Chapter 1

Introduction

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1.1. Background of impurity profiling

Human civilization has been experimenting and consuming drugs for many centuries, but it is only in last century that enormous systematic research has been done on development of drugs. The average life span of human is increased due to use of drugs to cure diseases. Today a majority of the drugs used are of synthetic origin. These are produced in bulk and formulated into convenient dosage forms for their therapeutic use. These formulations should deliver the drug substances in a stable, non-toxic and acceptable form, ensuring its bioavailability and therapeutic activity [1].

Safety and efficacy of pharmaceutical substances are two fundamental issues in drug therapy. The safety of a drug is determined by its pharmacological or toxicological profile as well as the adverse effects caused by the impurities in bulk and dosage forms. The drug should be safe, i.e. it should have acceptably low risk of adverse effects with doses of drug which provide the desired therapeutic effects. The impurities in drugs often possess unwanted pharmacological or toxicological effects by which any benefit from their administration may be outweighed. Thus the quality of the drug is directly related to safety. The quality and safety of a drug is generally assured by monitoring it using suitable analytical techniques and controlling the impurities effectively. Thus, the analytical activities concerning impurities in drugs are among the most important issues in modern pharmaceutical analysis [2].

An impurity as defined by International Conference on Harmonization (ICH) guidelines as “Any component of the medicinal product which is not the chemical entity defined as the active substance or an excipient in the product”, while impurity profiling is considered to be the analytical activities with the aim of detecting, identifying or elucidating the structure and quantitatively determining impurities in bulk drugs and pharmaceutical formulations [3].
Impurities have been named differently by various groups of scientists who deal with them. According to ICH guidelines, impurities can be broadly classified into the following three categories for the drug substance as given in Table 1.1

### Table 1.1. Classification of impurities

<table>
<thead>
<tr>
<th>Impurity</th>
<th>Origin</th>
<th>Example</th>
</tr>
</thead>
</table>
| Organic              | • Chemical process and/or storage of the API.  
                        • Volatile/ Non volatile                                              | • Starting materials  
                        • By-products  
                        • Intermediates  
                        • Degradation products                                               |
| Inorganic            | • Chemical process, Processing equipment, filter aids  
                        • Nonvolatile                                                       | • Reagents, ligands, catalysts  
                        • Heavy metals  
                        • Inorganic salts  
                        • Other materials e.g. filter aids, charcoal                       |
| Residual solvents    | • Solvents used during chemical reaction, crystallization, purification steps  
                        • Inorganic or Organic liquid                                         | • Benzene, CCl₄,  
                        • Acetonitrile, Chloroform, Methanol  
                        • Acetone, Ethanol                                                     |

### 1.2. Sources of impurities

Impurities can originate from various sources and accordingly they are classified as follows

#### 1.2.1. Process related impurities

Impurities in a drug substance or a new chemical entity (NCE) originate mainly during the manufacture of the drug substance from raw materials, solvents, intermediates, and by-products. The raw materials used for synthesis of API are generally of low purity and hence in turn affects the purity of the drug substance. Also chemist prefers doing one pot reaction in which the intermediates are not isolated as it is economical, easy and time saving but this increases the impurities in final product. Majority of the impurities are characteristic of the synthetic route of the manufacturing process. Since there are several possibilities of
synthesizing a drug, it is possible that the same product synthesized by different route may give rise to different impurities [4].

1.2.2. Degradation related impurities

Degradation product is a molecule resulting from a change in a drug substance brought about over time. Thus the degradants are the breakdown components of the drug substance formed during storage. Degradants may be chemically identical to process related impurities but the major difference is that degradants level may increase during storage while that of process impurities remain constant. So it is necessary to carry out stability studies to predict, evaluate and ensure drug product safety. The stability studies under various exaggerated conditions of temperature, humidity and light can help us to determine what potential impurities can be produced by degradation reactions. This stress study will help to optimize the storage conditions to avoid the formation of degradation products [5].

1.2.3. Formulation related impurities

A number of impurities in a drug product can also arise out of interactions with excipients that are used to formulate a drug product. Excipient related impurities are a major concern for low dose formulations specifically tablets as these contains more amount of excipient as compared to high dose formulations, hence it is important to identify non drug related impurities and is carried out by conducting stability studies[6].

1.2.4. Polymorphic impurity

Polymorphism is the term used to denote crystal systems where a substance can exist in different crystal packing arrangements, all of which have the same elemental composition. Different physical forms of a drug substance can display radically different solubilities, which affects the dissolution and bioavailability characteristics of the compound. The physical stability of polymorphs is also crucial. During various processing steps (grinding, mixing, tablet processing and so forth), the physical form of the drug substance may be compromised, subsequently leading to dissolution problems. Therefore the full characterization of polymorphic systems is critical to numerous groups within commercial drug development [7].
Ritonavir is Abbott's novel protease inhibitor, for human immunodeficiency virus (HIV), the causative organism of acquired immunodeficiency syndrome (AIDS). It is marketed as Norvir. Two years after the launch of Norvir to the market, some lots of Norvir capsules failed a dissolution specification. Investigation of this phenomena revealed the existence of a crystal form of ritonavir other than the one already known (Form I). This new crystal form was designated as Form II. The Form II was having less than 50% solubility as Form I resulting in poor dissolution behavior and eventually led to withdrawal of capsule from the market [8].

1.2.5. Enantiomeric impurity

Most of the chiral drugs of synthetic origin are racemates and in many cases one enantiomer may be less active, toxic in nature or may show totally different activity. Therefore many manufactures prefer the pure enantiomers over the racemates. When a pure enantiomer is used as drug then the other enantiomer is considered as an impurity. A famous case related to chiral impurity was thalidomide. This drug was approved as sedative in US during 1960s. The drug was prescribed to reduce morning sickness in pregnant women. The drug was withdrawn from market because of teratogenicity as nearly 8000 babies born with birth defect. Afterwards it was found that the R-isomer is sedative while that of S- isomer is having teratogenic property as shown Fig 1.1 [9].

![Fig.1.1 Thalidomide enantiomer](image)

1.3. Regulatory perspective of impurities

In the year 1990, International Conference on Harmonization (ICH), was established with the joint efforts of regulatory bodies and industry representatives of United States, Japan and
European Union with the main aim of harmonizing the efforts of registration agencies and pharmaceutical manufacturers organizations to improve the quality, safety and efficacy of drug therapy. The ICH ensures that the different countries have consistent requirement for the data that should be submitted to various regulatory agencies which obviate the need to duplicate the testing carried out during the research and development of new medicines required for product registration. It leads to a more economical use of human, animal, material resources and avoids the unnecessary delay in the availability of new medicines [10].

ICH has defined certain terminologies as below:

**Impurity profile**
- A description of identified and unidentified impurities

**Identified impurity**
- An impurity for which structural characterization has been achieved.

**Reporting threshold**
- It is the level above which an impurity reported to regulatory agencies to alert them to the presence of a specified impurity

**Identification threshold**
- A limit above which an impurity should be identified.

**Qualification of impurity**
- The process of acquiring and evaluating data that establishes the biological safety of an individual impurity.

**Qualification threshold**
- It is defined as the level above which specified impurity must be subjected to toxicological testing to demonstrate safety.

ICH has given some threshold limits for reporting, identification and qualification of impurities in drug substance and drug products as follows (Table 1.2) [11].
Table 1.2 ICH Reporting, Identification and Qualification threshold of impurities*

<table>
<thead>
<tr>
<th>Maximum daily dose</th>
<th>Reporting</th>
<th>Identification</th>
<th>Qualification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug substance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 2g</td>
<td>0.05%</td>
<td>0.10% or 1mg/day intake</td>
<td>0.15% or 1mg/day intake</td>
</tr>
<tr>
<td>&gt; 2gm</td>
<td>0.03%</td>
<td>0.05%</td>
<td>0.05%</td>
</tr>
<tr>
<td>Drug product</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 1 g</td>
<td>0.1%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 1g</td>
<td>0.05%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 1mg</td>
<td></td>
<td>1% or 5mg/day intake</td>
<td>1% or 50mg/day intake</td>
</tr>
<tr>
<td>1-10mg</td>
<td></td>
<td>0.5% or 20mg/day intake</td>
<td>1% or 50mg/day intake</td>
</tr>
<tr>
<td>10-100mg</td>
<td></td>
<td>0.2% or 2mg/day intake</td>
<td>0.5% or 200mg/day intake</td>
</tr>
<tr>
<td>100mg-2g</td>
<td></td>
<td>0.2% or 2mg/day intake</td>
<td>0.2% or 2mg/day intake</td>
</tr>
<tr>
<td>&gt; 2g</td>
<td></td>
<td>0.1%</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

* The table taken from the book- “Handbook of modern pharmaceutical analysis” by Satinder Ahuja

1.4. Role of impurity profiling

1.4.1. Impurity profiling in synthetic drug research

In the area of synthetic research, compounds are generally synthesized in small scale and only after the preliminary screening for activity the role of impurity profiling begins for selected molecules which have shown good activity. While reporting the impurities the organic chemist has to optimize the synthesis and purification of the substances so that these can be scaled up to prepare drug for formulation and for toxicological, preclinical and clinical trials. It is important to mention that at this stage of development, it is not necessary to identify the impurities, however it should be assured that same impurities are found in and same limit apply to all batches used for the trials [12].

1.4.2. Impurity profiling in production of bulk drugs

After the introduction of new active pharmaceutical ingredient (API), it has to be synthesized in bulk for formulation. It is very important that there should not be any new impurities appear during the scale up procedure and quantity of impurities in bulk drug material identified during the R&D phase remains below specification limit. It can happen that a new...
impurity appears during the scale up or the quantity of an impurity detected but not identified previously may exceed the threshold limit. Then it is mandatory to characterize that impurity so that an organic chemist make necessary step to avoid the formation of that particular impurity above threshold limit. Production of bulk drug should be done in strictly controlled conditions because a minor change in conditions can result in considerable changes in impurity profile. It is also important to estimate impurities in key intermediates in synthesis so that the origin of the new impurity in multistep synthesis can be rapidly identified [13].

1.4.3. Impurity profiling in Formulation and Development (F&D)

F&D people should have the idea of impurity profiling of bulk drug material used for development of formulation, which helps them in differentiating synthesis related impurities and degradation products. Stability studies are carried out to identify the degradation products from synthesis related impurities the quantity of degradation products increases over time while that of synthesis related impurities remain constant. These investigations also help the pharmaceutical technologist in preformulation studies. The preliminary stability studies reveal the degree of susceptibility of the molecule to different environmental conditions like heat, light, humidity, acidic, basic or oxidative condition. On the basis of these studies some of the potential degradation products in the drug formulation can be predicted. These studies will also help in identifying the possible interaction between the drug substance and the excipients [14].

1.4.4. Impurity profiling in drug registration

The manufacturers of the pharmaceutical substances have to submit a drug master file (DMF) to the regulatory authorities. The document provides the detailed information about the facilities; process used in the manufacture, packaging and storage conditions of the drug. It also contains profile of impurities along with the acceptance criteria for impurities. The main objective of DMF is to support regulatory requirements and to prove the quality, safety and efficacy of the drug product in order to obtain Investigational New Drug Application (IND), New Drug Application (NDA) and Abbreviated New Drug Application (ANDA). In the area of impurities, through the efforts of ICH and US-FDA a tremendous amount of information is available. The comparison of impurities in several batches of same product from same
manufacturer provides us a good indication for consistency in the manufacturing process while comparison with other manufacture of same drug leads us to the differences in the purity. The impurity profiling also gives the clue about the synthetic route used by different companies as some impurities are specific to the synthetic pathways [15].

Stability of active pharmaceutical ingredient (API) and drug product is also very important criteria as it is directly related to efficacy as well as safety of drug therapy. Forced degradation studies (also called stress degradation study) are performed to gain better understanding of API and drug product stability. It will help to identify the potential degradation products which, in turn, can help to establish the degradation pathway and intrinsic stability of the molecule. Thus stress testing is a predictive research tool used to identify stability of drug molecule and provide base for developing stability indicating method. Stress studies are carried out under various exaggerated conditions of temperature, humidity and light. This is explained in next section thoroughly.

1.5. Stress degradation study

Stress testing has long been recognized as an important part of the drug development process. Recent efforts by the International Conference on Harmonization (ICH) along with the pharmaceutical industry to reduce the time and cost that it takes to get products to market has made stress study as an important tool to predict the drug stability.

Previously there was confusion between accelerated stability study and stress degradation study which was then cleared in ICH guidelines. According to ICH guidelines accelerated stability study is designed to increase the rate of chemical degradation or physical change of an active drug substance or drug product using exaggerated storage conditions as part of the formal, definitive, storage program, while stress testing intended to “determine the intrinsic stability of the molecule by establishing degradation pathways in order to identify the likely degradation products and to validate the stability indicating power of the analytical procedure” [16].

Thus stress degradation study often termed as forced degradation study can be differentiated by the focus of study and the severity of conditions used from that of accelerated stability study. As this study is an investigation of intrinsic stability characteristics of the molecule,
provides the foundation for developing and validating analytical method and for developing a stable formulation. Stress testing studies are intended to discover stability issues, and are therefore predictive in nature which will affect different areas as follows

- Analytical methods development
- Formulation and package development
- Appropriate storage conditions and shelf-life determination
- Safety/toxicological concerns
- Manufacturing/processing parameters

1.5.1. Analytical method development

The purpose of stability studies is to monitor possible changes that occur in a product or material over a period of time and at different storage conditions. Therefore it is expected that all analytical methods applied in the study should be stability-indicating. A major challenge in developing a stability-indicating analytical method (SIAM) is the access to suitable degraded samples to aid in method development. As the stability depends on different parameters like processing conditions, quality of excipients used and different environmental conditions like heat, humidity and temperature, this is the reason why pharmaceutical chemists have to rely on forced degradation samples to develop SIAMs. The ability of forced degradation studies to forecast real-time degradation make this study as an important tool in analytical method development of trace analysis.

1.5.2. Formulation and packaging development

Well-designed stress-testing studies can determine the susceptibility of a compound to hydrolysis, oxidation, photochemical and thermal degradation. This information is then taken into consideration when developing the formulation and determining the appropriate packaging. For example if a compound is sensitive to hydrolysis, instead of wet granulation dry granulation is preferred and packaging can be developed such that protects from water vapor transmission from the outside of the package to the inside of the package to ensure long-term storage stability.
1.5.3. Storage conditions

Forced degradation studies will give us the adequate information that may be helpful to determine appropriate storage conditions. But the shelf life determination can only concluded by carrying the long term stability study.

1.5.4. Safety/ toxicological concern

Although much attention has been given to genotoxic impurities or potential genotoxic impurities arising from drug synthesis in the literature in recent years, genotoxic degradants have received less attention. In fact it requires special consideration since there is no opportunity for purification and their presence needs to be considered over the entire shelf life of the product. The fact that genotoxic or potentially genotoxic impurities can arise from degradation of the active ingredient in both the drug substance as well as drug product adds to the complexity compared with the control of process impurities. A systematic approach to the prediction of potential genotoxic degradants should be a valuable tool in support of effective and efficient drug development. If stress-testing studies indicate the formation of known toxic compound, steps can be taken early on to inhibit the formation of the toxic compound and to develop sensitive analytical methods to accurately detect and quantify [17].

1.5.5. Manufacturing/Processing parameters

There is a possibility of drug degradation during the manufacturing or processing step due to variety of conditions applied during the stage. Knowing the parameters that leads to degradation can help to design appropriate conditions which will reduce the probability of degradation. For example, if a compound is susceptible to degradation at low pH, then either the manufacturing steps under low pH conditions can be avoided or the time duration can be more carefully controlled to minimize the degradation.

1.6. Forced degradation timing and strategy

A novel drug candidate which enters in market had to pass through different stages of development like drug discovery, preclinical, clinical and finally market. In all this phases stress testing is a necessary with different goal.
1.6.1. Drug discovery stage

The main aim of stress study at this stage is to provide necessary information about the stability of new drug candidate. This information will help the discovery unit to design a compound with good stability and desired activity. Stress study at this stage is not very thoroughly designed hence many times degradation prediction software like CAMEO are used to save the time to carry such study.

1.6.2. Phases 1 and 2

The primary goal of stress study at this stage is development of stability indicating analytical method which should be specific to the compound of interest and should separate the desired compound from degradation products and impurities. Isolation and characterization of degradation products is rarely needed at this phases with the exception for degradation products that are suspected to be very potent. If the molecule is chiral and predominately one stereoisomer, the stereochemical stability of the API should be examined in the degraded samples [18].

The guidance recommends that drug products be stressed photolytically at phase 2. Scientifically, solid dosage forms should be stressed with heat, heat and high humidity, and with light. Liquid dosage forms should be stressed with heat, light, and possibly at different pH if the product contains water and is unbuffered. Stressed drug products should be examined for reactions between the API and excipients using hyphenated techniques. Clear differentiation between drug and non-drug related peaks should be made. Stressing placebo and product concurrently followed by comparison of the respective profiles can facilitate this determination. Degradation studies at this stage can readily afford insight for development of more stable formulations.

1.6.3. Phase 3

The FDA guidance states that stress studies should be conducted during phase 3 to demonstrate the inherent stability of the drug substance, potential degradation pathways, and the capability and suitability of the proposed analytical procedures. The stress studies should assess the stability of the drug substance in different pH solutions, in the presence of oxygen,
light and at elevated temperatures and humidity levels. The results should be summarized and submitted in an annual report.

With regard to the drug product studies during phase 3, the guidance states that, for certain drug products, one-time stress testing can be warranted to assess the potential for changes in the physical (e.g., phase separation, precipitation, aggregation, changes in particular size distribution) and/or chemical (e.g., degradation and/or interaction of components) characteristics of the drug product. The studies could include testing to assess the effect of high temperature, humidity, oxidation, photolysis and/or thermal cycling. The relevant data should be provided in an annual report.

1.6.4. New drug application (NDA)

There are possibilities of some changes in production of drug substance or in drug product after drug registration to reduce cost and to increase drug quality. Also sometimes there may be some changes in dosage form, dosage strength or in route of drug administration. So stress testing is necessary to detect any new impurities or degradation products.

Significant degradation products observed in drug products should also be isolated and characterized.

1.7. Experimental approach

Though the FDA and ICH guideline provides useful information and general comments about stress study it provides little information about strategies and principles for conducting the study. That’s why the protocol for conducting stress study varies tremendously in many pharmaceutical companies and they have their own internal guidelines which are based on the data and experience accumulated by skilled scientific persons [19].

1.7.1. General strategy for drug substance

One of the goals of forced degradation studies is to efficiently produce samples that contain the degradation products likely to be formed during manufacture, handling, and normal storage of the drug substance and drug product. Severity of stress conditions must be balanced with generation of realistic and representative impurity profiles. Overstressing a molecule can
lead to degradation profiles that are not representative of real storage conditions and perhaps not relevant to method development. Understressing, on the other hand, may fail to generate important degradation products. Therefore, stress-testing conditions should be realistic and not excessive. The extent of degradation targeted should be approximately 5-20%. Initial stress conditions can often be selected by considering the conditions used in previous studies of related compounds. For this reason, it is advisable to compile the quantitative and qualitative results of previous degradation studies in a substructure searchable database. In the absence of previous studies, a good rule of thumb is to start gentle and intensify the stress as required.

Forced degradation studies of API and formulations includes appropriate solid and solution state stress conditions like hydrolysis, thermal, oxidative and light exposure.

1.7.1.1. Hydrolytic degradation

As water is present in many drugs, excipients as well as in atmosphere, it is the most common degradation reaction. Hydrolysis reactions are generally acid and base catalyzed hence acid, base and neutral conditions are employed to carry out hydrolysis study. Acid hydrolysis is carried out using 0.1 to 1 M HCl or H₂SO₄. Some compounds which are insoluble in acidic media are made soluble using appropriate co-solvents like dimethyl sulfoxide, propionic acid and acetic acid. Methanol should be avoided in acidic conditions, specifically in compounds that contain carboxylic acid, ester or amide functional groups as methanol reacts with these groups [20]. Additionally sample may be heated to accelerate the degradation. It is recommended not to exceed the temperature beyond 70°C as there is possibility of deviation from real time stability. Neutral hydrolysis is generally carried out in phosphate buffer of pH 7.

1.7.1.2. Oxidative degradation

Oxidation is a common pathway for drug degradation in solid and liquid formulations. This generally occurs by 3 main mechanisms

- Autoxidation
- Nucleophilic/electrophilic process
Electron transfer process

Autoxidation is a radical initiated reaction which is a chain process, start with initiation phase followed by propagation phase and finally termination phase. AIBN (azabisisobutyronitrile) is generally used as free radical initiator. It is taken in molar ratio along with the drug in the range of 0.2-1. As this AIBN is not water soluble use of organic solvents like acetonitrile is advisable. The reaction is generally carried out at 40°C to avoid formation of secondary degradants [21].

The second major pathway by which oxidation occurs is electrophilic attack and nucleophilic addition, mediated by peroxides. Peroxides may present in many excipients (polyethylene glycol, povidone, polysorbates and hydroxypropyl cellulose) and its level may increase over time due to oxidation. Hence deliberate exposure to peroxides is done using hydrogen peroxide in 0.3% to 30% range. The reaction is carried out at room temperature because at high temperature there is risk of formation of highly reactive hydroxyl radical which may give rise to unrealistic degradants. The compounds having functional groups like amines, thiols, thioethers, pyrroles, and indoles are susceptible for oxidation and readily oxidized by peroxides.

The oxidative susceptibility of drug can also be evaluated by using transition metals like copper (II) and iron (III). These transition metals bind to the drug molecule and causes electron transfer where the oxidation state of metal get reduced and the drug molecule becomes an unstable radical cation, which further reacts with molecular oxygen to give oxidative degradation products.

1.7.1.3. Thermal degradation

Thermal degradation is carried out in solid state condition where the compound is exposed to high temperature sufficient enough to induce bond breakage. Thermal degradation follows Arrhenius equation which states that a 10°C increase in temperature doubles the rate of reaction. Hence the effect of temperature should be studied by 10°C increment (50°C, 60°C, 70°C etc). Humidity factor can also be included in thermal study. The sample is generally kept in humidity oven at 70°C/30% humidity and at 70°C/75% humidity. If humidity oven is not
available saturated NaCl solution is used to obtain 75% humidity at 70\(^\circ\) C and saturated MgCl\(_2\) is used to obtain 30% humidity at 70\(^\circ\) C.

1.7.1.4. Photolytic degradation

The pharmaceutical products may get exposed to different source of light like direct sunlight to a variety of artificial sunlight of which UV-Visible radiations are most potentially damaging. Hence the Photostability of drug substances and drug products should be evaluated to demonstrate that there is no loss of potency due to photodecomposition. There are two types of studies performed stress testing and confirmatory testing. Purposeful degradation study is performed to check the overall photosensitivity of drug molecule and to lay down its degradation pathway, while confirmatory study is performed to provide necessary information on handling, packaging and labeling. Photodegradation study could be performed by exposing samples to natural sunlight, but the problem with this is that the intensity of sunlight varies according to weather, latitude, time of day and season of year. To bring uniformity in the study ICH has given guidelines with systematic approach to study.

According to ICH guidelines any light source that is designed to produce an output similar to the D65/ID65 emission standard such as an artificial daylight fluorescent lamp combining visible and ultraviolet (UV) outputs, xenon, or metal halide lamp is used. D65 is the internationally recognized standard for outdoor daylight as defined in ISO 10977 (1993). ID65 is the equivalent indoor indirect daylight standard. For a light source emitting significant radiation below 320 nm, an appropriate filter(s) may be fitted to eliminate such radiation. Another option is that the same sample should be exposed to both the cool white fluorescent and near ultraviolet lamp. A cool white fluorescent lamp designed to produce an output similar to that specified in ISO 10977 (1993); and a near UV fluorescent lamp having a spectral distribution from 320 nm to 400 nm with a maximum energy emission between 350 nm and 370 nm; a significant portion of the UV should be in both bands of 320 to 360 nm and 360 to 400 nm. The sample must be exposed to both sources; however, the exposure can be in a sequential or simultaneous set-up [22].

ICH guidelines specify an exposure of 200 watt h/m\(^2\) for ultraviolet light confirmatory testing (1× ICH). Recommended stress conditions and time points to be tested are 5× and 10×
ICH for solid drug substances while an exposure of $1.2 \times 10^6$ lux hours for fluorescence (1× ICH). Recommended stress conditions and time points to be tested are 5× and 10× ICH for solid drug substances.

1.7.2 General strategy for drug product

As drug products contain various excipients, it is necessary to conduct stress stability studies on formulation to check the degradation due to drug-excipient interaction. This will in turn help to determine the physical and chemical compatibility of drug substance with excipients. The experiments are prone to vary depending upon the type of formulation. For solid dosage forms, the interaction between drug substance and water is critical; hence, thermal/humidity study is necessary, while for liquid dosage forms, hydrolysis, thermal, oxidative, and photolytic stress studies should be performed, giving more emphasis on hydrolysis. Semisolid dosage forms contain oil excipients; hence, more prone to oxidative degradation by autoxidation. Hence, oxidative degradation is studied carefully.

The following (table 1.3) explains the experimental details that should be performed on drug substance and various drug products.

Table 1.3 General protocol for forced degradation study of drug substance and drug product*

<table>
<thead>
<tr>
<th>Condition</th>
<th>Drug substance</th>
<th>Drug product</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Solid</td>
<td>Solution</td>
</tr>
<tr>
<td>Acid/Base</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Oxidative</td>
<td>×</td>
<td>✓</td>
</tr>
<tr>
<td>Photostability</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>Thermal</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Thermal/Humidity</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

✓ = recommended; X = optional, suggested for some compounds.

* The table taken from the book “Handbook of modern pharmaceutical analysis” Satinder Ahuja

1.8. Separation techniques

The estimation of organic impurities of the three types of impurities (inorganic impurities and residual solvents) is the most interesting and challenging task. Following are the different techniques by which the organic impurities and degradation products can be separated [23].
Capillary electrophoresis
- Gas chromatography
- High performance liquid chromatography (HPLC)
- Supercritical fluid chromatography
- Thin layer chromatography

The technique used will depend upon the nature of the sample and complexity of the separation. The chromatographic procedure used should be such that it resolves all the impurities and drug substance and allows accurate measurement of impurities.

Although capillary electrophoresis is electrophoretic method it is usually associated with chromatographic methods as it have some common requirements. This is a useful technique where very low quantity of sample available and high resolution is required.

Gas chromatography is one of the useful chromatographic techniques for separation and quantification of impurities in drug material, especially for volatile samples. This technique is practically useful for determination of organic volatile impurities.

Supercritical fluid chromatography is a form of normal phase separation that is used for the analysis and purification of low to moderate molecular weight compounds. But in recent times it has gained popularity for isolation of impurities. The main advantage of this method is use of supercritical CO$_2$ as mobile phase which facilitates faster analysis time and greater productivity. It is a low cost solvent and inert which reduces chemist exposure to harmful organic solvents.

HPLC is the most common technique used for detection of impurities because of its versatility. Availability of different columns like normal phase, reverse phase with different length, diameter and particle size ensures the identification and quantitation of trace level of impurities. The UV-Visible absorbance detectors are most commonly used because most of the pharmaceuticals have UV absorbance. With the advancement in detector technology the use of photo diode array detector, which provides the UV spectra of eluting peak while functioning as a multi wavelength. One of the advantages of PDA detector is peak purity evaluation. It compares the spectra at upslope, apex and downslope of peak and gives limited assurance that no trace of impurity is coeluting with the main peak.
UV/Visible absorbance detector is the choice for impurity estimation. Multiple detectors in series can also be used to deduce more information from single chromatographic run. Some compounds which do not have any UV absorbance, charged aerosol detector (CAD) evaporative light scattering detector (ELSD) and mass spectrometric detectors are mostly useful [24]. Gradient elution is generally preferred over isocratic elution which allows all the impurities get detected and increases the chances of resolution between drug and impurities.

1.9. Isolation techniques

To enable the complete characterization of impurities or degradants and to carry out the toxicity studies one needs to isolate it. Several chromatographic as well as non chromatographic methods are available. Few important methods routinely used are as follows

- Solid phase extraction
- Liquid- liquid extraction
- Supercritical fluid extraction
- Column chromatography
- Thin layer chromatography
- Preparative HPLC

The first three methods named above are although easy and time saving, they cannot isolate the impurities from a complex mixture. For this the chromatographic methods are the first choice.

TLC is an ideal technique of isolation because of its simplicity and less time required to develop it. For isolation band of sample is applied onto the plate then plate is developed with suitable solvent, the band of interest is scraped and the impurity is extracted from silica with an appropriate solvent. One of the disadvantages of preparative TLC is that only limited quantity of material can be isolated sufficient enough for LC-MS analysis but not for NMR analysis.

Column chromatography is very useful for isolation of gram quantity of material but not so useful when high resolution is required.
Preparative HPLC is most useful method for isolation of impurities and or degradants because of its resolving power. Before going to preparative scale one needs to develop and optimize method to maximize resolution on analytical HPLC and the scale up to preparative mode. This will determine the type of column, flow rate, mobile phase and sample loading capacity of the selected column. During scale up two important factors is loading capacity and flow rate which can be determined by following formula.

\[ M_p = M_a \times \frac{(D_p)^2 \times L_p}{(D_a)^2 \times L_a} \]

Where \( M_p \) is the maximum mass load on the preparative column and \( M_a \) is the maximum mass load on the analytical column, \( D_p \) is diameter of the preparative column, \( D_a \) is diameter of the analytical column, \( L_p \) is length of the preparative column, and \( L_a \) is length of the analytical column.

To maintain the same resolution when scaling-up a method, the flow rate also needs to be scaled proportionally. The preparative flow rate can be estimated by using formula

\[ F_p = F_a \times \frac{(D_p)^2 \times L_p}{(D_a)^2 \times L_a} \]

Where \( F_p \) is the preparative flow rate and \( F_a \) is the analytical flow rate.

### 1.10. Spectroscopic techniques

The following are the different spectroscopic techniques used for the characterization of impurities.

- Ultraviolet spectroscopy (UV)
- Infrared spectroscopy
- Mass spectroscopy
- Nuclear magnetic resonance (NMR)

With the advances in the techniques of mass and NMR spectroscopy, use of the UV spectroscopy in structure elucidation was decreased but with introduction of photo diode array
detector created entirely new possibilities in this field. The advantage of PDA detector over conventional UV detector is the rapid scanning of the entire UV-visible range simultaneously. The high sensitivity of PDA detectors made it useful to identify impurities at 0.01-0.02 % level. The determination of peak homogeneity i.e. peak purity by PDA detector helps to identify any coeluting impurity with the parent drug. Sometimes the small difference between impurity spectra and main component could be a diagnostic tool to characterize that impurity if the chemistry of the procedure and chromatographic behavior is carefully studied.

IR spectroscopy is also an important tool which gives valuable information about the functional groups present in the impurity, as well as we can make out that specific functional transformation took place.

As sensitivity is the most important parameter for determination of trace elements, Mass spectrometry has become a very important tool in characterization of impurities. With new developments in ionization methods like electron spray ionization (ESI), atmospheric pressure chemical ionization (APCI) enable to identify the molecular weight of impurities at pictogram level. By coupling chromatography with mass spectroscopy characterization of each peak from complex mixture is possible as resolving power of chromatography separates mixture into individual peak and mass spectroscopy identifies the mass of each separated peak. Further with the use of tandem mass spectrometry (MS/MS) the structure can be established by studying its fragmentation pattern. A component of mixture is separated by selecting a single ion at one specific m/z ratio by first analyzer and the fragments of this parent ion are monitored by second analyzer. This avoids the laborious isolation of trace impurity by preparative chromatography. The use of high resolution mass spectrometry gives the exact molecular weight up to 4 digits thus aids in molecular formula determination.

NMR spectroscopy is currently the most reliable, powerful and fast technique available for structural elucidation of API as well as impurities, degradants and metabolites. For characterization of degradants and metabolites generally $^1$H-NMR is sufficient to relate the unknown with the parent molecule but in case of process related impurities $^{13}$C-NMR is needed to obtain sufficient information on C-H connectivity data and provides good resolution of closely placed signals [25]. As the impurities, degradants and metabolites are present in trace quantities it represents one of the greatest challenges in NMR spectroscopy.
With the recent advances in techniques like high field strength, coupling of LC with NMR helped to characterize that at very low sample amount [26].

The general scheme of impurity profiling described in Fig. 1.2.

Fig. 1.2 Impurity profiling scheme

The figure taken from the book- “Analysis of drug impurities” by R.J. Smith and M.L. Webb
1.11. Qualification of impurity

Qualification is the process of acquiring and evaluating data that establishes the biological safety of an individual impurity or a given impurity profile at the level(s) being considered. When appropriate, it is recommended that one should provide a rationale for establishing impurity acceptance criteria that includes safety considerations [27].

An impurity is considered to be qualified when it meets one or more of the following conditions:

- When the observed level and proposed acceptance criterion for the impurity do not exceed the level observed in an FDA approved human drug product.
- When the impurity is a significant metabolite of the drug substance.
- When the observed level and the proposed acceptance criterion for the impurity are adequately justified by the scientific literature.
- When the observed level and proposed acceptance criterion for the impurity do not exceed the level that has been adequately evaluated in comparative in vitro genotoxicity studies.

Genotoxic compounds have ability to induce genetic mutation or chromosomal aberration causing damage to DNA which may contribute to cancer. Although there are few pharmaceutical API having genotoxic and carcinogenic potential, specially used in cancer chemotherapy are still in use because of its risk to benefit ratio. It is not the case with impurities as it carries only risk and is not having any benefit from its administration [28]. ICH guidelines were considered sufficient for qualification of impurity, but the major concern was about unusually toxic impurities causing genotoxicity, mutagenicity and carcinotoxicity at level lower than the qualification threshold. To address this issue european medicine evaluation agency (EMEA) published guidelines in June 2006. The EMEA guideline recommends that any potentially genotoxic impurities (PGIs) in the drug substance should be identified, either from existing genotoxicity data or through the presence of structural alerts. On the basis of analysis of over 700 carcinogens in rodents it was found that a dose of 1.5μg/day is safe and unlikely to increase the lifetime cancer risk by more than 1 in million. This threshold limit is called threshold of toxicological concern (TTC) [29].
1.11.1. *in silico* toxicity prediction

Many drugs have been failed in clinical trials due to its unacceptable toxicity, causing expensive loss to the pharmaceutical companies. So there was an urgent need for techniques capable to identify the toxicity in early stage of product development. Computer based (*in silico*) techniques are particularly appealing for this purpose, because they are extremely fast and cost efficient and can be applied even without a physical availability of compound[30]. As there is more and more information is available on toxicity dataset, this information has been used to develop sophisticated QSAR software based systems to predict *in silico* genotoxicity. These expert systems generally use topographical, sub structural and electronic descriptors to predict the carcinogenicity, mutagenicity as well as acute and chronic toxicity. As in vivo toxicity testing are expensive, time consuming and increased pressure to reduce animal experiments has led to increased acceptance of these studies. The results of in silico studies are accepted by many regulatory authorities including USFDA for registration. The decision tree for qualification of genotoxic impurities is as shown in Fig 1.3.
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Fig. 1.3 Decision tree for qualification of genotoxic impurity

The figure taken from EMEA Guidelines for genotoxic impurities
1.12. Summary

Impurity profiling is an important part of drug development process which reduces the unnecessary delay in drug reaching to the market. ICH has issued guidelines regarding the threshold of impurities in drug substances and drug products. Any impurity, if present above the identification threshold should be characterized and if it is above qualification threshold the biological safety of that impurity should be evaluated.

Stress testing is an integral part of drug development which helps to identify the intrinsic stability of the drug to various environmental factors like temperature, heat and oxidation. The information obtained from these studies gives the prediction of potential degradation products likely to arise during storage, thus helps to maintain appropriate storage conditions.

Thus overall the impurity profiling plays a very important role in drug quality, safety and efficacy of pharmaceuticals to human health.