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A Comprehensive review on Isolation, Characterization and Identification of degradant impurities in Active Pharmaceutical Ingredient

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ABSTRACT

The various drug inventions consist of active pharmaceutical ingredient (API), and its excipients. However, API often contain unwanted impurities that can affect their purity, safety, and effectiveness. Impurity profiling identifies and characterizes all possible impurities, which are generally related to the API, its manufacturing process, as well as stability. The impurities from API can arise from factors such as stereochemistry, crystallization, or functional groups. While related to process impurities contains chemicals, catalysts, residual solvents, intermediates, and by-products formed during synthesis or formulation. Stability-related impurities result from the degradation or interaction of APIs with excipients over time. These impurities can significantly influence the drug's bioavailability and overall performance. Regulatory authorities like the ICH and USFDA have set strict limits on impurity levels in APIs. Hence, isolating and characterizing impurities using advanced analytical techniques is vital to ensure biological safety and therapeutic efficacy. With growing regulatory emphasis, impurity profiling has now become a mandatory step in new drug development and approval processes, ensuring the production of safe and high-quality pharmaceuticals. This review outlines the potential sources of respect impurities, the need to the impurity profile and the procedures for characterizing as well as identifying impurity. [1]

INTRODUCTION:

The undesirable component that is left in the formulation is known as a drug impurity. These impurities are created throughout formulation process otherwise as a result of aging of API. The reagents, intermediates, also byproducts of the chemical reaction of the API throughout the manufacturing process, as well as degradation and excipient interaction products that may arise in the API or the final drug product, are likely source of impurities. [2]

Impurities in pharmaceuticals are undesirable chemicals that uniform in minor amount might affect the efficacy as well as safety of the therapeutic products. Impurity profiling is the process of describing, characterizing, and quantifying both known and unknown impurities that are present in drug substances. [3]

These impurities can arise from [12] contact between the main packaging as well as drug product. It can also presents through storage of compound as well as from overwrap, ink, label, boxes cardboard. The assessment of organic, inorganic, residual solvent as well as elemental impurities are done as per ICH guideline Q3.[4,5,6]

These impurities are the organic impurities, inorganic impurities and its residual solvents etc.

1) **Organic impurities:**

Organic impurities in API are unwanted substances which is formed during manufacturing, it may be key raw material, intermediate, genotoxic impurities, by-product or degradant products. Sometime it may be formed during storage time. The brief information about the organic impurities is given below

- **Key Raw Material (KSM):**
  Residual chemicals left over from the raw materials used to make the API.
- **Intermediates**
  These are the residual compounds from earlier stages of multi-step synthesis.
- **Genotoxic Impurities:**
  These impurities are highly potent, like some nitrosamines (NDMA, NDEA), that can cause cancer and require extreme control.
- **By-Product:**
  By-products formed during the chemical reactions used to create the API.
- **Degradation Products:**

Impurities that form when the API itself breaks down over time or due to exposure to light, heat, or moisture.

- **Catalyst, ligands and reagent:**
  Residuals from chemicals used to speed up or control the reactions that create the API.

2) Inorganic Impurities:

Inorganic impurities are non-carbon based. It may be heavy metals, inorganic salts, filter aids, ligands etc.

- **Heavy metals (Elemental impurities):**
  These impurities are the toxic elements. As per ICH guideline Q3D [7], these metals are classified in to class -1, 2 and 3.
  These metals are classified based on toxicity. The class-1 consists of 4 elements such as Cd, As, Hg, and Pb which are human toxicants and no use in the manufacturing of pharmaceuticals. The class-2 elements, commonly called as route-dependent human toxicants. The class 2 elements are further divided in to classes mainly, A and B. The class A have comparatively increased possibility of occurrence in the drug product and thus require risk assessment across all potential sources of elemental impurities and routes of administration. In this generally comes Co, V and Ni elements. The class B elements have lesser possibility of occurrence in the drug product linked to their low abundance and lower potential. Generally, in class B we have 10 elements such as Pd, Ru, Se, Au, Ag, Ir, Os, Tl, Rh, and Pt. The class-3 are have comparatively lower toxicities by the oral route of administration. In this class have 7 elements such as Cu, Ba, Cr, Sn, Li, Sb, and Mo.
- **Inorganic Salt:**
  The remaining salt after purification steps. These may the sulphates, chlorides etc.

3) Residual solvents:

The solvents used throughout the manufacturing process to dissolve the API and other ingredients. Ideally, all the solvent is removed during purification. However, trace amounts may remain. Based on toxic nature residual solvents are divided in to 3 classes.

- **Class-1 Solvents:**
  These solvents are unacceptable due to their environmental hazards, known human carcinogens, it highly suspected human carcinogens. These solvents are not used in the manufacturing of pharmaceuticals. In this class we have 5 solvents such as Benzene, carbon tetrachloride, Carbon tetrachloride, 1,2-Dichloroethane, 1,1-Dichloroethane and 1,1,1-Trichloroethane.
- **Class-2 Solvents:**
  In this class solvents are very limited usage in therapeutic product due to their carcinogenic potential, neurotoxicity and teratogenicity.
- **Class-3 solvents:**
  In this class, solvents have little toxicity to man. There is not much health-based exposure. Class 3 solvents have PDEs of 50 mg or more per day.

Sources of Impurities:

The resulting is the various source of impurities

- **Key Starting Material:**
  The impurities existing in the raw materials used to synthesize the API can carry over into the final product. Rigorous quality control of starting materials is crucial to minimize this.
- **Manufacturing Process:**
  By products formed during chemical reactions used to create the API can become impurities. Incomplete reactions or side reactions need to be controlled to minimize their formation. Residuals from reagents and catalysts used in the synthesis can contaminate the API if not adequately removed during purification. Inorganic salts introduced during purification steps or present in the starting materials can become impurities.
  Trace amounts of metals like lead or arsenic can leach from manufacturing equipment or catalysts, requiring strict control measures. Contamination from filtration materials used to purify the API can happen if not properly managed.
- **Degradation of the Drug Substance:**
  The API itself can break down over time due to exposure to factors like light, heat, moisture, or air. These degradation products can become impurities and affect the drug's potency or safety.
- **Formulation and Packaging:**
  Excipients added to the final drug product, such as fillers, binders, or disintegrants, might contain trace impurities that need to be controlled. Leaching from containers used for storage or packaging can introduce impurities if not made from suitable materials.
- **Environmental factors:**
  Improper storage conditions during manufacturing or distribution can expose the drug to temperature, humidity, or light fluctuations, leading to degradation and impurity formation.
- **Limits of impurities in API:**
  Limits for impurities, particularly in pharmaceuticals, are stringent guidelines set by bodies like ICH and USP, defining maximum acceptable levels (often <0.1% to 0.5%) for known/unknown substances, elemental as well as degradant impurities, confirming safety by requiring identification and toxicological studies such as identification, reporting and qualification threshold to protect public health from probable damage.
- **Forced Degradation Study (FDS):**
  The pharmaceutical industry performs forced degradation [8,9,10] experiments, which expose drugs to a variety of stress environments in order to accurately mimic potential effects that the product may have throughout its shelf life. The goal is to produce degradation products and thoroughly analyse them to fix how they affect the superiority of the final product.
  The FDS process generates a degradation product that can be examined to determine the molecule's stability. It involves the degradation of drug substances and drug products under severe conditions. As per ICH recommendation, stress testing is meant to validate the stability-indicating techniques employed and to identify the expected degradation products, which makes it easier in determining the intrinsic stability of the molecule and outlining degradation pathways. Studies on forced degradation helps in the production of degradants in a shorter period of time, within few weeks. The samples produced by forced Degradation can be utilized to establish the stability indicating technique that can be used in the future for sample analysis.

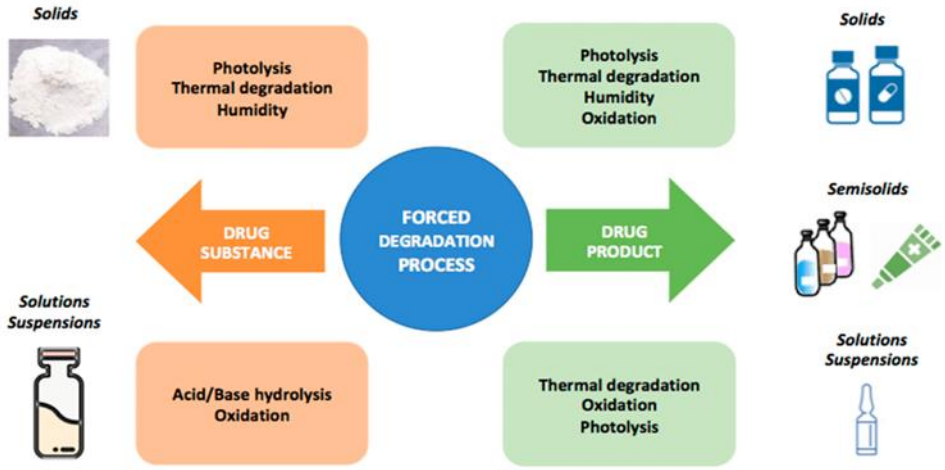

Figure 1: A general procedure of degradation conditions applied for the API and its product[10]

- **Selection of degradation conditions:**
  In the guidelines, the regulatory bodies have specified the upper limit of degradation products. It is noted that 5–20% degradation is permitted for validation of chromatographic tests. For small molecules, the stability limit should be greater than 90%, so a degradation of roughly 10% is sufficient. Usually, the stability of drug products is monitored using spiked samples of a combination of known degradation products and drug substances. This makes it relaxed to categorize the products that are realized during the degradation process. If the API shows any changes in its physical and chemical properties, as well as changes in its activity during its shelf life, the drug molecule is said to have undergone degradation. If no such degradation is observed, the study will either be stopped or the drug sample will be put under more stress in order to determine the type of secondary degradants that are likely to be formed during the process. The API will be exposed to additional energy in order to evaluation the stability of molecules if, in any case, little or no degradants are formed as a result of increased stress. The FDS conditions briefly described below:
- **Hydrolytic Conditions:**
- **Acid hydrolysis:**
  In Acid hydrolysis condition, breakdown the API by using Acid such as diluted hydrochloric acid. During acid hydrolysis study sample is dissolved in respective solvent and added some mL of diluted hydrochloric acid. After exposing sample, it is neutralized with base such diluted sodium hydroxide and analyzed on HPLC system. The control degradation condition is achieved based on the degradation product.
- **Base hydrolysis:**
  In base hydrolysis, hydrolysis is carried out using sodium hydroxide. During base hydrolysis study sample is dissolved with respective solvent and added some mL of diluted sodium hydroxide. After exposing sample, it is neutralized with acid and analyzed on HPLC system.
- **Oxidation Condition**
  The oxidation degradation is carried out using hydrogen peroxide (H_2O_2). The choice of oxidizing agent, such as hydrogen peroxide, and its concentration depend on the drug's properties. Typically, 1-5% hydrogen peroxide solution at neutral pH and room temperature is used for 20% degradation is achieved. Certain functional groups are prone to oxidation, including benzylic carbons (next to aromatic rings), allylic carbons (next to double bonds), tertiary carbons (with three alkyl groups), and α-positions (near heteroatoms like oxygen or nitrogen). These groups can form degradation products like hydroperoxides, hydroxides, or ketones. Understanding these factors helps researchers predict and study potential degradation pathways for drugs.
- **Photolytic degradation**
  In photolytic degradation, samples is exposed to 1.2 million lux hours. Photolytic degradation may happen over non-oxidative. The non-oxidative reactions involve changes like isomerization, dimerization, and bond cleavage. Essentially, light can catalyse oxidation reactions, leading to drug degradation. Understanding these mechanisms helps predict and mitigate photolytic degradation in pharmaceuticals.
- **Thermal condition**
  Higher temperatures can accelerate drug degradation, posing challenges for heat-sensitive APIs. Thermal degradation contains reactions such as hydrolysis, decarboxylation, pyrolysis, rearrangement, isomerization, and polymerization. The degradation studies in thermal conditions are typically conducted between 40°C to 80°C, with 70°C at varying humidity levels for 1-2 months being a standard condition. Precautions are necessary when using extreme temperatures to ensure accurate and relevant degradation data.
- **Humidity Condition**
  Humidity plays a significant role in drug degradation. In forced degradation studies, exposing drugs to 90% humidity for 1 week can induce degradation, helping identify potential degradants in APIs and finished products. This stress testing provides valuable insights into stability and degradation pathways.
- **Factors Affecting Degradation**
  Drugs can degrade due to various factors like moisture, temperature, pH, oxygen, and light.
  - **Moisture:**
    The water-soluble materials might become melted due to occurrence of moisture. This can lead to

physical as well as chemical changes, especially in water soluble substances.

- **Temperature:**
  Increased temperature can accelerate degradation reactions like hydrolysis. Change in temperature do have effect on stability of drug.
- **pH:**
  The pH can also affect degradation rates, especially through hydrolysis. Buffering to optimal pH can help stabilize drugs.
- **Oxygen:**
  Oxygen can cause oxidation; removing oxygen or using inert gases like nitrogen can help stabilize sensitive drugs.
- **Light:**
  Generally, some of the drugs are light sensitive and degraded once exposed to the light; storing them in amber glass containers or dark conditions can help prevent degradation.

Isolation of Impurities

The isolating contaminants is essential. However, because of the instrumental methods, it directly characterizes the contaminants, separation of impurities is avoided. Impurities are typically isolated using both chromatographic as well as non-chromatographic procedures before being characterized.

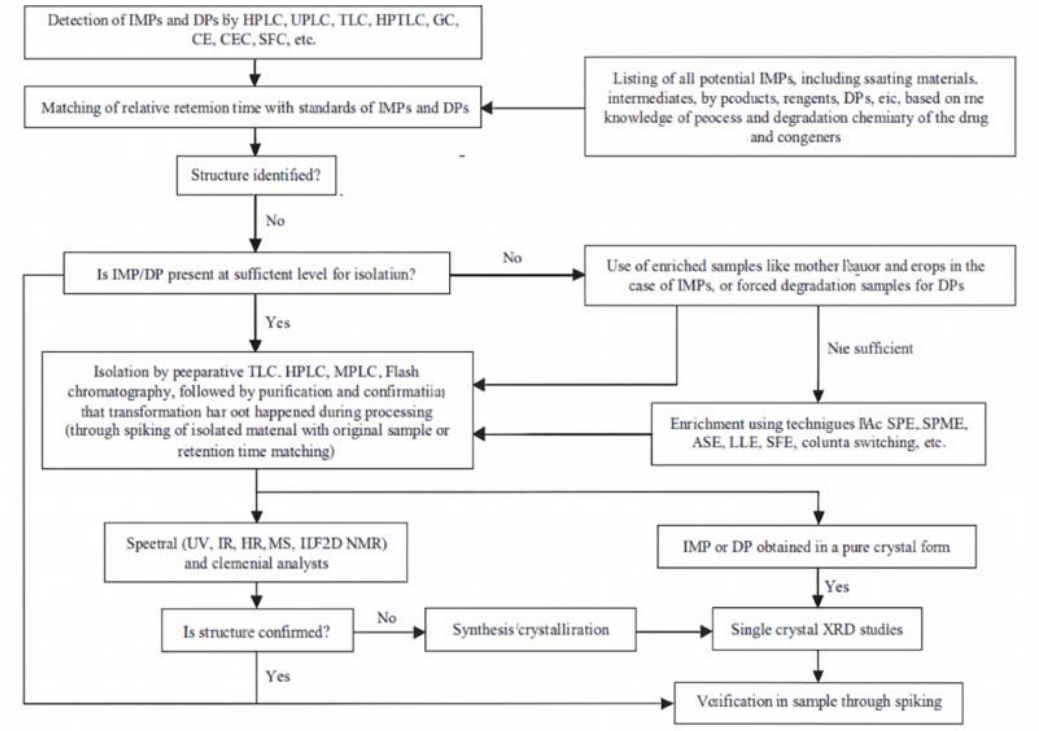

Figure 2: Approach for impurities characterization[11]

- **Chromatographic techniques for separation and isolation of impurities**
  The various chromatographic techniques are applied for isolation of impurities.[12] The selection of chromatographic techniques for isolation of impurities depends on its nature as well as stability.
- **Column Chromatography:**
  Impurities weighing between milligrams and kilograms can be quantitatively separated with it. UV-Visible spectrophotometry is applied to identify the eluent by periodically monitoring the collected fractions from a specific sample. The separation steps in column chromatography[13] shown in below flow diagram.

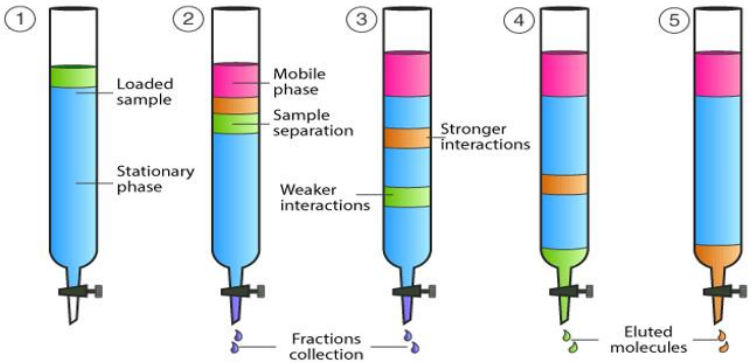

Figure 3: Flow diagram of separation steps in column chromatography [13]

- **Thin Layer Chromatography:**

Thin Layer Chromatography (TLC)[14,15] is a versatile as well as cost-effective laboratory method used for quickly separate the non-volatile components within the mixture. The process relies on differential migration, where a sample is useful to the stationary phase (usually a silica-coated plate) and then introduced to a mobile phase (a solvent). Components of the mixture separate as a solvent travel up the plate through capillary action: polar compounds adhere more strongly to the polar plate and travel slowly, whereas fewer polar compounds move faster through the solvent. After the solvent reaches the top, the separated components are visualized and identified.

- **High-performance Liquid Chromatography:**

High-performance liquid chromatography (HPLC)[16,17] is an analytical chemistry method that is used to separate and assess liquid sample components. This technique relies on the interaction between a solid matrix, flowing solvent, and instrumentation for sample handling, detection, and analysis. The process relies on pumping a pressurized liquid solvent, called the mobile phase, over a column packed through a solid material, stationary phase. The components of mixture transfer through the column at various speeds based on their chemical and physical interaction with the column and flowing solvent. Molecules that interact more elute slower, while those that prefer the solvent elute faster, effectively separating the mixture into its pure components. A detector then senses these separated compounds as they exit the system. In HPLC analysis various detectors are used based on the component properties. If the component has chromophore, UV-Visible detector is selected for the analysis. The refractive index detector is called as universal detector and specially used for analysis of alcohols, polymers and sugars.

- **Gas chromatography:**

Gas chromatography (GC)[18] is a useful analytical technique that separates mixtures of volatile compounds to determine what they are and how much of each is present. The sample is inserted into a heated chamber, vaporized, and passed by an inert gas (the mobile phase) over a coiled column covering a stationary phase. The separation happens because each compound interacts differently through the column and moves at a unique speed depending on its chemical properties, particularly its volatility and polarity. The separated components departure the column, the detector measures them, creating a chromatogram (a graph of peaks over time) that allows for their identification and quantification respectively. capillary action, separating the mixture's components according to their interactions with both phases. After development, the individual substances can be detected using methods such as UV absorption, fluorescence, or chemiluminescence.

- **Capillary electrophoresis (CE):**

Capillary electrophoresis [19] is an analytical procedure that separates molecules based on their size and charge by applying an electric field across a thin capillary filled with an electrolyte. The molecules migrate at different velocities depending on their electrophoretic mobility, which is influenced by their charge-to-size proportion. The electroosmotic flow is the bulk movement of the buffer solution through the capillary, caused by the negatively charged silanol groups on the capillary walls attracting positive ions in the buffer. When an electric field is applied, these positive ions move toward the cathode, dragging the entire liquid along, enabling even neutral molecules to migrate. The net velocity of an analyte is the sum of its electrophoretic mobility and the electroosmotic flow. By controlling pH, voltage, and capillary surface characteristics, CE can achieve highly efficient separation of ions and molecules based on their charge and size. Capillary electrophoresis is widely used in pharmaceutical formulation to analyse and quantify active ingredients, excipients, and impurities due to its high resolution, speed, and minimal sample consumption. CE is especially valuable for separating and characterizing chiral drugs, and is often used in research and development, and stability studies of pharmaceutical products. Its versatility allows for impurity profiling, enantiomeric purity assessment, and detection of degradation products, making it an essential tool for ensuring drug quality and regulatory compliance in pharmaceutical analysis.

- **Supercritical fluid chromatography:**

The SFC [20] works by using a special kind of fluid—called a supercritical fluid as the moving part in the separation process. The most common one is carbon dioxide, which is turned into a supercritical state by applying high pressure and temperature. In this state, the fluid acts like both a liquid and a gas, making it great for separating mixtures quickly and efficiently. The sample is forced through a column by this supercritical fluid, and different parts of the mixture stick to the column more or less, depending on their properties. The parts that stick less come out first, and the ones that stick more come out later, so they get separated. This method is fast, uses less solvent, and is good for sensitive or hard-to-separate substances. Impurities in pharmaceutical products, whether organic, inorganic, or degradation-related, can significantly affect their quality, safety, and effectiveness. Therefore, identifying and characterizing these impurities is crucial for ensuring the product meets the strict requirements of regulatory agencies.

The typical flow diagram of Supercritical fluid chromatography is shown in figure-4.

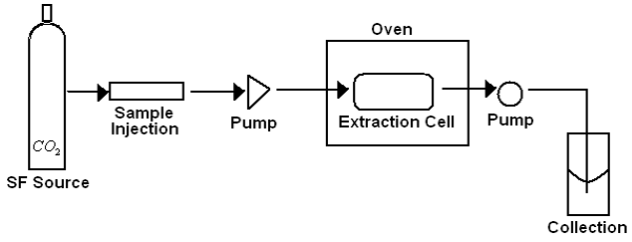

Figure 4: The typical flow diagram of Supercritical fluid chromatography

- **Characterization techniques for identification of impurities:**

Impurity identification and characterization involve a multi-technique approach combining separation and spectroscopic methods to determine the structure and quantity of impurities. Key techniques include LC-

MS/MS for molecular weight/structure, NMR for detailed structural elucidation, and FTIR/UV for functional groups. These tools ensure compliance with regulatory standards (e.g., ICH guidelines > 0.1% for pharmaceuticals).

- **UV-Visible Spectroscopy:**

UV–Visible spectroscopy [21] is an analytical technique based on the absorption of ultraviolet (200–400 nm) and visible (400–800 nm) radiation by molecules containing chromophores. When UV or visible light passes through a sample, electrons in the molecule absorb energy and undergo electronic transitions (mainly $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$). The amount of light absorbed is directly proportional to concentration (Beer–Lambert law). In this technique radiation source is Deuterium lamp for UV and Tungsten lamp for visible region. The prism or diffraction grating is as monochromator. UV–Visible spectroscopy helps to detecting chromophores impurities, monitoring related substances, studying degradation products as well as estimating impurity concentration. This is a simple and rapid technique, non-destructive, also requires less sample for analysis.

- **Infra-Red Spectroscopy (IR):**

The branch of spectroscopy that studies the IR portion of electromagnetic spectrum is known as IR spectroscopy. It involves a number of methods, the greatest frequent being a sort of absorption spectroscopy. Impurity profiling, especially in the chemical and pharmaceutical sectors, makes great use of IR spectroscopy [22] to identify and characterize impurities in samples according to their molecular structure and functional groups. Impurities can be discovered by recognizing unexpected peaks or shifts in the spectrum, which correlate to the presence of extra functional groups or contaminants. It can be used to detect substances by scanning into sample compositions, much like any other spectroscopic method. The typical flow diagram of IR spectroscopy is shown in figure-5.

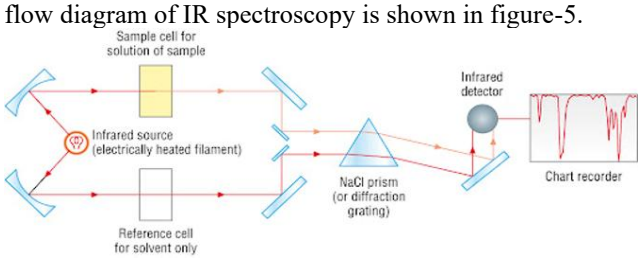

Figure 5: The typical flow diagram of IR spectroscopy

- **Mass Spectroscopy:**

Mass spectroscopy [23] is an analytical technique used for the identification and characterization of impurities by measuring their mass-to-charge ratio (m/z) of ionized molecules. In this technique, the sample is converted into ions, which are separated according to their m/z values using electric or magnetic fields. The resulting mass spectrum shows the molecular ion peak and fragment ions, providing molecular weight and structural information. In mass spectroscopy, EI, CI, ESI, APCI, MALDI are the ionization sources and Quadrupole, TOF and Ion trap are the mass analyser. The Electron multiplier is as a detector. In this technique, identifies unknown impurities and degradation products, determines molecular mass of impurities and provides fragmentation patterns for structure elucidation. This is a very highly sensitive and selective technique and can be coupled with HPLC (LC-MS) as well as GC (GC- MS). In Pharmaceutical industries it is used to identified for degermation of mass of unknown impurities, degradation products etc.

- **Liquid Chromatography–Mass Spectrometry (LC-MS):**

LC–MS [24] is a hyphenated analytical technique that combines the separation power of liquid chromatography (LC) along with the identification capability of mass spectrometry (MS). It is one of the most important tools for impurity profiling. In liquid chromatography separates components of a mixture based on their interaction with the stationary and mobile phases. The eluted compounds are ionized (commonly by ESI or APCI) and analysed by mass spectrometry, which measures their m/z values. The LC–MS is especially useful for separating and identifying unknown impurities and characterizing degradation products. It detecting very trace-level impurities (ppm–ppb). It determining molecular weight of impurities. This is highly sensitive and selective technique and suitable for non-volatile and thermally labile compounds

- **Gas Chromatography–Mass Spectrometry (GC-MS)**:

The GC–MS [25] is a hyphenated analytical technique which combines both, the separation capability of gas chromatography (GC) with the identification power of mass spectrometry (MS). It is widely used for volatile and semi-volatile impurity analysis. In gas chromatography, the sample is vaporized and separated based on boiling point and interaction with the stationary phase. The separated components enter the mass spectrometer, where they are ionized (usually by Electron Ionization, EI) and analysed based on their m/z ratio. The GC-MS technique is highly sensitive and specific and used for identify volatile and residual solvent impurities. It detects process-related impurities and characterize unknown volatile compounds.

- **Nuclear Magnetic Resonance Spectroscopy (NMR)**:

The NMR spectroscopy [26] is an advanced analytical technique used for the identification and structural characterization of impurities, especially organic impurities. The certain nuclei such as ^{1}H and ^{13}C possess nuclear spin. When placed in a strong magnetic field and irradiated with radio-frequency energy, these nuclei absorb energy and undergo resonance. The resonance frequency depends on the chemical environment, producing a characteristic NMR spectrum.

- **Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR):**

The ^{1}H NMR spectroscopy is a widely used analytical technique for the identification and structural characterization of organic compounds and impurities by studying the behaviour of hydrogen nuclei in a magnetic field. The hydrogen nuclei (^{1}H) possess nuclear spin when they are placed in a strong magnetic field and irradiated with radio-frequency energy, they absorb energy and resonate. The exact resonance

depends on the chemical environment of each proton, producing a characteristic spectrum.

- **Carbon-13 Nuclear Magnetic Resonance Spectroscopy (^{13}C NMR):**

The ^{13}C NMR spectroscopy is an analytical technique used to study carbon atoms in organic compounds, providing detailed structural information and helping identify impurities. The ^{13}C NMR is a naturally occurring isotope of carbon (≈1.1% abundance) with nuclear spin. When placed in a strong magnetic field and exposed to radio-frequency radiation, ^{13}C nuclei absorb energy and resonate. The chemical shift depends on the carbon's electronic environment, producing a spectrum with peaks corresponding to different carbon atoms.

CONCLUSION:

The presence of degradant impurities in Active Pharmaceutical Ingredients (APIs) poses significant challenges in terms of drug safety, efficacy, and regulatory compliance. Isolation, characterization, and identification of these impurities are essential steps in pharmaceutical quality control.

A combination of analytical techniques-including chromatography (HPLC, LC-MS, GC-MS), spectroscopy (UV, IR, NMR), and mass spectrometry—provides complementary information for the detection, structural elucidation, and quantification of impurities. Chromatographic methods allow separation and quantification of impurities. Spectroscopic techniques provide structural and functional group information. Hyphenated techniques (LC-MS, GC-MS, LC-NMR) are particularly powerful for identifying unknown degradants at trace levels. Advancements in analytical instrumentation, coupled with regulatory guidelines (ICH Q3A/B, Q3C), have enhanced the ability to monitor, control, and minimize degradant formation during drug development and storage.

Overall, a systematic approach combining isolation, detection, and structural characterization ensures that APIs are safe, effective, and meet stringent quality standards. Continuous research and method optimization remain crucial to addressing the evolving challenges of degradant impurities in pharmaceutical products.

CONFLICT OF INTEREST:

Conflict of interest declared is none

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