1.1 Importance of Analytical method development in Pharmaceutical research and development:

Analytical method development and validation is an important area during the development of drug substance and drug product in the pharmaceutical industry.

Method development should be based on various considerations. It is appropriate to have maximum sample information to make an effective development desired for intended analytical method application and also on available resources for chromatography.

1.1 Steps involved in analytical method development

1.1.1 Collection of literature:

Analytical method development starts with literature search where in various pharmacopeias like USP, EP, BP, JP etc and chromatographic journals are referred to check the availability of suitable analytical methods. If any suitable method is found, it is still necessary to perform method optimization and validation to prove that the method can be successfully adapted for its intended use.

1.1.2 Sample information

The synthetic route from raw material to finished dosage forms (structures) of the molecule should be collected and the impurities originating from starting materials of synthesis, degradation products,
excipients, solvents etc. need to be considered. The method should be designed based on the closely related structures and get the best resolution between the closely related compounds. The structures of impurities, starting material, intermediates and degradation products are compared with the structure of drug substances and arrive at the polarity whether they are less polar or more polar than the compound of interest.

1.1.3 Selection of diluent

The diluent should be chosen based on the solubility of impurities, degradation products, starting materials, intermediates and the analyte. The diluent should be compatible with the mobile phase to get a better peak shape of analyte.

1.1.4 Selection of Chromatographic technique

Depending on the nature of the analyte, various chromatographic techniques like Thin layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), gas chromatography(GC), HPLC (High Performance Liquid Chromatography) and newer technique like capillary electrophoresis (CE) can be selected.

1.1.5 Selection of stationary phase:

The selection of bonded phase can be based on the polarity of molecule and its by-products. For RP-LC, a wide variety of columns are accessible covering a large range of polarity by cross-linking Si-OH
groups with linear alkyl chains like C\textsubscript{8}, C\textsubscript{18} and phenyl groups (-C\textsubscript{6}H\textsubscript{6}), nitrile groups (-CN), different embedded hybridized groups amino groups (-NH\textsubscript{2}) etc (Fig.1.1)

**Fig 1.1: Different alkyl chains attached to Si-OH**

Silica based columns with various cross linkings in the increasing order of polarity are as follows:

\[\text{Non-polar} \rightarrow \text{moderately (mid) polar} \rightarrow \text{Polar}\]

\[C\textsubscript{18} < C\textsubscript{8} < C\textsubscript{6} < C\textsubscript{4} < C\textsubscript{2} < \text{Phenyl} < \text{Amino} < \text{Cyano} < \text{Silica}\]

**1.1.5.1 Particle shape:**

The particles can be either spherical or irregular [Fig.1.2]. Irregular particles include in general higher surface areas and higher carbon
loads. Spherical particles confer higher efficiency, improved column stability, quick stabilization and lower back-pressure when compared to irregularly shaped particles.

**Fig. 1.2: The shapes of spherical and irregular particles**

![Spherical vs Irregular Particles](image)

**1.1.5.2 Particle size:**

Particle size for HPLC/UPLC column packing denotes the average diameter of the packing particles [Fig.1.3]. Particle size have an effect on the back-pressure and the separation efficiency of column. The column efficiency (performance) and column back-pressure are inversely proportional to the square of the particle diameter. A well packed column with 3 µm packings produces almost twice the separation efficiency of a comparable 5 µm column. However, the 3 µm column will have about a three-fold higher back-pressure compared to the 5 µm column when operated with the same mobile phase and at the same flow rate.
Fig.1.3: Different particle sizes of HPLC/UPLC column packing

1.1.5.3 Surface Area:

The surface area is sum of interior pore surface and particle outer surface in square meters per gram [Fig.1.4]. A high surface area normally present high retention times (RT) and resolution (Rs) for separating complex, multi component samples. The physical structure of particle substrate establish the surface area of the packing material in LC column stationary phases. A packing material with a thin pore size will contain a big surface area and vice versa. Surface area is determined by pore size. Pore size and surface area are inversely related.

Fig.1.4: The schematic diagram of surface area
1.1.5.4 Pore size:

The pore size of a packing material specify the average size of the pores within each particle [Fig.1.5]. Generally pore size of 150 Å or less is selected for samples with molecular weights less than 2000 and a pore size of 300 Å or larger for samples with molecular weights greater than 2000. In general, the range is from 60 Å to 10,000 Å. Larger pores permit larger solute molecules to be retained through maximum exposure to the surface area of particles.

Fig. 1.5: A representative diagram of pore size

1.1.5.5 Carbon load:

The carbon load is the quantity of bonded phase attached to the base material expressed as the percentage of carbon [Fig.1.6]. High carbon loads usually offer greater resolution and greater retention times for hydrophobic samples. Low carbon loads shorten run times and often show dissimilar selectivity.
1.1.6 Selection of Mobile phase:

The mobile phase selection is one of the critical parameters as it encourages the solute and the stationary phase interactions. An appropriate care must be taken while selecting the mobile phase like use of strong acids, strong bases, and halide solutions should be avoided.

1.1.6.1 Selection of Buffer pH and type of buffer:

Buffers are usually employed to attain consistent chromatographic results. Buffers are employed to control the retention of ionic analytes. When the analyte is in ionic form, it usually attains polar in nature and spends shorter time on the stationary phase and elutes quickly. To control the selectivity of the ionic analytes, the buffer pH plays a significant role.

In general, when buffer pH increases, the acidic analytes get ionized and become more polar in nature, and conversely, when buffer pH decreases, the basic analytes get ionized. [Fig. 1.7]
The pH of the mobile phase selected should be at least ± 1.0 pH units from the analyte pKa value. This confirms that the analytes are either as 100% ionized or 100% non-ionized and it helps in controlling peak shape and the run to run reproducibility.

It is always use buffer in aqueous portion of the mobile phase and it increases the ruggedness of the method. The most commonly used buffers are tabulated in Table 1.1
Table 1.1: Commonly used buffers for HPLC

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pKa</th>
<th>Buffer Range</th>
<th>UV Cutoff (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate</td>
<td>2.1</td>
<td>1.1 – 3.1</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>6.2 – 8.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12.3</td>
<td>11.3 – 13.3</td>
<td></td>
</tr>
<tr>
<td>Formic acid*</td>
<td>3.8</td>
<td>2.8 – 4.8</td>
<td>210</td>
</tr>
<tr>
<td>Acetic acid*</td>
<td>4.8</td>
<td>2.8 – 5.8</td>
<td>210</td>
</tr>
<tr>
<td>Citrate</td>
<td>3.1</td>
<td>2.3 – 4.1</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td>4.7</td>
<td>3.7 – 5.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.4</td>
<td>4.4 – 6.4</td>
<td></td>
</tr>
<tr>
<td>Trias</td>
<td>8.3</td>
<td>7.3 – 9.3</td>
<td>205</td>
</tr>
<tr>
<td>Triethylamine*</td>
<td>11.0</td>
<td>10.0 – 12.0</td>
<td>200</td>
</tr>
<tr>
<td>Pyrrolidine</td>
<td>11.3</td>
<td>10.3 – 12.3</td>
<td>200</td>
</tr>
</tbody>
</table>

* Volatile buffers

1.1.6.2 Selection of buffer concentration:

The mobile phase concentration has little effect on retention time of analyte in HPLC. A concentration of buffer in the range of 5-100mM is usually sufficient for the most applications. The concentration should be low enough to avoid the pumping problems due to the precipitation of buffer in HPLC.

1.1.6.3 Selection of organic additives:

The addition of mobile phase additives are usually to control the secondary interactions of the residual silanols on the stationary phase with the analyte. Triethyl amine (TEA) or Diethyl amine (DEA) are preferred in the mobile phase to control the peak tailing of basic analytes and acetic acid was preferred for acidic analyte.
1.1.6.4 Selection of organic modifiers in mobile phase:

Three solvents are usually employed as modifiers to control the selectivity and separation of peaks in HPLC.[Fig. 1.8] They are Acetonitrile, Methanol and Tetrahydrofuran. Out of these three, Acetonitrile is the preferred choice as it has low UV cut-off and less viscosity than the other solvents. A minimum of 10% aqueous portion need to be used to avoid the pumping problems due to 100% Acetonitrile.

**Fig. 1.8: Typical chromatogram on different solvent strengths**

1.1.6.5 Selection of ion pair reagents:

To analyse the ionic compounds which are highly polar in nature and difficult to retain on the stationary phase, ion pair reagents are helpful to control the selectivity.
Acidic ion pair reagents like alkyl sulphonates are used to retain positively charged ionic bases. Basic ion pair reagents like alkyl amines are used to retain negatively charged ionic acids. In general, ion pair method should be considered as the last option when there is no alternative available. The most commonly used ion pair reagents are shown in Fig. 1.9.

Fig. 1.9: Most commonly used ion pair reagents in HPLC

1.1.7 Selection of Detector:

The selection of detector is based on the presence or absence of chromophores in the analyte. But, majority of pharmaceutical compounds exhibit UV spectra in the range of 200-400nm. The non-chromophoric compounds can be analysed by using RI/ELSD/MS. If the analyte fluoresce, it can be analysed by using FLD. To confirm the peak
purity, PDA detector is employed. It collects the spectra at each point of
the peak starting from the elution to peak end and compares the spectra
of peaks collected at all points versus the peak apex and thus confirms
the homogeneity of the peak.

1.2 Source of impurities in pharmaceutical drug substances and
drug products:

An impurity in a drug substance as defined by the International
Conference on Harmonisation (ICH) Guidelines is any component of the
drug substance that is not the chemical entity defined as the drug
substance. Similarly, an impurity in a drug product is any component of
the drug product that is not the chemical entity defined as the drug
substance or an excipient in the drug product.[1-2]

The impurities are generated in drug substances from various sources
and phases of the synthetic process and preparation of drug products.
The accurate difference between the process related impurities and
degradation products cannot be justified. But, most of the impurities are
specific to synthetic process.

The “impurities” in a drug substance are categorized as

(a) organic impurities

(b) inorganic impurities and

(c) residual solvents
(a) **Organic impurities:** They can arise during the production process and/or storage condition of a drug substance. They include starting materials (can be isomeric impurities), impurities in the starting materials, by-products which can be formed due to incomplete and over reactions, intermediates, degradation products. A proper understanding of the chemical transformation from the starting material to the finished product is required.

(b) **Inorganic impurities:** They can result from the manufacturing process. These include reagents, ligands and catalysts, heavy metals or other residual metals, inorganic salts and materials like filter aids, charcoal etc. They are usually present in less amount but it should be controlled carefully during the manufacture of the drug substance.[3]

(c) **Residual solvents:** They are the solvents used for the preparation of the drug substance.[4] In general, the solvents are not completely removed during the preparation of drug substance. They may sometimes become a critical parameter and it has to be controlled based on the toxicity and according to the ICH classification of the solvents. They are:

**Class 1:** Solvents to be avoided

**Class 2:** Solvents to be limited

**Class 3:** Solvents with low toxic potential
The impurities in drug product are generated especially in the form of solutions and the impurities can be noticed significantly that are susceptible to both degradation and microbial contamination.

1.2.1 Control and qualification of impurities:

The control and qualification of impurities in drug substances and drug products are established in ICH. ICH provided the guideline and laid down the series of thresholds that are based on the daily dose of the drug substance and drug product. The substances that are present above ‘reporting threshold’ must be recorded and need not to be characterized unless they are present in greater amount than ‘identification threshold’. If impurities are unidentified, an appropriate justification need to be given and should be controlled in the specification as a known impurity.

Qualification is the process of establishing the biological safety of an individual impurity or an impurity profile at the specified level. Organic impurities present above the ‘qualification threshold’ must go through this method to reduce the amount present in the drug substance. The rationale established for impurity acceptance criteria should include safety considerations. The impurity that was present at any level when adequately tested for safety and clinical studies would be considered as qualified.
A lower qualification threshold of impurities is appropriate when certain drug substances are associated with adverse reactions in patients. Conversely, a higher qualification threshold can be justified based on patient population, drug class effects, clinical considerations.

The various threshold criteria provided by ICH for drug substances and drug products are presented in Table 1.2-1.3 [a-c]

**Table 1.2: Impurity thresholds in drug substances**

<table>
<thead>
<tr>
<th>Maximum daily dose</th>
<th>Reporting threshold</th>
<th>Identification threshold</th>
<th>Qualification threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤2g/day</td>
<td>0.05%</td>
<td>0.10% or 1.0 mg/day intake (whichever is less)</td>
<td>0.15% or 1.0 mg/day intake (whichever is less)</td>
</tr>
<tr>
<td>≥2g/day</td>
<td>0.03%</td>
<td>0.05%</td>
<td>0.05%</td>
</tr>
</tbody>
</table>

**Table 1.3: Impurity thresholds in drug products**

**Table 1.3 (a): Reporting thresholds in drug products**

<table>
<thead>
<tr>
<th>Reporting Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum daily dose</td>
</tr>
<tr>
<td>≤1g</td>
</tr>
<tr>
<td>&gt;1g</td>
</tr>
</tbody>
</table>
Table 1.3 (b): Identification thresholds in drug products

<table>
<thead>
<tr>
<th>Identification threshold</th>
<th>Maximum daily dose</th>
<th>Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;1mg</td>
<td>1.0% or 5 µg TDI, whichever is lower</td>
</tr>
<tr>
<td></td>
<td>1mg-10mg</td>
<td>0.5% or 20 µg TDI, whichever is lower</td>
</tr>
<tr>
<td></td>
<td>&gt;10mg-2g</td>
<td>0.2% or 2mg TDI, whichever is lower</td>
</tr>
<tr>
<td></td>
<td>&gt;2g</td>
<td>0.10%</td>
</tr>
</tbody>
</table>

Table 1.3 (c): Qualification thresholds in drug products

<table>
<thead>
<tr>
<th>Qualification threshold</th>
<th>Maximum daily dose</th>
<th>Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;10mg</td>
<td>1.0% or 50 µg TDI, whichever is lower</td>
</tr>
<tr>
<td></td>
<td>10mg-100mg</td>
<td>0.5% or 200 µg TDI, whichever is lower</td>
</tr>
<tr>
<td></td>
<td>&gt;100mg-2g</td>
<td>0.2% or 3mg TDI, whichever is lower</td>
</tr>
<tr>
<td></td>
<td>&gt;2g</td>
<td>0.15%</td>
</tr>
</tbody>
</table>

1.2.2 Pharmacopeial norms:

The control of impurities in various monographs like USP, EP, BP were conducted by a set of tests defined in each individual monograph. These monographs are periodically updated with respect to scientific progress and regulatory developments.
Two general chapters (<466> & <1086>) of the US Pharmacopoeia (USP) deal with organic impurity testing. The definitions and concepts are clearly explained although different terminology from that of ICH is used.

The USP divides analytical methods into four individual categories: Quantitation of major components or drug substances; determination of impurities or degradation products; determination of performance characteristics (e.g., dissolution, drug release); identification tests.

1.2.3 ICH Quality guidelines:

The ICH classified impurities as specified and unspecified.

Specified impurities are those with specific acceptance criteria included in the specification for the drug substance. They can be identified or unidentified.

Specified identified impurities should be reported along with specified unidentified impurities that are present at a level greater than (> ) identification threshold. The procedure used and assumptions made in establishing the level of unidentified impurities should be clearly stated and should be referred by an appropriate label like ‘Unidentified A with relative retention of 0.9’

In summary, the new drug substance specification should include, where applicable, the following list of impurities:
Organic Impurities

- Each specified identified impurity
- Each specified unidentified impurity
- Any unspecified impurity with an acceptance criterion of not more than (≤) the identification threshold
- Total impurities

Residual Solvents

Inorganic Impurities

1.3 Importance of stability indicating analytical methods (SIAM):

USFDA provided definition of SIAM in the draft guideline of 1998 reads as: ‘quantitative validated analytical methods that can identify the changes with time in the physical, chemical, or microbiological properties of drug substance and drug product, and that are specific so that the contents of active ingredient, degradation products and other components of interest can be accurately calculated without interference.’

The analytical methods developed for the estimation of impurities should be stability indicating. It means the method should be able to separate the impurities, degradants and monitor the stability samples of either drug substances or drug products.
The core purpose of stability testing of pharmaceutical drug substances and pharmaceutical dosage forms is to provide information on how the quality and efficiency of an active pharmaceutical ingredient (API) or dosage form varies with time under the influence of various environmental conditions like temperature, light, and humidity, which subsequently led to the establishment of recommended storage conditions, re-testing time, and product shelf life.

The developed SIAMs can recognize the changes ultimately in the chemical and physical properties of API and dosage forms [1], so that the components of analyte, impurities, and degradants can be accurately predicted without interference. The need for the development of SIAMs during the analysis of drugs is increasing with respect to regulatory viewpoint which can have impact on the marketing of the product.

1.3.1 Stability testing of the new drug substance and drug product:

1.3.1.1 Drug substance:

The objective of the stability study for drug substance is to ensure the quality to remain within specification until retest period. At least three batches are to be selected that are manufactured at pilot-scale or commercial level and should use the same synthetic process and manufacturing method. The testing should include chemical, physical, and microbiological attributes that are likely to alter during storage and show impact on product quality, safety, and efficacy. The duration of
study and storage conditions will depend on the nature of the drug substance. During the submission to regulatory authorities, atleast 12 months long term and 6 months accelerated data is required. If significant variation in the accelerated data is observed, an additional intermediate condition is required.

The storage conditions are tabulated in Table 1.4.

**Table 1.4: Storage conditions as per ICH**

<table>
<thead>
<tr>
<th>Storage condition</th>
<th>Temperature/Humidity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long term</td>
<td>25±2°C/60±5%RH</td>
</tr>
<tr>
<td>Accelerated</td>
<td>40±2°C/75±5%RH</td>
</tr>
<tr>
<td>Intermediate</td>
<td>30±2°C/65±5%RH</td>
</tr>
</tbody>
</table>

**1.3.1.2 Drug product:**

The stability study of drug product is based on the knowledge of drug substance and understanding of formulations. The requirements for the drug substance usually applies to drug product unless specified. In the study of antimicrobial preservatives, the preservative efficacy testing and assay is usually required. In the study of solid oral dosage form, storage at high relative humidity is usually required. The drug products that have high water content stored in semi permeable containers, a low relative humidity is appropriate.
1.4 Advanced development in chromatography:

During the last two decades, there is a tremendous development in application of chromatography with respect to novel development of stationary phases, versatile development of detectors, various hyphenated techniques and development of new LC system that can have more applications.

1.4.1 New technology of stationary phases:

1.4.1.1 Fused core silica columns:

Fused-core silica stationary phases correspond to a key technological advancement in the arena of fast HPLC separations. [5]

The development of fused-core or ‘superficially porous’ silica particles was believed as an advance in column technology aimed at decreasing analysis times while maintaining column efficiencies and required relatively low back pressures.

Fused core columns contain solid inner core and porous outer core. The outer core is adequately thin to allow rapid mass transfer into and out of stationary phase and because the inner core is solid fused silica, analytes cannot penetrate any further. So the diffusion path length is shorter than the porous particles of around the same diameter and approximately equivalent to that of the sub 2µm columns
The schematic diagram of the fused core structure is represented in Fig. 1.10

**Fig. 1.10: Structure of Fused core silica particle**

These phases are prepared by fusing a 0.5µm porous silica layer onto 1.7µm nonporous silica cores. A major benefit of the fused-core particles is the small diffusion path (0.5µm) compared to fully porous particles (e.g., 1.8µm).

The reduced intra-particle flow path of the fused particles present superior mass transfer kinetics and better performance at high mobile phase velocities, while the fused-core particles provide lower pressure than sub-2µm particles which become successful to use fused-core columns on conventional HPLC systems.
Since the fused-core particles produce about 45% less back pressure as compared to the sub-2µm particle column, it was possible to increase to double the length of fused-core column to achieve a better separation on Agilent 1100 HPLC system.

Another important feature that is distinctive to the fused-core particles is their narrow particle size distribution which provide packing of these columns with great ruggedness.

The fused-core column was proved to provide better analyte shape selectivity and similar hydrogen bonding, hydrophobic, and acidic ion-exchange selectivities as compared to sub-2µm particle. As predicted, the sample loading capacity and retention factors on the fused-core particle column were slightly lower than those for the sub-2µm particle column. Ideal column for run time reduction without going for UHPLC techniques as this column can be used with regular HPLC instruments.

An example of Fused core columns: Ascentis Express (Sigma - Aldrich)

1.4.1.2 Monolith Columns:

The monolith columns consists of a single piece of solid made of either porous silica or porous cross-linked polymer.[6] Interconnected skeletons and interconnected flow paths called ‘through-pores’ or ‘Macropores’ that go through these skeletons and small pores on the surface area of the monolithic skeleton are called Mesopores. The schematic diagram of monolith silica is represented in Fig.1.11 [a-c]
The important characteristics of monolith columns are:

- Monolith is a recent development of column technology and is expected to play a major role in separation science in the coming days.
- An innovative new “Gel-Sol” technology is followed to prepare a silica gel polymer.
Monoliths have high permeability and porosity, large number of theoretical plates and can be operated at high flow rate with low back pressure. Better mass transfer properties that leads to higher efficiency.

The material has a silica gel skeleton that contains mesopores with diameters of approximately 13 nm and macropores with diameters of approximately 2 µm.

The disadvantages include limited availability and reproducibility, lack of wide range pH applications and expensive.

Example of Monolith column: Chromolith (Merck)

1.4.2 Versatile development of detectors:

1.4.2.1 Evaporative Light Scattering Detector (ELSD):

The evaporative light detection system has revolutionized the analysis in HPLC since its introduction around 1980.[7]

This type of detector can be utilized for all solutes having lower volatility than the mobile phase. Detection of semi-volatile solutes can be achieved by new generations of instruments that can operate at relatively low temperatures. The majority of non-volatile solutes can have the detection limits in the nanogram range per injection.
1.4.2.1.1 Principles of Operation

The ELSD principle of operation employs three distinct stages: [Fig. 1.12-1.13]

a) Nebulisation

b) Evaporation

c) Detection

**Fig. 1.12: Evaporative Light Scattering Detector**

**Fig. 1.13: Mechanism of operation of ELSD**
The operation of ELSD is based on three steps.

**a) Nebulisation:** Inside the nebulizer, the target substances separated in a column are mixed together with the mobile phase passes through a needle, mixes with nitrogen gas and forms a dispersion of droplets and these are carried to a drift tube.

**b) Evaporation:** The droplets pass through a heated “drift tube” where the mobile phase only evaporates leaving a fine mist of dried sample particles in solvent vapor and are carried to the detection unit.

**c) Detection:** The sample particles pass through a flowcell where they are hit with a laser light beam. Light scattered by the sample particles is detected by a photomultiplier generating an electrical signal.

The intensity of the signal detected in the ELSD can be represented by the following equation:

\[
\text{Signal intensity} = a \times (\text{Quantity of target substance})^b
\]
Here, "a" and "b" are constants that are determined by a variety of factors, such as the size of the particles, the concentration and type of the target substances, the gas flow rate, the mobile phase flow rate and the temperature of the drift tube.

The response in ELSD is based on the scattering of light by solute particles and strongly depends on the size of the formed particles which depends on the sample concentration, the vapour pressure of the solutes, the gas flow and the physico-chemical properties of the mobile phase. During gradient elution in LC, that is common practice in pharmaceutical analysis, the composition of the mobile phase changes. This strongly affects the particle size and consequently ELSD sensitivity. The higher the organic modifier concentration, the higher the response.

In principle, ELSDs are capable of analyzing all substances that have an evaporation temperature lower than that of the mobile phase, and can attain roughly the same level of detection sensitivity for any compound. For this reason, they are well-suited to the detection of components such as sugars, fats, surfactants, synthetic macromolecules, and steroids, as these components have low light absorbance, making them difficult to detect with UV detectors.
1.4.2.1.2 Characteristic properties of the detector:

1. Low background signal
2. Reproducibility
3. Low band broadening
4. Detector response and quantification

1.4.3 Application of hyphenated techniques:

Due to increase in stringency of the regulatory authorities in the control of impurities, characterization of impurities with conventional approach that are time consuming and sometimes difficult if present in trace levels led to the importance of the hyphenated techniques.[8]

The available hyphenated instruments have LC, GC or CE on the front end and MS, NMR or IR on the detection side, e.g., LC-MS, GC-MS, CE-MS, LC-NMR, LC-NMR/MS, CE-NMR, LC-IR etc.

1.4.3.1 The development of MS based hyphenated techniques:

GC-MS was the first hyphenated technique chosen and it was unique for the confirmation of organic volatile IMPs and residual solvents present in a sample. However, the limitations include estimation of non-volatile and thermally labile impurities and degradation products.

In particular, LC-MS instruments are becoming popular among the hyphenated techniques. Also available are combined LC-MS-NMR
systems. These are slowly reaching into analytical laboratories worldwide and find their increasing use and hence enhancement in the number of publications on characterization of IMPs/DPs coming out every year.

The range of instruments that are commercially available listed below:

- LC-MS (Single Quad).
- LC-MS-MS (Triple Quad).
- LC-TOF.
- LC-MS-TOF (Q-TOF, Triple TOFTM).

1.4.3.2 The development of NMR based hyphenated techniques

1.4.3.2.1 LC-NMR:

The coupling of LC effluent to NMR was first time reported in 1978. Since then, several instruments have been set up in industry and research laboratories. Modern LC-NMR systems are allied with multiple technological advancements like utilization of strong field magnets, microprobes and cryoprobe technology to enhance instrument sensitivity and resolution. Magnets with 500 MHz and above field strength are regular as attachments to LC.
1.4.3.2.2 CE-NMR

Hyphenation of CE to NMR spectrometer offers comparable benefits as LC-NMR with respect to chemical identification, separation and ability to provide structural information on analytes present in relatively small amounts. Similar to LC-NMR, both continuous flow and stopped flow modes are used in CE-NMR. However, the typical problem in CE hyphenated NMR is the small sample volume output from CE that results in shorter residence time in NMR, thus effecting detection sensitivity.

Yet, the hyphenated techniques are anticipated to remain in the mainstay and are going to get an increased popularity for the characterization of IMPs and/or DPs, once the technology gets improved offering still higher resolution and sensitivity, and as the machines become affordable.

1.4.4 Development of Ultra Performance Liquid Chromatography (UPLC):

In 2004, separation science was transformed and further progress in instrumentation and column technology were achieved significantly to increase resolution, sensitivity and speed in liquid chromatography.[9] Columns with smaller particles (sub-2µm) and instrumentation with specific capabilities designed to deliver the mobile phase at 15,000 psi
(1,000 bars) were needed to realize the new level of performance. A new system was created now called ultra-performance liquid chromatography.

The principle of UPLC is based on Van Deemter equation which denotes the importance of small particle size which leads to smaller plate height. According to Van Deemter, the relationship between plate height ‘H’ and velocity ‘u’ is: \[ H = A + \frac{B}{u} + Cu \]

Where A denotes the eddy diffusion

B denotes the longitudinal diffusion and

C denotes the resistance to mass transfer.

Smaller the value of H, more is the column efficiency.

The comparison data of the plate height with velocity for various particle sizes are shown in Fig.1.14.

**Fig.1.14: Development of particle sizes over the last three decades**

After studying the above data, it is concluded that as the particle size decreases to less than 2.5 µm, there a considerable gain in efficiency and
the efficiency did not diminish at increased flow rates or linear velocities. By using smaller particles, speed and peak capacity (number of peaks resolved per unit time) can be increased. This introduction and review traces some of the developments and technological advancements made in producing the first commercially available UPLC instrument.

In 2000, novel second generation bridged ethyl hybrid (BEH) technology was introduced. The manufacture of this type of stationary phase is shown in Fig. 1.15.

**Fig. 1.15: Particle synthesis using BEH technology**

Waters introduced ACQUITY BEH columns with four different bonded phases ACQUITY BEH C\(_{18}\) and C\(_{8}\) (a straight chain alkyl columns), ACQUITY BEH Shield RP\(_{18}\) (embedded polar group column) and
ACQUITY BEH Phenyl (phenyl group attached to the silyl functionality with a C₆ alkyl). All the bonded phases are made with particle size of 1.7µm. These columns will have high efficiency and can operate with wide pH range.

The major advantages of UPLC are faster separations with increased sensitivity and more sample throughput and less operational cost and solvent consumption. The limitations of UPLC include more back pressure and decrease of the lifetime of column and more maintenance.

1.5 Development of validated SIAMs that are expected to meet regulatory requirements

1.5.1 A preliminary study of drug structure to evaluate the likely decomposition route(s)

This is the initial step for the development of SIAM.[10] Much information can be obtained form the structure by study of functional groups and the other key components. For example functional group categories like amides, esters can undergo hydrolysis.

1.5.2 Collection of information on physicochemical properties

Before starting method development, it is necessary to obtain the physicochemical parameters like pka, solubility logP, wavelength maxima of the drug substance. The knowledge of pka facilitates in controlling the mobile phase pH such that the value remains ±1.5 units of the pka value. The solubility profile in organic, aqueous and generally used HPLC
solvents and their combinations can thus demonstrate to be very helpful in the selection of the sample solvent and mobile phase.

1.5.3 Stress (forced degradation) studies

This involves stress studies to generate the forced degradation products. According to ICH Stability testing of new drug substances and products Q1A (R2) current step 4 version dated 6 February 2003, the following guidelines are suggested:

(i) 10 °C increments above the accelerated temperatures (e.g. 50 °C, 60 °C, etc.),
(ii) humidity where appropriate (e.g. 75% or greater),
(iii) hydrolysis across a wide range of pH values,
(iv) oxidation and
(v) photolysis.

The guideline does not provide information on how actually photolytic, hydrolytic and oxidative studies need to be performed.

1.5.4 Preliminary separation study on stressed samples

The stress samples are analysed preliminarily to study the various types of degradation products produced under various conditions. The analysis can be initiated in reverse phase chromatography using a regular octadecyl column and a simple mobile phase that is compatible with LC-MS to identify any degradation products. The selection of wavelength should be based on the spectra of the drug substance, impurities and degradation products.
1.5.5 Method development and Optimization

After preliminary chromatographic study, the RT and RRT of all products produced should be tabulated for each reaction condition. Special interest is then paid to those components whose RT and RRT is very close. PDA spectra or LC-MS profile of such components are acquired and critically evaluated to determine whether the products are same or different.

The method is optimized to separate closely eluting peaks by changing the pH, mobile phase ratio, flow rate, gradient, solvent type, temperature, and the column and its type.

1.5.6 Identification and characterization of the degradation products and preparation of their standards

Before initiating to the validation of a SIAM, it is required to identify the drug degradation products and prepare for their standards. These are necessary to establish specificity/selectivity of method. The work can even be initiated once a thought on nature and number of degradation products produced under different degradation conditions is achieved from preliminary separation studies.

1.5.7 Validation of SIAMs

The main objective of validation at this stage is on establishing the specificity/selectivity, followed by other validation parameters like precision, accuracy, linearity, range, robustness, etc. This validated
method can be applied in the analysis of bulk drug stability samples for
determination of its retest or expiry period.

Validation is the process of presenting the documented evidence that
something does what it is intended to do. A precise and documented
validation procedure given to regulatory agencies with a proof that the
system and method is appropriate for its intended use. By impending
method development, optimization, and validation in a reasonable,
stepwise manner, laboratory capitals can be employed in a more capable
and fruitful manner.

The USP was made available specific guidelines for method validation
for compound evaluation. USP elaborated following analytical
parameters for analytical method validation.

(a) Precision

(b) Accuracy

(c) Limit of Detection

(d) Limit of Quantitation

(e) Specificity

(f) Linearity and Range

(g) Ruggedness

(h) Robustness
1.5.7.1 Precision

The precision of an analytical method is the degree of agreement between individual test results acquired when the method is applied to multiple sampling of a homogenous sample under the approved conditions. It is usually expressed as the % RSD for a statistically significant number of preparation.

As per ICH, precision should be established at three different levels namely repeatability, intermediate precision and reproducibility. Repeatability conveys the results of method operating over a short time interval under the same conditions (inter-assay precision). It should be calculated from a minimum of nine determinations covering the particular range of the procedure (for example, three levels with three replications each) or from a minimum of six determinations at 100% of the test or target concentration. Intermediate precision conveys the results from within the same lab variations due to random events such as differences in days, analysts, equipment and so on. Reproducibility conveys the results of precision studies between different laboratories. This data is used for the inclusion of procedures in pharmacopeias.

In precision study, the documentation should include the standard deviation, relative standard deviation and confidence interval.
1.5.7.2 **Accuracy**

Accuracy is closeness of test results achieved by an analytical method to the true value. It indicates deviation from mean value found to the true value.

Accuracy is calculated by percentage recovery of known quantity of impurity spiked to the analyte with the concept of impurity added and impurity found in the method.

1.5.7.3 **Limit of Detection**

The limit of detection (LOD) is the lowest concentration of an analyte in the sample that can be detected but not essentially quantitated as an exact value.

It can be calculated based on signal-to-noise ratio (between 2 to 3), visual evaluation and standard deviation of response and slope of the calibration curve.

1.5.7.4 **Limit of Quantitation**

The limit of quantitation (LOQ) is the lowest concentration of an analyte in the sample that can be calculated with acceptable precision and accuracy. It can be calculated based on signal-to-noise ratio (about 10), visual evaluation and standard deviation of response and slope of the calibration curve.
1.5.7.5 Specificity/Selectivity

The terms selectivity and specificity are often used interchangeably. The term specificity denotes the ability to evaluate the analyte free from the interference of components that may be expected to present. The term selectivity denotes the ability to measure accurately the analyte in presence of components that may be expected to present.

In general, specificity is carried out during the validation of identification test and in determination of impurities. As part of identification test, the specificity should be demonstrated by its capability of discriminating the closely related structures and the confirmation by obtaining negative results when spiked with related compounds and positive results when spiked with analyte.

1.5.7.6 Linearity and Range

Linearity is the ability of an analytical method to provide test results that are directly proportional to the concentration of analyte within a given range.

Linearity is calculated by determining the correlation coefficient and y-intercept of the linear regression line for the response of analyte versus concentration plot.

Range is the interval between the higher and lower levels of analyte that was established with acceptable linearity, precision and accuracy in
the method. The range usually depends on the intended application of
procedure.

1.5.7.7 Ruggedness

Ruggedness is the degree of reproducibility of end results
accomplished by the analysis of same sample under different test
conditions i.e., different analysts, laboratories, instruments and days. In
ICH, though the term is not described separately, it is covered in the
topic of precision.

1.5.7.8 Robustness

Robustness is the capability of method to remain unchanged by little
intentional variations in method parameters.

The variations may include change of organic solvent in mobile phase,
pH of buffer in mobile phase, column oven temperature and flow rate.
According to ICH, the robustness should be initiated in the early phase of
method development. In addition, if the results are susceptible to
variation in method parameters, the parameters should be controlled and
the precautionary statement should include in the documentation of the
method.
1.5.8 Importance of mass balance in SIAM development:

The mass balance is a method of adding together the assay value and degradation products obtained to see how closely these add up to 100% of initial value with due indication of analytical error. Regulatory authorities apply mass balance to be appropriate in evaluating the analytical method as a stability-indicating method and decide whether all degradants have been taken into consideration.

1.6 Importance of Genotoxic impurities evaluation:

Genotoxic impurities (GTI) are the chemical substances that impact genetic material through mutations. Mutations include chromosomal breaks, insertion or covalent binding into DNA during replication, chromosomal rearrangements and they have the potential to cause cancer in human.[11]

GTI with respect to pharmaceuticals can arise from many places including reagents, starting materials, solvents, intermediates or undesired side reactions from the synthetic process of API that can get carried over up to the final product. Moreover, the API itself can degrade to form GTI or they can arise in the drug product by reaction between containers or excipients and API. There is a potential risk of damaging DNA if GTI is carried over into a product taken by patients.

1.6.1 Regulatory guidelines:

The European Medicines Agency (EMEA) was the initial regulatory body to provide comprehensive guidelines to handle GTIs which was
started operation at the beginning of 2007. The USFDA later released a
draft guideline in Dec 2008. In fact, both of these guidelines reveal the
recommended approaches to deal with GTIs, in particular its control
limits in the form of Threshold of Toxicological concern (TTC) wherein 1.5
microgram per day daily intake of impurity is taken as practically safe
dosage, whereas low and high limits are specific to the case depending
on the toxic potential of a given compound. Hence, GTIs need to be
controlled below the TTC limit. The TTC limit of 1.5 µg/day is based on
85% of the tested carcinogens that had a calculated risk lower than
1:100000.

1.6.2 Classification of GTI:

Muller classified GTI into five groups.

(a) Class-1: Known Genotoxic carcinogens

(b) Class-2: Genotoxic but with unknown carcinogenic potential

(c) Class-3: Alerting structure unrelated to API with unknown Genotoxic
potential

(d) Class-4: Alerting structure related to API

(e) Class-5: No alerting structure or indication of Genotoxic potential

Muller categorized these five classes in the form of a decision tree.

[Fig. 1.16]
In the above decision tree, Class 1 compounds need to be eliminated as possible as they are known carcinogens and if not possible need to be controlled on a staged TTC concept. Class 2 compounds are evaluated based on toxicity threshold mechanisms. If a toxicity threshold establish, this level will be applicable to the compound based on permitted daily exposure (PDE) calculations incorporated from ICH guidelines. If a toxicity threshold cannot be established, the default TTC will be applicable. Class 3 compounds need to be evaluated to determine if they are positive for genotoxicity. If they are proved genotoxic, the conclusion for the impurity follow Class 2 path, but if it is found as negative the impurity is controlled as an ordinary impurity according to
ICH guidelines. Class 4 compounds can be evaluated and qualified based on API genotoxicity. If the API is genotoxic, then the Class 2 path is applicable, or else it is treated like an ordinary impurity. Class 5 has no structural alerting groups and is also like controlled as an ordinary impurity.

### 1.6.3 Staged TTC concept:

The TTC default value hinders the development of pharmaceuticals. PhRMA suggested the staged TTC concept in which patients can be exposed to more than 1.5µg/day. The staged TTC concept is based on the maximum daily dose of the drug and duration of the exposure. The TTC value is also dependant the indication of drug, patient life expectancy, pediatric patient populations, highly potent structural alerts. The common structural alerts to genotoxicity is presented in Fig.1.17.

**Fig.1.17: Structural alerts for Genotoxic impurities**

Muller proposed staged TTC approach in terms of maximum daily dose and ADI is calculated based on the duration of the exposure and
the GTI limit is given in terms of percentage and ppm at each combination of maximum daily dose and duration of exposure. [Fig.1.18]

**Fig.1.18  Staged TTC: Concentration to be controlled in % and ppm vs maximum daily dose**

<table>
<thead>
<tr>
<th>Daily dose of API (mg)</th>
<th>Concentration of impurity (%)</th>
<th>Acceptable (maximum) daily intake (ADI) and duration of exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤4 weeks</td>
<td>1-3 months</td>
</tr>
<tr>
<td>ADI = 120 µg</td>
<td>ADI = 40 µg</td>
<td>ADI = 20 µg</td>
</tr>
<tr>
<td>3000.0</td>
<td>0.004</td>
<td>0.001</td>
</tr>
<tr>
<td>2000.0</td>
<td>0.006</td>
<td>0.002</td>
</tr>
<tr>
<td>1500.0</td>
<td>0.008</td>
<td>0.003</td>
</tr>
<tr>
<td>1200.0</td>
<td>0.010</td>
<td>0.003</td>
</tr>
<tr>
<td>1000.0</td>
<td>0.012</td>
<td>0.004</td>
</tr>
<tr>
<td>900.0</td>
<td>0.013</td>
<td>0.004</td>
</tr>
<tr>
<td>800.0</td>
<td>0.015</td>
<td>0.005</td>
</tr>
<tr>
<td>700.0</td>
<td>0.017</td>
<td>0.006</td>
</tr>
<tr>
<td>600.0</td>
<td>0.020</td>
<td>0.007</td>
</tr>
<tr>
<td>500.0</td>
<td>0.024</td>
<td>0.008</td>
</tr>
<tr>
<td>400.0</td>
<td>0.030</td>
<td>0.010</td>
</tr>
<tr>
<td>300.0</td>
<td>0.040</td>
<td>0.013</td>
</tr>
<tr>
<td>200.0</td>
<td>0.060</td>
<td>0.020</td>
</tr>
<tr>
<td>100.0</td>
<td>0.120</td>
<td>0.040</td>
</tr>
<tr>
<td>90.0</td>
<td>0.133</td>
<td>0.044</td>
</tr>
<tr>
<td>80.0</td>
<td>0.150</td>
<td>0.050</td>
</tr>
<tr>
<td>70.0</td>
<td>0.171</td>
<td>0.057</td>
</tr>
<tr>
<td>60.0</td>
<td>0.200</td>
<td>0.067</td>
</tr>
<tr>
<td>50.0</td>
<td>0.240</td>
<td>0.080</td>
</tr>
<tr>
<td>40.0</td>
<td>0.300</td>
<td>0.100</td>
</tr>
<tr>
<td>30.0</td>
<td>0.400</td>
<td>0.133</td>
</tr>
<tr>
<td>25.0</td>
<td>0.480</td>
<td>0.160</td>
</tr>
<tr>
<td>20.0</td>
<td>0.600</td>
<td>0.200</td>
</tr>
<tr>
<td>10.0</td>
<td>1.20</td>
<td>0.400</td>
</tr>
<tr>
<td>9.0</td>
<td>1.33</td>
<td>0.444</td>
</tr>
<tr>
<td>8.0</td>
<td>1.50</td>
<td>0.500</td>
</tr>
<tr>
<td>7.0</td>
<td>1.71</td>
<td>0.571</td>
</tr>
<tr>
<td>6.0</td>
<td>2.00</td>
<td>0.667</td>
</tr>
<tr>
<td>5.0</td>
<td>2.40</td>
<td>0.800</td>
</tr>
<tr>
<td>4.0</td>
<td>3.00</td>
<td>1.00</td>
</tr>
<tr>
<td>3.0</td>
<td>4.00</td>
<td>1.33</td>
</tr>
<tr>
<td>2.0</td>
<td>6.00</td>
<td>2.00</td>
</tr>
<tr>
<td>1.0</td>
<td>12.00</td>
<td>4.00</td>
</tr>
<tr>
<td>0.9</td>
<td>13.33</td>
<td>4.44</td>
</tr>
<tr>
<td>0.8</td>
<td>15.00</td>
<td>5.00</td>
</tr>
<tr>
<td>0.7</td>
<td>17.14</td>
<td>5.71</td>
</tr>
<tr>
<td>0.6</td>
<td>20.00</td>
<td>6.67</td>
</tr>
<tr>
<td>0.5</td>
<td>24.00</td>
<td>8.00</td>
</tr>
<tr>
<td>0.4</td>
<td>30.00</td>
<td>10.00</td>
</tr>
<tr>
<td>0.3</td>
<td>40.00</td>
<td>13.33</td>
</tr>
<tr>
<td>0.2</td>
<td>60.00</td>
<td>20.00</td>
</tr>
<tr>
<td>0.1</td>
<td>&gt;100%</td>
<td>40.00</td>
</tr>
</tbody>
</table>
The upper shaded area of Fig.1.18 represents the impurity concentrations that are \( \leq 100 \) ppm relative to the maximum API dose. At
these very low concentrations, analytical method development becomes extremely challenging. In contrast, the lower shaded area represents concentration of impurity ≥ 0.5%. But, in general the quality of the drug substance need to be controlled as it may have impact at impurity concentrations higher than 0.5%. Hence, there is a critical need to develop analytical methods to monitor the GTI at low level with appropriate sensitivity and selectivity.

1.7 Scope and Objectives of research work:

The present research study was focuses on the development of new stability-indicating analytical methods for two active pharmaceutical ingredients (API) and their dosage forms, two key intermediates and the potential genotoxic impurities evaluation of one API. The research work also reveals the validation of the developed LC methods as per ICH requirement and shows the fitness of developed methods to monitor the stability study of the pharmaceutical dosage forms. The list of API and key intermediates taken for research study was listed in the below Table 1.5.
Table 1.5: The Chemical names, structure of Active pharmaceutical ingredients (API) and key intermediates.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>API/key intermediate chemical names</th>
<th>Structure</th>
<th>Therapeutic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>Eslicarbazepine acetate&lt;br&gt;(S)-(-)-10-Acetoxy-10,11-dihydro-5H-dibenz[b,f]azepine-5-carboxamide</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>It is an antiepileptic drug</td>
</tr>
<tr>
<td>2.0</td>
<td>Asenapine maleate&lt;br&gt;(3aS,12bS)-5-chloro-2-methyl-2,3,3a,12b-tetrahydro-1H-dibenzo[2,3:6,7]oxepino[4,5-c]pyrrole maleate</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>a new atypical antipsychotic developed for the treatment of schizophrenia and acute mania associated with bipolar disorder.</td>
</tr>
<tr>
<td>3.0</td>
<td>L-VCR&lt;br&gt;(2S)-2-((2-amino-6-oxo-1H-purin-9(6H)-yl)methoxy)-3-hydroxypropyl 2-((benzyloxy)carbonyl)amino)-3-methylbutanoate</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>Key intermediate of Valganciclovir hydrochloride which is an antiviral medication used for the treatment of cytomegalovirus (CMV) infections.</td>
</tr>
<tr>
<td>4.0</td>
<td>Spiro alcohol (1R,3r,5S)-3-hydroxyspiro[bicyclo[3.2.1]octane-8,1’-pyrroloidin]-1’-ium chloride</td>
<td>Key intermediate of Trospium chloride that has antispasmodic, antimuscarinic effects</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>Quetiapine fumarate 4-dibenzo[b,f][1,4]thiazepin-11-yl)-1-(2-(2-hydroxyethoxy)ethyl)piperazin-1-ium fumarate</td>
<td>atypical antipsychotic approved for the treatment of schizophrenia, bipolar disorder and depression</td>
<td></td>
</tr>
</tbody>
</table>

**REFERENCES:**


