CHAPTER 1

Introduction

&

Literature review
INTRODUCTION

Analytical chemistry may be defined as the science and art of determining the composition of materials of the elements or compounds contained.

Pharmaceutical analysis deals with the qualitative and quantitative analysis of the raw materials such as Active Pharmaceutical Ingredients (API) & excipients and various dosage forms like Tablets, Capsules, Parenteral, and Liquid & Topical Preparations etc.

The importance of analytical chemistry with respect to various scientific areas could be illustrated by considering its impact on pharmaceutical research quality control, in environmental analysis and clinical analysis. In pharmaceutical industry the quality and quantity of drug in dosage form must be carefully controlled. Even a small change in purity of drug can affect the therapeutic value. Pharmaceutical analysis also deals with analyzing biological sample in support of biopharmaceutical and pharmacokinetic studies.

Before a drug is approved and made available in the market. It is necessary to establish the qualitative properties and therapeutic value of the drug. Pharmaceutical analytical studies are applied to establish dosage levels of drug and in the determination of their metabolites at various stages of metabolism.

Accurate, specific and sensitive tests must be developed since the compounds are often determined at nanogram levels in biological samples. Presently, the laws of many governmental agencies including US FDA are working on, how a new drug must be tested? The procedures are very strict and considerable amount of time and finance needs to be spent on establishing the quality of drug.

It should be emphasized that the present day analyst should be aware and capable of developing new methods for this purpose and also keep the pharmaceutical industries and quality control laboratories in viable position.
Importance of Analytical Methods

Quality is important in every product or service, but it is vital in medicine as it involves life. Unlike other consumer goods, there can be and there is no second quality. Therefore analytical methods which are a measure of quality of the drugs play a very comprehensive role in drug development and follow up activities, to assure that a drug product meets the established standard, is a stable and will continue to meet purported quality throughout its shelf life (2)

These methods should be selective and sensitive to monitor the known and unknown impurities, have to be written in a format such that they can be produced over a period of time and from laboratory to laboratory, i.e. these methods should be validated.

Analytical methods are required for characterization of drug substance and drug product composition during all phases of pharmaceutical development (3). Early phase methods must support changes in synthetic routes and dosage form and elucidate the structures and levels of impurities. In later phases, goals change to the development of rapid and robust methods for release and stability evaluation. Analysis includes a wide range of simple and instrumental analytical methods, but the most widely most used analytical methods for quality assurance are spectroscopy and chromatography based. Most quantitative analysis require, measuring specified components in the presence of sample matrix and/or related substances, therefore isolation or separation of the components are required preceding quantitative analysis. In such cases chromatographic techniques are used for quantitative analysis. In cases where matrix interference is not observed quantitative measurements are made using spectroscopic or titration methods directly. (4) For the present studies analytical methods based on Reversed Phase High Performance Liquid Chromatography (RP-HPLC), and Mass Spectroscopy (LC-MS) have been developed.
Importance of Quality of Pharmaceuticals

The term “Quality” as applied to drug product has been defined as the sum of the all factors that contribute directly or indirectly to the safety, effectiveness and reliability of the product. Quality assurance may be defined as the responsibility of an organization to determine that the system, facilities and written procedure are adequate and are followed in order to assure that product quality is controlled and will meet all the requirements in its final dosage form. Thus quality assurance not only reviews quality but also determines the purity of the relative amount of components present in the sample. (5)

Pharmaceutical analysis deals with the scientific and technical aspects of measurement of compositional and constitutional features of the sample. It can be broadly divided into

- **Qualitative analysis** (Identification)
- **Quantitative analysis** (Estimation)

Qualitative analysis reveals the identity of species i.e. the identification of compounds, elements or impurities in the sample.

Quantitative analysis helps to find the relative amount of one or more of these species or analyze in numerical terms. Qualitative information is required before a quantitative analysis is undertaken.

The various steps involved in a typical quantitative analysis are

- Chemical nature of the sample
- Obtain adequate amount of sample
- Selection of method
- Preparation of laboratory sample
- Number of samples to be analyzed
- Elimination of possible interferences
- Measurement of analyte
- Estimation of the reliability of the results (validation)
- The importance of newer analytical methods
**Chromatography Techniques:**

In chromatographic methods, separation is based on variation in the distribution of different compounds between two dissimilar phases - a stationary phase and a mobile phase. The sample components are separated into fractions based on their relative affinity towards the two phases.

**High Performance Liquid Chromatography**

High-performance liquid chromatography (HPLC) is an advanced form of liquid chromatography used in separation of the complex mixture of molecules which come across in chemical and biological systems, in order to understand better the role of individual molecules. It is the fastest growing analytical technique for the analysis of drug substances. Due to its simplicity, wide range of sensitivity and high specificity, HPLC is being considered as ideal technique for the analysis of many drugs in both dosage forms and biological fluids. The rapid growth of HPLC has been facilitated by the development of reliable, moderately priced instruments and efficient columns.

In classical column chromatography, the mobile phase flows slowly through the column by means of gravity. But in HPLC, the separation is about 100 times faster than the conventional liquid chromatography due to packing of very small particles and thus differs from other Liquid chromatography technique. This small particle size results in more rapid approach to distribution equilibrium and smaller plate height (HETP) so that given length of column is highly efficient and peaks are narrow. However the close packing of these small particle reduces the flow rate of mobile phase through the packed bed and in order to achieve a reasonable flow rate it is necessary to apply pressure to the mobile phase, and hence the technique is referred as high pressure liquid chromatography.

This analytical method has prime importance for those compounds, which are non-volatile or thermally unstable so that they are not amenable to GLC analysis. The compounds which are not analyzed by GLC, but can be analyzed by HPLC are carbohydrates, nucleoside, Steroids, alkaloids, peptides, amino acids and antibiotics. Prior to the development of HPLC, compounds are analyzed by GLC,
Quantitative TLC, Paper chromatography and Liquid- Liquid or Liquid- solid chromatography.

The Selection of Suitable Chromatographic (HPLC) system for various solutes needs to be verified and confirmed by experiment as the same cannot be made with certainty.

1. By knowing the chemical nature of the sample components, the suitable phase system can be selected from the literature reference.

2. If the Chemical nature of the Sample is not known, then the sample solubility studies provide indications as to which chromatographic method to be employed.

**Principle**

The liquid chromatography, technique is used to separate molecules and ions for analysis. the separation is based on polarity of compounds, a mixture of molecules dissolved in a solution (mobile phase) is separated into its constituent parts by passing through a column of tightly packed solid particles (stationary phase). Some compounds in the sample mixture will have greater preference for stationary phase then the mobile phase and will be retained in column for longer. The separation occurs because each component in the mixture interacts differently with the stationary phase. Molecules that interact strongly with the stationary phase will move slowly through the column, while the molecules that interact less strongly will move rapidly through the column. This differential rate of migration facilitates the separation of the molecules.

HPLC utilizes different types of stationary phases, a pump that carries the mobile phase(s) and analyte through the column, and a detector to signal the characteristic retention time for the analyte. (6)
The essential parts of high performance Liquid chromatographic system are,

- Solvent reservoir
- Pump
- Injection port
- Column
- Detector
- Recorder

A block diagram of HPLC System is as given below.

**Figure No. 1 Block diagram of HPLC System**

**Pumps**

In HPLC, a pump provides the higher pressure to carry the mobile phase and analyte through the heavily packed column. The increased density which arises from smaller particle size allows better separation on shorter length columns as compared to ordinary length columns.
There are two types of stationary phases mainly reverse phase and normal phase.

Whether a column can be used for normal phase or reverse phase chromatography will be determined from the nature of the stationary phase.

**a. Normal phase HPLC:**

Normal phase chromatography consists of a non-polar mobile phase and polar stationary phase. Polar compounds in mixture will stick longer to polar silica than non-polar compounds, therefore more polar compounds elute later than non-polar compounds.

**b. Reversed phase HPLC:**

About 75% of current HPLC analysis is performed using the reverse phase (7). In reversed phase, chromatography the stationary phase is mainly silica chemically bonded through a siloxane (Si-o-Si-C) linkage to a low polar functional group. These phases are prepared by treating the surface silanol groups of silica with an organochlorosilane (8). The polarity of the column can be changed by varying the alkyl chain length in R

\[
\begin{align*}
\text{Si-OH} + \text{Cl} & \rightarrow \text{Si-} \quad \text{R} + \text{HCl} \\
\text{CH}_3 & \quad \text{Si-} \quad \text{R} + \text{HCl}
\end{align*}
\]

Where R = C6H13 (Hexyl), C8H17 (Octyl) or C18H17 (Octadecyl).

**Figure No. 2 Carbon chains in Stationary phase**

*For our studies mainly we have used C-8 columns*

Retention time can be increased by adding more water to the mobile phase; thereby making the affinity of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to the now more hydrophilic mobile phase. Similarly, an investigator can decrease retention time by adding more organic solvent to the eluent.
HPLC Detectors (9)

Based on the method or principle used in detection various types of detectors are available. For the present study UV and PDA detectors are mainly used. Other detectors commonly found on HPLC instruments are RI detectors, electrochemical detectors and Fluorescent detectors.

UV Detectors

In these systems detection depends on absorption of UV ray energy by the sample. The equipment comprises of accessories in order as UV source, grating (for light defraction), sample passing through a tubing exposed to rays, photo cell, charge conductor etc. When the UV rays emitted by lamp pass through gratings, rays split into different wavelengths.

One specific wavelength rays are passed through sample. Some amount of light is absorbed by sample and the unabsorbed rays which fall on photo cell.
These rays on collision on photo cell produce electrons whose current is recorded. This is indicative of nature and quantity of sample. This UV wavelength range of absorption is specific for sample. These are the HPLC detectors used in general, unless there is requirement for analysis of special compounds. They are capable to detect very wide range of compounds. The sensitivity ranges till microgram quantity of estimation.

PDA detectors:

These are detectors which are similar to UV detectors but the only advantages are higher sensitivity and measure the entire absorption range i.e. it gives scan of entire spectrum.

HPLC method development (10)

The wide range of equipment, columns, eluent and parameters involved makes high performance liquid chromatography (HPLC) method development seem challenging. The process is influenced by the nature of the analytes and generally follows the following steps:
Step 1 – Initial studies

Step 2 - Selection of initial conditions

Step 3 - Selectivity optimization

step 4 - System optimization

**Step-1 Initial studies**

When developing an HPLC method, the first step is always to consult the literature to know the physico chemical properties of analyte. The properties like solubility, polarity, molecular weight and partition coefficient are key parameters in selecting the right column and mobile phase for a successful chromatographic separation.

**Sample preparation.** The sample matrix determines whether the sample requires dissolution, filtration, extraction, preconcentration or clean up. Chemical derivatization may be required to assist detection sensitivity or selectivity.

**Types of chromatography.**

Reverse phase is the choice for the majority of samples, but if acidic or basic analytes are present then reverse phase ion suppression (for weak acids or bases) or reverse phase ion pairing (for strong acids or bases) should be used. The stationary phase should be C18 bonded. For low/medium polarity analytes, normal phase HPLC is a potential candidate, particularly if the separation of isomers is required.

**GRADIENT HPLC. (11)**

This is only a requirement for complex samples with a large number of components due to the maximum number of peaks that can be resolved with a given resolution is much higher than in isocratic HPLC.

Gradient HPLC also give greater sensitivity, for analytes with longer retention times (for a given peak area, peak height is inversely proportional to peak width). Detectors UV detectors, single channel, multi-channel or Photodiode array are the most common detectors used in HPLC
UV wavelength. The analytical sensitivity is maximum at $\lambda_{\text{max}}$, but the wavelengths showing high absorbance can be used as analytical wavelengths. The UV wavelengths below 200 nm are avoided because mobile phase interferences and detector noise increase in this region. Higher wavelengths give greater selectivity.

**Step 2 - Selection of chromatographic conditions.**

This step determines the optimum conditions to adequately retain all analytes; that is, ensures no analyte has a capacity factor of less than 0.5 (poor retention could result in peak overlapping) and no analyte has a capacity factor greater than 10–15 (excessive retention leads to long analysis time and broad peaks with poor detectability). Selection of the following is then required.

*Mobile phase solvent strength.*

The solvent strength is a measure of its ability to sweep analytes from the column. It is generally controlled by the concentration of the solvent with the highest strength; for example, in reverse phase HPLC with aqueous mobile phases, the strong solvent would be the organic modifier; in normal phase HPLC, it would be the most polar one. The aim is to find the correct concentration of the strong solvent.

*Gradient HPLC.* With samples containing a large number of analytes (20–30) or with a wide range of analyte retentivities, gradient elution becomes necessary to avoid excessive retention.

**Step 3 - Selectivity optimization.**

The aim of this step is to achieve adequate selectivity (peak spacing). The mobile phase and stationary phase compositions need to be taken into account. To select these, the nature of the analytes must be considered.

Once the analyte types are identified, the relevant optimization parameters may be selected. The optimization of mobile phase parameters is always considered first as this is much easier and convenient than stationary phase optimization.
**Step 4 - system parameter optimization.**

This is used to find the desired balance between resolution and analysis time after satisfactory selectivity has been achieved. The parameters involved include column dimensions, column-packing particle size and flow rate. These parameters may be changed without affecting capacity factors or selectivity.

**Step 5 - method validation.**

Proper validation of analytical methods is important for pharmaceutical analysis when assurance of the continuing efficacy and safety of each batch manufactured relies solely on the determination of quality. The ability to control this quality is dependent upon the ability of the analytical methods, as applied under well-defined conditions and at an established level of sensitivity, to give a reliable demonstration of all deviation from target criteria.

**METHOD VALIDATION**

“Validation is the process of collecting documented evidence that the method performs according to its intended purpose”. This is based on analytical experiments performed according to the validation protocols that comply with the international guidelines i.e. ICH guidelines on method validation. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) is a unique project that brings together the regulatory authorities of Europe, Japan and the United States and experts from the pharmaceutical industry in the three regions to discuss scientific and technical aspects of product registration.(12) (13)

Forced degradation (FD) study is a process in which the natural degradation rate of a pharmaceutical product is increased by the application of an additional stress. FD studies (i) help to identify reactions that cause degradation of pharmaceutical product, (ii) are part of the development strategy and an integral component of validating analytical methods that indicate stability and detect impurities which are formed during manufacture, storage, or use and their properties are different from the desired product with respect to activity, efficacy and safety, and (iii) are designed to generate product-related variants and develop analytical methods to
determine the degradation products formed during accelerated and long-term stability studies. Any significant degradation product should be evaluated for characterization and quantization for its potential hazard.

The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity and light, enables to establish a retest period/shelf lives for a drug substance and a recommended storage condition. Methods can be developed which measure the amount of drug remaining, the amount of drug lost (or the appearance of degradation products), or both. The expert Working Group of the International Conference on Harmonisation of the Technical Requirements for Registration of Pharmaceuticals for Human Use developed a guideline on stability testing for registration application within the European Union, Japan and the United States. The goal of the ICH stability guideline was to exemplify the core stability data package required for new drug substances and products in the European Union, Japan and the United States such that the data generated in any of the regions is mutually acceptable in the other two. The guideline applies to the information required for the registration applications of new molecular entities and drug products, but not to abbreviated or abridged applications, clinical trial applications, and so on. The test conditions were selected based on the climatic conditions in three areas so that test data provides evidence on the variation in quality with time under the influence of a variety of representative environmental factors. These data in turn allow recommended storage conditions and shelf lives to be established.
All analytical procedures require some form of validation, regardless of whether the method is used for stability, in-process analysis, release, or acceptance testing. (14)(15)(16)

Method validation has received considerable attention in literature from various industrial committees and regulatory agencies. There is a wide variety of information and guidance available, as mentioned below;

List of Literature from industrial committees and regulatory agencies

<table>
<thead>
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<th>Committees and regulatory agencies</th>
<th>Guidelines available</th>
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| ICH                               | a) Q2R1 Guidelines are guidelines for new method development and its validation.  
b) Q1R1 Guidelines are for development and validation of stability indicating analytical methods includes methodology |
| The United States Food and Drug Administration | Two industry guidelines:  
a) for the validation of analytical methods  
b) For the validation of bio-analytical methods  |
| IUPAC (17)                         | “Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis” |
| EURACHEM (18)                     | detailed guide for method validation primarily developed for ISO/IEC accredited laboratories but because of its completeness it is also a good source for (bio)pharmaceutical laboratories |
| AOAC(19)                          | technical document for the verification of analytical methods for the ISO 17025 accreditation. |
| Huber (20)                        | Has published a technical document for the verification of Analytical methods for the ISO 17025 accreditation. |
| Viswanathan and co-authors (21)   | An overview for validation of bio-analytical methods. |
Parameters for Method Validation

The parameters as defined by the ICH and by other organizations and authors are summarized in below and described in brief in the following paragraphs. (22)(23)

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<tbody>
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<td>1</td>
<td>Specificity</td>
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<td>13</td>
<td>Solution stability</td>
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1. Specificity
Specificity is the ability of the analytical method to assess unequivocally the analyte in the presence of components that may be expected to be present in the sample.

**Determination**
Blank solution, Placebo solution, known impurity (if included in specification) solution at specification limit and standard solution shall be prepared and injected to check the interference.

**Acceptance criteria**
No peak should be observed due to blank solution, placebo solution and known impurity solution at the retention time of principle peak as observed in the standard solution.
2. Precision
The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple samplings of the same homogeneous sample under prescribed conditions.

System precision
Determination
Standard solution to be prepared as described in proposed method and to be injected (measured) six times. The relative standard deviation of the response shall be calculated.
Acceptance criteria
Relative standard deviation should not be more than 1.0 %

Method precision
Determination
Six different sample solutions shall be prepared from the homogeneous sample and shall be analyzed using the proposed method over a short period of time by same analyst, on same equipment, on same day. The assay results shall be calculated. The relative standard deviation of the results shall be calculated
Acceptance criteria
Relative standard deviation should not be more than 2.0 %

Intermediate precision (ruggedness)
Determination
Six different sample solutions shall be prepared from the homogeneous sample and shall be analyzed using the proposed method by different analyst, on different equipment, on different day. The result shall be calculated. The absolute difference in the assay results obtained in Method precision (Mean value of six results) and Intermediate precision (Mean value of six results) shall be calculated.
Acceptance criteria
The absolute difference in the assay results obtained in Method precision (Mean value of six results) and Intermediate precision (Mean value of six results) should not be more than 2.0 %.
3. **Accuracy (Recovery)**

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the found value.

**Determination**

Recovery solutions shall be prepared by spiking the drug substance into the volumetric flask containing placebo powder to obtain the solutions at 50%, 100% and 150% of target concentration of drug substance as in sample solution described in proposed method. The concentration of placebo in recovery solutions at 50%, 100% and 150% shall remain constant as in sample solution described in proposed method. If the spiking amount of drug substance is less than 10 mg than stock solution of drug substance shall be used to prepare the recovery solutions. The recovery solution at all the three concentration levels shall be prepared in triplicate and analyzed. The recovery in mg and finally as % recovery shall be calculated for each level and mean recovery of all solution shall be calculated.

**Acceptance criteria**

All the individual recoveries should be within 97.0% to 103.0% and the mean recovery should be within 98.0% to 102.0% for Assay and 80% to 120% for Impurities.

4. **Linearity and range**

The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration of analyte in the sample.

**Determination**

Linearity solutions shall be prepared from the stock solution of working standard to obtain the solutions at 10%, 50%, 80%, 100%, 120%, and 150%. The prepared solutions shall be analysed in duplicate.

A graph of mean peak area vs. concentration (ppm) shall be plotted. The slope, intercept and correlation coefficient of the regression line shall be reported. For range, record the concentration levels over which the results are linear.
Acceptance criteria
Correlation Coefficient should be not less than 0.999.

5. Limit of detection and quantitation

Limit of detection
The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated as an exact value.

Limit of quantitation
The Quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy.

Determination
A series of solutions shall be prepared by quantitative dilutions of the stock solution of impurity standard to obtain solutions at 5 %, 10 %, 15 %, 20 %, 25 % and 30 % of specification limit. Each solution will be injected into the chromatograph in duplicate (single injection to be done if impurity solution are not stable) and the mean peak areas will be calculated.

The slope and residual standard deviation shall be determined for each impurity using the respective value of peak response and concentration (ppm). The value of limit of detection and limit of quantitation shall be determined for each impurity using the following formula:

Calculation:

\[
\text{LOD} = \frac{3.3 \times \sigma}{S} \quad \text{LOQ} = \frac{10 \times \sigma}{S}
\]

Where,
\[
\sigma = \text{Residual Standard Deviation}
\]
The solution at LOQ level for each impurity shall be prepared and injected in six replicates and the relative standard deviation of the peak areas for each impurity peaks shall be calculated.

Acceptance criteria
Relative standard deviation of the peak area due to each impurity in six replicate injections of LOQ solution should not be more than 10.0 %.

6. Robustness
The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Determination
One set of analysis shall be carried out using the same homogeneous sample by making individual small deliberate changes in the analytical procedure. Those changes shall be done as described below if any of the applicable
Change in flow rate (Flow rate specified in method ± 0.2)
Change in column oven temperature (Temperature specified in method ± 5°C)

The result of assay shall be calculated for each set of analysis. The absolute difference in the results obtained in robustness study and method precision (set 1) shall be calculated.

Acceptance criteria
The absolute difference in the results obtained in robustness study and method precision (set 1) should not be more than 2.0 %.

S = Slope
LOD = Limit of detection
LOQ = Limit of quantitation
7. Solution stability

Determination
Standard solution and sample solution shall be prepared as described in proposed method. The prepared solutions shall be tightly closed and stored at room temperature and at 10°C and analysed at 12 hours and 24 hours if the drug substance tends to be stable in solution (Based on the information obtained from development report). If the drug substance tends to be unstable in solution then analysed the stored solution at every 1 hour up to 8 hours. The % difference in response at time point with respect to initial response shall be calculated for standard solution and test solution.

Acceptance criteria
The % difference in response at time point with respect to initial response should not be more than 1.0 %. If it is out of the set criteria, appropriate recommendation shall be made.
Impurity in pharmaceuticals

Impurities in pharmaceuticals are the unwanted chemicals that remain with the active pharmaceutical ingredients (API’s) or develop during formulation or upon aging of both API and formulations. (24)

Many potential impurities arise during the synthesis of API. Sources of impurity includes, starting materials, intermediates, reagents, solvents, catalysts and reaction by products.

Impurities can endanger the human health by affecting quality, safety and efficacy of the product. The presence of these unwanted chemicals even in small amounts may influence the efficacy and safety of the pharmaceutical products. Due to the noticeable impact of impurities on quality of pharmaceuticals, impurity control in pharmaceutical products is a primary goal of drug development.

These potential impurities should be identified to find out their origin and how to minimize these impurities at initial level itself. The amount of these impurities present in drug substance (API) will determine the safety of drug product. Therefore identification, quantification, qualification and control of impurities are now crucial part of drug development.(25)

To assure the quality of drugs, impurities must be monitored carefully. Stringent international regulatory requirements have been in place for several years as outlined. The various regulatory authorities emphasizing on the impurity profiling includes ICH, USFDA, Canadian Drug and Health Agency and Guidelines given by department of health and ageing therapeutic goods administration of Australian government(26). The different official compendia, such as British Pharmacopeia (BP), United States Pharmacopeia (USP), and European Pharmacopeia (EP) are incorporating limits to restrict levels of impurities present in API as well as in formulations(27)(28). Table 1 shows the thresholds of impurities specified by ICH guidelines. (29)
Different Sources of Impurities:
Impurities observed in API may arise during synthetic route and they can be of different types such as organic impurities, organic volatile impurities or inorganic impurities. During DP development, impurities may observed either as degradation products because of inherent instability of drug substance, or as a result of incompatibility of drug with added excipients, or from interaction of drug with primary packaging materials used(30) (31)

Figure 2 shows various sources of pharmaceutical impurities.
In general according to ICH guidelines, identification of impurities below 0.1% level is not considered necessary unless the potential impurities are expected to be unusually potent or toxic According to the ICH guidelines, impurities related to API’s are classified in to the following main categories (32)(33)(34)
C. Residual solvents:
Residual solvents are the organic volatile chemicals used during the manufacturing process or generated during the production. Organic solvents play a crucial role in the synthesis of pharmaceuticals. But these solvents also have disadvantage, as many of the solvents used have toxic or environmentally hazardous properties. Complete removal of these solvents is very difficult. To meet the safety requirement necessary changes should be done in manufacturing process to reduce their use wherever feasible. Toxic solvents must be avoided during manufacturing process. The final purification step in most of the pharmaceutical drug-substance processes involves a crystallization step and as a result the crystals formed can entrap a finite amount of solvent from the mother liquor that may cause degradation of the drug or that entrapped solvent acts as a residual impurity.

**Figure No. 3 Different Sources of Impurities**

Different methods are available to keep an eye on impurities. The most important criterion is the ability of method to selectively identify and quantify the compound of interest. Main tools for impurity analysis mainly includes spectroscopic and chromatographic (separation) methods or a combination of both. The
Different types of instrumental techniques, which are employed in impurity profiling of pharmaceuticals can be divided in three major categories (35)

A. Spectroscopic Techniques.
B. Chromatographic Techniques.
C. Combination of Spectroscopic and chromatographic techniques. (i.e. Hyphenated techniques)

For present work mainly LC-MS technique has been used. LC-MS uses the separation efficiency of LC with the ability of an MS simultaneously to selectively detect and confirm identity of analyte. The mass spectrometer is a ‘compound specific detector’ providing information directly related to molecular structure of an analyte. In parallel to this evolution in LC–MS instrumentation, there has been incredible growth in LC–MS techniques for pharmaceutical analysis. Now a day’s LC–MS is accepted as a routine tool within the pharmaceutical industry. (36)(37)

The recent improvements in LC–MS technology have been remarkable. LC–MS is used in pharmaceutical analytical development mainly for exploring products for impurities and identifying those that are detected. In this technique a detection limit of a few hundred ppm is achievable where all impurities above 0.1% can easily be identified. (38)(39)

The ionization techniques used in LC-MS are generally soft ionization techniques that mainly give the molecular ion species with only a few fragmentations. The two most commonly used interfaces include Electrospray Ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI). With these ionization techniques, various types of mass analyzers, such as Quadrupole, Ion Trap and Time of Flight (TOF) are available. These mass analyzer provides varying degree of mass accuracy and resolution so that exact mass for the compound can be found out. (40)

The information obtained from a single LC-MS run, is not sufficient for confirmation of identity of compound. Nevertheless, this problem has now been resolved by the introduction of tandem mass spectrometry (MS-MS), which provides fragments through collision-induced dissociation of the molecular ions produced. Use of LC-MS-MS is increasing speedily day by day. (41)
Drug Profile

Anastrozole:

1,3-Benzeneacetonitrile, alpha, alpha, alpha', alpha'-tetramethyl-5-(1H-1,2,4-triazol-1-ylmethyl)-2-[3-(2-Cyanopropan-2-yl)-5-(1,2,4-triazol-1-ylmethyl)phenyl]-2-methylpropanenitrile

Molecular Formula: C17H19N5
Molecular Weight: 293.3663
Solubility in water: Insoluble

Density: 1.08 g/cm³
PKa/pKb: 11.38 (pKb)
Partition Coefficient: .969

Anastrozole (an-ASS-troh-zole) is a medicine, used extensively in the treatment of breast cancer. Anastrozole is a potent and selective non-steroidal aromatase inhibitor indicated for the treatment of advanced breast cancer in post-menopausal women with disease progression following tamoxifen therapy.

Anastrozole (INN) marketed under the trade name (Arimidex) by AstraZeneca, is a drug used to treat breast cancer after surgery and for metastases in both pre and post-menopausal women. Some breast cancer cells require estrogen to grow, and eliminating estrogen suppresses their growth. (42) (43) (44)
Drug Profile

Temozolomide:

(4-methyl-5-oxo- 2, 3, 4, 6, 8-pentazabicyclo [4.3.0] nona-2, 7, 9-triene- 9-carboxamide)

Molecular Formula: C₆H₆N₆O₂

Molecular Weight: 194.15
Solubility in water: Slightly soluble
Density: 1.97 g/cm³ (20 C)
PKa/pKb: 15.29 (pKa)
Partition Coefficient: -1.15

Temozolomide is a chemotherapy drug that works by slowing cancer cell growth. Temozolomide is an imidazotetrazine derivative and an antineoplastic agent. It is a prodrug that has little to no pharmacological activity until it is hydrolyzed in vivo to 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC). After administration, temozolomide undergoes rapid, nonenzymatic hydrolysis at physiological pH to MTIC, which is the active form of the drug. MTIC is generated through the effect of water at the highly electropositive C4 position of temozolomide, causing the ring of temozolomide to open, release carbon dioxide, and generate MTIC. Temozolomide is administered orally and penetrates well into the central nervous system. Temozolomide (45)(46)(47)
Literature review

From the Literature review it is unable to find the combined HPLC methods for their respective formulations of Temozolomide and Anastrozole are present in publications however, an analytical methods for individual analysis for Temozolomide and Anastrozole was published. It is felt necessary that to develop quantitative LC method for simultaneous determination of Temozolomide and Anastrozole for their respective formulations. It was also found that there are some analytical methods reported for the Temozolomide and Anastrozole separately and most of the works reported were done on the biological fluids.

With growing concerns over food safety and the need to increase sample-throughput in analytical testing laboratories, there is a constant requirement for accurate, simpler, faster and improved analytical methods. The complexity of food matrices and the presence of much potential interference, require specific and selective methods of analysis.

S. Kumar et al reported method which developed and validation of spectrophotometric method for estimation of anastrozole bulk and pharmaceutical dosage formulation. Method was developed and validated by using a simple solvent system for anastrozole bulk as well as tablet dosage form. In this method, water and ethanol are used as mobile phase and detection wavelength to be 221nm. The procedure was validated as per ICH rules for Accuracy, Precision, Detection limit, Linearity, Reproducibility and Quantitation limit. The linearity concentration was 40-60µg/mL with the correlation coefficient of 0.9971. The percentage recovery for anastrozole was found to be 98.6 to 100.8%. Limit of detection and limit of quantitation values were found to be 1µg/mL and 3µg/mL. The method has been successfully used to analyze commercial solid dosage containing 1 mg of anastrozole with good recoveries and proved to be robust. This provides shorter analysis time and conserves the solvent system. (48)

S. Kumar et al reported reverse phase isocratic HPLC method. Which describe quantitation of anastrozole in tablet dosage form. The quantification was carried out using Grace smart RP18, 5 µ (100 mmx4.6 mm) with UV detected at 215 nm. The elution was achieved isocratically with a mobile phase comprising a mixture of buffer (pH 6.0) and acetonitrile (1:1, v/v). The flow rate was 1.0 mL/min the
procedure was validated as per ICH rules for Accuracy, Precision, Detection limit, Linearity, Reproducibility and Quantitation limit. The linearity concentration range was 10-20 mcg/mL with the correlation coefficient of 0.9935. The percentage recovery for Anastrozole was found to be 97.31±2.2%. Limit of detection and limit of quantitation values were found to be 0.351 mcg/mL and 1.053 mcg/mL the method has been successfully used to analyze commercial solid dosage containing 1mg of anastrozole with good recoveries and proved to be robust. (49)

Hiriyanna S. & Basavaiah K. (2008) reported that chromatographic method for Isolation and Characterization of Process Related Impurities in Anastrozole Active. Three impurities ranging from 0.08%-0.12% by peak area in anastrozole pharmaceutical ingredient were detected by simple isocratic reverse-phase high performance liquid chromatography (HPLC). These impurities were isolated by prep-HPLC and were characterized by LC-MS/ MS, GCMS and NMR experimental data. Based on the results obtained from different spectroscopic experiments, these impurities have been characterized (Impurity I), (Impurity II) and (Impurity III). This work reveals HPLC method for detection, separation of three process related impurities from anastrozole and prep-HPLC method for isolation of these impurities from the anastrozole bulk drug. All the three impurities detected were characterized using GC-MS, LC-MS/MS and NMR experimental data. (50)

Saravanan G et al reported that the stress stability behavior and development of a liquid chromatographic method for the quantitative determination of anastrozole. A separation was achieved on a Hichrom RPB18 (250 · 4.6 mm, 5 µm) column using water and mixture of acetonitrile and methanol (1:1 ratio) as mobile phase. Forced degradation studies performed on bulk samples of anastrozole using acid, base, hydrogen peroxide, heat and UV light. Degradation of the drug substance was observed in base hydrolysis. Degradation product formed under base hydrolysis was found to be Imp-C. The sample solution and mobile phase were found to be stable up to 48 h. The developed method was validated with respect to linearity, accuracy, precision, robustness and forced degradation studies prove the stability indicating power of the method. (51)

Reddy Y R et al reported stress stability method for Anastrozole tablets formulation subjected to different ICH prescribed stress conditions of thermal,
hydrolysis, humidity, photolysis and oxidation stress. The drug was found to be stable for all the stressed conditions except for oxidation. Separation of anastrozole from its potential impurities, degradation products and five anastrozole related compounds as main impurities were achieved. Chromatographic separation achieved on Inertsil ODS-3V, 250 mm x 4.6 mm i.d, 5 micron analytical column. The elution of impurities with time dependent gradient programmed. Mobile phase consisting of water and acetonitrile as at column flow rates of 1 ml/min and at 215 nm UV detection. The same method was also extended to LC-MS/MS studies which were carried out to identify the degradation product. (52)

Jangid A. et al reported that method for estimation of anastrozole in human plasma was validated using letrozole as internal standard. The analyte and internal standard were extracted from plasma using simple solid-phase extraction. The compound were separated on a reverse-phase column with an isocratic mobile phase consisting of 0.1% formic acid in water and acetonitrile (12 : 88, v/v) and detected by tandem mass spectrometry in positive ion mode. The ion transitions recorded in multiple reaction monitoring mode were m/z 294.1 --> 225.1 for anastrozole and m/z 286.1 - --> 217.1 for internal standard. (53)

Bock M et al reported that validated assay for the quantification of anastrozole in human plasma by capillary gas chromatography-63Ni electron capture detection. An assay was developed for the quantification of anastrozole [2,2′-[5-(1H-1,2,4-triazol-1-ymethyl)-1,3-phenylene]bis(2-methylpropiononitrile)] in human plasma using liquid-liquid extraction. Anastrozole and an internal standard were chromatographed and detected by gas chromatography with electron capture detection, using a combination temperature-pressure program. The range of the assay is 3 to 100 ng/ml. Anastrozole was quantified by comparing its peak area to that of an internal standard. A cross-validation of this assay was also successfully performed between several laboratories. (54)

R Mansel et al reported that Cost-effectiveness analysis of anastrozole vs. tamoxifen in adjuvant therapy for early stage breast cancer in the United Kingdom: the 5-year completed treatment analysis of the ATAC ('Arimidex', Tamoxifen alone or in combination) trial Results from the completed treatment analysis of the ATAC
Arimidex, Tamoxifen alone or in combination) trial indicated that anastrozole was significantly superior to tamoxifen in terms of efficacy and safety in the adjuvant treatment of postmenopausal women with hormone receptor-positive (HR+) early breast cancer. On the basis of these results, this study estimated the cost-effectiveness of anastrozole vs tamoxifen, from the perspective of the UK National Health Service (NHS). (55)

C Sitaram et al reported that Determination and characterization of degradation products of Anastrozole by LC–MS/MS and NMR spectroscopy. Two new degradation products for Anastrozole active pharmaceutical ingredient (ANZ) have been identified and reported in this paper. The ANZ was subjected to thermal, photolytic, oxidative and base stress conditions prescribed by ICH guidelines. Separation of ANZ from its existing impurities and the two new impurities was achieved by using on Oyster ODS-3 (100 mm × 4.6 mm × 3.0 µm) column with an isocratic mixture of 10 mM ammonium formate and acetonitrile in the ratio 60:40 (v/v). The flow rate was 0.5 ml min⁻¹. The elution was monitored at 215 nm. An isocratic stability indicating reverse phase liquid chromatographic (RP-LC) and LC–MS/MS method was developed for the determination of purity and assay of ANZ through forced degradation studies. The degradation products were well resolved from main peak and its impurities thus proved the stability, indicating power of the method. The developed method was validated as per International Conference on Harmonization (ICH) guidelines with respect to specificity, limit of detection, limit of quantitation, precision, linearity, accuracy, robustness and system suitability. (56)

Gustavo D et al reported that Anastrozole quantification in human plasma by high-performance liquid chromatography coupled to photospray tandem mass spectrometry applied to pharmacokinetic studies. Anastrozole quantification in human plasma by high-performance liquid chromatography coupled to photospray tandem mass spectrometry applied to pharmacokinetic studies. A rapid, sensitive and specific method for quantifying the aromatase inhibitor (anastrozole) in human plasma using dexchlorpheniramine as the internal standard (I.S.) is described herein. The analyte and the I.S. were extracted from 200 µl of human plasma by liquid–liquid extraction using a mixture of diethyl ether:dichloromethane (70:30, v/v) solution. Extracts were removed and dried in the organic phase then
reconstituted with 200 µl of acetonitrile:water (50:50; v/v). The extracts were analyzed by high performance liquid chromatography coupled with photospray tandem mass spectrometry (HPLC–MS–MS). Chromatography was performed isocratically on a Genesis, C18 4 µm analytical column (100 mm × 2.1 mm i.d.). The method had a chromatographic run time of 2.5 min and a linear calibration curve ranging from 0.05–10 ng ml–1. The limit of quantification (LOQ) was 0.05 ng ml–1. This HPLC–MS–MS procedure was used to assess pharmacokinetic studies. (57)

Fabio G et al reported that development and validation of high performance liquid chromatographic method for the simultaneous determination of anastrozole, bicalutamide, tamoxifen, and their synthetic impurities A simple and sensitive analytical method for simultaneous determination of anastrozole, bicalutamide, and tamoxifen as well as their synthetic impurities, anastrozole pentamethyl, bicalutamide 3-fluoro-isomer, and tamoxifen e-isomer, was developed and validated by using high performance liquid chromatography (HPLC). The separation was achieved on a Symmetry C-8 column (100×4.6 mm i.d., 3.5 µm) at room temperature (±24 °C), with a mobile phase consisting of acetonitrile/water containing 0.18% N,N dimethyloctylamine and pH adjusted to 3.0 with orthophosphoric acid (46.5/53.5, v/v) at a flow rate of 1.0 mL min–1 within 20 min. The detection was made at a wavelength of 270 nm by using ultraviolet (UV) detector. No interference peaks from excipients and relative retention time indicated the specificity of the method. (58)

O Sherikar et al reported that various approaches for impurity profiling of pharmaceuticals, tremendous attention was given for the impurity profiling of pharmaceutical products in present time. Impurities can endanger the human health by affecting quality, safety and efficacy of the product. The presence of these unwanted chemicals even in small amounts may influence the efficacy and safety of the pharmaceutical products. Due to the noticeable impact of impurities on quality of pharmaceuticals, impurity control in pharmaceutical products is a primary goal of drug development. To assure the quality of drugs, impurities must be monitored carefully. Stringent international regulatory requirements have been in place for several years as outlined in USFDA, Canadian Drug and Health Agency,
International Conference on Harmonization. In this review, a description of different types of impurities and their origin in relation to ICH guidelines are presented. The article further throws light on to different methodological aspects of impurity profiling such as NMR, MS, TLC, HPLC, HPTLC, GC, CE and other hyphenated techniques like, LC-MS, GC-MS,LC-NMR, CE-MS and ICP-MS which are routinely used for monitoring impurities.. (59)

Kim H et al reported that Temozolomide (SCH 52365; TEMODAL) is currently used for the treatment of patients with glioblastoma multiforme and anaplastic astrocytoma, which are serious and aggressive types of brain cancers. A high-performance liquid chromatographic (HPLC) method was developed and validated for the analysis of temozolomide in human plasma. The determination of temozolomide involved extraction with ethyl acetate followed by separation on a reversed phase C-18 column and quantification by UV absorbance at 316 nm. The calibration curve was linear over a concentration range of 0.1-20 microg/ml. The limit of quantitation was 0.1 microg/ml, where the coefficient of variation (CV) was 0% and the bias was 10.0%. The method was precise with a coefficient of variation ranging from 2.5 to 6.9% and accurate with a bias ranging from 5.0 to 10.0%. Temozolomide was unstable at 37 degrees C in human plasma with a degradation t1/2 of 15 min; however, it was stable at 4 degrees C for at least 30 min. Temozolomide was stable in acidified human plasma (pH < 4) for at least 24 h at 25 degrees C, and for at least 30 days at -20 degrees C. Moreover, temozolomide was stable in acidified human plasma after being subjected to three freeze thaw cycles. The assay was shown to be specific, accurate, precise, and reliable for use in pharmacokinetic studies. (60)

Kunithala V et al reported reverse phase HPLC method has been developed for the estimation of Temozolomide in dosage form. It was resolved by using a mobile phase of Potassium dihydrogen phosphate: acetonitrile in the ratio 40:60 v/v at a flow rate of 1.0 ml/min. on HPLC system using UV -Visible detector at the wavelength of 287 nm. The column used was C18 (4.6 x 150mm, 5µm, Make: ODS) or equivalent .The linearity range was found to be 10-50 µg/ml. The proposed new method is found to be economic, sensitive, precise, rapid and reproducible. (61)
Saravanan G et al reported that a Stability-Indicating LC Assay and Degradation Behavior of Temozolomide Drug Substances. A chromatographic separation was achieved on an Inertsil ODS 3V, 250 × 4.6 mm ID, 5 µm column using mobile phase A (buffer 5 mL glacial acetic acid in 1,000 mL of Milli Q water) and mobile phase B (methanol). Forced degradation studies were performed on bulk sample of Temozolomide using acid (0.5 N hydrochloric acid), base (0.5 N sodium hydroxide), oxidation (10% v/v hydrogen peroxide), heat (60 °C) and UV light (254 nm). Degradation of the drug substance was observed in base hydrolysis and oxidation. Degradation product formed under these conditions was found to be Imp-A. When the stress samples were assayed, the mass balance was close to 99.5%. The sample solution was stable up to 48 h at 5 °C and mobile phase was found to be stable up to 48 h at 25 °C. The developed method was validated with respect to linearity, accuracy, precision, robustness and forced degradation studies prove the stability indicating power of the method. (62)

H Nygren et al reported that Temozolomide (TMZ) is an alkylating agent with a broad spectrum of antitumor activity, including brain tumors in children. A powder for preparation of a TMZ solution for intravenous administration (2.5 mg/mL) has recently been approved. A possibility to use this formulation for oral administration would facilitate TMZ administration. The degradation of TMZ was studied photometrically at 330 nm in two solutions, 2.5 mg/mL at room temperature (RT; 22°C) and 1.25 mg/mL at 5°C, prepared from the intravenous formulation. More than 90% of TMZ remained intact after storage for 9 days at RT (2.5 mg/mL) and 13 weeks at 5°C (1.25 mg/mL). The high stability of a TMZ solution prepared from the powder for infusion formulation makes it suitable for oral administration. (63)

S Sairam et al developed and validated method for reverse phase - high performance liquid chromatography (RP-HPLC) have been for the estimation of Temozolomide in its pharmaceutical dosage form and estimation was carried out on a Develosil ODS MG.5 (150x4.6mm),5µm, with mobile phase containing (0.5% w/v glacial acetic acid) named as Solution-A : Methanol [90 : 10, v / v] was used. The flow rate was 1 ml min-1 and separation was monitored by UV
detection at 254 nm. Chromatogram showed peak at a retention time of 7.306 ± 0.009 min. validation of the method for linearity and range, intra-day and inter-day precision, accuracy, specificity, recovery, ruggedness, robustness and limits of detection and quantification were obtained as 0.598 µg / ml and 1.81 µg / ml respectively. The calibration plot was linear from 20-60 µg ml-1 and the correlation coefficient was 0.999. The proposed method is fast, accurate and precise for the quantitative determination of Temozolomide capsules. (64)

Treudler R et al reported that Ten patients with malignant melanoma and phototoxic reactions under dacarbazine or 5-(3,3-dimethyl-1-triazeno) imidazole-4-carboxamide (DTIC) chemotherapy were investigated. All patients available for testing showed increased ultraviolet A-sensitivity (n = 5); patch testing revealed no type IV allergies (n = 6). In 5 patients intravenous DTIC was replaced by oral temozolomide, and no phototoxicity occurred. Temozolomide may represent an alternative for patients with DTIC-induced phototoxic skin reactions. (65)

Chowdhury S et al reported that A HPLC/electrospray ionization tandem mass spectrometric (LC/ESI/MS/MS) method for the quantitative determination of MTIC (5-(3-N-methyltriazen-1-yl)-imidazole-4-carboxamide), a pharmacologically active hydrolysis product of temozolomide, was developed and validated over a linear range from 10 to 400 ng ml(-1) in dog plasma and from 10 to 500 ng ml(-1) in rat plasma. This HPLC method utilized small plasma volumes (70 microl), Samples were processed and analyzed one at a time every 4.5 The lower limit of quantitation (LLOQ) was 10 ng ml(-1) in the plasma from both species. Inter-assay accuracy and precision of all calibration standards and quality control (QC) samples were within +/- 11 and 12%, respectively, with the exception of the LLOQ in rat plasma (17%). The validated method was used to determine the time dependent plasma concentration of MTIC in rats and dogs following a single oral dose of temozolomide. The standard curve and the quality control data indicate that the method performed acceptably throughout the sample analysis period. (66)

Jakubowicz-Gil J et al investigate the effect of Temozolomide (an alkylating chemotherapeutic agent) and quercetin (natural flavonoid) on cell death in the
human astrocytoma cell line MOGGCCM (WHO grade III). Our results indicate that Temozolomide induces autophagy, while quercetin promotes severe necrosis in the cell line in a manner dependent on the drug concentration. Demonstrated for the first time that combinations of both drugs were much more effective in programmed cell death induction in glioma cells. At a low (5µM) drug concentration, quercetin potentiated a pro-autophagic effect of Temozolomide, while after treatment with a higher drug concentration (30µM), autophagy switched to apoptosis. (67)

Shen F. et al reported that a high-performance reversed-phase liquid chromatography analysis was developed to quantify temozolomide in plasma and urine of patients undergoing a chemotherapy cycle with temozolomide. All samples were immediately stabilized with 1 M HCl (1 + 10 of biological sample), frozen and stored at -20 degrees C prior to analysis. The clean-up procedure involved a solid-phase extraction (SPE) of clinical sample (100 microliters) on a 100-mg C18-endcapped cartridge. Matrix components were eliminated with 750 microliters of 0.5% acetic acid (AcOH). Temozolomide was subsequently eluted with 1250 microliters of methanol (MeOH). The resulting eluate was evaporated under nitrogen at RT and reconstituted in 200 microliters of 0.5% AcOH and subjected to HPLC analysis on an ODS-column (MeOH-0.5% AcOH, 10:90) with UV detection at 330 nm. The method reported here was validated for use in a clinical study of temozolomide for the treatment of metastatic melanoma and high grade glioma. (68)

A Razak, et al reported that Temozolomide (TMZ), an Imidazotetrazine derivative is an alkylating agent that exhibits broad-spectrum antitumor activity against murine tumors research work was to develop and validate the UV spectrophotometric method for the quantitative determination of temozolomide (TMZ) in bulk and capsule formulation. UV spectroscopic determination was carried out at an absorption maximum of 268 nm using methanol as solvent. In this method the linearity of TMZ was found over the concentration range of 2-24 µg/ml with a correlation coefficient 0.995. The parameters like precision, accuracy, limit of detection and limit of quantitation and robustness were studied according to International Conference on Harmonization (ICH) guidelines. The
limit of detection and limit of quantification of TMZ were found to be 0.2135 and 0.679 ng/ml respectively. (69)

E Gilantet al develops a bio-analytical method for the determination of temozolomide (TMZ) in human plasma. Plasma concentration of TMZ was determined on a C18 column after liquid-liquid extraction. Isocratic elution was applied with the mixture of aqueous acetic acid and methanol. Theophylline was used as the internal standard. To prevent chemical degradation of TMZ at physiological pH, plasma samples were acidified to pH < 3. (70)

M Andrasi et al reported that The applicability of micellar electrokinetic capillary chromatography for the analysis of temozolomide (TMZ) and its degradants, 3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MTIC) and 5-amino-imidazole-4-carboxamide (AIC) has been studied. Using short-end injection, the analysis of TMZ and its degradants could be performed within 1.2 min. The therapeutic concentration of TMZ in blood samples can be determined after direct sample injection and conventional on-capillary UV detection. The proposed MEKC method was applied to study the stability of TMZ in water and serum at different pH values. It was established that the half-life of the TMZ in vitro serum at room temperature was 33 min, close to the half-life (28 min) obtained in water at pH 7.9. (71)

Y Wang et al reported that Novel 3-substituted imidazo[5,1-d][1,2,3,5]tetrazinones 3 have been prepared by two routes: reaction of 5-diazoimidazole-4-carboxamide 2 and isocyanates, and nitrosative cyclisation of 5-amino-1-carbamoyl-imidazole-4-carboxamides 7. The latter cyclisations do not proceed efficiently when the 1-carbamoyl group bears an electron-donating alkyl group. 5-Amino-1-carbamoylimidazole-4-carboxamides 7 cyclise with triethyl orthoformate or triethyl orthobenzoate to yield imidazo[1,5-a][1,3,5]triazinones 15. A 1H NMR study of the decomposition of 8-carbamoyl-3-ethylimidazo[5,1-d][1,2,3,5]tetrazin-4(3H)-one 3c in Deuteriated phosphate buffer has shown that its ethylating capacity is attenuated by the unproductive generation of ethene. This observation explains why the ethylimidazotetrazine possesses weaker antitumour properties than the clinically-used congener temozolomide (72)
Bhosale S. et al reported that, RP-HPLC Method for Simultaneous Determination of Butenafine Hydrochloride and Betamethasone Dipropionate in a Cream Formulation. An RP-HPLC method has been developed for the simultaneous determination of butenafine hydrochloride and betamethasone dipropionate on an Inertsil C18 column (250cm4.6mmid) using a mobile phase gradient consisting of methanol and water at a flow rate of 1 mL/min. Detection was carried out at 254 nm. The method was validated with respect to specificity, linearity, accuracy, precision, ruggedness, and robustness. (73)

M Annapurna et al reported that a fast, sensitive and accurate reverse phase liquid chromatographic method was developed and validated for the simultaneous determination of Dorzolamide and Timolol maleate in ophthalmic preparations. Chromatographic separation was achieved on Inertsil ODS 3V C18 column (250 X 4.6 mm, 5 μm particle size) with mobile phase consisting of Acetonitrile and 1-Octane Sulphonic acid buffer (0.02M) pH adjusted to 3.5 ± 0.05 with orthophosphoric acid (36:64 V/V) at a flow rate of 1.0 mL/min. The analytes were detected at 254 nm and 295 nm for Dorzolamide and Timolol maleate respectively by PDA detector. Brimonidine was used as internal standard (IS). (74)

Rodney et al reported that simultaneous analysis of common antineoplastic agents potentially hazardous to healthcare workers is of much interest for the evaluation of the overall health risk to these workers. Such analysis could be applied to both air and surface monitoring samples to provide a broader indication of risk to combinations of these agents. A reverse-phase high performance liquid chromatograph (HPLC) with a Waters Symmetry C8 column and a UV wavelength of 195 nm was selected for method development. The mobile phase was 22.75 percent acetonitrile in water buffered to a pH of 6.0. The HPLC analytical method developed is able to detect all five agents of interest, and at minimum detectable concentrations of 0.5-μg/mL for each of the five agents. (75)
ANALYTICAL METHOD DEVELOPMENT AND OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

Selection of Wavelength
The sensitivity of the HPLC depends upon the selection of detection wavelength. An ideal wavelength is one that gives good response for the drugs to be detected. As per Literature review and based on the PDA Scan chromatogram, the wavelength of 215 nm was selected for Anastrozole and temozolomide analysis.

Selection of Chromatographic Method
Proper selection of the method depends upon the nature of sample (ionic or ionisable or neutral molecule), its molecular weight and solubility. The drug (Anastrozole and temozolomide) selected in the present study was polar in nature and thus either Reversed phase or Ion-pair or Ion exchange chromatography can be used. The Reversed phase (Gradient) chromatography was selected.

Selection of Mobile Phase
Ammonium acetate and Methanol (24:76, 34:66, 44:56) with PH 5.5 was adjusted with orthophosphoric acid were analysed in HPLC with standards of Anastrozole and temozolomide. The Anastrozole and temozolomide peaks were eluted but tailing was observed. So, this composition was not selected.
Potassium dihydrogen orthophosphate and Methanol (24:76, 34:66, 44:56) with pH 2.5 was adjusted with orthophosphoric acid were analysed in HPLC with standard Anastrozole and temozolomide. The Anastrozole and temozolomide peak was eluted but peak broadening was observed. So, this composition was also not selected.
Ultimately the Ammonium Acetate buffer (0.1%) was selected as Mobile Phase A. Ammonia is more volatile than acetic acid. The salt is in equilibrium with free base; however ammonium acetate comes in large amounts in small bottles. As ammonia vaporizes pH drops slowing ammonia loss. Method can be used for Mass spectroscopy analysis.
Acetonitrile was used as Mobile Phase B. A gradient Program was used in HPLC with standard Anastrozole and temozolomide. The Anastrozole and temozolomide peak was eluted and thus this composition was selected.
compared to all three, this showed better elution of with sharp peak. So, this composition was selected for this study.

**Selection of Flow Rate**

0.8 ml/min, 1.0 ml/min and 1.2 ml/min as flow rates on C8 column using Buffer, Methanol with pH 2.5, the chromatographs of standard solution were recorded. At 1.0 ml/min as flow rate, the peaks were symmetrical and well resolved. But, when 0.8 ml/min and 1.2 ml/min were used, the Anastrozole and temozolomide were observed with tailing and broadening respectively. Hence, 1.0 ml/min was selected (back pressure 3000 psi) for further studies.
OBJECTIVE

A patient taking a pharmaceutical product expects the product to be safe and efficacious. Due to abundance of pharmaceutical agents available in the pharmaceutical market in various dosage forms either as a single drug component or in combination with other drugs and also due to potency of the most of the drugs, it becomes necessary to quantitate these agents in their formulations in a precise manner.

Pharmaceutical regulatory agencies worldwide demand that the product retains its quality, purity, and potency for the time the product is commercially available. Consequently the agencies expect to see stability data supporting the proposed expiration date of the product in the marketing submission. In the broader sense the stability studies that are conducted should provide evidence of how the quality of the drug substance and drug product changes over time when subjected to various environmental conditions, such as temperature, humidity, and light. It has also been observed that mainly in case of the cream formulations, interference by cream components by cream base components hamper the analysis of active constituents, which makes it a challenging task. Therefore there is always a need to develop validated analytical methods which are precise, accurate, selective, and sensitive and can be used for routine analysis and stability studies of the drug products.

The objective of the present work was to develop validated analytical methods with the help of which we can separate and simultaneously quantitate drug components from the pharmaceutical formulations.

The specific aim was to develop a method, which can be used simultaneously for the estimation of Anastrozole & Temozolomide and also for the estimation of their related compounds and degradation products in bulk as well as in marketed formulations. Temozolomide and Anastrozole are not official in any pharmacopoeia and not a single analytical method was reported for the simultaneous estimation of these drugs in the formulation and Related compounds using the same method.

- To validate developed analytical method which is simple, reliable, workable and economical under routine conditions of analysis.
- To evaluate and study, stability indicating nature of the developed and validated method.
- To apply developed analytical methods for the estimation of Anastrozole and temozolomide in marketed formulations.
• To optimize and further validate the developed HPLC method for quantitative determination of Anastrozole in human plasma by LC/MS/MS.
• To overcome typical challenges encountered while developing and validating methods for pharmaceutical products containing single and more than one active ingredient and impurities.

In this study it was decided to carry out project work by utilizing the following instrumental techniques to analyze Bulk and Formulation:
1) High Performance Liquid Chromatography (HPLC).
2) High Performance Liquid Chromatography (LC/MS/MS)