurately determining the component of interest in the sample. The most commonly used analytical techniques in pharmaceutical industries are Potentiometric titrations, Differential scanning calorimetry, Infrared spectroscopy, In vitro dissolution test, Liquid chromatography, Gas chromatography etc. Out of these and many other analytical techniques, chromatographic methods play a distinctive role in determining the quality and characterization of pharmaceutical products during their development and commercial manufacturing activities.

Quality by Design (QbD)

QbD is an organized way of method development that consists of objectives beforehand with weightage on product, understanding on process and its control, created on scientific and “quality risk management” (ICH Q9 2005:2). Whenever a new product is developed for regulated markets like United States (US), the company will take appropriate regulatory approvals to market the product in that region. During the application review process, the regulatory bodies realized that the data provided by the companies is not scientific. In many cases, the development was unscientific and there was less flexibility in the process and had
very less scope for improvement in the process (Timothy et al, 2011: 545). Hence, the regulatory bodies were under the view that there need to be a change in the development approach and practice to produce better quality products. Subsequently the United States Food and Drug Administration (USFDA), a body that regulates quality of medicines in US came up with QbD concept. Further evolution of QbD model for drug products development and practices of manufacturing directed to three International conference on harmonization (ICH) guidance documents namely “Pharmaceutical development” (Q8 (R2), 2009), “Quality risk management”(Q9,2005) and “Pharmaceutilcal quality system”(Q10, 2008).

QbD for drug development necessities a thorough and scientific and thoughtful identification of critical quality attributes (CQA’s) affecting ultimate product quality ((ICH Q8 (R2), 2009). The industry bodies are involved in product developments using QbD in communication with the FDA. Hence, the principles of QbD are used extensively in industry for drug product development. Though the ICH guidance documents discuss in details about QbD for product development, they do not explain analytical method development by QbD. Hence, the present approaches for method evaluation, method development, inter laboratory method transfers and direction for use of methods during the stability studies have not changed. Now, it is time to evaluate the technique of method development and subsequent steps of method in light of QbD.

The traditional way of method development is termed “One Factor at a Time” (OFAT) approach. In this approach, the liquid chromatographic methods are developed by changing individual variables of method one at a time and observe its impact on the separation. In the next step, one more variable is changed again and again record the quality of separation and so on. This approach of method development is laborious and takes lot of time and results in many development trials. The data from many trials makes it difficult for interpretation. Many a times, it so happens that the separation obtained based on trial and error method and OFAT approach leads to a development of non-robust methods. The final separation obtained could be “by chance” and not based on scientific evidence. It is the personal experience of analytical scientist that is used in method development but not scientific rationale. Moreover, interaction effects of variables are not studied by scientist for the final separation. Because of these reasons, the
analytical chemists would have problems during the inter laboratory transfer of methods and routine use of the method in same laboratory. By practice, we have seen that some of the methods had to be redeveloped during the course of validation or inter-laboratory method transfers because of lack of understanding on the method.

The other disadvantages of OFAT method development approach include (Timothy et al, 2011: 546)

- Difficulty in out of specification (OOS) investigations because of lack of knowledge on the method.
- Frequent out of trend (OOT) and OOS observations.
- Improper and lack of timely support for the product development and improvement because of poor quality of methods.
- Repeating OOS observations for different products across the industry and a less understanding on cause of variability.
- Any change in the existing method calls for post approval change, resulting in regulatory approval delays and in the availability of product in market.

ICH Q8 (R2), Q9 and Q10 does not discuss more specific approach towards the analytical method developments. Hence, in the present work; the QbD principles have been used in latitude of analytical method development. The definitions for QbD elements (ICH Q8 (R2), 2009) in analytical method development are included in following section.

**Critical quality attributes (CQA)**

By definition, it is the “most probable collation of quality characteristics of a method that preferably would be attained to confirm the anticipated quality and performance, in consideration of safety and efficiency of the drug product”.

**Critical Process Parameter (CPP)**

It is a method factor whose changeability has an influence on a CQA’s. CPP’s should be identified, controlled and observed to ensure that the method produces reproducible results.
Design of experimentation (DOE)

It is a statically constructed experimental plan. By definition, DOE is “A planned and systematized way of performing the experimentation for determining the relationship between CPP’s affecting CQA’s. Through DOE, multiple CPP’s can be varied at a time using a well-constructed statistical model. Also, the main effects and interaction effects of CPP’s can be determined accurately through DOE.

Quality target product profile (QTPP)

“Quality target product profile is an ultimate goal of the quality physiognomies of a drug product that typically will be achieved to confirm the expected quality with safety and efficacy of drug product in consideration.”

Design space (DS)

By definition it is “The multidimensional combination for interaction of input variables and process parameters that have been demonstrated to provide assurance of quality. Working within the design space is not considered as change. Movement out of the design space is considered to be a change and would normally initiate a regulatory post approval change process.” From the analytical method perspective, DS is a working area for method in which the quality of method is unaffected by changing the study variables within that space.

Analytical Target Profile (ATP)

Analytical target profile defines the purpose of measurement and determines the reason for method development. By definition it is a “methodical way to development that is initiated with predefined purposes with considerations of product and process control and understanding, created on scientific background and quality risk management”. Generally, ATP for method could be quantification of impurities, assaying the main analytes or measurement of release rate of drug from drug product. The ATP for method development will be defined based on the knowledge on QTPP for drug product and composition details of drug product. ATP is projected as a new way of explaining analytical methods for regulatory compliances to eliminate post approval variation.
The steps involved in method development by UPLC (Ultra Performance Liquid Chromatography) can be presented in the form of a flow chart given in figure 1.1.

Figure 1.1: Flow chart representing analytical method development by UPLC

**Step 1:** The feasibility phase is for the analyst to check whether the development can be productively done by using available resources.

**Step 2:** Research phase is for describing the activity aimed at ascertaining information on the sample of interest and understand QTPP with an intention that the data will be advantageous in developing the method. (ICH Q8 (R2) 2009)

**Step 3:** Development phase is a conversion of findings of research into an analytical method and to use the understandings obtained from research towards the analytical procedures. Further, the development phase also includes risk analysis parameters and design space (DS) into the method.

**Step 4:** The optimization stage involves development of method through well-constructed DOE by different statistical models. Also, DOE will boost analyst confidence on the quality of data along with information on main and interaction effects. The DS for method is established in order to cater the day to day laboratory variations without affecting the overall method performance.
**Step 5:** Validation is an essential and very critical step that a method should achieve as a whole to prove its suitability for quality control use. Validation by definition “Is the procedure of establishing the methods suitability for intended application”. For regulated markets like US (United States) and European Union (EU), the method validation should be done meeting ICH Q2 (R1) guideline requirements.

**Step 6:** Method transfer is a process for transfer of technology of newly developed and validated method to other laboratory (Receiving laboratory). The other laboratory could be within the same organization or in the different organization. The analytical method must meet the validation requirements in order to have successful transfer to quality control or testing lab. (United States pharmacopoeia 2015:1224)

Having understood this, method development is an imperative task. With multiple factors that can influence the final results that are obtained, the analytical chemist must acquire cutting-edge knowledge and understanding on QTPP, CQA’s, ATP and CPP’s of the method that is yet to be developed. Thus, using a systematic and scientific approach of QbD can make the assignment simple resulting in resources saving for company and subsequently in a method meeting intended purpose.

**Quality requirement of pharmaceutical drug products**

The quality requirement of drug product depends on the class to which it belongs. UPLC methods are used extensively in an analytical laboratory for quality assessment of pharmaceutical drug products. The classification of pharmaceutical product is given in the following section.

**Pharmaceutical products and their classification**

A pharmaceutical product is a dosage form that is combination of active substance formulated with excipients (United States pharmacopoeia 2016:1151). The active substance is the main component of dosage form that performs the intended pharmaceutical activity. The dosage forms are formulated with active substance to ease dosing, delivery at the intended site and administration to patient. With the progress of science in the pharmaceutical industry, novel drug delivery systems are developed.

There are different ways of classification for pharmaceutical drug products that are established on the properties of active moiety, route of administration and drug
delivery technique like tablets, capsules, injections, ointments, gels, transdermal patches etc. These pharmaceutical dosage forms manufactured would be tested for the quality before it is available for human consumption. The quality requirement for each of these pharmaceutical products varies depending on specific intended application. Also, the overall characteristic of pharmaceutical product is expressed in terms of safety, effectiveness and quality. The safety and efficacy of pharmaceutical product shall be demonstrated by clinical, bioavailability and bioequivalence studies. The quality of pharmaceutical product is determined by its compliance to predetermined set of specifications. Specification by definition “is as a list of tests in references to analytical procedures and suitable limit criteria”. A pharmaceutical product is considered to be acceptable for the intended purpose, if meets these specifications (ICH Q6A 1999:1). Further, the specifications for drug product are proposed and justified by manufacturers based on knowledge on product, regulatory requirements and is in turn approved by regulatory authorities. ICH Q6A guidance provides general recommendations of specifications for products at the time of release and throughout the shelf life. Depending on the chemical composition, pharmaceutical products quality could get affected by general climatic factors such as light, temperature and humidity. The interaction of drug product with environmental factors leads to generation of impurities. Hence, strict control of storage conditions are necessary for maintaining quality and integrity of the product.

Impurity

An impurity of a drug product is defined as “element of the pharmaceutical drug product that is not either the chemical entity called as drug substance itself or an excipient in the drug product” (ICH Q6A 1999:18). The impurity content of a pharmaceutical product depends on the nature of drug substance used in drug product. The impurities does not have intended pharmaceutical application and should be controlled in the drug product.

Therapeutic application of many drugs raise need in giving the impurity profile, minor and major degradants and their amounts to ensure safety of administration as well as concentration levels. The aim of regulatory agencies monitoring pharmaceutical product quality is that customers often not able to independently assess the quality of drugs they use. Although certain physical quality failures like
change in appearance and shape can be detected easily by a pharmacist or consumer, many of the more serious failures cannot be distinguished. Hence, Food and drug administration (FDA) stands in for customers by implementing its statutorily based quality regulation.

The pharmaceutical products manufactured are tested in an analytical laboratory using various analytical techniques to check their compliance to the specifications before releasing to the market for human consumption. Hence, the analytical technique such as UPLC play a crucial and critical role in determining and ensuring the quality of pharmaceutical products.

**Classification of Impurities**

ICH guidelines (ICH Q3A (R2): 2006, Q3B (R2): 2006, Q3C (R5): 2011 and Q3D: 2013) classify the impurities to three categories as summarized in table 1.1.

<table>
<thead>
<tr>
<th>Type of impurity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic</td>
<td>Key starting materials, Reagents, Intermediates, side reaction / By-products, Degraded products and Catalysts</td>
</tr>
<tr>
<td>Inorganic</td>
<td>Inorganic salts, Reagents, Ligands, Catalysts, Heavy metals or other residual metals</td>
</tr>
<tr>
<td>Residual solvents</td>
<td>Inorganic or organic liquids</td>
</tr>
</tbody>
</table>

The starting materials used in manufacturing of drug substance can contribute to impurities in the drug substances (Muehlen. E, 1992: 837–841, Palleros et al, 2000:2). The classification of impurities can be explained by taking Aspirin (Acetylsalicylic acid) tablet as an example. Acetylsalicylic acid is an active ingredient used in treatment of pain, fever and inflammation. On prolonged usage, Aspirin also acts as a blood thinning agent in case of strokes and heart attacks to avoid death ( Brayfield A et al:2014). Based on the route of synthesis (Palleros et al, 2000: 494) and chemical properties, it is known that Acetylsalicylic acid in Aspirin tablets readily degrades in moist conditions to give salicylic acid and...
Acetic acid which are considered as degradation impurities of Aspirin tablets. (Figure 1.2)

![Figure 1.2: Hydrolysis reaction of acetylsalicylic acid in Aspirin tablets in moist condition](image)

In the route of synthesis for the preparation of Acetylsalicylic acid from acetic anhydride, phosphoric acid is used as a catalyst. The traces of phosphates can remain in active ingredient and get carried to the drug product as an inorganic impurity.

ICH has defined different thresholds for impurities and specifications will be derived based on these thresholds. The different type of thresholds for impurities are discussed in the following sections.

**Thresholds for degradation impurities in new drug products**

Considering the Total Daily Intake (TDI) of drug product, ICH guideline (ICH Q3B (R2), 2006:7) has derived three types of thresholds for impurities namely Identification, Reporting and Qualification. The threshold for each type are given in tables 1.2 and 1.3.

<table>
<thead>
<tr>
<th>Maximum Daily Dose</th>
<th>Reporting Thresholds</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 1g/day</td>
<td>0.1%</td>
</tr>
<tr>
<td>&gt; 1g/day</td>
<td>0.05%</td>
</tr>
</tbody>
</table>

Table 1.2: Reporting thresholds for impurities in new drug products
Table 1.3: Identification and Qualification thresholds for new drug products

<table>
<thead>
<tr>
<th>Maximum Daily Dose(^1)</th>
<th>Identification Thresholds(^2,3)</th>
<th>Maximum Daily Dose(^1)</th>
<th>Qualification Thresholds(^2,3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 1mg</td>
<td>1.0% or 5 µg TDI, whichever is lower</td>
<td>≤ 10mg</td>
<td>1.0% or 5 µg TDI, whichever is lower</td>
</tr>
<tr>
<td>1 mg - 10 mg</td>
<td>0.5% or 20 µg TDI, whichever is lower</td>
<td>10 mg - 100 mg</td>
<td>0.5% or 20 µg TDI, whichever is lower</td>
</tr>
<tr>
<td>&gt;10 mg - 2 g</td>
<td>0.2% or 2 mg TDI, whichever is lower</td>
<td>&gt;100 mg - 2 g</td>
<td>0.2% or 3 mg TDI, whichever is lower</td>
</tr>
<tr>
<td>&gt; 2 g</td>
<td>0.10%</td>
<td>&gt; 2 g</td>
<td>0.15%</td>
</tr>
</tbody>
</table>

1 The amount of drug substance administered per day.

2 Thresholds for degradation products are expressed either as a percentage of the drug substance or as TDI of the degradation product. Lower thresholds are appropriate if the degradation product is unusually toxic.

3 Higher thresholds should be scientifically justified.

Further, identification of unknown impurities is necessary when it is beyond the identification threshold. (Qiu F, 2005; 15-17)

**Different types of analytical methods**

Many types of analytical methods are used to determine the quality requirements of pharmaceutical drug product. For the purpose of method validation, methods are classified into four common types. Each has different validation requirements based on the intended application, importance and purpose of the method.

Commonly four kinds of analytical tests are defined in ICH (ICH Q2 (R1), 2005) guidelines. They are,

- Identification tests
- Impurity content quantification tests
- Limit tests for monitoring of impurities
- Quantification tests for assay of active drug in samples of drug substance, drug product or any other component(s) of in the drug product.
Identification tests are planned to check and ensure the identity of analyte of interest in a test sample. The identity of analyte would be achieved normally by comparing the properties of the material by its Ultraviolet (UV) spectra, Chromatographic retention time, Colorimetric methods, mass number from a Mass spectral analysis, Infrared spectrum and melting points in comparison to a reference standard. In case of impurities, identification test could be a quantitative test like clarity of solution or a limit test like heavy metals test. The validation requirement for methods vary from the procedures used for quantitative test to a limit test. Assay methods are proposed to quantitate the analyte in a particular sample. In this scenario, the assay indicates a quantitative measurement of analyte of interest or most essential constituent in the drug substance. Similar method validation is required for quantifying one or more drug substances in the drug product.

**Assay method**

The assay methods are used in quantification of one or more active components in a sample. Some of the products require separate quantification of active analyte (compound or substance) and some require simultaneous estimation of analytes. The dosage and content of active in drug product determines the working concentration for the analytical technique. The products like vitamin tablets, hormone tablets and anticancer drugs will have low concentration of active component. In these cases the sample pretreatment should be performed to enrich the active analyte in the sample solution for accurate determination. Also, for simultaneous determination, the diluent should be capable of completely dissolving all the analytes of interest. In order to accurately quantify the active analytes in a sample, they should completely dissolve in the sample diluent and “retention range” for the analytes should be acceptable. Further, degradation impurities, sample matrix (placebo) and blank peaks should be well resolved from active analyte peak. Therefore, a scientific approach for the simultaneous estimation of active components in drug products is necessary. Hence, this was one of the research objective of this thesis.
Impurities (Related substances) method

The impurities in pharmaceutical dosage form can be derived from the drug substance or may be generated in the process of preparation of the drug product or generated during its storage (Degradation products). Also, different sources and routes of synthesis can lead to a different impurity profile (Gavin et al, 2006: 1251–1259). A detailed evaluation of known and unknown impurities in a dosage form would result in an Impurity profile (Go¨ro¨g, S et al 1988: 697–705, Go¨ro¨g, S, 2000: 12-13). Degradation product may be defined as “An impurity resulting from a chemical change in the drug substance brought about during manufacture and/or storage of the new drug product by the effect of factors like light, temperature, pH, water or by a chemical reaction with an excipient and/or the immediate container closure system” (ICH Q3B(R2) 2006: 6). The quantification and limit of impurities in pharmaceutical drug products is presently a critical issue for pharmaceutical industry. Global regulatory guidance for impurities describe the critical requirements for pharmaceutical products and their impurity levels. More specifically, ICH guidelines namely Q3A (R2), Q3B (R2), Q3C (R5) and Q3D discuss about different kind of impurities and their thresholds in pharmaceutical products. Further, pharmacopoeias like British Pharmacopoeia (BP), European Pharmacopoeia (Ph.Eur), Japan Pharmacopoeia (JP) and United States Pharmacopoeia (USP) have incorporated the limits for permissible levels of impurities in the drug substances or pharmaceutical products. In reference to limiting and monitoring the toxic impurities include having sensitive and specific analytical technique and process capabilities for removal of impurities. Special considerations are necessary for stereo-chemical impurities. A key aspect of related substances method development is to develop and use reliable technique for separating and detecting impurities to provide a high degree of confidence that all impurities that are already present. Thus, the related substances method is used for accurate estimation of these process and degradation impurities of drug product.

The correct estimation of impurities by a related substances method is possible only if the method is sensitive at the expected level of impurities, reproducible and accurate. Hence, the method development for estimation of related substances in the drug product requires a systematic and scientific approach. Thus, this was also one of the research objective of this thesis.
As per the current regulatory requirements, newly developed analytical method must be stability indicating and specific. Stability demonstrating nature of method can be established through forced degradation/stress testing by subjecting the pharmaceutical drug product to different degradation conditions like acid treatment, base treatment, photolysis, oxidation, dry heat etc. (ICH Q2 (R1) 2005: 7). Under each degradation condition, it is necessary to show that the method is capable to quantify the analyte of concern without any interference from sample matrix and degradation impurities. Alternatively different methods of stress testing are also established (Baertschi S.W et al 2005, 90 - 99, Reynolds D.W, 2002).

**Method validation**

It is a practice established in the laboratories to demonstrate the suitability of any analytical method for intended application (United States pharmacopoeia, 2015: 1225). The results obtained from a non-validated method are not accepted by the regulatory authorities, as results obtained by using a method that is not properly validated are not reliable. Hence, ICH has classified analytical methods into different types and defined the general validation requirement for each of them. The method validation parameters required (ICH Q2 (R1), 2005:3) for each type of method are presented in the table 1.4.

### Table 1.4: Typical validation parameters for different type of analytical methods

<table>
<thead>
<tr>
<th>Type of analytical procedure characteristics</th>
<th>Identification</th>
<th>Testing for Impurities</th>
<th>Assay - dissolution content/potency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Quantitative test</td>
<td>Limit test</td>
</tr>
<tr>
<td>Accuracy</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Precision</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Repeatability</td>
<td>-</td>
<td>+(1)</td>
<td>-</td>
</tr>
<tr>
<td>Intermediate Precision</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Specificity (2)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Detection Limit</td>
<td>-</td>
<td>-</td>
<td>+(3)</td>
</tr>
<tr>
<td>Quantitation</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Linearity</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Range</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
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- signifies this characteristic is not normally evaluated
+ signifies that this characteristic is normally evaluated

(1) In cases where reproducibility (see glossary) has been performed, intermediate precision is not needed.

(2) Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s).

(3) May be needed in some cases.

In case of UPLC methods used in the estimation of Assay and Related substances/impurities, the detailed description of method validation parameters along with acceptance criteria are provided in the following section

Specificity

By definition “It is the capability of a method to detect the sample component of interest in the presence of other sample components such as excipients, additives, preservatives, other impurities etc.,”. The specificity of method would be established in many ways which include injection of placebo, blank (Diluent) and sample matrix containing the analyte of interest. Non-interference blank, sample matrix and placebo peaks with analyte would demonstrate specificity of the method. Further, specificity can also be demonstrated by forced degradation studies.

In forced degradation studies, the sample matrix containing the analyte is exposed to different degradation conditions like acid degradation, base degradation, oxidative degradation, thermal stress, neutral degradation with water, exposure to light for a given duration of time. Conditions for forced degradation of an individual sample will be established keeping in mind the properties of the drug substance(s) in the drug product like melting point, chemical reactivity towards the degradation reagents and temperature. These conditions would be determined during the early development/method development phase. The degraded and undegraded samples will be analyzed on UPLC with a Photo Diode Array detector (PDA) and check for “Peak purity”. Peak purity is a measure of peak homogeneity. If the peak purity is more than threshold value, then the peak is treated as pure and do not have any co eluting peaks with analyte of interest (“NO” purity flag). Depending on the software, type of detector and equipment manufacturers, Different techniques are available commercially for determining the peak purity.
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Accuracy
It is expressed as the “closeness of agreement between the value that is accepted either as a conventional true value or an accepted reference value and the determined value”. In other words, it is the difference between measured and the actual value and is generally interpreted as percent of recovery. ICH guidelines (ICH Q2 (R1), 2005: 9-10) recommend using confidence range for documenting accuracy results. Accuracy without precision has no significance. Accuracy in combination with precision study will be used in determination of range for the method. Further, it suggests testing minimum three replicates at three different concentrations. The mean accuracy value at each level and over all accuracy covering all levels should be reported along with relative standard deviation.

Precision
Precision for an analytical procedure “is a measure of closeness of a series of values (degree of scatter) acquired from multiple sample preparations of the same batch of material under prearranged conditions” (ICH Q2 (R1), 2005: 4). The Precision study for an analytical method is very important as it supplements the privileges of accuracy and linearity.

Precision for any analytical method would be established by synthetic mixture of sample. Precision is classified into three sub types that is Repeatability (Method precision), Intermediate precision and Reproducibility. Repeatability deals with precision of method in same working conditions in a small interval of time (precision within the laboratory). Intermediate precision also deals with variation with in the laboratory. The variations considered are like different days, chemist, instrument, analytical column etc. Repeatability would be performed by preparation of minimum 9 measurements including the indicated range or at least 6 measurements at working concentration of method. The same procedure is followed for intermediate precision except with the changes as discussed above.

Data interpretation should consider the repeatability of sample set performed as part of intermediate precision and sum of the values obtained from repeatability and intermediate precision. Reproducibility deals with the precision of method across different laboratories and is done as part of method transfer between the laboratories. Reproducibility is evaluated by data generated between the two different laboratory trials and demonstrates the precision between laboratories (collective studies done generally on a standardized methodology).
Detection limit and Quantitation limit

The Detection limit (LOD) for an analytical method “is the smallest quantity of analyte that can be detected in a sample but not in essence quantitated as a precise value”. The Quantitation limit (LOQ) of an analytical method “is the smallest amount of analyte that can be estimated in a sample with necessary precision and accuracy” (ICH Q2 (R1), 2005: 5-6). The LOQ would be proved by analysis of appropriate number of test samples that have analyte at or near the quantitation limit. It would be necessary to provide quantitation limit and procedure utilized for demonstrating the quantitation limit. Based on type of procedure like non-instrumental or instrumental, different ways for determining the LOD have been practiced. ICH guidelines for method validation (ICH Q2 (R1), 2005: 11-12), gives three different approaches for establishing the detection and quantification limits. They are by signal-to-noise method, visual observation and slope method.

Visual evaluation approach

This approach in general is adapted for non-instrumental methods and a few cases of instrumental methods. LOD is performed by analysis of samples with known amount of analyte and by checking the lowest level at which the analyte can be reliably detected.

Signal-to-Noise method

This method would be adapted in cases of analytical procedures using instrumental methods of detection that exhibit baseline noise like in case UPLC methods. Signal-to-noise ratio would be calculated by matching measured signals of samples with pre-established low levels of analyte with that of blank. Generally signal-to-noise ratio at 3 or 2:1 is termed as satisfactory for establishing the detection limit and a signal-to-noise ratio between of 10:1 is considered acceptable for quantitation. Signal-to-noise technique is most widely used method for UPLC methods.

Mathematically, the LOD and LOQ values are calculated using the formula as given below.

Standard Deviation of Response and the Slope

The detection limit (LOD) can be mathematically stated as

\[
\text{LOD} = \frac{3.3\sigma}{s} \quad \text{Eq (1)}
\]
The quantitation limit (LOQ) may be presented as

\[ \text{LOQ} = \frac{10 \sigma}{S} \text{Eq (2)} \]

Where,
\( \sigma \) = Standard deviation of signal
\( S \) = Slope of the calibration curve

The standard deviation, residual standard deviation, y-intercept of regression lines shall be determined by construction of a calibration curve by analyzing samples having an analyte in the ranges of LOD.

**Linearity**
The capability of an analytical method within a given range to obtain test results that are unswervingly or via mathematical modification relational to the concentration of analyte in the sample is termed as linearity. Generally, least squares regression method is used for expressing the linearity of the method. Linearity would be demonstrated by visual inspection of a calibration curve constructed by plotting analyte area counts with that of content in percentage or µg/mL. The data will be assessed by suitable statistical methods like calculation of a regression line by technique of least squares. (ICH Q2 (R1), 2005: 8-9)

A plot of the data with statistical terms should be presented along with study of unconventionality of actual data points in regression line to help and in evaluation of linearity.

**Range**
The range for an analytical method is generally demonstrated from linearity and accuracy studies that depending on the proposed application of method. Further, it is established by acceptable linearity, accuracy and precision data.

Generally a range of 80 to 120 percent of test concentration is acceptable for assay of drug product. In case of content uniformity, usually 70 to 130% of test concentration or a broader range depending on the type and characteristics of dosage form with proper justification. Further for impurity methods, linearity should be generally from reporting levels to minimum 120 percent of specification limit.
Robustness
The robustness by definition “is a measure of methods capability to produce reproducible results by minor but intentional variations in method conditions”. This further shows an indication of methods dependability during normal usage.
USP recommends robustness as part of method development (United States pharmacopoeia, 2014: 1225). If the method development is based on QbD, robustness will be a part of method development and separate evaluation is not necessary. For QbD based method developments, Robustness and Intermediate precision (Ruggedness) are not necessary to be performed as part of formal method validation.

System suitability testing
System suitability measurement is a vital part of many analytical procedures and especially the case with liquid chromatography methods. System suitability testing is run to verify that the chromatographic system is adequate for planned analysis. (United States pharmacopoeia, 2016: 1225).
During method validation, each parameter should be performed along with system suitability criteria.

Pre-defined acceptance criteria (tolerance limits) for validation parameters
The upper limit and lower limits for the population gives information about the fraction of population values with a fixed confidence and in turn be used to set suitable specifications. The acceptance criteria for each method validation parameter relays on type of method being validated. As there are many types of methods and diversified approaches employed for each type of method validation. Currently neither USP nor ICH Q2 (R1) guideline on method validation describes the acceptance criteria for method validation parameters. However, the following limits (Table 1.5 and 1.6) are considered to be acceptable/suitable for different validation parameters based on industry standards for assay and related substances methods by UPLC.
Table 1.5: Acceptance criteria for related substances method validation parameters

<table>
<thead>
<tr>
<th>Test Parameter</th>
<th>Acceptance Criteria’s</th>
</tr>
</thead>
<tbody>
<tr>
<td>System</td>
<td>All system suitability parameters should comply the requirements as per test method.</td>
</tr>
<tr>
<td>Specificity</td>
<td>-----</td>
</tr>
<tr>
<td>Diluent/blank, Placebo interference</td>
<td>There should be no interference from the peaks due to placebo/diluent at Retention time (RT) of principal peak of analyte and impurity peaks.</td>
</tr>
</tbody>
</table>
| Test spiked with known Impurities       | • All the known impurities should be well separated from each other and from main peaks.  
• The purity of impurity peaks in samples of spiked and un-spiked sample should comply.  
• For Waters “Empower 3 software”, the purity angle have to be less than purity threshold and there should be “NO” purity flag. |
| Forced degradation studies (stress studies) | • All the degradation peaks should be well separated from impurities and main components of interest.  
• For Waters “Empower 3 software”, the purity angle should be less than purity threshold and there should be “NO” purity flag. |
| Accuracy                                | • The percentage recovery of each individual and mean of determinations at each level of spiking study for peaks of interest should be within 100.0±10.0.  
• The percentage RSD at each level of spiking study should be ≤ 10.0.  
• The percentage RSD of all levels of spiking study should be ≤ 10.0. |
| Precision                               | -----                                                                                |
| System precision                        | The percentage RSD of peak responses of analyte should comply as per test method.     |
**Method precision**
- The results should be within specification limits.
- Percentage RSD for any known impurity and the highest unknown impurity concentration (C in percent) is as follows (6 determinations)
  - $C > 0.5$: not more than 5.0
  - $0.05 < C \leq 0.5$: not more than 7.0
  - $C \leq 0.05$: not more than 10.0
- The percentage RSD for total impurities should be not more than (NMT) 10.0.

**Intermediate precision**
- The results should be within specification limits.
- RSD for any known impurity and the highest unknown impurity for concentration (C in percent) is as follows (6 determinations)
  - $C > 0.5$: not more than 5.0
  - $0.05 < C \leq 0.5$: not more than 7.0
  - $C \leq 0.05$: not more than 10.0
- RSD for any known impurity and the highest unknown impurity for concentration (C in percent) is as follows (12 determinations)
  - $C > 0.5$: not more than 5.0
  - $0.05 < C \leq 0.5$: not more than 7.0
  - $C \leq 0.05$: not more than 10.0
- The percentage RSD for total impurities should be NMT 10.0.

**Detection limit (LOD) and Quantitation limit (LOQ)**
- A clear peak should be seen at LOD level for each component.
- Percentage RSD for area response at LOQ level is NMT 10.0 for 6 injections.
- The accuracy of each known impurity should be between 80.0 to 110.0 percent.

**Linearity**
- The regression coefficient and correlation coefficient should be $\geq 0.99$. 
### Range

The range of method should be between 50 to 300 percent of working concentration of impurities.

### Solution stability

- The area difference of standard preparation at each interval should be within ± 5.0 percent from the initial value.
- The difference for area of impurities in sample preparation at each interval should be within ± 5.0 percent from the initial value.
Table 1.6: Acceptance criteria for assay method validation parameters

<table>
<thead>
<tr>
<th>Test Parameter</th>
<th>Acceptance criteria’s</th>
</tr>
</thead>
<tbody>
<tr>
<td>System Suitability</td>
<td>All system suitability parameters should comply the requirements as per the test method.</td>
</tr>
<tr>
<td>Specificity:</td>
<td>-----</td>
</tr>
<tr>
<td>Diluent/blank, Placebo Interference</td>
<td>There should be no interference from the peaks of placebo/diluent/blank at the RT of principal peak.</td>
</tr>
</tbody>
</table>
| Stress testing studies (degradation studies) | • All the peaks of interest must be well resolved.  
  • Peak purity of principal peak in sample preparation should comply.  
  • For Waters “Empower 3 software”, the purity angle has to be less than the purity threshold for acceptance of no interference and there should be “NO” purity flag. |
| Accuracy                        | • The percentage recovery should be between 100±3.0 at each level.  
  • The percentage RSD for accuracy should not be more than 2.0 at each level.  
  • The percentage RSD of accuracy across all the levels (3x3) should be NMT 2.0.                                                                                                                                   |
| Precision                       | -----                                                                                                                                                                                                                   |
| System precision                | • The percentage RSD for area response from 6 repeated injections of system suitability solution/standard solution must be NMT 2.0 or as defined in the system suitability.  
  • The percent RSD for retention time from 6 repeat injections of system suitability solution/standard preparation ought to be NMT 1.0.                                                                 |
<table>
<thead>
<tr>
<th>Method precision</th>
<th>The percent RSD for 6 replicate sample preparations should be NMT 2.0.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermediate Precision</td>
<td>• The percentage RSD for 6 sample determinations ought to be NMT 2.0.</td>
</tr>
<tr>
<td></td>
<td>• The percentage RSD for 12 sample preparations must be NMT 2.0. (6 from each)</td>
</tr>
<tr>
<td></td>
<td>• Percentage difference of mean value of assay result from each type of precision ought to be NMT 2.5.</td>
</tr>
<tr>
<td>Linearity</td>
<td>• The regression coefficient and correlation coefficient should be ≥ 0.998.</td>
</tr>
<tr>
<td>Range</td>
<td>The range of method should be between 80 to 120 percent of working concentration.</td>
</tr>
<tr>
<td>Solution stability</td>
<td>• The area difference for sample solution at each interval should be within ± 2.0 percent from the initial value.</td>
</tr>
<tr>
<td></td>
<td>• The area difference for standard solution at each interval should be within ± 2.0 percent from the initial value.</td>
</tr>
</tbody>
</table>

**Chromatography**

Chromatographic technique was primarily invented and used by a Russian botanist Mikhail Tswett (Tswett, M. S, 1905: 20-39). He used this technique to separate colour pigments of plant leaves by passing the petroleum ether extract of plant leaves through a column packed with fine powder of calcium carbonate. The separated colour (pigments) appeared as colored bands or zones on the column of packing material. Since different coloured bands appeared in the column, the term Chromatography (chroma meant colour and graphy is writing) was given by the inventor. Thus, Chromatography is a partitioning method wherein a sample gets distributed between stationary phase (such as calcium carbonate) and movable phase (Liquid such as petroleum ether) depending on their affinity towards them.
A pictorial representation for separation of sample colour bands inside the column is as given in figure 1.3.

Figure 1.3: Pictorial representation of chromatographic separation with different colour bands inside the column (Courtesy: http://www.chemicool.com)

There are different type of chromatographic techniques based on the type of stationary phase and mobile phase. The regularly used techniques are planar chromatography (Thin layer and Paper chromatography), High pressure or performance liquid chromatography (HPLC), Gas chromatography (GC) etc. The summary of different types of stationary phase and mobile phase are listed in table 1.7.

Table 1.7: Different chromatographic techniques with details on type of stationary phase and mobile phases

<table>
<thead>
<tr>
<th>Technique</th>
<th>Stationary phase</th>
<th>Mobile phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thin layer chromatography (TLC)</td>
<td>Solid coated on surface like aluminum or glass plate</td>
<td>Liquid</td>
</tr>
<tr>
<td>High performance liquid chromatography HPLC/ Ultra performance liquid chromatography (UPLC)</td>
<td>Solid or Liquid coated on solid</td>
<td>Liquid</td>
</tr>
<tr>
<td>Gas chromatography</td>
<td>Solid or Polymer liquid immobilized on solid</td>
<td>Gas</td>
</tr>
</tbody>
</table>

Liquid chromatographic technique is further classified into two sub classes namely Normal phase and Reverse phase. In normal phase chromatography, the polar material like silica is used as a stationary phase and nonpolar solvent like hexane is
used as mobile phase. In reverse phase, a less polar material like octadecylsilyl (C<sub>18</sub>) or octylsilyl (C<sub>8</sub>) coated on to silica support is used as stationary phase and polar solvent such as water or buffer prepared by water in combination with organic solvents is used as mobile phase. Reverse phase is the best separation technique in liquid chromatography due to its feasibility & versatility. Ultra performance or Ultra pressure liquid Chromatography (UPLC) is the most advanced version of liquid chromatographic techniques used in pharmaceutical industry for determining the identity, purity and quality of pharmaceutical products. UPLC has many advantages in comparison with the conventional HPLC and is explained with the help of Van Deemter equation (Van Deemter et al 1956: 271-289) and plot as given in figure 1.4.

![Van Deemter plot of HETP as a function of mobile phase linear velocity](image)

Figure 1.4: Van Deemter plot of HETP as a function of mobile phase linear velocity (Courtesy: Restek Corporation, USA)

Van Deemter equation

\[ HETP = A + \frac{B}{u} + Cu \]  

Eq (3)

Where,

A = Eddy diffusion term or numerous pathway distribution that is contributed from column packing.
B = Molecular transmission, subject to the flow rate of mobile phase.
C = Effect of mass transmission, governed by the flow rate of mobile phase.
U = Rate of flow of mobile phase.
HETP = Height equivalent to theoretical plates.
This thesis elaborates the analytical method activities to achieve separation for active ingredients and impurities in pharmaceutical drug product using UPLC instrument.

**Principle of UPLC**

In UPLC, the particle size of stationary phase is less than HPLC and is tightly packed into the column. Mobile phase is pushed into the stationary phase at pressures as high as 15000 psi (pressure). The technique is relatively new in the pharmaceutical industry in comparison to HPLC and is now a days used extensively for quantifying active ingredients, impurities, characterization of drugs etc., In UPLC, The sample components in a mixture gets separated by a variety of physical-chemical interactions between the analyte and stationary phase in the chromatographic column. The sample (liquid) to be analyzed is introduced into the column by a stream of mobile phase under with high pressure. Due to partition of sample components between stationary phase and mobile phase inside the column, sample components are retarded by stationary phase as it traverses the length of column. The retardation of analyte molecules in the column are based on the nature of analyte, chemistry of stationary phase and mobile phase composition. The time spent by analyte from injection point to its elution out of the column is called the retention time (RT). RT is considered a reasonably unique identifying characteristic for a given analyte. By increasing the flow of mobile phase, the retention time of analyte can be controlled without affecting the chromatographic performance as given in the Van Deemter equation. Water or aqueous buffer along with combination of organic solvents like acetonitrile, methanol are used as mobile phases. Water may contain buffers or salts to assist in the separation of complex analyte components by maintaining a constant pH during the separation and providing selectivity requirements.

Even though the working principles of HPLC and UPLC are similar; there are a few differences as well. The variances are tabulated in table 1.8.
Table 1.8: Summary of variances between HPLC and UPLC

<table>
<thead>
<tr>
<th>Criteria</th>
<th>HPLC</th>
<th>UPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>HETP</td>
<td>Changes with change in linear velocity is high</td>
<td>Changes with change in linear velocity is less</td>
</tr>
<tr>
<td>Operating pressure</td>
<td>Up to 5000 psi</td>
<td>Up to 15000 psi</td>
</tr>
<tr>
<td>Particle size of stationary phase</td>
<td>Between 1.7 to 2.5 µm</td>
<td>Between 5 µm to 10 µm</td>
</tr>
<tr>
<td>Sensitivity levels</td>
<td>Sensitivity levels are low</td>
<td>Sensitivity levels are very high</td>
</tr>
</tbody>
</table>

A typical image of modern UPLC instrument is shown in Figure 1.5.

![Modern UPLC Instrument](image)

Figure 1.5: Modern Ultra performance liquid chromatography (UPLC) instrument (Make, courtesy: Waters corporation, USA)

The description and function of each component of UPLC are given below

**Pump**

The pump in UPLC is used for pumping the mobile phase through the chromatographic column at a desired pressure. The mobile phase from mobile phase reservoir is pumped into the column without generating the pulse in the flow to maintain a constant flow rate and pressure.
Auto sampler

In the auto sampler, the samples will be stored in trays provided inside the sample compartment. As per the software program, the samples from auto sampler will be taken to the injector and injected into the column. The temperature of auto sampler can be programmed as per requirement.

Injector

Injector in UPLC injects a required volume of sample solution into the column. The sample from a vial placed inside the auto sampler will be collected into injector loop through injector needle. Most modern UPLC instruments use FTN (Flow through needle) technology where in the mobile phase flows through the injector needle during the injection. The advantage with this technology is that the sample carryover and contamination is minimized to a larger extent.

Column compartment

The analytical column is housed inside the column compartment and its temperature can be set as per the method conditions. Working temperature range of the column compartment is between 10°C and 85°C. The column compartment is insulated to avoid heat loss and temperature fluctuations during the chromatographic run.

Detector

Detector is connected next to the column compartment in UPLC instrument. Based on the nature of the analyte to be detected, there are different type of detectors used such as; Ultra violet (UV) detector, Photodiode array detector (PDA), Fluorescence detector (FLD), Evaporating light scattering detector (ELSD) and Refractive index detector (RI). Further, most extensively used detector is UV detector because of its versatility and sensitivity. In case the analyte is not sensitive to UV detector due to absence of auxochromes and chromophores in the molecule, usage of other type of detector shall be explored. ELSD is popularly known as “universal detector” but has limitations in terms of sensitivity.

In the present thesis work, The UPLC instruments used for experimentation are Waters Acquity UPLC (Binary) and its latest model Waters Acquity UPLC H-Class (Quaternary) equipped with PDA detector. The “dwell volume” of instruments is less than 400 µL, injection cycle time is less than 30 seconds and operated on “flow through needle” technology. The instruments were operated through computers installed with Waters Empower 3 Software.
CHAPTER 1 | INTRODUCTION

With all these advanced features, UPLC methods are helpful in accurate determination and quantification of active components, impurities in the pharmaceutical drug products even at trace levels. Further, UPLC methods are used in the identification of unknown impurities as well as the composition of an unknown sample. Thus, to meet the goals mentioned above; it is very necessary to development methods that are precise, accurate and robust. Also, the method development should be based on effective scientific back ground. Hence, a more systematic and scientific way of method development called Quality by Design (QbD) as explained before is very much necessary.

The main objectives of this study

Dimenhydrinate orally disintegrating tablets (ODT) is available as over the counter drug product. It is a combination of two drugs namely Diphenhydramine and 8-Chlorotheophylline. Both belong to a different class of medicines and differ in their chemical properties. Diphenhydramine belongs to a class of antiemetic drugs and 8-Chlorotheophylline is a chlorinated derivative of theophyllines. It is predominately used by young children and requires correct dosing to eliminate all safety and efficacy issues. Further, a more systematic; QbD based methods are required to accurately determine active ingredients and impurities (related substances) in sample matrix. Hence the aim of present work is QbD based reversed phase UPLC methods in

• Development of method for concurrent estimation of Diphenhydramine and 8-Chlorotheophylline in Dimenhydrinate orally disintegrating tablets.
• Validation of newly developed method for estimation of Diphenhydramine and 8-Chlorotheophylline in Dimenhydrinate orally disintegrating tablets.
• Development of a method for quantification of “related substances” of Dimenhydrinate in Dimenhydrinate orally disintegrating tablets.
• Validation of newly developed method for quantification of related substances of Dimenhydrinate in Dimenhydrinate orally disintegrating tablets.
Organization of the thesis
The research work carried out for this doctoral thesis has been organized into 6 chapters. The summary of contents of each chapter is as given below.

Chapter-1
Provides general information about the pharmaceutical industry and role it plays in maintaining the public health. Further, it provides information on pharmaceutical drug products and their quality requirements during manufacture, handling, and storage. This chapter briefly covers various analytical techniques used for quality assessment of drug products. Also, the basic principles of chromatographic techniques and comparison between UPLC and HPLC is provided. The drawbacks of traditional method development and the need for usage of QbD principles for analytical method development are explained. Finally, the validation requirement for each type of method and details of each method validation parameter are explained.

Chapter-2
This chapter is subdivided into two sections based on the type of method development experiments performed. Section A: Details the procedure followed for identification of critical process parameters (CPP) to obtain separation between 8-Chlorotheophylline and Diphenhydramine. Section B: Discuss about the optimization step performed for quantification and separation between 8-Chlorotheophylline and Diphenhydramine by design of experimentation (DOE), Central composite design (CCD) and Design space.

Chapter-3
Presents the details of experiments for assay method validation parameters for simultaneous estimation of Diphenhydramine and 8-Chlorotheophylline followed by results and discussion.

Chapter-4
Consists of two sections
Section A: Screening experimentation for identification of vital few factors for separation of Dimenhydrinate impurities.
Section B: Factorial designs, gradient slope and run time optimization for impurities quantification method.
CHAPTER 1 | INTRODUCTION

Chapter-5
Describes the details of experimentation performed for method validation for related substances of Dimenhydrinate ODT followed by results and discussion.

Chapter-6
Presents a conclusion to the thesis along with recommendations. It highlights the results that were obtained from the thesis research and discuss about the future scope of work.

Bibliography
A list of references mentioned in the different chapters are provided here according to alphabetical order.

Publications and Conferences
Here the details of published papers and conferences attended are listed.