CHAPTER 1

Role of liquid chromatography and mass spectrometry in pharmaceutical analysis
1.1 Introduction

Identification, determination and quantification of different types of related substances have always been key tasks in pharmaceutical drugs process development for their quality, efficacy and safety concerns. Related substances are the unwanted components which remain present in pharmaceutical drugs after their synthesis, or developed during storage and transit or during the preparation of formulations or upon aging of both API and formulated APIs in medicines. The presence of these unwanted components even in trace amounts may influence the efficacy and safety of the pharmaceutical products by possessing unwanted pharmacological and toxicological effects by which any benefit from their administration may be outweighed [1]. The pharmaceuticals must be of acceptable quality, safe and effective, in order to produce the desired therapeutic effect in patients. The use of poor quality and ineffective drugs will not only endanger the therapeutic treatment but also erode the public confidence in a country’s health program. Quality of pharmaceutical drugs commands special attention because these are affecting the life of human beings directly. The terms related components, related substances, and related impurities terms are synonyms for impurities; the use of these terms at any phrase means one and the same (i.e. impurities).

As defined by the International Conference on Harmonisation (ICH), impurity is “Any component of the medicinal product which is not the chemical entity defined as the active substance or an excipient in the product”. Analytical methods using for the estimation of impurities in API should be of stability indicating to monitor the stability of pharmaceutical formulations during the investigational phase of drug development, and once the drug is marketed, the ongoing stability studies must be conducted. The purpose of stability testing is to provide an evidence on how the quality of a drug substance/product varies with time under the influence of various environmental factors such as humidity (acidic/alkaline/neutral), temperature and light, which enables us to establish a retest period/shelf lives for a drug substance and a recommended storage conditions.

Various analytical methodologies have been employed in the literature for determination of related substances in pharmaceutical drugs. There is a great need for development of new analytical methods for quality evaluation of newly developed drugs. The drug discovery and development process has undergone dramatic changes
particularly over the last two decades. Progress in drug discovery has been fueled by improvements in methodologies and technologies including high performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS), nuclear magnetic resonance (NMR), hyphenated techniques like LC-MS, LC-NMR, and GC-MS, and high throughput purification methods.

**Need for development of new analytical methods**

Until the beginning of 20th century drug products were produced and marketed with no control over purity and safety. Quality of the most of drugs were generally very poor and patented with dubious value.

In 1937, a manufacturer used ethylene glycol as a vehicle for an elixir of sulfanilamide, which caused more than 100 deaths [2]. Another example was thalidomide, a sedative and sleep inducing medicine introduced in the early 1960s, which caused serious malformation in newborn babies of women who consumed it during early days of pregnancy. Later it was found that the S-(-)-enantiomer of thalidomide possessed teratogenic action and has no importance for the desired sedative or sleeping inducing property [3]. It has caused great concern for the pharmaceutical industry as well as various regulatory agencies. There upon the Food, Drug, and Cosmetic (FD&C) act was revised requiring advance proof of safety and various other controls for new drugs. An example for the necessity of change in the attitude of drug manufactures involves the case of one of the most classical drugs, aspirin. The quality of it was tested by titrimetric assay (saponification by sodium hydroxide and back titration of consumed base) using US, British as well as Indian pharmacopoeia [4].

A colour test for free salicylic acid as the main impurity/decomposition product was included. Later, when HPLC become widespread in pharmaceutical analysis, in addition to salicylic acid, three more impurities were found in both bulk drugs as well as formulations of aspirin synthesized from various manufacturers as shown in Figure 1.1 [5, 6]. It was supposed that these impurities are capable of reacting with protein amino functions that are responsible for allergic reactions of aspirin [7]. It is apparent that all of the above impurities consume sodium hydroxide in the titration method and their presence could not be detected by conventional techniques. It is clear that more sophisticated methods are necessary which can identify, characterize, determine, and quantify all the possible related substances.
present in drug substance/product. The presence and type of related substances in a pharmaceutical drug generally depend upon the origin from where it is coming out.

![Chemical structures of aspirin and its impurities detected by HPLC](image)

**Figure 1.1: Chemical structures of aspirin and its impurities detected by HPLC**

1.2 Origin of impurities in pharmaceutical drugs

According to the International Conference on Harmonisation (ICH) of technical requirements for registration of pharmaceuticals for human use guidelines, impurities are classified as organic impurities, inorganic impurities and residual solvents. Organic impurities may arise from starting materials, by-products, synthetic process intermediates and degradation products. Inorganic impurities may derived from the manufacturing process and are normally known and identified as reagents, ligands, inorganic salts, heavy metals, catalysts, filter aids and charcoal etc. Residual solvents are the impurities introduced with solvents [8-13].

**Last intermediate of synthesis**

Impurities fall in to this category are often called as most probable impurities. For example the last step in the synthesis of darifenacin is the condensation of intermediates I with II, these unreacted intermediates could be probable impurities in the bulk drug material [14, 15].
**Products of over reactions**

During the synthesis some steps of the reaction are not selective in many cases and the reagent attacks last intermediate at an undesired site in addition to the desired site lead to the formation of undesired product. An example of this is final step in the synthesis of darifenacin (2 eq. of intermediate II reacts with Intermediate I) leads to the formation of dimer impurity in addition to drug substance [14, 15].

![Dimer of Darifenacin](image)

**Products of incomplete reactions**

It is quite possible that partial reactions lead to the formation of impurities in the synthesis of most number of drugs. An example of such case is the catalytic hydrogenation step in the synthesis of lisinopril. The functional group \(-\text{CO}\) present in the molecule transformed to \(-\text{CH}_2\) by catalytic hydrogenation to form enalapril/lisinopril. Unreduced and/or partially reduced oxo group leads to the presence of hydroxyl derivatives of enalapril/lisinopril as impurities [16].

![Enalapril and Impurity](image)

**Impurities originating from starting materials**

Impurities present in the starting materials can also become the source for impurities of many drug substances. In such cases the impurities also undergo same reactions as that of main component leading to impurities. An example of such case is the appearance of the ethyl substituted impurity in Pramipexole bulk drugs, formed by
the reaction of acetic anhydride present in propionic anhydride (II) with the starting material (I) [17].

\[
\begin{align*}
&\text{H}_2\text{N}_2\text{NH}_2 + \text{I} \\
&\quad \text{NaBH}_4 \quad \text{BF}_3\text{O(Et)}_2 \\
&\quad \text{II} \\
&\quad \text{Pramipexole} + \\
&\quad \text{Impurity}
\end{align*}
\]

**Impurities originating from solvents**

In some cases either the solvents or the impurities formed with solvents could be transformed during the synthesis leading to a host of impurities in the drug substances. As an example, during the synthesis of Imatinib mesylate, methane sulphonic acid reacts with solvents and gives rise to two impurities [18].

\[
\begin{align*}
&\text{N} \quad \text{N} \\
&\quad \text{Imatinib mesylate} \\
&\quad \text{MeOH} \quad \text{CH}_3\text{SO}_3\text{H} \\
&\quad \text{CH}_3\text{SO}_3\text{CH}_3 \quad \text{Impurity-I} \\
&\quad \text{EtOH} \quad \text{CH}_3\text{SO}_3\text{C}_2\text{H}_5 \quad \text{Impurity-II}
\end{align*}
\]

**Impurities originating from catalysts**

The use of catalysts may also sometimes lead to the formation of impurities. During the synthesis of Fluoxetine, Potassium t-butoxide is used as a catalyst, which reacts with one of the intermediates Chlorobenzotrifluoride and forms Benzotrifluoride t-butyl ether as an impurity [19].

\[
\begin{align*}
&\text{OH} \quad \text{NHCH}_3 + \quad \text{CF}_3 \\
&\quad \text{CHlorobenzotrifluoride} \\
&\quad \quad \text{CF}_3 \quad \text{OC} \\
&\quad \quad \text{Fluoxetine} \quad \text{Benzotrifluoride t-butyl ether} \\
&\quad \quad \text{(Impurity)}
\end{align*}
\]
**Products of side reactions**

Most of the chemical reactions are not 100% selective. In such cases the side reactions are common and unavoidable in the course of a synthesis of drug substance. In the synthesis of Agomelatine, a melatonin analog represents a new class of antidepressants, the formation of dimeric impurity along with drug substance is a common phenomenon, which was resulted as a side product due to reaction of intermediate with itself as shown below [20].

\[ \text{Intermediate} \xrightarrow{\text{CH}_3\text{COCl}} \text{Agomelatine} \]

\[ \text{Impurity 1} \xrightarrow{\text{CH}_3\text{COCl}} \text{Impurity 2} \]

**Degradation products**

Impurities can also be formed by the degradation of final product (drug substance) during its manufacturing process. Degradation products resulting from storage conditions or formulation to different dosage forms or aging are common impurities in the medicines [13]. According to the ICH guidelines the *degradation product* is a molecule resulting from a chemical change in the substance brought about by overtime and/or action of e.g. light, temperature, pH of water or by reaction with excipient and/or the intermediate container closure system [9, 21]. For example in the case of aspartame, in the presence of moisture, hydrolysis occurs to form the degradation products i.e. L-aspartyl-L-Phenylalanine, 3-benzyl-6-carboxymethyl 2, 5 diketopiperazine and β-L-aspartyl-L-phenylalanine methyl ester. Aspartame degradation also occurs during prolong heat treatment [9]. Another example of such
case is eslicarbazepine acetate, an epileptic drug on exposure to moisture undergo degradation and one degradation product was formed. These degradation products were considered to be the impurities of finished products [22].

![Eslicarbazepine acetate and degradation product](image)

**Enantiomeric impurities**

In the case of a chiral drug administered as a pure enantiomer, the antipode is considered to be an impurity [23]. The majority of therapeutic chiral drugs using as pure enantiomers are natural products. The high level of enantioselectivity of their biosynthesis procedure excludes the possibility of the formation/presence of enantiomeric impurities [13]. The reason for its presence in synthetic drugs can be either the incomplete enantioselective syntheses or incomplete resolution of the enantiomers of the racemate [10, 24]. Although the ICH guideline excludes enantiomeric impurities, all pharmacopoeias consider them as ordinary impurities [9]. Some examples of pharmaceuticals where one isomer has the desired effect and the other has harmful properties include, thalidomide, where the $R$-enantiomer is a sedative and the $S$-enantiomer is teratogenic; ethambutol, where the $S,S$-enantiomer is tuberculostatic and the $R,R$-enantiomer causes blindness. Some examples of the chiral drugs whose antipodes possess undesired effects are listed in Table 1.1.

**Table 1.1:** Chiral antipodes having undesired side effects

<table>
<thead>
<tr>
<th>Drug</th>
<th>Structure</th>
<th>Configuration</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thalidomide</td>
<td><img src="image" alt="Thalidomide structure" /></td>
<td>R, S</td>
<td>Sedative</td>
</tr>
<tr>
<td>Ethambutol</td>
<td><img src="image" alt="Ethambutol structure" /></td>
<td>S,S, R,R</td>
<td>Tuberculostatic, Blindness</td>
</tr>
</tbody>
</table>
Residual solvents

Residues of solvents are usually present at trace levels in bulk drugs and their formulations as those are commonly used in all phases of activities in the pharmaceutical industries. Based on the safety considerations, solvents are placed in one of the classes given below [25].

Class 1: These solvents are known as human carcinogens and to be avoided completely. Examples are benzene (2 ppm), carbon tetra chloride (4 ppm), 1,2-dichloroethane (5 ppm), 1,1-dichloroethene (8 ppm) and 1,1,1-trichloroethane (1,500 ppm), etc. These suspected human carcinogens and environmental hazards should not be used in any activities in pharmaceutical industries. If their use is unavoidable, the levels should be in compliance with the specifications prescribed by regulatory authorities.

Class 2: These solvents are non-genotoxic carcinogens and should be limited due to their inherent toxicity. Some examples include acetonitrile (410 ppm), cyclohexane (3880 ppm), and methanol (3000 ppm) etc. The values given in parenthesis of class 1 and class 2 solvents are threshold values.

Class 3: These solvents are generally low toxic to the human beings. No health based exposure limit is needed since the Per Day Exposures (PDEs) can generally be limited by Good Manufacturing Practices (GMP) or other quality-based requirements. Some examples include acetic acid, ethyl acetate, ethanol, and tetrahydrofuran etc.
Inorganic impurities

These are less commonly found in drugs formed from various possible sources. The starting materials, catalysts, solvents and reagents could be the main sources of inorganic salts. Many chemical reactions are promoted by metal based catalysts. For instance, a Ziegler-Natta catalyst contains titanium, Grubb’s catalyst contains ruthenium, and Adam’s catalyst contains platinum.

Impurities from excipients

The most frequently used excipients in pharmaceutical formulation preparations are carbohydrates e.g. starch, cellulose derivatives, lactose, sucrose, polymeric materials such as polyethylene glycol, various oils of plant origin, povidone, stearic acid, its magnesium salt, inorganic materials such as calcium phosphate and various forms of silicic acid etc. The quantity of these materials are quite higher than that of the active ingredient in all most all cases. Traces of metals in the excipients can catalyze the active pharmaceutical ingredients to form degradation products. For example, peroxide impurities in polyethylene glycols may cause the degradation of oxidizable drugs [26].

1.3 Identification of impurities

The identification, characterization (structure elucidation) and quantitative determination of impurities including degradation products of a drug substance are of prime importance in the course of all the phases of development of drug formulations. Stability indicating analytical methods have to be used in the course of development of drug formulations which indicate the presence of all possible impurities including degradation products in bulk drugs. During the course of stability studies generally the content of the degradation products increases while the process related impurities may likely to remain constant. The comparison of the impurity profiles of several batches of a drug substance from the same manufacturer provides a good indication for the consistency of the manufacturing process while the comparison of impurity profiles of samples originating from different manufacturers can give a clear depiction about the differences between their purity and the levels standards of the manufacturing procedures. Figure 1.2 shows the general scheme for detection, identification, structure elucidation and determination of related organic impurities in pharmaceutical drugs [27].
Figure 1.2: Schematic flowchart for detection, identification, structure elucidation and determination of related organic impurities in drugs.

Chromatography, spectroscopy and hyphenated techniques are most commonly used for the identification of impurities in drugs. HPLC plays an important role in the separation, identification and quantitation of both samples and complex components present in the raw materials, intermediates, bulk drugs and their
formulations [28]. Thin layer chromatography (TLC) is also a powerful technique for rapid screening of unknown materials in bulk drugs [29, 30]. Gas liquid chromatography (GLC) has also a promising role in analysis of pharmaceutical products for analyzing volatile impurities and residue solvents [31]. Several approaches towards the establishment of organic impurity profiles of synthetic drugs have been made, which involve the prediction of likely impurities within the synthetic process, their isolation and identification by suitable analytical techniques [32-34]. Inductively coupled plasma-mass spectrometry (ICP-MS) coupled to HPLC has also been widely used for analyzing inorganic impurities. An overview of recent applications of (ICP-MS) in determination of inorganic impurities in pharmaceutical drugs and formulations has also been reported [35].

1.4 ICH Guidelines

According to ICH Guidelines, each impurity of a drug substance and drug product must be investigated with respect to both chemistry and safety aspects. Chemistry includes the identification (including structural characterization), reporting and quantitation using suitable analytical procedures, while a process of acquiring and evaluating data concerning the biological safety of an impurity (qualification) to patients comes under safety issues. Individually listed impurities, listed with limited specific acceptance criteria, are referred to as specified and they can either be identified or unidentified. Unspecified impurities are limited by a general acceptance criterion. A decision tree for the identification and qualification of impurities along with their corresponding thresholds depending upon the maximum permitted daily dose (MDD) is given by ICH. Specified unidentified impurities are generally referred to by an appropriate qualitative analytical description (e.g. relative retention time) [10, 11, 21, 24, and 36]. Together, the following list of organic impurities must be presented in the specification of a synthetic drug substance:

- Each specified identified or unidentified impurity
- Any unspecified impurity
- Total impurity

The complete list of topic, codes and their corresponding quality guidelines developed by ICH is shown below in Table 1.2.
Table 1.2: List of topics, codes and their corresponding quality guidelines developed by ICH

<table>
<thead>
<tr>
<th>Topic/ Code</th>
<th>Quality guidelines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1A(R2)</td>
<td>Stability Testing of New Drug Substances and Products</td>
</tr>
<tr>
<td>Q1B</td>
<td>Stability Testing: Photo stability Testing of New Drug Substances and Products</td>
</tr>
<tr>
<td>Q1C</td>
<td>Stability Testing for New Dosage Forms Annex to the ICH Harmonised Tripartite Guideline on Stability Testing for New Drugs and Products</td>
</tr>
<tr>
<td>Q1D</td>
<td>Bracketing and Matrixing Designs for Stability Testing of Drug Substances and Drug Products</td>
</tr>
<tr>
<td>Q1E</td>
<td>Evaluation of Stability Data</td>
</tr>
<tr>
<td>Q1F</td>
<td>Stability Data Package for Registration in Climatic Zones III and IV</td>
</tr>
<tr>
<td>Q2(R1)</td>
<td>Validation of Analytical Procedures: Text and Methodology</td>
</tr>
<tr>
<td>Q3A(R2)</td>
<td>Impurities in New Drug Substances</td>
</tr>
<tr>
<td>Q3B(R2)</td>
<td>Impurities in New Drug Products</td>
</tr>
<tr>
<td>Q3C(R4)</td>
<td>Impurities: Guideline for Residual Solvents</td>
</tr>
<tr>
<td>Q4B</td>
<td>Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions</td>
</tr>
<tr>
<td>Q4B ANNEX 1</td>
<td>Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Residue on Ignition/Sulphated Ash General Chapter</td>
</tr>
<tr>
<td>Q4B ANNEX 2</td>
<td>Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Test for Extractable Volume of Parenteral Preparations General Chapter</td>
</tr>
<tr>
<td>Q4B ANNEX 3</td>
<td>Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Test for Particulate Contamination: Sub-Visible Particles General Chapter</td>
</tr>
<tr>
<td>Q4B ANNEX 4A</td>
<td>Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on MICROBIOLOGICAL EXAMINATION of Non-Sterile Products: Microbial Enumerations Tests General Chapter</td>
</tr>
<tr>
<td>Q4B ANNEX 4B</td>
<td>Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Microbiological Examination of Non-Sterile Products: Test for Specified Micro-Organisms General Chapter</td>
</tr>
<tr>
<td>Q4B ANNEX 4C</td>
<td>Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Microbiological Examination of Non-Sterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for</td>
</tr>
<tr>
<td>Q4B A\text{N}N\text{E}X 6</td>
<td>Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Uniformity of Dosage Units General Chapter</td>
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<td>-------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Q4B A\text{N}N\text{E}X 7</td>
<td>Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Region on Dissolution Test General Chapter</td>
</tr>
<tr>
<td>Q4B A\text{N}N\text{E}X 8</td>
<td>Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Sterility Test General Chapter</td>
</tr>
<tr>
<td>Q4B A\text{N}N\text{E}X 9</td>
<td>Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Tablet Friability General Chapter</td>
</tr>
<tr>
<td>Q4B A\text{N}N\text{E}X 10</td>
<td>Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions on Polyacrylamide Gel Electrophoresis General Chapter</td>
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<tr>
<td>Q5A(R1)</td>
<td>Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human or Animal Origin</td>
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<tr>
<td>Q5B</td>
<td>Quality of Biotechnological Products: Analysis of the Expression Construct in Cells Used for Production of R-DNA Derived Protein Products</td>
</tr>
<tr>
<td>Q5C</td>
<td>Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products</td>
</tr>
<tr>
<td>Q5D</td>
<td>Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products</td>
</tr>
<tr>
<td>Q5E</td>
<td>Comparability of Biotechnological/Biological Products Subject to Changes in Their Manufacturing Process</td>
</tr>
<tr>
<td>Q6A</td>
<td>Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances</td>
</tr>
<tr>
<td>Q6B</td>
<td>Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products</td>
</tr>
<tr>
<td>Q7</td>
<td>Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients</td>
</tr>
<tr>
<td>Q8(R2)</td>
<td>Pharmaceutical Development</td>
</tr>
<tr>
<td>Q9</td>
<td>Quality Risk Management</td>
</tr>
<tr>
<td>Q10</td>
<td>Pharmaceutical Quality system</td>
</tr>
<tr>
<td>Q8/Q9/Q10</td>
<td>Quality Implementation Working Group on Q8, Q9 and Q10</td>
</tr>
<tr>
<td>Q&amp;As</td>
<td>Questions &amp; Answers</td>
</tr>
</tbody>
</table>
1.5 Quality control of organic impurities

According to ICHQ3A guideline, control of the organic impurities in a new drug substance is based on its maximum daily dose and total daily intake (TDI) of the impurities present in it. The ICH threshold for control of the organic impurities in new drug substances are shown in Table 1.3. Depending on the maximum daily dose, whether it is higher or lower than 2g, organic impurities in a new drug substance at or greater than 0.05% or 0.1% requires identification.

**Table 1.3**: Organic impurity threshold in new drug substances based on ICHQ3A [11, 12 and 36]

<table>
<thead>
<tr>
<th>Maximum Daily Dose(^1)</th>
<th>Reporting Threshold(^2,3)</th>
<th>Identification Threshold(^2,3)</th>
<th>Qualification Threshold(^2,3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤2 g/day</td>
<td>0.05%</td>
<td>0.1 or 1.0 mg/day intake (whichever is lower)</td>
<td>0.15% or 1.0 mg/day (whichever is lower)</td>
</tr>
<tr>
<td>&gt;2 g/day</td>
<td>0.03%</td>
<td>0.05%</td>
<td>0.05%</td>
</tr>
</tbody>
</table>

\(^1\) The amount of drug substance administered per day
\(^2\) Higher reporting thresholds should be scientifically justified
\(^3\) Lower thresholds can be appropriate if the impurity is unusually toxic

According to ICHQ3B guideline, control of organic impurities in new drug products is also based on their maximum daily dose and TDI of impurities present in them. The identification thresholds for organic impurities present in new drug products are divided into four groups to give more consideration to low dose drug products as shown in Table 1.4. The maximum daily dose of most of new drug products is between 10 mg–2 g/day, therefore, any impurities at or greater than 0.2% would have to be identified [12, 37, and 21].

**Table 1.4**: Organic impurity threshold in new drug products based on ICHQ3B [11, 12 and 40]

<table>
<thead>
<tr>
<th>Reporting Thresholds</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maximum Daily Dose(^1)</strong></td>
</tr>
<tr>
<td>≤1 g/day</td>
</tr>
<tr>
<td>&gt;1 g/day</td>
</tr>
</tbody>
</table>

**Identification Thresholds**
### Maximum Daily Dose

<table>
<thead>
<tr>
<th>Maximum Daily Dose&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Threshold&lt;sup&gt;2,3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1mg/day</td>
<td>1.0% or 5 µg TDI, whichever is lower</td>
</tr>
<tr>
<td>1-10 mg/day</td>
<td>0.5% or 20 µg TDI, whichever is lower</td>
</tr>
<tr>
<td>&gt;10mg-2g/day</td>
<td>0.2% or 2 mg TDI, whichever is lower</td>
</tr>
<tr>
<td>&gt;2g/day</td>
<td>0.10%</td>
</tr>
</tbody>
</table>

### Qualification Thresholds

<table>
<thead>
<tr>
<th>Maximum Daily Dose&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Threshold&lt;sup&gt;2,3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 10mg/day</td>
<td>1.0% or 50 µg TDI, whichever is lower</td>
</tr>
<tr>
<td>10-100 mg/day</td>
<td>0.5% or 200 µg TDI, whichever is lower</td>
</tr>
<tr>
<td>&gt;100mg-2g/day</td>
<td>0.2% or 3 mg TDI, whichever is lower</td>
</tr>
<tr>
<td>&gt;2g/day</td>
<td>0.15%</td>
</tr>
</tbody>
</table>

<sup>1</sup> The amount of drug substance administered per day.

<sup>2</sup> Thresholds for degradation products are expressed either as a percentage of the drug substance or as total daily intake (TDI) of the degradation product. Lower thresholds can be appropriate if the degradation product is unusually toxic.

<sup>3</sup> Higher thresholds should be scientifically justified.

#### 1.5.1 Pharmacopoeial status

The quality of a drug substance (active) with respect to organic impurities is controlled by a set of tests within a pharmacopoeial monograph. In order to keep pace with scientific progress and regulator developments these individual monographs are periodically updated. Based on the revised ICH Q3A(R2) impurity testing guideline major pharmacopoeias will continue publishing new or revised relevant monographs and general chapters. Active drug substances found to contain organic impurities not detected by the relevant pharmacopoeial tests prescribed below are not of pharmacopoeial quality, unless the amounts and the nature of those impurities are compatible with GMP [38].

Organic impurities testing was discussed in two general chapters (<466> & <1086>) of the US Pharmacopoeia (USP), and concepts and definitions of various terms are clearly described although different terminology from that of ICH is used. Until now, one of three types of following tests in bulk pharmaceutical chemicals is ordered:

i. A chromatographic purity test coupled with a non-specific assay

ii. A chromatographic purity indicating method that also serves as an assay
iii. A specific limit test for known impurities, that requires reference standards of those impurities [39].

The upcoming new and revised USP monographs will include quality tests that actually control specified and unspecified organic impurities. Different routes of synthesis yield different impurity profile, in those cases different analytical procedures will be proposed. All known/specified impurities will be separately listed with a further limit of 0.1% for any unspecified/unknown impurity. Total impurities above the disregard limit should be less than 1.0%. According to USP, a suitable test for detection of impurities that may have been introduced from extraneous sources should be employed in addition to tests provided in a specific monograph [24, 38, and 39].

The European Commission had decided that the principles and terminology of the revised ICH Q3A guideline should be implemented in all (both new and already published) monographs of European Pharmacopoeia (EP) for the active substances [40]. In the fifth edition of EU, a new general chapter dealing with the control of impurities in pharmaceutical substances was introduced. According to EP, control of organic impurities in synthetic drug substances is often accomplished by the test of related substances, which is currently a limit test involving the comparison of peak areas, but will progressively be changed to utilize a quantitative acceptance criterion in future monographs [10, 41]. Some individual monographs already satisfy this demand. In case of the failure of general test in controlling a given impurity, more tests are ordered [44]. As per EU, potential impurities with defined structures which are detected by the tests in a monograph, but not present in medicinal substances above the identification threshold, are referred to as detectable impurities. These detectable impurities are limited by a general acceptance criterion [45]. Individual monographs were published by EU in a new format include a separate section in which all impurities (specified and detected) are listed. Specified unidentified impurities are not listed in this section, but their specific acceptance criteria along with their appropriate analytical description (e.g., retention time) are reported in the text, wherever it is applicable [25].

1.5.2 Stability testing of new drug substances and drug products

The developed methods for the estimation of impurities should be with stability indicating ability to monitor the stability of dosage forms during the investigational phase of drug development, and once the drug is marketed, for
the ongoing stability studies which must be performed. The purpose of stability testing is to provide evidence on how the quality of a drug substance or product varies with time under the influence of a variety of environmental factors such as humidity, heat, and light, which enables us to establish a retest period/shelf lives for a drug substance and a recommended storage condition. The analytical methods should be developed which measure the amount of drug remaining, the amount of drug lost (or the appearance of the formed degradation products), or both. The International Conference on Harmonisation (ICH) of the Technical Requirements for Registration of Pharmaceuticals for Human Use had developed a guideline on stability testing for registration application within Japan, the European Union and the United States. The goal of this ICH stability guideline was to exemplify the core stability data 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 also 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. In the ICH stability guideline, 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. The data obtained from stability studies in turn allows us to establishing the recommended storage conditions and shelf lives.

1.5.3 Stress testing route to the development of stability-indicating analytical methods (SIAMs)

As per ICH stability guideline,

**Stress testing (drug substance):** Studies undertaken to elucidate the intrinsic stability of the drug substance. Such testing is part of the development strategy and is normally carried out under more severe conditions than those used for accelerated testing.

According to ICH stability guideline for drug substance “*Stress testing of the drug substance can help identify the likely degradation products, which in turn can help to establish the degradation pathways and the intrinsic stability of the molecules and validate the stability indicating power of the analytical procedures used*”. The nature of the stress testing will depend on the individual drug substance/product involved.
Stress testing of a drug substance is likely to be carried out on single batch sample, which should include the effect of temperature (in 10 °C increments (e.g., 50 °C, 60 °C, 70 °C etc.) above that for accelerated testing), humidity (e.g., 75% RH or greater) where appropriate, photolysis, and oxidation. The susceptibility of the drug substance to hydrolysis across wide range of pH values should also be evaluated in solution or suspension. Photo stability testing of drug substance should be an integral part of stress testing and the standard conditions for photo stability testing are described in ICH Q1B photo stability guideline. Examination of the degradation products formed under various stress conditions is useful in establishing the degradation pathways, and developing and validating suitable analytical procedures.

Results from these stress studies will form an integral part of the information provided by the regulatory authorities. The description of stress testing was slightly modified in the revised stability guideline from the original description in ICH Q1A. The original ICH Q1A description contains this additional paragraph: Stress testing is conducted to provide data on forced decomposition products and decomposition mechanisms for the drug substance. The severe conditions that may be encountered during distribution can be covered by stress testing of definitive batches of drug substance.

As per ICH stability guideline, **Stress testing (drug product):** Studies undertaken to assess the effect of severe conditions on the drug product. Such studies include photostability testing (see ICH Q1B) and specific testing of certain products (e.g., metered dose inhalers, creams, emulsions, refrigerated aqueous liquid products).

From the ICH definition, it is clear that there is now a regulatory differentiation between “accelerated testing” and “stress testing”. Stress testing is distinguished from accelerated testing by both severity of the conditions and the focus or intent of the results. Stress testing also often referred as “forced degradation” is an investigation of the “intrinsic stability” characteristics of the molecule. It also provides the foundation for development and validation of analytical methods and for development of stable formulations. Drug substance/product stress testing studies are intended to discover its stability issues, and are therefore predictive in nature. Stress testing study is not part of the “validated” formal stability program. Rather, it is a research investigation requiring a scientific expertise and judgment.
1.5.4 Practical aspects

No regulatory authority (e.g. ICH, FDA, etc) provide information on how to carry out stress testing studies on pharmaceutical drugs even though they mentioned the information about reporting the data of stress studies. However, considerable amount of literature can be found on stress testing from which one can select the conditions of stress testing. Many authors have proposed their own different approaches to conduct stress testing on pharmaceutical drugs, which include Singh and Bakshi’s approach [42], Reynold’s approach [43], and S. Klick’s generic approach [44]. Among these proposals Singh and Bakshi approach gives the most detailed information on the execution of stress testing which is given below.

Important practical aspects of Singh and Bakshi approach in conduction of stress testing are

**Nature of drug:** While conducting stress studies, the new drug should be assumed to be labile in specific nature and accordingly, subjected to corresponding stress conditions. Initially, drug should be subjected to intermediate stress conditions such as 0.1N HCl/NaOH 8h reflux, water 12 h reflux (i.e., acid, base and neutral hydrolyses); 3% H₂O₂ 6h RT (oxidation); 1.2 x 10⁶ lux h light exposure (photolytic degradation). If considerable decomposition of drug not occurs then harsher conditions mentioned in the approach should be applied; however, if the drug decomposes excessively, mild conditions should be applied.

**Drug concentration:** The stress testing studies should be carried out at a concentration of 1 mg/mL. It is also suggested that some stress studies should be conducted at a concentration at which the drug is expected to be present in the final formulations.

**Design of the studies:** For every stress study, it is advised to generate four different samples and report the results of each. These include 1) blank solution (stored under normal conditions i.e., without stress), 2) blank solution (subjected to stress in the same manner as that of drug solution), 3) zero time sample (containing the drug stored under normal conditions i.e., without stress) and 4) drug sample subjected to stress treatment.

**Equipment for stress testing:** The stress testing reactions can simply be carried out in containers like volumetric flasks or stoppered culture tubes and stored in water bath set at the desired temperature. For reactions carried out above 80°C and under reflux conditions, a boiling water or oil bath equipped with a temperature
regulator may be used. In case of oxidative stress, studies should be carried out in a leak proof stoppered container. For photolytic stress, the study should be carried out in a petri dish in case of solid state stress, where drug samples are spread evenly as a thin layer. In case of liquid or dissolved sample, any transparent container can be used which gives maximum exposure to light.

**Preparation of samples for HPLC studies:** Processing of stress samples is necessary for making proper concentration of drug, acid, base or oxidizing agent to make them compatible with HPLC for analysis. For this, one approach is diluting the sample (should preferably done in mobile phase) enough so that the concentration of reagent falls within the tolerable range. The second approach involves neutralization of acidic and basic solutions to a tolerable pH. The dilution approach is simpler and preferable method compared to neutralization as degradation product may further degrade by changing pH. If stress sample contains solid particles then those solid particles should be separated and dissolved in appropriate solvent which may help to detect insoluble degradation product formed during stress testing.

**1.5.5 Regulatory aspects**

The ICH Q1A(R2) guideline on Stability Testing of New Drug Substances and Products emphasizes that the testing should cover the features which are susceptible to change during storage and are likely to influence quality, safety and/or efficacy must be done by validated stabilityindicating test methods [45]. The ICH Q3B(R2) guideline on ‘Impurities in New Drug Products’ clearly emphasizes on providing documented evidence of validated analytical procedures which are suitable for the detection and quantitation of degradation products [21]. These validated analytical methods should also demonstrate that impurities unique to the new drug substance do not interfere with it and separated from specified and unspecified degradation products in the drug product. The ICH Q6A guideline provides note for guidance on specifications, and mention the requirement of stabilityindicating assays under Universal Tests/Criteria for both drug substances and drug products [46]. Unfortunately, no ICH stability guideline provides an exact definition of a stability-indicating method. However, it is provided in the US-FDA stability guideline of 1998. According to this guideline Stability-indicating methods were defined as ‘validated quantitative analytical methods that can detect the changes with time in the chemical, physical, or microbiological properties of the 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 measured without interference’ [47]. The United States Pharmacopeia (USP) has also a requirement listed under ‘stability studies in manufacturing’, which says that samples of the products should be assayed for potency by the use of a stability indicating assay [48]. The ICH Q7A guideline on Good Manufacturing Practices for Active Pharmaceutical Ingredients also clearly mentions that the test procedures used in stability testing should be validated and be stability indicating [49].

As per regulatory authorities, performing and reporting of stress testing does not required explicitly at the Phase 1 and Phase 2 of Investigational New Drug (IND) stages although it is encouraged to facilitate selection of stability indicating methods [50]. However, regulatory authorities may still ask questions concerning results from stress testing as early as a Phase 1 IND, especially where the formation of potentially toxic degradation products are possible. At Phase 3 of IND for drug substances require stress testing and guidance suggests that these studies be conducted on drug products. At Phase 3, the guidance strongly suggests, but not always require, that degradation products detected above the ICH identification thresholds during formal stability testing trials should be identified.

The guidance requires a summary of stress studies of both drug substance and drug products including elucidation of degradation pathways, demonstration of the stability indicating nature of analytical methods, and identification of significantly formed degradation products while submitting NDA [51]. Stressing the drug substance under various conditions such as hydrolysis, oxidation, thermolysis, and photolysis in solution and the solid state is required. The design of stress studies is formulation dependent in case of drug products and is left to the discretion of the applicant.

The guidance suggests the analytical assumptions made when determining mass balance should be explained in the registration application [46]. In some cases failure of demonstrating mass balance may also be acceptable where a thorough investigation has been conducted to understand the chemistry of the molecule. Examining mass balance in stressed samples of drug substance/product can reveal the need for better analytical methodology from the start.
Generally formation stereoisomers of the drug substance and their degradation products is of very limited possibility during stress studies. This can obviate the need for testing for these potential impurities during formal stability trials. As per guidance for these impurities ICH identification and qualification thresholds may not apply [46]. Drug-related and non-drug-related degradation products can be distinguished by conducting stress studies on the drug substance, drug product, and placebo. These stress studies should allow the discrimination between synthetic process related impurities, excipients, degradation products derived from active substance/excipients. In case of combination tablet formulations, stress testing should be conducted on active substances together and formation of degradation products and reactions between active ingredients should be investigated [51]. The guidance specifies that identification thresholds for degradation products observed in formal stability samples of the drug substance and product are dose dependent.

1.6 Analytical techniques

Literature reports indicates that titrimetric, spectrophotometric, chromatographic and hyphenated techniques have been commonly employed in the analysis of stability samples. Among these chromatographic and hyphenated techniques (especially HPLC and LC-MS) have gained prime importance.

1.6.1 Chromatography

Chromatography is a technique used to separate and analyze complex mixtures. Liquid chromatography is a powerful tool in separation science. The components to be separated are distributed between two phases: a stationary phase and a mobile phase, which percolates through the stationary bed. The classification and types of liquid chromatography is shown in Figure 1.3.

The phases are chosen such that components of the sample have differing affinities towards each phase. A component, which has quite affinity towards stationary phase, will take longer time to travel through it than a component, which has low affinity. As a result of these differences in mobilities, sample components separate from each other as they travel through the stationary phase. The separated molecules leave the column and get detected by one or more on-line electrical devices with signals proportional to the concentration of the analytes [52-54].
1.6.1.1 High performance liquid chromatography (HPLC)

HPLC is the common analytical technique applied at all stages of drug discovery, and development. Throughout the drug discovery and development paradigm, rugged HPLC separation methods are developed and validated. It is useful in the evaluation and control of purity of chemical components used in the manufacture of bulk drugs [55, 27]. A block diagram showing the all key components of HPLC is shown below in Figure 1.4.
A variety of approaches in establishing the impurity profiles of drugs have been outlined [56]. Which involve the prediction of likely impurities of synthetic process, their isolation and identification by suitable analytical methods. Impurities in bulk drug substances at levels of 0.1% or even less can be detected by HPLC. In most of the cases, reversed phase (C18, C8, etc) columns and UV detector are preferred for HPLC method. In case of UV detection where different impurity components display different absorption maxima, a multiple wavelength scanning program capable of monitoring several wavelengths simultaneously can be used which is possible with Photo diode array (PDA) detector. PDA detector is generally used not only to scan the components through the entire UV range, but also record the spectra and chromatograms of all the components in a drug. PDA detection also useful in checking purity of chromatographic peaks (i.e., specificity). Several approaches using HPLC for determination of impurities in bulk drug substances and their formulations have been reported [57, 58]. In addition, HPLC is also found useful in understanding the stability of drug and identification of degradation products [59, 60]. Reduction in analysis time is always preferable for high throughput analysis. Different columns are available to achieve such goals. HPLC in combination with PDA and other detectors is useful for determination analytes in trace level in
biological samples [61]. LC-MS is not only an alternative for such cases, but also useful in characterization of impurities or degradation products.

1.6.2 Hyphenated techniques

Structural characterization of degradation products is one of the important aspects of drug development. Unfortunately it is associated with several limitations while following conventional approaches. These processes are time consuming and may become very difficult if several degradation products are present. The problems become still more difficult if the components of interest are present in trace levels and where enrichment efforts are unsuccessful. In such cases, an usual practice is to resort to multiple cycling experiments on a preparative column till the desired purity of components suitable for spectral studies is achieved. The difficulties become more worse when DPs are unstable, or if secondary reactions occur during processing and analysis [62, 63] based on various mass tools. The structural characterization of degradation products by conventional approach based on fragmentation pattern is shown in Figure 1.5.

Figure 1.5: Detailed strategies for laying down mass fragmentation pattern of the drug and its degradation products

In overcoming the difficulties of conventional methods, more sophisticated hyphenated techniques are preferred for the trace level identification and characterization of impurities and degradation products. The available hyphenated
instruments have LC, GC or CE on the front end (for separation of components) and MS, NMR or IR on the later end (i.e., detection side). Some examples of hyphenated techniques are LC-MS, GC-MS, CE-MS, LC-NMR, LC-NMR/MS, LC-IR, CE-NMR, etc. In particular, LC-MS technique found more useful in pharmaceutical drug development and these instruments are commercialized in multiple variants.

1.6.2.1 Liquid chromatography-mass spectrometry (LC-MS)

Liquid chromatography combined with mass spectrometry (LC-MS) and tandem mass spectrometry (LC-MS/MS) techniques have been proven to be the best analytical techniques of choice in various stages of drug discovery and development. In combination with chromatographic separation techniques LC and GC, mass spectrometer in the form of LC-MS and LC-MS/MS have gained prime importance for the mixture analysis in pharmaceutical research and development. Mass spectrometric analysis generates complete mass spectral data which inturn provides valuable information regarding the molecular weight, structure, identity, quantity, and purity of a sample. In addition to LC-PDA data, mass spectral data also adds specificity to method which increases confidence in the results of both qualitative and quantitative analysis. It is also important to take in consideration that for most of the components mass spectrometer is more sensitive and far more specific than all other available LC detectors. PDA and UV are oftenly used detectors as most of the compounds exhibits absorptions because of the presence of chromophores in their molecular structures. Compounds with no chromophores can also be analyzed by a LC-MS which are not possible by LC-PDA/UV. It is also found useful in the identification and quantification of components which are unresolved in LC, which inturn reduce the time need to optimize chromatographic conditions as well as method run time. In most of the cases, an HPLC method developed using other available detection techniques for determination of components of interest can be transferred to LC-MS. However, some of the factors should be kept in mind always while setting up an efficient LC-MS method for regular operation [64]. Nonvolatile buffers such as phosphates, citrates and borates should be avoided in LC-MS methods. The most suitable buffers for LC-MS analysis are volatile ammonium acetate and ammonium formate [65].

In general, the functioning of all mass spectrometers involves four steps

(i) Introduction of the sample in to source,
(ii) Ionization of the sample molecules (neutral) to convert them to ions in the gas phase (ionization method),

(iii) Sorting of the resulting gas-phase ions by mass-to-charge ratios (mass analyzer), and

(iv) Detection of separated ions.

The conceptual illustration of the mass spectrometer showing the all major components of mass spectrometer is shown in Figure 1.6. The components are sample inlets (depends upon sample and ionization technique; ion source (origin of gas phase sample ions); mass (m/z) analyzer (responsible for separation of ions according to their individual m/z values); detector (generates the signals that are a recording of the m/z values and abundances of the ions); vacuum system (the components that remove molecules, thereby providing a collision-free path for the ions from the ion source to the detector); and the computer system (coordinates the functions of the individual components and records and stores the data). Critical components of a mass spectrometer include ion source, mass analyzer, and detector.

Various ionization methods have been developed over the years depending upon the state and nature of sample. The ionization methods include electron impact (EI), chemical ionization (CI), matrix-assisted laser desorption/ionization (MALDI), desorption ionization (DI), electrospray ionization (ESI), fast atom bombardment (FAB) and atmospheric pressure chemical ionization (APCI). A list of ionization methods and their application to various sample types is given in Table 1.5.

![Figure 1.6: The conceptual illustration of the mass spectrometer](image-url)
### Table 1.5: Types of molecular ionizations in mass spectrometry

<table>
<thead>
<tr>
<th>Type of Ionization</th>
<th>Ionizing Agent</th>
<th>Source Pressure</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electron ionization (EI)</td>
<td>50–70 eV electrons</td>
<td>$10^4$–$10^6$ torr</td>
<td>Extensive fragmentation allows structure determination; GC/MS</td>
</tr>
<tr>
<td>Chemical ionization (CI)</td>
<td>Gaseous ions</td>
<td>~1 torr</td>
<td>Molecular mass determination; GC/MS</td>
</tr>
<tr>
<td>Desorption ionization (DI)</td>
<td></td>
<td>$10^5$–$10^6$ torr</td>
<td>Molecular mass and structures of high mass, nonvolatile compounds in condensed phase</td>
</tr>
<tr>
<td>Fast atom bombardment (FAB)</td>
<td>Energetic Ar or other neutral atoms</td>
<td></td>
<td>For large biopolymers</td>
</tr>
<tr>
<td>Laser desorption (LDI) and matrix-assisted LDI (MALDI)</td>
<td>Energetic photons</td>
<td></td>
<td>For large biopolymers</td>
</tr>
<tr>
<td>Electrospray (ES) ionization</td>
<td>Electric field; ions in solution</td>
<td>Atmospheric or slightly reduced pressure</td>
<td>HPLC/MS and CE/MS</td>
</tr>
<tr>
<td>Atmospheric pressure chemical ionization (APCI)</td>
<td>Corona discharge; gaseous ions</td>
<td>Atmospheric</td>
<td>HPLC/MS</td>
</tr>
</tbody>
</table>

The ESI process is currently the softest ionization technique by which even non-covalent interactions in proteins can be measured. It is commonly employed in LC-MS analysis. Thus ESI-MS is an ideal mode for the analysis of biomacromolecules as well as small molecules, since it allows fine structural information to be obtained directly from the biomolecules. Accurate molecular weight (10-100 kDa) of large proteins can also be determined by ESI-MS. The basic ESI source consists of a spray needle at high electrical potential (4–5 kV), a thermal/pneumatic desolvation chamber, and a vacuum interface. The ionization process is carried out at atmospheric pressure. It involves spraying the sample solution out of a needle. Mechanism of formation of ions in ESI source is shown in Figure 1.7.
Figure 1.7: A Schematic representation of ion formation in ESI source

This process involves the formation of small charged droplets of sample, followed by the evaporation of solvent to leave the sample molecules in gas phase and ionized state. The ions formed in source are then ‘swept’ into a mass analyzer held essentially in vacuum where they are separated based on their mass to charge (m/z) ratio which are then detected by a detector.

In ESI, two types of ionization are possible based on the nature of molecule which are positive ionization mode and negative ionization mode. In positive ionization mode the protonated molecular ions \([M+H]^+\) are usually the dominant species, although they can be accompanied by salt adducts (\([M+Na]^+, [M+K]^+\) etc). Sometimes, doubly charged molecular ion at approximately half the m/z value, and/or a trace of a dimeric species at approximately twice the m/z value are also possible. Positive ionization is used in general for pharmaceuticals, protein and peptide analyses.

In negative ionization mode the deprotonated molecular ions \([M-H]^-\) are usually the most abundant species and exclusive. Negative ionization is generally useful for analysis of pharmaceuticals containing carboxylic acid group. It is also used for the analysis of oligonucleotides and oligosaccharides.

The mixture of charged ions (molecular ions formed in the ion source and fragmentation ions from a collision chamber) contains information that would be lost as these ions are not separated and identified in some meaningful way. In particular, the mass-to-charge ratio (m/z) of each of these ions must be measured and separated. To do this, the mass analyzer which is the heart of mass spectrometer must be able to
take advantage of some unique property of the ion that results from the imposition of an electrical or magnetic field. Although quadrupole m/z filters \((\text{single quadrupole analyzer, triple quadrupole analyzer, and ion-trap analyzer})\) are commonly used in LC-MS and GC-MS, other types of m/z analyzers are currently used for both these and other applications, which include the \textit{time-of-flight (TOF) and magnetic sector analyzers}. TOF analyzer is also useful for accurate mass measurements. The main purpose of mass analyzer is to hold ions, select specific mass ions as the radio frequency is scanned, and move the selected ion into the ion detector for counting. The separated ions are the transferred to and detected by \textit{electron multiplier} or \textit{photomultiplier} detectors. The analyzers used in the present studies are depicted in Figures 1.8 to 1.10.

**Figure 1.8:** Schematic representation of Quadrupole mass analyzer.

**Figure 1.9:** Schematic representation of Triple quadrupole mass analyzer.
1.6.3 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy is the most widely used and reliable technique for structural elucidation of synthesized organic molecules and degradation products. It plays an important role in identifying even impurities at low level in bulk drug materials with or without preparative chromatographic isolation. For identification and characterization of impurities, modern NMR offers various ranges of experiments [66]. Structural elucidation of impurities in drug materials mostly involves the use of $^1$H and $^{13}$C experiments. The information obtained from these experiments is sufficient to ascertain the structure of an unknown impurity or degradation product of a drug material. In some cases, particularly $^{19}$F and $^{31}$P could be powerful markers [67]. Two-dimensional experiments [60] such as double quantum filtered correlation spectroscopy (DFC-COSY), and heteronuclear single quantum coherence (HSQC) etc., are also found very useful in structure elucidation studies.
1.6.4 Other techniques

UV-Visible spectroscopy for identification and determination of impurities in drug substances without chromatographic separation is of very little importance. Nevertheless, for determining certain impurities in bulk drugs, UV-Visible spectrometric method is recommended in pharmacopoeias. IR spectroscopy is generally used to ascertain the functional groups present in the impurities of interest. Capillary electrophoresis (CE) has its ability to provide a different selectivity to characterize the impurity content and profiles in drug substances [68]. Future approaches for studying impurity profiling of drugs may include the use of LC-NMR (now using rarely), LC-NMR-MS, CE-NMR, SFC-NMR etc. Thus, each technique has its own unique identity and importance in the impurity profile of drug materials.

1.6.5 Isolation of impurities by preparative HPLC

Application of preparative HPLC comes in to picture when the identification of impurities (including degradation products) cannot be carried out with reliability by means of applying simple analytical techniques such as LC, GC, LC-MS, etc. The quantity of isolated impurity should be sufficient for subsequent spectroscopic studies. In such cases preparative HPLC isolation followed by spectroscopic (NMR, MS) investigation provides a lot of information to carry out structural elucidation of an unknown impurities. It is quite reasonable to state that preparative HPLC technique is very much useful in the drug development process.

1.7 Steps involved during the development of stability-indicating analytical methods (SIAMs)

A SIAM is an analytical procedure used to estimate the decrease in the amount of the active ingredient (i.e., API) present due to degradation. According to FDA guidelines, a SIAM is defined as a validated analytical procedure that accurately and precisely measures active ingredients (drug substance or drug product) free from potential interferences like degradation products, process related impurities, excipients, or other potential impurities, and it recommends that all assay procedures for stability studies should be stability-indicating [69]. Liquid chromatography (LC) is used routinely to separate and quantitate the analytes of interest during stability studies.

Development of a SIAM generally involves the following three steps

i) Sample generation

ii) Method development, and
iii) Method validation.

**Step 1: Generation of the sample**

Stressing of the drug substance in both solution and solid forms generates the sample that contains the products most likely to form under various most realistic storage conditions, which is in turn used to develop the SIAM. The goal of the SIAM is to achieve baseline separation of all the resulting products in stress (i.e., API and all the degradation products) without any coelution. In generally, the goal of the stress studies is to get the degradation of API by 5-20%. Any more than this may result irrelevant degradation products (i.e., degradation products of the degradation products). Any less than this may because the miss of important degradation products. Experience and data obtained from previous studies on related compounds also should be used when developing new SIAM protocols.

**Step 2: Development of LC Method**

After the generation of stress sample through the use of a properly designed and executed forced degradation, it can be used for the development of the LC method. Nowadays, LC method developments are often performed on gradient systems capable of automated switching of column and mobile phases, and temperature control. Selectivity (i.e., both Resolving power, and specificity), and rapidness are key chromatographic method attributes to be kept in mind always during method development.

**Selectivity during Method Development**

Selectivity of the method can be manipulated by any one or a combination of different factors which include the type of stationary phase, composition of mobile phase, and pH of buffers. Most part of the method development generally involves changing of mobile phases and column stationary phases to get a good separation. However, recent advances in stationary phase technology have made possible the use of pH as a true selectivity tool for the separation of ionisable compounds [70, 71]. These hybrid columns take advantage of the best of both the silica and polymeric stationary phase worlds. These columns are manufactured using a classical sol-gel synthesis that incorporates carbon in the form of alkyl groups, resulting them as mechanically strong with high efficiency and operate over an extended pH range. Figure1.11 illustrates that why pH can be such a useful tool in selectivity.
Acidic compounds are more retained at lower pH, while basic compounds showed more retention at higher pH (neutral compounds are of course unaffected). For a traditionally used tested range of pH between 4 and 8 a slight change in pH would result in a dramatic shift in retention (up-slope or down-slope of curves) as shown in Figure 1.11. However, by operating at pH extremes, not only is there a 10-30-fold difference in retention that can be exploited in method development, the method can be made more robust as well, a desirable outcome with validation in mind. Indeed, the selectivity differences afforded by a change in pH of buffer are the equivalent to a 20% change in the organic solvent in mobile phase composition, and often are underutilized.

**Evaluating Specificity during Method Development**

Another important parameter that must be evaluated during method development is specificity. The USP and ICH guidelines define specificity as the ability of a method to assess unequivocally the analyte of interest in the presence of potential interferences [72, 73]. Earlier, specificity had been accepted from the evaluation of resolution, peak shape, and tailing factors. But now, starting with USP 24, and as a direct result of the ICH process, it was recommended that a peak purity test based on photodiode-array (PDA) or mass spectrometry (MS) detection should be conducted to demonstrate that a given peak was pure or not. Modern PDA detection technology has proven it as a powerful tool for evaluating peak purity (i.e., specificity). PDA detectors can collect UV-Visible absorption spectra across a range of wavelengths. At each data point the spectra collected across a chromatographic...
peak will be processed through a software manipulations involving multidimensional vector algebra, and finally compared to determine purity of that chromatographic peak. In this manner, PDA detectors today can distinguish even minute spectral and chromatographic differences that are not readily observed by simple overlay comparisons [74-75]. To get a successful specificity test, PDA detector requires

i. A UV chromophore, or some absorbance in the wavelength range selected

ii. Some degree of chromatographic resolution

iii. Some degree of spectral difference

**Step 3: Validation**

According to ICH guideline on validation of analytical methods [76], *the objective of an analytical procedure is to demonstrate that it is suitable for its intended purpose*. Different methods have different requirements when it comes to validation studies. Based on method type, the USP recognizes four categories and defines the analytical performance characteristics that must be investigated during validation [77].

Category 1: Analytical methods for the quantification of major components of bulk drug substances or active ingredients.

Category 2: Analytical methods for the determination of impurities in bulk drug substances or degradation products.

Category 3: Analytical methods for the determination of performance characteristics.

Category 4: Identification tests

The complete list of analytical performance characteristics that must be measured for each category are summarized in Table 1.6.

According to the USP guidelines, SIAM falls into Category 2. All analytical performance parameters must be determined for this category as SIAMs need to be quantitative. One should keep in mind that stress testing methods are only screening methods used to help in understanding the degradation chemistry of the drug substance and therefore, do not need to be validated to the extent of final control methods.
Table 1.6: Data elements required for validation

<table>
<thead>
<tr>
<th>Analytical performance characteristics</th>
<th>Category I</th>
<th>Category II</th>
<th>Category III</th>
<th>Category IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Quantitative</td>
<td>Limit tests</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Accuracy</td>
<td>Yes</td>
<td>Yes*</td>
<td>*</td>
<td>No</td>
</tr>
<tr>
<td>Precision</td>
<td>Yes</td>
<td>Yes*</td>
<td>Yes*</td>
<td>No</td>
</tr>
<tr>
<td>Specificity</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes*</td>
<td>Yes</td>
</tr>
<tr>
<td>Detection limit</td>
<td>No</td>
<td>No</td>
<td>Yes*</td>
<td>No</td>
</tr>
<tr>
<td>Quantitation limit</td>
<td>No</td>
<td>Yes*</td>
<td>Yes*</td>
<td>No</td>
</tr>
<tr>
<td>Linearity</td>
<td>Yes</td>
<td>Yes*</td>
<td>No*</td>
<td>No</td>
</tr>
<tr>
<td>Range</td>
<td>Yes</td>
<td>Yes*</td>
<td>*</td>
<td>No</td>
</tr>
</tbody>
</table>

The concepts in the ICH validation guideline (for analytical methods) are a good starting point for validation of stress testing methods, however, the overall validation should be significantly abbreviated when compared to the validation of final control methods, as stress testing methods are investigational methods. As per ICH, validation parameters which must be investigated are

- Accuracy
- Precision
- Specificity
- Linearity and range
- Limits of detection (LOD and quantitation (LOQ)
- Robustness

Generally accuracy is not a problem in stress testing methods as the response of the detector is linear, but samples needs to be completely dissolved prior to analysis. Accuracy of the assay and impurities can be determined by recovery studies over concentrated ranges. A typical range to be tested for assay of drug substance might be 50 to 150% of its nominal concentration, while for impurities tested range might cover LOQ as starting level or sometimes 50 or 80 % of nominal concentration to a few percent (generally 120 or 150%).

The specificity of the methods cannot be fully validated because one does not know all the possible degradation products during initial stress testing studies. It can be addressed to some extent by using any available known impurities and degradation products.
Precision of the assay (repeatability) of the main component and impurities can be evaluated by preparing a limited number of assay and impurity samples at nominal concentrations and using simple statistical analysis to estimate the standard deviation of results. Estimation of intermediate precision and reproducibility should normally not be necessary for stress testing methods or they can be done by using similar samples used for repeatability prepared and analyzed by different analyst on different day.

Detection and quantitation limits for degradation products (unknown) can be determined by using parent compound (drug substance) and assuming that the response of all degradation products will be similar to their parent compound. Although there is no requirement to reach any specific detection limit, a reasonable is 0.1% since the aim of stress testing is to detect the major degradation products in samples which is approximately 5–20% degraded.

The linearity of the method should be validated over the ranges for both assay and impurities determination. A typical assay range to be tested might be 50 to 150% of nominal sample concentration, while for impurities tested range should be LOQ to a few percent (generally 150 or 200 or 250%) to its nominal concentration.

In addition, other parameter might be validated is robustness. According to ICH, 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. It can be checked by deliberately varying the optimized chromatographic conditions to a small extent, which normally include column temperature, mobile phase composition, flow rate, and buffer pH.

System suitability test should be conducted prior to the study of each validation parameter to check whether the instrument used for experiments or not. System suitability parameters are retention time, tailing factor, theoretical plates, and capacity factor. It can be done by injecting the sample containing drug and impurities at their nominal specification concentration. System suitability also confirms the selectivity of the method (i.e., each analyte is individually specific).

One of the most important aspects of stress testing is the analysis of samples using a suitable analytical method, which, in many cases, usually a reversed phase HPLC (RP-HPLC). This necessitates the development of a RP-HPLC (stability indicating) method capable of measuring the loss of parent compound,
possible process related impurities as well the levels of degradation products or impurities formed in stress testing studies.

1.8 Role of Mass Balance in SIAMs

Mass balance correlates the measured loss of a parent drug to the measured increase in the amount of degradation products during stress. It is a good quality control check in analytical methods to show that all degradation products formed are adequately detected and do not interfere with the quantitation of parent drug (i.e., stability-indicating methods). All the regulatory agencies use mass balance to assess the appropriateness of the analytical method as a stability-indicating method and determine whether all degradants have been accounted for [78].

In mass balance calculations, the loss of parent drug (or the amount of drug remaining) is determined from a sample assay, and the measured increase in degradation products (known) is determined by a related substances method. The fundamental approach for determining mass balance is to quantitate the decomposition peaks (consider all are known) using degradation products and then reconcile the measured loss in the parent drug with the measured amount of degradation products. If the loss in potency can be reasonably accounted for by the amount of degradation products measured, then it is said that the mass balance is achieved.

The assessment of degradation in pharmaceutical products involves two aspects of analytical measurement.

i. A specific or selective analytical method must be available for accurate assay measurement of parent drug in order to measure any of its loss.

ii. Methodology should be in place for quantification of the degradation products formed.

Ideally, when degradation occurs, the measured amount of parent drug loss should be in well correlation with the measured increase in degradation products. This correlation is referred to as “mass balance” [45]. Recently, ICH has provided a definition for “mass balance; material balance” as “The process of adding together the assay value and levels of degradation products to see how closely these add up to 100% of initial value, with due consideration of the margin of analytical precision.” The concept is useful scientific guide for evaluating data, but it is not achievable in all circumstances [79].
Mass balance in pharmaceutical analysis is very important for several reasons. By demonstrating the degardative loss of parent drug correlation with the measured increase in degradation products unaccounted for. Conversely, if one observes, for example, a 20% loss of parent drug but only measures a 10% increase in degradation products, it is likely that additional degradation products (unknown) formed are not accurately determined by the given method(s). These unknown degradation products might be potentially toxic. It is very important to have methods that detect all major degradation products (both known and unknown) to correlate well with the loss of parent drug. That means safety is the major reason for the study of mass balance.

Mass balance checking is also very useful in method validation [80]. In order to demonstrate that the analytical methods are stability-indicating, both unstressed and stressed samples should be compared. Correlation of any increase in degradation product with loss of parent drug aids in demonstrating that the methods can accurately assess degradation or not. It is also important in understanding degradation pathways [81]. For example, consider a situation where both acidic and oxidative stress results a substantial loss of parent drug. If good mass balance is achieved for the acidic stress, but not for oxidative stress. In such a case further work to better understand the oxidative degradation pathway is warranted. It may be that the poor mass balance in the oxidative stress results from important oxidative degradation products that are unaccounted for, which need to be more fully elucidated to understand response factor differences. Mass balance is an important consideration in assessing degradation pathways of pharmaceutical products. Often, response factor differences between degradation products and the parent compound are responsible for mass balance problems. Relative response factors (RRF) should, therefore, be incorporated, when possible (where mass balance is not achieved in general calculation), in the quantification of degradation samples.

1.9 Statement of the problem

It is evident from the preceding sections that the assurance of quality and safety of pharmaceutical drugs is quite important because these are considered to be not only healthcare products but also lifesaving substances. In development of new drugs, the determination of purity is important in order to establish the acceptability of batches for safety assessment, clinical trials, and formulation preparations. These
activities are considered to be the primary objectives of drug manufacturers. The stability indicating analytical methods (SIAM) for the separation, identification (including characterization), and determination of assay of drug substance and content of each impurity is highly desirable. Modern analytical techniques, particularly HPLC permit the analysis (separation and quantitation) of analytes even in complex matrices. Application of Mass spectrometry (MS), and hyphenated technique (LC-MS) found useful in the characterization of unknown compounds.

Now a days HPLC and other analytical techniques (MS, LC-MS, NMR, etc) are increasingly popular in the field of pharmaceutical analysis. Keeping this in view, the present work has been proposed as “application of liquid chromatography and mass spectrometry in the analysis of some drugs”. It includes development and validation of new stability indicating HPLC methodologies for lacosamide, carisbamate, ambrisentan and rivaroxaban in the presence of their process related impurities and characterization of degradation products by MS. In addition, a chiral LC method has also been included for the determination of pramipexole in the presence of its process related impurities.

i. The anticonvulsants are very important therapeutic agents in treating patients with epilepsy, diabetic neuropathic pain, infantile spasms and other neurological disorders. There are several kinds of anticonvulsants depending upon mode of action. Lacosamide and carisbamate are two anticonvulsant drugs used for the treatment of epilepsy and infantile spasms, respectively. As the complete stability protocol of lacosamide and carisbamate are necessary for patients’ safety, these were selected in the present study.

\[
\begin{align*}
\text{Lacosamide} & \quad \text{Carisbamate} \\
\end{align*}
\]

ii. Pulmonary arterial hypertension (PAH) is a rare, chronic, and life-threatening disease. Treatment of PAH includes one of the three main pathways, which are endothelin (ET-1), prostacyclin, and nitric oxide (NO). Ambrisentan is an orally active endothelin type-A (ETA) selective ERA approved for the
treatment of PAH. As the complete stability protocol of ambrisentan is necessary for patients’ safety, it was selected in the present study.

![Ambrisentan](image1)

Ambrisentan

iii. Rivaroxaban, an anticoagulant, is the first and only oral selective Factor Xa (FXa) inhibitor used for the treatment of the versatility of multiple indications includes deep vein thrombosis (DVT), pulmonary embolism (PE). As the complete stability protocol of rivaroxaban is necessary for patients’ safety, it was selected in the present study.

![Rivaroxaban](image2)

Rivaroxaban

iv. Chirality plays an important role because the enantiomers exhibit different pharmacological and toxicological properties in living systems. Chiral chromatography is an important analytical tool for separation and determination of inactive/active enantiomers present in enantiomerically pure drugs and finished products. Pramipexole hydrochloride, a non-ergot selective dopamine receptor agonist, used to reduce the depressive symptoms in patients with Parkinson's disease was selected for present work.

![Pramipexole](image3)

Pramipexole
1.10 Aims and Objectives

1. To develop and validate a selective stability indicating RP-HPLC method for determination of process-related impurities as well as assay of an anti-epileptic drug lacosamide. The work involves characterization of the degradation products by using ESI-MS/MS, LC-ESI-MS/MS, HRMS, and NMR. The work also involves isolation and confirmation of isomer of drug formed during degradation.

2. To develop and validate a selective stability indicating RP-HPLC method for determination of process-related impurities as well as degradation products and assay of an anticonvulsant drug carisbamate. The work involves forced degradation studies of carisbamate, synthesis, isolation and characterization of degradation products and process impurities using modern spectroscopic techniques viz., ESI-MS/MS, HRMS, and NMR. The work also involves the confirmation of formation of isomer of drug during degradation.

3. To develop and validate a selective stability indicating RP-HPLC method for the determination of process related impurities as well as assay of an antihypertensive drug ambrisentan. The work involves characterization of degradation products by LC-ESI-MS/MS and HRMS.

4. To develop and validate a selective stability indicating RP-HPLC method for the determination of process related impurity as well as assay of an anticoagulant rivaroxaban. The work involves characterization of the degradation products by using LC-ESI-MS/MS, and HRMS. The work also involves isolation and characterization of process related impurity of rivaroxaban by NMR and mass spectrometry.

5. To develop and validate a normal phase LC method for enantioselective resolution of pramipexole. The work involves the resolution of pramipexole enantiomers in the presence of process related impurities by using a polysaccharide based chiral stationary phase.
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