1. INTRODUCTION

1.1. Introduction to TB pathology

Bacteria of Tuberculosis are killing human beings and animals since year 1700s and years ago before them. During year 1700s it was known as "the white plague" as patient gets pale due to TB and during 1800s it was known as "Consumption", as large number of people died due to TB. Now TB can be named from affected organ e.g. pulmonary TB, lymph node or extra pulmonary TB. The bacteria causing TB *Mycobacterium tuberculosis* was identified by German scientist Dr. Robert Koch on March 24, 1882, therefore that day is declared as **"World TB day".** [1-11]

TB is contagious disease; it can be transferred from person to person or from animal to human by air transmission or by close contact with affected person or animal. A special ventilated room and good diet along with the medicines is necessary for TB patients.

1.1.1. Development for TB diagnosis, vaccine and treatment

Earlier there was no proper diagnostic method for TB; the first method introduced for diagnosing TB was TB skin test in 1907. In TB skin test small amount of tuberculin was inserted under the skin to see the response of body towards tuberculin. Fig. 1.1 shows microscopic image of TB bacterium *Mycobacterium tuberculosis.*

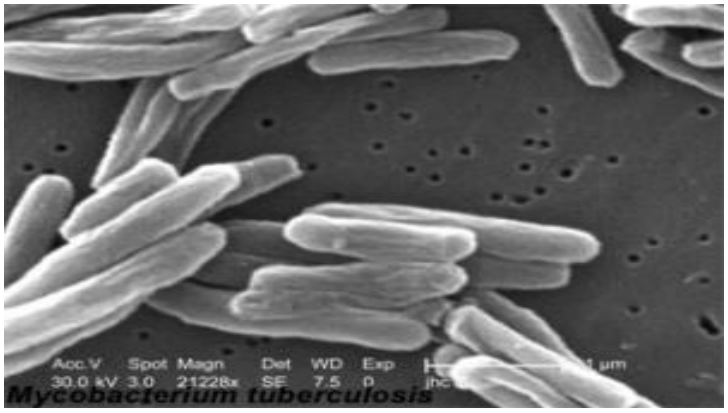


Figure 1.1.Microscopic image of *Mycobacterium tuberculosis* [12]

In 1908 needle and syringes were started to use for injecting tuberculin under the skin. In 1930 American scientist developed protein named purified protein derivatives for tuberculin for skin test and US government since 1940 using that derivative protein for TB skin test,

after that various tests were introduced but TB skin test is still a preferred test used along with chest X-ray and TB blood test(Interferon gamma release assay) [13].

TB vaccine is still under development area, a completely efective TB vaccine is not available in world but in some regions like India where TB is common; children are given BCG vaccine (as an alternative option) to prevent meningitis due to TB. Initially when antibiotics were not discovered, people with TB were cured by good food, good air and rest only, soon after that house hold remedies were came up for cure. Cod liver oil, massage of vinegar and inhalation of turpentine were accepted as common cure for TB. Streptomycins were introduced for TB treatment in year 1943, Waksman who discovered streptomycin later on rewarded with the Nobel Prize. During the years 1951-1966 more antibiotics were introduced for TB treatment, those were, Isoniazid, Pyrazinamide, Ethambutol and Rifampin.

TB can be cured by completing course of antibiotics, rest and healthy diet. Common therapy for TB is use of combination of antibiotics, e.g. Rifampicin and isoniazid along with streptomycin or ethambutol. This treatment is being given for eighty years and still working without any change in it. Patients may have developed resistance to these antibiotics so drug resistant TB required different treatment, another category of drugs for multi drug resistant TB (Delamanide and Bedaquiline), TB along with HIV. For TB patients DOT (directly observed therapy) campaign is carried out by governments so that internal or non internal failure to take medicines can be avoided [14, 15].

1.1.2. Global TB status- 2019

World Health organization (WHO) provides every year global TB status from Africa, America, Eastern Mediterranean, Europe, south-east Asia and western Pacific region. In 2019, approximately 10 million people fell ill with TB globally. Drug resistant TB cases are increasing which is threat to public health. In 2019 half million cases of multidrug resistant TB were recorded. Three countries had largest share in global data which are India (27%), China (14%) and Russian federation (3%). These countries are included in END TB by 2025 campaign by WHO. European Region is about to achieve goal END TB by 2020. [16]

1.1.3. TB Regulations

TB is a highly contagion disease it is necessary to control spreading of it. Worldwide World health organization (WHO) is working with national health governing body of each country to get control and end TB worldwide. Globally data for death from TB are estimated for each year and actual data are reported at global health observatory (GHO). General program of

work (GPW) is planned, from 2019-2023 thirteenth GPW is going on with plan to work against TB worldwide. WHO is working with United Nations (UN) and states of UN to fight against TB with involvement of non-state actors. Non state and state actors play vital role to make plan more effective. Collaboration with research institute and academies, UN volunteer and emergency partners also play lead road. [16]

1.1.4. Why is TB drug development important?

Ancient disease, TB is still killing thousands of people of developing countries. Novel treatment for TB is research area of interest till date. As the era changes TB bacteria also getting resistant to some antibiotics, so newer and stronger antibiotics are needed to be use for treatment. Most of ancient diseases are controlled or eliminated but controlling TB is even in today's high-tech era is difficult that is increasing the importance of drug development for TB.

1.1.5. Future Prospects

Antibiotics therapy, multidrug therapy and newer antibiotics (for treatment of multidrug resistance) are the treatment for TB. Vaccines are in pipeline so that TB risk can be reduced, new diagnosing techniques for simple and rapid diagnose of TB and therapy that reduce drug dosage course may be in reality we will see in future.

1.1.6. Classification of TB drugs [17-20]

Based on their chemical structures, antibiotics for TB treatment are classified by WHO as following;

Group A: Fluroquinolones:

Levofloxacin, Moxifloxacin and Gatifloxacin

Group B: Second line injectable agents:

Kanamycin,Amikacin and Capreomycin

Group C: Other core second line agents:

Ethionamide/Proethionamide, Cycloserine/Terizidone, Linezolide, Clofazimine

Group D: add on agents:

D1: Pyrazinamide, Ethambutol and High dose isoniazide

D2: Bedaquiline, Delamanide

D3: p-amino salicylic acid, Imipenam-cilastatin, Meropenam, Amoxiciline-clavulanate.

1.2. IMPURITY PROFILING

Impurity in universal way: any substance which is not of use or may be useful elsewhere but unwanted in particular substance is referred to as an impurity. Profiling means creating a short report on a broad topic. From pharmaceutical point of view, impurity is an additional or redundant substance those are found with active pharmaceutical ingredients (APIs). Impurity profiling means systemic detection, isolation and identification of possible/already present impurities. Quantification is additional analytical activity which may be preferred. Pharmaceutical industry is concerned with purity of substances; either it is a solvent, formulation or API, for that reason many regulatory authorities have issued guidelines on impurities in substance. While working with API it is essential to know the quality and quantity of impurities present in it. [21]

1.2.1. Definition and synonyms

According to International Conference for Harmonization (ICH) the globally accepted guidelines, for maintaining quality and safety of drug substance and drug product, an impurity is defined as "any component of medicinal product or new drug substance which is not the chemical entity defined as the active drug substance or an excipient in a drug product." [22] ***Impurity*** is an entity of the drug API (bulk material) or drug product (Marketed final product) that is not the chemical entity defined as the drug API, an excipient or any other chemical addition of drug product. Degradation product is also a type of an impurity.

ICH defines, ***Degradation products*** as a molecule resulting from a change in the drug substance brought about over time. For the purpose of stability testing of the products in this guidance, such changes could occur as a result of processing or storage (e.g hydrolysis, oxidation, and pyrolysis.) [23]

Common terms used for impurities in pharmaceutical industries are *by-products* (accidental compound generated in reaction with final product), *degradation products* (a product formed due to reaction between degradant and API or molecule of interest), *interaction products* (unwanted reaction between two entities present in formulation), *intermediates* (intended compound results from reaction and generated with product), *penultimate intermediates* (last

compound of synthetic chain that leads final product to be stable or generated), *related products* (impurities those are analogous or have akin biological activity of compound of interest or API) and *transformation products* (intended or unintended compound formed during reaction, term similar to *by product*).

U.S pharmacopeia uses different terminology for impurities, they are *Concomitant compound* (geometrical or optical isomers), *Foreign substance* (unintentionally added or adulteration or contamination), *ordinary impurities* (harmless substance present with compound of interest) , *Organic volatile impurities* (similar to residual solvent), *signal impurities* (these are similar to degradation product or by product, identification of them can help to solve reaction mechanism), *toxic impurities* (these impurities are very harmful even in small quantity).

ICH uses three main terminologies for impurities they are,

- *Organic impurities* (starting material, process related products, intermediates and degradation products).
- *Inorganic impurities* (salts, catalyst, ligands, heavy metals and residual metal)
- *Residual solvents* (organic and inorganic liquids used during reaction or purification or crystallization).

1.2.2. Regulatory requirements for Impurity study [24-27]

To control the impurities, experts from European Union (EU), Japan Union and The US Food and Drug administration (USFDA) have jointly prepared guidelines which can broadly cover aspects of impurity and its related studies. The guidelines are *the International Conference of Harmonization* (ICH). ICH covers almost all aspects of impurity profile and study. For impurity studies following guidelines are followed:

1. *ICH Quality guideline Q1A* deals with stability testing of new drug substance and products, Q3A and Q3B deals with impurities in new drug substance and new drug product respectively. Q3C deals with residual solvent.

2. *US-FDA guideline* of NDA (New drug application) and ANDA (abbreviated new drug application) deals with impurities. FDA generated an impurity guidance to submit New Drug Application (NDA) and Abbreviated New Drug Application (ANDA) in which it requires to submit impurity data along with applications so that review, interpretation and implementation of NDA and ANDA can be regulated.

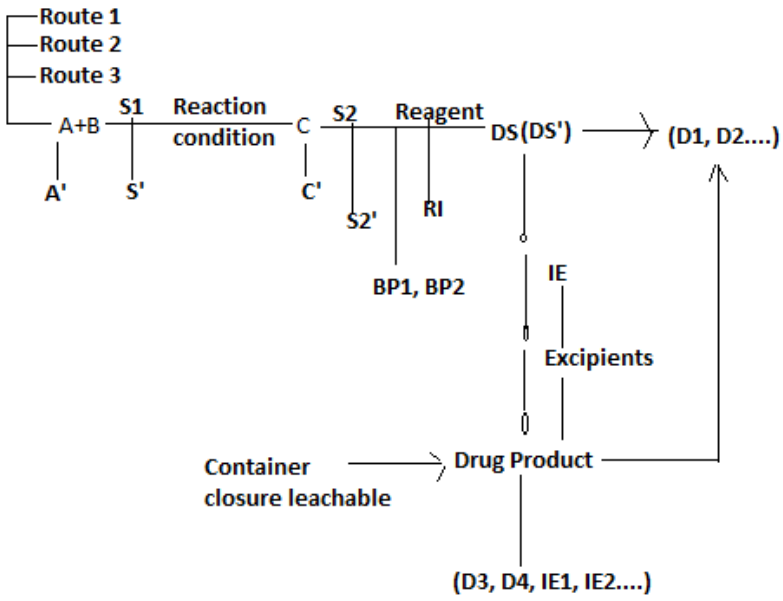
Investigational exemption for a drug should clearly state the methods, facility; controls used during manufacturing and maintain appropriate standards for identity, strength, quality and purity as needed for safety and give significance to clinical investigation made with the drug.

NDA demands stability data which contains identity, purity and stability of compound till expiration date to maintain quality of product. FDA can withdraw a product from market if product fails to meet impurity or stability criteria during commercial usage.

3. *TGA* (Therapeutic Governance Authority) of Australian regulatory guideline for prescription medicine.

1.2.3. Sources of Impurities[28-31]

Impurities may arise from a number of sources; Fig. 1.2 shows a potential source of impurities in proceeding of reaction for synthesis of material.



A+B: Starting material = C : Intermediate = DS: Drug substance
A' : Starting material impurity that can form C' and DS'
BP : By Product of reaction
D: Degradation product
S': Solvent impurity
RI: reagnet impurity
IE: Excipient Impurity

Figure: 1.2. Sources of impurities in synthetic reaction

1. **Crystallization related impurities**: polymorphism and solvatomorphism both terms are used for crystal structures. When substance has different crystal packing arrangement of

same elemental composition it is called polymorphism and when different elemental composition is present in same crystal packing is called solvatomorphism. Both terms are important for regulatory authorities as it can affect properties of material like conductivity, density, dissolution rate, hygroscopicity, rate of reaction, solubility etc.

2. Stereochemistry related impurities: stereochemistry related impurities include the substance having same chemical structure but different spatial orientation, which can be stereoisomer. Chirality of substance can be referred to as one or more asymmetric carbon, its mirror image is not super imposable. This chiral molecule or enantiomers have same chemical structure but different spatial arrangement so that they differ in their optical rotation. Both the d-isomer and l-isomer have different pharmacological or toxicological effect. The isomer which has undesired effect is considered as chiral impurity. As a number of chiral carbon increases in molecule there are more chances of chiral impurities.

3. Residual solvents: Solvents are used during synthesis of API and excipient; it can incorporate in final formulation and may lead to hydrate form. Solvent impurities are shown in figure 2 as S'. Most common solvent is water used in synthesis reaction but in some products it is not even considered as impurity until it can lead to form a hydrate or causing hydrolysis of material. Moisture content is important in product it should not be in equilibrium with atmosphere. Solvents may lead to drug degradation, loss of crystalline integrity and chemical instability. Pharmacopeia had listed test for organic volatile impurities to detect them. Impurities found in end product of APIs during synthesis have source of reaction material. It may contain residual solvent, byproduct, chain product or traces of starting material. Some of these impurities can be eliminated by purification of end product but some time solvent used for purification is carried over as residual solvent in end product.

4. Synthetic intermediate and By-product: Inherent impurities can be further classified in starting material and intermediates, impurity in starting material, by product of synthesis, reagent, catalysis and ligand, product of overreaction and product of side reaction. When reaction starts from starting raw material to end product a number of planned or unplanned intermediate and byproducts can be formed. *Fig. 1.2* shows complete explanation of sources of impurities in synthesis of drug product.

5. Formulation related impurities: Formulation may contain impurities those are method related, environment related, cross contaminations or excipient. Method used to prepare formulation generates impurity in formulation and if formulation is not stored

properly that can be source of impurity. If formulation contains two of more APIs that are reacting with each other can generate impurity or interaction of API with excipient, or excipient itself containing impurity. Methods used to prepare formulation are also important; the use of heat or water can cause degradation of drug. Oxidation or photolytic degradation can be occurring if proper precautions are not taken.

6. Impurities arising during storage: Storage related impurities (degradation) are formed during storage or aging of API or forced degradation of API. According to ICH guidelines degradation product can be defined as," molecule resulting from a chemical change in the substance brought about by overtime or due to the action of light, temperature, pH or water or by reaction with excipient and/or the intermediate container closure system.

7. Genotoxic impurities: In ICH guidelines genotoxic impurities falls in special category as they are potential carcinogenic even in trace amount. They can react with DNA and mutate it, thus mutation lead to cancer. These functional groups can be present in API e.g organohalides. The guideline for testing such impurities is shown in ICH S2 guideline for Genotoxicity testing and data interpretation intended for human use. Another guideline for control of genotoxic impurities is ICH M7 guideline Assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk.

ICH prescribed threshold values for acceptance of impurities: The control and acceptance limit for impurities in new drug substance or formulation are given in ICH guidelines. The control of impurity is discussed in *section 1.2.5.*

1.2.4. Approach to Impurity profiling[32-38]

To study an impurity in drug product and substance various methodologies can be applicable. Steps included in study of impurities are

- Stability study,
- Forced degradation study,
- Degradation product (DP) identification,
- DP separation and isolation,
- Characterization of DPs.

Stability study includes long term stability of drug substance and drug product at different temperature, different humidity and different time duration. From these studies details of long term drug stability, effect of humidity and temperature on drug can be obtained. By pursuing such details, effect of packaging and closure leachables can be distinguished from drug degradation. Table 1.1 describes long term stability testing conditions for drug substances and drug products.

	Conditions	**Time for testing (Month)**	**Data submission**
Long Term or Ambient	$25^0C \pm 2^0 C$, 60% RH ± 5%	0,3,6,9,12,18,24,36,48,60	12 month
Intermediate or Controlled	$30^0C \pm 2^0 C$, 60% RH ± 5%	0,3,6,9,12	6 month
Accelerated or Short term	$40^0C \pm 2^0 C$, 75% RH ± 5%	0,1,2,3,6,9	6 month

Table: 1.1. Formulation and API stability testing conditions [39]

Forced degradation or stress degradation method is used to stimulate atmospheric effect similar to long term stability of API and formulation. Forced degradation is normally performed at room temperature. The temperature, pH, light, acid, alkali, water and oxidative agent have major role in production of degradation product. All these parameters are needed to study during impurity profiling. Forced degradation study should be carried out in mild to moderate to severe conditions. Mild conditions are at room temperature with low concentration of degradant, moderate condition is 50-60^{0}C temperature with same or increased concentration of degradant than mild condition. The conditions may be made harsher so as to try to achieve at least 20.0% degradation for stress studies. *Fig 1.3* shows types of stress degradation for drug substance/product.

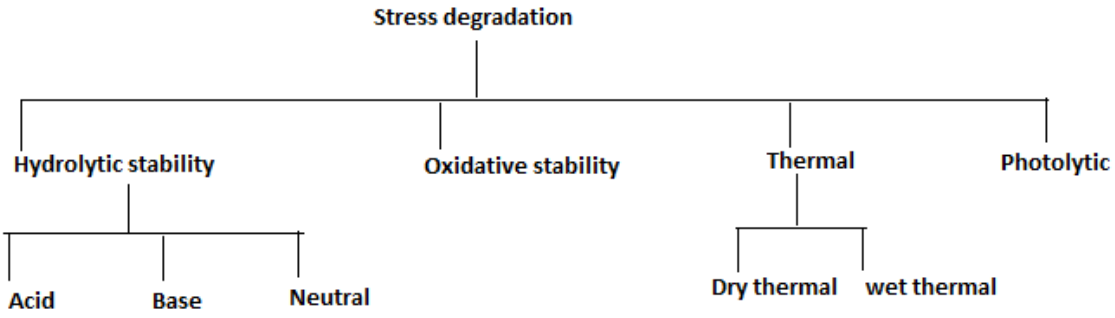


Figure: 1.3. Drug substance and drug product stability study flow chart

Stress degradation study is carried out with degradants like acid, base, water, peroxide, light and heat. Drug stability is checked in mentioned medium, starting with mild condition like 0.01N HCl or 0.01N NaOH at room temperature. If drug of formulation degrades in mild condition than milder condition requires and degradant concentration is decreased than mild condition. If no degradation of API or formulation occurs it shows stability in mild condition so further study can be carried out using temperature slight higher than room temperature. 10^0C rise in temperature is carried out till 80^0C and if still no degradation than increase concentration of reagents and study start again at room temperature with gradually increase in temperature. Concentration of Degradant can be increased till harsh condition or degradation of API or formulation appears but not to the level that is too harsh. Likewise study should be carried out with water and in oxidative medium also. Thermal condition requires 21 days of drug substance (DS) or drug product at 80^0C with sampling at different day interval. Similarly DS or drug product exposed to direct UV light in sun light or photo degradation chamber for 21 days with different day interval testing. Degradation study can be carried out using different analytical methods such as RP-HPLC, TLC, HPTLC, UV, IR, and Spectroflurometry and any other suitable analytical method. Once drug degrades and forms degradation product stability study completes with particular degradant. Next step is to separate drug and degradation product and to isolate the degradation product. Method used to separate drug substance and degradation product is called stability indicating method. Isolation can be carried out choosing suitable method from TLC, HPTLC, Column chromatography, Preparative HPLC or flash chromatography. It is not necessary to isolate degradation product in solid form unless and until it requires further study in solid phase.

Degradation product identification can be done using reference standard method or analytical methods. In reference standard method impurity standard is required, further study

is carried out by standard impurity with that of obtained after degradation of drug. This step is required only when impurity is known and listed in authorized reference.

Various spectroscopic methods to characterize impurities are used, most of them are combined as detector with chromatographic system, they are; UV (Ultraviolet), IR (Infrared), Raman spectroscopy, MS (mass spectroscopy), NMR (nuclear magnetic resonance). *Fig. 1.4* shows process of characterization of degraded product and impurity profiling.

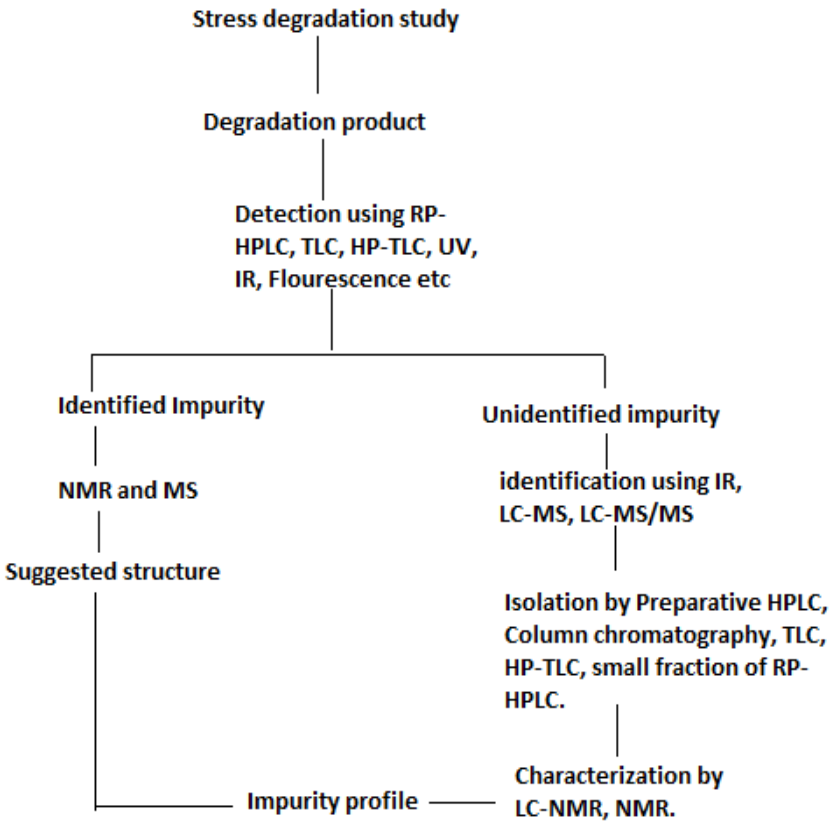


Figure: 1.4.Flow chart for impurity profiling.

A number of analytical methods are available in high tech era to perform stability study and impurity profiling. The analytical methods which can be considered as conventional methods are TLC (Thin layer chromatography), UV method and titrimetric methods. HPLC can be considered as conventional and new method. Recent advanced analytical techniques for stability study includes IR, UHPLC (Ultra HPLC), HPTLC, various hyphenated techniques (LC-MS, LC-NMR, LC-MS/MS).

Degradation product separation and isolation is critical step for characterization of DP, only identification or marking presence of DPs is not enough. Separation of DP is essential to isolate them and characterize particular DP individual. Separation can be achieved using RP-

HPLC, CE (Capillary electrophoresis), chiral separation, gas chromatography, SFC (Super critical fluid chromatography). Once separation of DP's established either by isocratic or by gradient elution method, it can be transferred to preparative HPLC for isolation. Isolation can also be carried out by column chromatography, flash chromatography, TLC (Thin layer chromatography), HPTLC, solid phase extraction, liquid-liquid extraction and super critical fluid extraction.

For separation various methods are used they are; CE (capillary electrophoresis), Chiral separation, GC (Gas chromatography), HPLC (High performance liquid chromatography), SFC (super critical fluid chromatography), TLC (thin layer chromatography).

Among all the techniques for separation TLC and HPLC are the widespread techniques used by researchers. Both are type of chromatography based on different principle of chromatography. **HPLC** (high pressure/performance liquid chromatography) is based on separation principle of polarity and affinity. System suitability testing (SST) parameters are used to determine column efficiency, resolution and repeatability of chromatographic system for the analysis. SST parameters for applied chromatographic system of assay of active ingredients or impurity determination or dissolution testing must pass in order to start sample analysis to be approved and further proceedings. SST parameters are resolution (R), Repeatability, Column efficiency (N) and tailing factor (T). Other parameters are capacity factor (k) and signal to noise ratio (S/N). Accepted limit criterion for SST parameters are shown in *Table 1.2.*

SST parameters	Limits
Repeatability of peak response	RSD ≤1.0 % for 5 replicates
Resolution	Value > 2.0
Tailing factor	Value ≤ 2.0
Column efficiency (N)	Plate count > 2000
Capacity factor	Value > 2

Table: 1.2. SST parameters and limits for parameters. [40]

ICH Q2 (R1) guideline is used to validate the chromatographic method; parameters for method validation are Linearity, Range, Precision, Accuracy, Specificity, Sensitivity (LOD

and LOQ) and robustness. Thus, chromatographic system and column efficiency can be determined using SST parameters.

Characterization of DP is concluding step for impurity profile. Some hyphenated techniques are available that eliminate isolation step unless solid status of DP required. LC-NMR and LC-MS/MS or LC-MS can characterize DP without isolation of DP. Mass spectrometry gather data related to molecular weight or m/z ratio, while NMR data confirms stereochemistry of structure data obtained by mass spectroscopy. Both the data are interrelated and supportive to each other.

Sometimes safety data for DP are also collected to know whether DP is having genotoxic effect or biological effect in body.

1.2.5. Control of impurity

Impurities can produce lethal consequence or may diminish biological effect of main substance. Impurities have unwanted and unpredictable effect, while enantiomeric impurity can reduce or remove biological effect of main substance as one enantiomer is biologically active and another may be inactive or toxic.

Pharmacopeial status

Major Pharmacopeias revise their editions from time to time and update the monograph. New impurities may be added with their assay method. U.S pharmacopeia has two specific chapters for impurities <466> and impurity in drug substance and drug product <1086> dealing with organic impurities. Definitions are described but terminology used in USP is completely different then that is used in ICH. Pharmacopeias using three methods for test of substance, a chromatographic purity test coupled with non specific assay, chromatographic purity indicating method that also serves as an assay and specific limit test for known impurity, in this test reference standard of impurity is required. European Pharmacopeia and Indian Pharmacopeia also refer ICH and use the same terminology for impurities described by ICH.

ICH guidelines [41, 42]

ICH was established in order to make uniform guidelines for marketing and registration of pharmaceutical product in other country. In 1980 such stability guidelines were issued later on regulatory bodies and industrial bodies of European Union, Japan and USA gave their input to prepare harmonized guideline. The drawback of ICH guidelines was not dealing with

climatic conditions, worldwide different climatic zones are there and ICH guideline conditions were not applicable to each and every climatic zone. To correct that problem World Health Organization (WHO) developed another guideline for stability study according to different climatic zones in 1996. USFDA also issued guidelines for iron containing dosage form in 1997. ICH guidelines were later prepared for veterinary product also. In India Drug Manufacture Association issued guidelines for stability testing of drug substance and drug testing based on technical monograph available.

According to ICH guidelines impurities should be studied chemically and biologically. Chemical study is gathering of data like identification, chemical structure, and quantitation and reporting by suitable analytical method. Biological study includes biological or toxicological effect of impurity concerned with safety aspects. Impurities those are listed and in specified amount are specific impurities. They can be specified or unspecified. Unspecified impurities followed general acceptance criterion.

Following ICH guidelines are appropriate to refer for stability testing of drug substance or drug product:

Q1A Stability testing of new drug substances and product

Q1B Stability testing: Photo stability testing of new drug substance and product

Q1C Stability testing of new dosage form

Q1D Bracketing and Matrixing designs for stability testing of drug substances and products

Q1E Evaluation of stability data

Q1F Stability data package for registration application in climatic zones III and IV

Q3A (R2) Impurities in New drug substances

Q3B (R2) Impurities in new drug products

Q3D (R1) Guidelines for elemental impurities

Q3E Impurity: assessment and control of Extractable and leachable for pharmaceuticals and biological

Q5C Quality of Biotechnological products: Stability testing of Biotechnological /Biological product.

ICH guidelines for stability study describe limits for identification, reporting and qualification of impurities. Table 1.3 shows ICH limits for impurity to report identify and qualify based on daily dose limit.

Maximum Daily Dose	Reporting Thresholds	Identification Thresholds	Qualification Thresholds
≤2 g/ Day	0.05%	0.10% or 1.0 mg TDI, Whichever is lower	0.15% or 1.0mg TDI, whichever is lower
>2 g /Day	0.03%	0.05%	0.05%

*TDI- Total daily intake

Table: 1.3. Impurity Thresholds in new drug product [40]

1.2.6. Degradation kinetic Study of API[43-50]:

Degradation kinetic study is performed to predict the stability of API in a number of degradation conditions applied to API to know the stability and degradation behavior of API. These variations can be useful during product development and during evaluation of storage condition, the results show that how long the API can withstand to be in theraputical or biological specification. The degradation kinetics study is a drug characteristics and shelf life study; stressor, drug and excipient interaction in terms of degradation product formation. Degradation kinetics can be performed by accelerated degradation of drug in different conditions such as at different temperature, different time interval and at different reagent concentrations. Kinetic study is based on rate of reaction; speed with which reaction between two or more component to form product.

There are four main components that can increase reaction rate; concentration of reagent, time, temperature and catalyst. Degradation kinetics study of API can be useful in pre-formulation study during formulation development as degradation kinetics studies include the drug-excipient interaction study. The most favorable storage condition and probable shelf life of API or formulation can be predicted using degradation kinetics study data. The stability of API can be determined in different stressors, different stressor concentration and different conditions during degradation kinetics study.

The **order of reaction** is an important parameter for kinetic study. E.g. A is component 1 and B is component 2 both are reacting to produce product C, for successful reaction to produce

product c number of molecule for product C, a number of molecule required of A to react with b number of molecule of B.

$$aA + bB = cC$$

So from above equation rate of reaction can be calculated by, $r = k [A]^a [B]^b$

Where k is rate constant, a and b is power raised by its coefficient of component A and B respectively. Now order of reaction is a+b.

Order of reaction can be calculated by sum of power raised to its co efficient of respective component. Depending on it order of reaction can be zero order, first order, and second order or x number.

Zero order of reaction is independent of initial concentration of drug. It takes place when in reaction one component is in large amount compared to another component. While reaction is completed using catalyst it probably following zero order reaction. Practically zero order reactions are very fast and it changes order of reaction once reaction is completed.

First order of reaction is depending on concentration of reactant. E.g. Radioactive decay follows first order reaction. Similarly **second order reaction** is depending on product of concentration of two reactants.

2.6.1 Kinetic parameters

When drug kinetic study is performed, it is always assumed that drug is following one compartmental model. Kinetic parameters are calculated for different temperature, different concentration and different time, so at specific time and temperature what amount of drug is degraded can be identified. Kinetic parameters can be calculated using graph plotted for concentration versus time or using equations.

Following Pharmacokinetic parameters are determined for drug degradation kinetic study:

The enthalpy of activation ($\Delta H^{\updownarrow}$) and entropy of activation ($\Delta S^{\updownarrow}$) were calculated using following equations; equation 1 and equation 2, respectively.

Equation 1

$$\Delta H \updownarrow = Ea - RT$$

Where, Ea is activation energy in KJ/mol, R is universal gas constant (8.314J/K/mol) and T is absolute temperature (K).

Equation 2

$$(\Delta S \updownarrow) = \frac{\Delta H \updownarrow}{T} - R \ln \frac{T}{k} - R \ln \frac{k}{h}$$

Where, R is universal gas constant (8.314K/J/mol), T is absolute temperature (K), k is Boltzmann constant (1.3807×10^{-23} J s) and h is Plank's Constant (6.626×10^{-34} J s).

- **Activation energy (E_a)** is minimum energy required to undergo degradation and to form degradation products. Activation energy of first order reaction can be calculated from slope (m) of regression equation of Arrhenius Plot (*ln k* versus 1/t (K)) using equation 3.

Equation 3

$$Slope\ (m) = -Ea/Rt$$

R is universal gas constant (8.314J/K/mol)

- **Rate constant** for drug is coefficient that is proportionally related to concentration of reactant or product at given temperature. Rate constant of first order reaction can be calculated by equation 4 and 5, equation and graphical method respectively.

Equation 4

$$Rate\ Constant\ (k) = \frac{2.303/t}{\log(\frac{Ao}{A})}$$

The slope of regression equation obtained from plot *ln C* versus time can be used to calculate rate constant (k), shown in equation 5,

Equation 5

$$Slope(m) = \frac{-k}{2.303}$$

For zero order reactions rate constant can be calculated using slope of plot %drug versus time, the slope is negative

- **Half life (t_{50})** is a time required to degrade half concentration of drug. At specific temperature half life remains constant. Half life of first order reaction can be calculated by equation 6

Equation 6

$$Half\ Life\ (t50) = \frac{0.693}{k}$$

- **Shelf life (t_{90})** is time when 90% of drug is degraded than original concentration. Shelf life predicts expire date for drug. Shelf life of first order of reaction can be calculated using equation 7.

Equation 7

$$Shelf\ life\ (t90) = \frac{0.105}{k}$$

For zero order reaction,

$$Shelf\ life\ (t90) = \frac{0.1(C0)}{k}$$

- **Order of reaction** is sum of power raised to co efficient to respective component.

Study of degradation kinetics helps to understand reaction mechanism and can give insight of reaction. Some reaction can be controlled by concentration of reaction like if reaction is following second order than adding more amount of reactant can speed up reaction.

1.3. DESIGN OF EXPERIMENTS (DoE) [51-70]

Concept of quality was introduced by Joseph. M Juran in his publication, he proposed that quality in products and procedures can be achieved by designing the experiments using statistical tools. Quality by Design (QbD) can be defined as," a systemic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management".[51-58] The arrangement of field experiments (1926) and the design of experiment (1935) books were written by Ronald Fisher who is a pioneer of Design of experiment. DoE is most

popular approach in pharmaceutical industry for product development and for analytical procedures. [52]

Design of experiment is set of experiments conducted to understand effect of different parameters on response variable. Response can be physical property, instrumental response or any process related effect on product. DoE is mathematical tool that can create a set of experiments, predict parameter effect within predefined limits and analyze effect of each parameter on individual response, even though DoE is useful in product development we used it in degradation kinetics study to reduce number of experiments to perform and can get more knowledge about kinetic parameters and their responses.[53-54] To generate set of experiments three basic requirements are, predefined limits, selection of parameters and mathematical analysis to predict between set limits. The result of set of experiment can optimize parameters in order of most affecting to least affecting parameters and interaction of those parameters with product or with parameters. [55-57]

DoE can be cost effective and it can save consumable of these experiments. When there are number of parameters affecting same product, DoE is chosen as an option to solve technical problem by giving summarized result, accessibility of results in selected range, accuracy, prediction and optimization. [58-60]

1.3.1. Design of experiment steps:

DoE can be performed using software, Design Expert®, Minitab® and JMP etc. Before starting practical experiment it is essential to define a problem which needed to be solved. Next step is to select set of variables from number of variables affecting to problem. Selection of variable is based on initial trial and error based experimentation or screening of variable in DoE model is done by using screening methods. Variable levels are selected from highest to lowest; sometimes middle point is also selected. Design is created using suitable software and experiments are performed to get data. Once data collected, they are analyzed and report is created. Final optimization of process and conclusion is depends on analysis of data. Prediction of data can be possible for any variable between range of highest to lowest selected for variable. Optimized variables are also given with predicted response to compare actual values with predicted values of software, so that one can check accuracy of software. [61-63]

1.3.2. Experimental Designs:

Experimental design is a design of experiment trial using replication, randomization and level of factors. Different experimental designs have specific uses. There are 3 types of experimental designs,

1. Mixture design
2. Factorial design
3. Response surface

In these studies the factorial designs were used which are of two level or more, among which one is highest point indicated by +1 and one is lowest point indicated by -1, if center point is selected it is indicated by 0. Factorial designs are selected for screening of variable to check either independent variable is having positive, negative or no effect. Interactions between response and variable can also be studied by this design. If variable number is too high and experimentation number is also high fractional factorial design is also available but it will provide limited information regarding variable and response. Interaction of variables can't be studied by fractional factorial design. It is easy to understand that as the number of variable increases, number of experiment will also increase. Factorial design are generated by L^f term, where L is level (-1,0,+1). [64-67]

After screening process, the interaction of variable and their curvature can be studied by response surface design. Response surface design can give extensive interaction data of variables in experimental space. Data obtained here is represented in cubic design in 3D or 2D cubic design. Response surface design is preferred when it is compared to three level factorial designs; even mixture design is a special form of response surface design. Response surface design like Box- Behnken and Central composite design are more practical.[68]

When model is based on dependant variable it is called statistical model, when independent variables are evaluated and dependent response is obtained is called observation. Observation and error helps to develop statistical model. DoE is mathematical model and is using mathematical equations like mean, median, range, standard deviation and coefficient of variance. Factors may have different positive, negative or no effect when compared from low level to high level of factor. Design space is the space for individual factor which cover low level to high level of factor effect on response. When two factors for example factor 1 and factor 2 , both are at different level: factor 1 at high and factor 2 at low, response obtained on

y axis is different, there is no parallel line and they are crossing each other indicates both factors are having interaction. Replications are part of experimental design to avoid experimental error in trail. There are chances of error during experimental design; some of the error can be identified during performance of experimental trial, as factor variation interrupts in producing same result. Some remains unidentified as those errors are obtained during measurement of response or error in measurement technique or method. Others are unknown errors which don't allow getting reproducible result without any known reason. [69] Analysis of data is important step while performing DoE, mathematical techniques are used to analyze data. Analysis of variance (ANOVA) is tool used to establish statistical significance and interaction or comparison of response data with error data. Another mathematical tool for analysis is 'F' test; F test uses probability level, it will analyze at selected probability level what is significant of factor and where can be interaction. Whether Data responses are significant or not can be predicted using comparison of F value obtained and F value of table. To get significant data response F value should be higher. ANOVA gives interpretation of response data whether response obtained is in limit of fluctuation or error, ANOVA uses F-test to verify error and response. It is difficult to use ANOVA equation and term manually, it is preferable to use software and computer generated terms for ANOVA. [70]

During these studies, DoE was utilized for prediction purpose in degradation kinetics study rather than optimization of formulation or procedure. The predicted values were compared with the values obtained by conventional degradation kinetic study to check the efficiency of DoE multi factorial tool.

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