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Review Article

Development and Validation of Stability Indicating Methods by Chromatography: A Review

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ABSTRACT

High Performance Liquid Chromatography has become a powerful technique in analytical chemistry which evaluates drug product stability. This is most accurate method for quantitative and qualitative determination in the analysis of drug product. Forced degradation has a most crucial role in the development of stability indicating HPLC methods to separate various drug related impurities found during production or synthesis of drug product. This article will focus on strategies and areas of concern relevant to development of stability indicating methods by HPLC. Forced degradation studies provide us with degradation pathways of drug substances and products

Keywords: HPLC; Forced Degradation; Degradation Pathways; Impurities; Stability Indicating

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1.**Introduction**

Stability indicating methods (SIM) are approved quantitative analytical techniques that identify the characteristics of drugs and substances. Drug stability testing offers precise analytical methods for quantifying the active ingredients in pharmaceutical formulations while preventing interference from potential impurities, degradation products, and process impurities [1]. Stability of drug products can be ascertained analytically using high performance liquid chromatography. studies of forced degradation carried out in a variety of settings, including light, oxidation, pH, and dry heat, and separated from the degradation product. Research on forced degradation not only improves manufacturing development, formulation, and packaging, but also advances pharmaceutical development [2].

1.1 *When to perform forced degradation studies?*

- During optimisation of lead
- Method development and validation of Active Pharmaceutical Ingredient (API)
- While filing Investigational new drug (IND)/New Drug Application (NDA).

1.2 *Regulatory status of stability indicating assays*

As per requirement of regulatory authority for stability study of drugs and its formulations following guidelines were implemented as per Table 1.

Table 1: Regulatory guidelines of International Council of Harmonisation (ICH)

Standard	Title and Reference
ICH Q1A(R2)	Stability testing of new drug substance and products
ICH Q1B	Photostability of new drug substances and products

1.3. Requirement of stability studies

- Determines shelf life of drug substance and product.
- Explains storage conditions of drug product
- Provides information about degradants or impurities which appear due to aging.
- Evaluate inherent stability of drug and improve formulations and manufacturing process.
- Identifies reactions responsible for degradation of product [3].

*1.4. Objectives of forced degradation***:**

1) To determine the pathways by which drug substances and drug products degrade and to produce a degradation profile.
2) Differentiating between degradation products associated with drug substances and those associated with non-drug substances (such as excipients) in formulations.
3) Clarification of degradation products' structures.
4) Determining a drug substance's intrinsic stability in both its solid and solution states.
5) Identify the mechanisms of drug substance and drug product degradation, including thermolytic, hydrolytic, oxidative, and photolytic processes.
6) To create and assess a method for stability indication.
7) To address issues with stability (such as mass balance) [4]

2. Strategies for Stability Indicating Method Development

To create representative samples for the purpose of creating stability-indicating techniques for drug substances and drug products, forced degradation is used. The selection of stress conditions ought to align with the normal manufacturing, storage, and use conditions of the product, which vary depending on each [5]. General concepts of designing stability indicating analytical methods are discussed below:

2.1 Understanding Physicochemical Properties of Drug

Knowledge of the physicochemical properties of API and formulations are extremely important to help in the development of the method. Information about these properties can be gathered through search of the literature, company drug profiles, spectral libraries, and reports. Information on dissociation constants, spectrophotometric properties, oxidation-reduction potentials are helpful in determining the best means of measuring and quantifying the analyte of interest. Structure of analyte particularly functional group and active sites demonstrate the likely degradation products and drug susceptibility towards oxidation, hydrolysis, thermal degradation etc. Compatibility studies performed by assessing the stability when mixed with common lubricants and excipients to find out reaction between the drug and inactive materials [6].

2.2 Setup preliminary High performance Liquid Chromatography (HPLC) conditions

These conditions can be adapted from official, unofficial or from literature. New methods need to be established if there are no suitable methods available. Establishment of experimental conditions must be based on properties of API and impurities. Selection of proper column and mobile phase is crucial. A proper condition for experiments initially will save much time for subsequent developmental stages [7].

2.3 Sample preparation for method development

SIMs are developed daily by stressing under different conditions for API above those normally used for accelerated stability conditions. Apart from determining specificity in SIMs, stress testing also known as forced degradation, provides information of degradation pathways and products which form during storage and promote formulation, development, manufacturing, and packing. Stressing the API generates the products likely to form under real storage conditions which are further used to develop the SIM. Goal of these studies is degrading the API by 5 to 10. Each forced degraded sample analysed using suitable detector especially PDA [8].

2.4 Developing Separation-Stability indicating chromatographic conditions

For selecting initial chromatographic conditions for SIM of new molecule, critical thing to ensure is degradants are in solution state easy to separate and detect. For this diluent of water: organic phase in the ratio of 1:1 provides the increase in likelihood of solubility for most related materials ensuring proper disintegration of solid dosage forms [9].

The second step is obtaining degradation conditions that resolve many distinct peaks as possible from a set of test samples. The variables common for separation include solvent type, mobile phase, pH, column type and temperature [10].

2.4.1 Isocratic or Gradient mode

Usually, it is preferred to work in isocratic conditions wherein mobile phase composition remains constant. There are never any abrupt chemical changes in the system or column because they are always in an equilibrium state. Still, the needs for HPLC analysis have grown, and the samples are typically complicated, therefore the HPLC systems have developed into extremely durable, dependable apparatuses, and the columns are produced to give thousands of injections; consequently, in recent times most of the chromatographic runs have been founded on the mobile phase composition in gradient mode. The strength of the solvent is raised in a gradient type throughout the chromatographic run, with time. Choosing of the gradient or isocratic mode relies on the quantity of active elements that need to be divided or resolved [11].

2.4.2 Type of solvent

The relative polarity of the solvent and the sample can be compared to determine which starting solvent is best for a particular separation. By matching the solvent to the most polar functional group on the sample molecule (alcohols for OH, amines for NH2,

etc.), this is done as a first approximation. Following this procedure, the separation can be further refined from this attempt: The solvent is too polar to permit the adsorbent to retard the sample if the sample is visible at the solvent front. Choose a solvent that is less polar and located higher on the scale [12]. On the other hand, switch to a solvent or solvent blend that is lower down (higher polarity) on the scale if the sample does not appear in a reasonable amount of time.

Selectivity will be impacted by the type of solvent used (methanol, acetonitrile, and tetrahydrofuran). The analyte's solubility and the buffer being used may influence the decision between methanol and acetonitrile. Of these three solvents, tetrahydrofuran is the least polar. It frequently causes significant shifts in selectivity and is incompatible with the low wavelength detection needed for most pharmaceutical compounds [13].

2.4.3 Mobile phase pH

Ionisable functional groups like amino, pyridine, and carboxylic acid are present in most pharmaceutical compounds. The introduction of new packing materials that are stable up to pH 12 allows a mobile phase pH to be used as a retention/selectivity adjustment parameter in a wider range of applications. Since the sample is eluted in the void volume, there is no separation when it is eluted with a 100% (organic) mobile phase. This is because retention is seen when the solvent strength of the mobile phase is lowered to permit solute molecules to compete in equilibrium between the bonded phase and the mobile phase, rather than when the sample is retained. By replacing C18/C8 with cyano or phenyl, mobile phase optimisation can be improved alongside bonded phase optimisation [14].

2.5. Role of the column and column temperature

2.5.1 Selecting an HPLC column

The column is the main component of an HPLC system. During method development, changing a column will have the biggest impact on analyte resolution. Particle size, retention capacity, stationary phase chemistry, and column dimensions all need to be taken into account when selecting the ideal column for a given application. An HPLC column is made up of three main parts: the stationary phase, the matrix, and the hardware (column housing) [15].

2.5.2 Column temperature

Since temperature can have an impact on selectivity, column temperature control is crucial for the long-term reproducibility of a method. Generally speaking, a target temperature of between 30 and 40°C is adequate for good reproducibility. For varying the solvent strength throughout the run, temperature-programmed HPLC can be utilised instead of solvent gradient elution, and this is anticipated to be especially helpful with small-bore columns that have low thermal mass 18. Many studies have examined how temperature changes affect retention [16].

2.6 Peak purity

The ability to confirm the purity of the separated species—that is, to make sure no coeluting or comigrating impurity contributes to the peak response—is a crucial requirement of a separation analysis. Prior to using quantitative data from chromatographic or electrophoretic peaks for additional computations, peak purity should be confirmed. The comparison between the normalizing signals of the pure and impure peak is displayed in "figure 1" below [17].

An integral component of a SIM's validation is peak purity (also known as peak homogeneity) analysis of the main peak, which determines whether impurities are present beneath the main peak. PDA detection can be used to perform direct evaluation in-line. By altering one or more chromatographic parameters (column, mobile phase, gradient composition, etc.) that have a substantial impact on the separation

selectivity, one can indirectly evaluate peak purity [18].

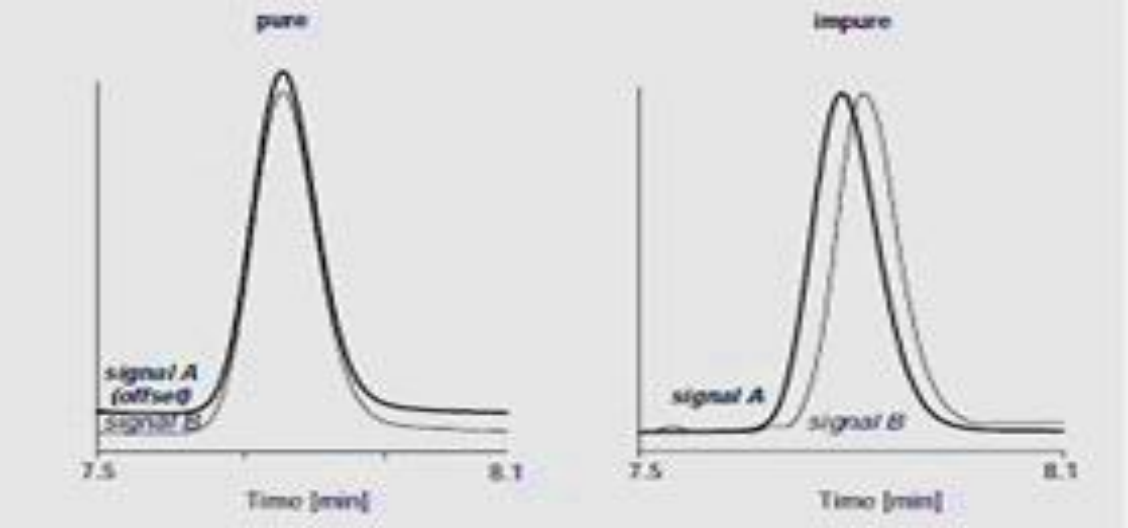

Fig.1: Signal normalisation for pure and impure peak

2.7. Method optimisation

Improved sensitivity is obtained after separation through method optimisation. It is necessary to consider the compositions of the stationary phase and mobile phase. The optimisation of mobile phase parameters is prioritised over stationary phase optimisation due to its ease of use and convenience. It is necessary to examine only those parameters in the optimisation that are most likely to have a significant impact on selectivity to reduce the total number of trial chromatograms. Improved sensitivity is obtained after separation through method optimisation. It is necessary to consider the compositions of the stationary phase and mobile phase [19].

2.8. Validation of Analytical methods

Although they can occur simultaneously, validation and method development are two separate processes that follow method selection. Analytical techniques used in quality control should guarantee a sufficient degree of confidence in the viability of the findings of the analyses of raw materials, excipients, intermediates, bulk products, or finished products. Before a test procedure is validated, the criteria that will be applied must be chosen. Analytical procedures must be developed in compliance with the International Conference on Harmonisation (ICH) guidelines (Q2A and Q2B), and they have to be used in settings that follow good manufacturing and laboratory practises (GMP and GLP). The US Food and Drug Administration (FDA) and US Pharmacopoeia (USP) both refer to ICH guidelines.

3. Forced Degradation Studies in Stability-Indicating Method Development

Chemical stability in pharmaceutical molecules is a serious issue since it affects the efficacy and safety of the medication. The FDA and ICH guidelines specify the requirement for stability testing data in order to comprehend how the quality of a drug substance and drug product changes over time under the influence of various environmental conditions. Regulatory documentation needs to be aware of the molecules' stability in order to provide proper formulation, packaging, storage conditions, and shelf life [20]. Along with establishing specificity, forced degradation of the drug's substance and product will yield the following data:

1) Determining the pathways by which drug substances and drug products degrade;

2) Differentiating between formulations relating to drug substances and those relating to non-drug substances (e.g., excipients);
3) Clarifying the structure of degradation products;
4) Determining the intrinsic stability of a drug substance molecule in both solution and solid state; and
5) Identifying the mechanisms by which the drug substance and drug product degrade by thermolysis, hydrolysis, oxidation, and photolysis [21].

3.1. Time to perform forced degradation

Knowing when to perform forced degradation studies is crucial for the development of new drug substances and products. FDA guidelines recommend that stress testing be carried out in phase-3 of the regulatory submission procedure [22]. Stress studies ought to be carried out in various pH solutions, with light and oxygen present, and at high levels of humidity and temperature to ascertain the stability of the medication. These stress tests are carried out on just one batch. The outcomes ought to be compiled and presented in a yearly report. But beginning early stress testing in preclinical phase I of clinical trials is strongly recommended and ought to be carried out on drug substances to obtain adequate time for identifying degradation products, structural information, and optimisation of the stress conditions. An early study of stress also provides timely suggestions for enhancing the manufacturing process and the stability-indicating properties of an analytical procedure [24].

3.2. Limits for degradation

Pharmaceutical scientists have had numerous conversations about the issue of how much degradation is sufficient. Drug substances between 5% and 20% have been accepted as reasonable for chromatographic assay validation. For small pharmaceutical molecules with acceptable stability limits of 90% of label claim, some pharmaceutical scientists believe that 10% degradation is ideal for use in analytical validation [24].

3.3. Strategy for selection of degradation conditions

To create representative samples for the purpose of creating stability-indicating techniques for drug substances and drug products, forced degradation is used. The selection of stress conditions ought to align with the normal manufacturing, storage, and use conditions of the product, which vary depending on the circumstances [25]. The “figure 2” illustrates a general protocol of degradation conditions used for drug substance and drug product.

For forced degradation studies, the following stress factors are a minimum requirement: oxidation, photolysis, thermal degradation, acid and base hydrolysis, freeze-thaw cycles, and shear [26]. The pH, temperature, and specific oxidising agents that must be used are not specified in regulatory guidelines. Although Q1B specifies that the light source should produce combined visible and ultraviolet (UV, 320–400 nm) outputs and that exposure levels should be justified, the applicant is free to design the photolysis studies as they see fit [27]. Finding the circumstances that cause the medication to deteriorate by roughly 10% should be the goal of the first trial. "Table 2" lists some of the most common conditions encountered in forced degradation studies. According to some scientists, it makes sense to start with extremely harsh circumstances. To assess the rate of degradation, tests should be conducted at shorter (2,5,8,24h, etc.) intervals and at temperatures of 80^0C or even higher. Testing at early stages allows for the differentiation of the primary degradants and their secondary degradation products, which improves the degradation pathway determination. In a different method, degradation begins under the conditions listed in “Table 2” and assumes that the drug substance is labile [28].

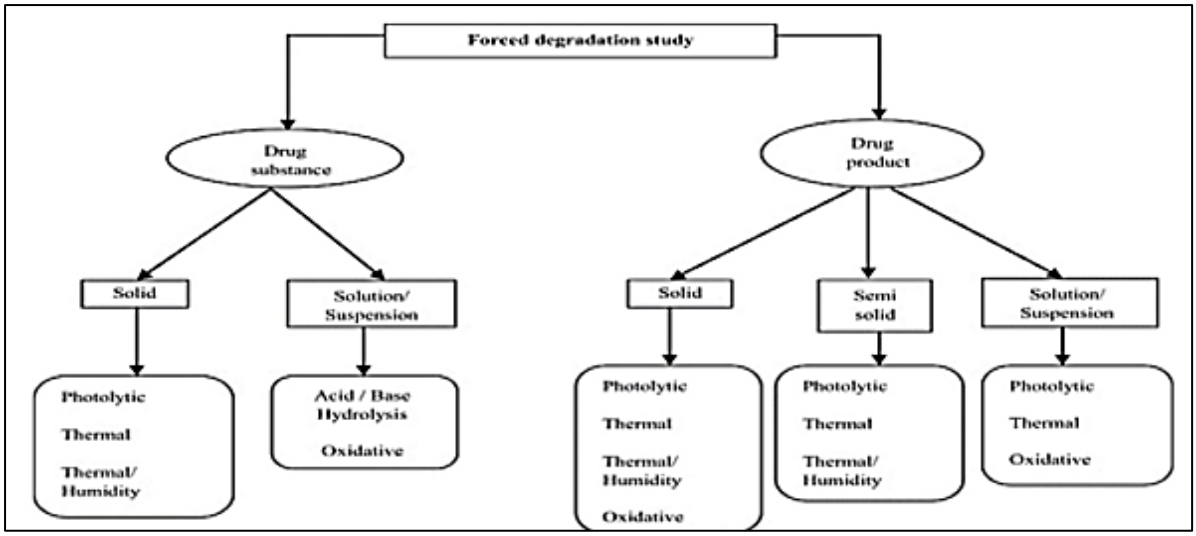

Fig. 2: General protocol for forced degradation conditions

Table 2: Degradation Studies and Conditions

Degradation type	Experimental conditions	Storage conditions	Sampling time(days)
Hydrolysis	Control API	40^0C, 60^0C	1,3,5
	0.1M HCl	40^0C, 60^0C	1,3,5
	0.1M NaOH	40^0C, 60^0C	1,3,5
	Acid control (no API)	40^0C, 60^0C	1,3,5
	Base control (no API)	40^0C, 60^0C	1,3,5
	pH: 2,4,6,8		
Oxidation	3% H_2O_2	25^0C,60^0C	1,3,5
	Peroxide control	25^0C,60^0C	1,3,5
	Azobisisobutyronitrile (AIBN)	25^0C,60^0C	1,3,5
	AIBN control	25^0C,60^0C	1,3,5
		25^0C,60^0C	1,3,5
Photolytic	Light 1 x ICH	NA	1,3,5
	Light 2 x ICH	NA	1,3,5
	Light 3 x ICH	NA	1,3,5
	Heat chamber	NA	1,3,5
Thermal	Heat chamber	NA	1,3,5
	Heat chamber	NA	1,3,5
	Heat chamber	NA	1,3,5

3.4. Degradation conditions

3.4.1 Hydrolysis

For a broad range of pH conditions, the common chemical reactions for degradation are hydrolysis. The chemical compound's degradation upon reaction with water is called hydrolysis. For acidic and basic hydrolysis, catalysis of ionisable functional groups occurs. For acid hydrolysis sulphuric or hydrochloric acid are most suitable while sodium or potassium hydroxide for base hydrolysis. Co-solvents are used if compounds are poorly soluble in water. Forced degradation is initiated at room temperature and further temperature shall be increased if no degradation occurs [29].

3.4.2 Oxidation

For oxidative degradation, hydrogen peroxide is widely used. Apart from these, metal ions, oxygen and radical indicators such as azobi-isobutyl-nitrile (AIBN) may also be used. Drug structure permits selecting concentration and criteria of oxidation. An electron transfer mechanism occurs in this process [30].

3.4.3 Photolytic conditions

Since light exposure do not hamper the drug substance, photolytic studies are carried out. These studies are carried out to generate primary degradants in presence of UV or fluorescent light for drug substance. ICH guidelines recommend some conditions for photostability [31]. Drug substance and solid/liquid drug product must be exposed to minimum of 1.2 lx hrs and 200 W h/m^2 light. Suitable wavelength for photolytic degradation is 300-800nm. Photo-oxidation by stress conditions is induced by free radical mechanism. Groups causing photolysis include carbonyls, nitroaromatic, sulphides and polyenes [32].

3.4.4 Thermal conditions

Carried out at more severe conditions than described in ICH Q1A guidelines for accelerated testing. The samples involving solid-state drug substance and drug product are exposed to dry and wet heat. For liquid products only dry heat is applicable. Arrhenius equation 1 explains the effect of temperature on thermal degradation.

$$K = Ae^{-Ea/RT} \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

In the equation;

where k is specific rate constant, Ea is energy of activation, R is the gas constant (1.987 cal/degree mol). T is absolute temperature. Thermal degradation usually performed at 30-40^{0}C [33].

4. Stability Indicating Assay Method

A SIM is an analytical technique used to quantify the amount of an API that is lost because of deterioration in a drug product. A stability-indicating method, according to the FDA guidance document, is a validated quantitative analytical technique that can detect changes with time in the relevant properties of the drug substance as well as products. A stability-indicating approach precisely evaluates variations in the concentration of the active ingredients without the impact of extra degradation products, contaminants, or excipients.

4.1. Sample Preparation

The proper preparation of the sample is an essential step in the stability indicating assay procedure. Knowing the structure of the drug and how it breaks down is crucial to selecting the right strategy. Stress testing is done on the prepared sample, so understanding its constituent parts, their rate of degradation, and any associated contaminants from earlier samples will help in the creation of a reliable stability-indicating assay technique [34].

4.2. Choice of Method

The method's selectivity and specificity, or how sensitively it can assess the given material, determine which approach is best. The methodology is selected after a careful analysis of the literature for a sample that is anticipated to be used and for which procedures have already been developed. It relies on the method's ability to discriminate between the drug's degraded

result and its API, along with any associated contaminants.

4.3. Development and optimisation of methods

Finding the pKa value, log P, solubility, and max of the pertinent drug is the first step in developing a technique. It is standard procedure to use HPLC in a reverse phase approach for pharmaceutical separation. In a mobile phase consisting of acetonitrile, water, and methanol in different ratios can be used for the initial stages of separation. Methanol or acetonitrile should be selected for the organic phase based on the solubility of the analyte. The water: organic phase ratio can be initially set at 50:50 and pertinent adjustments can be made as trials progress to achieve a satisfactory separation of peaks [35].

4.4. Validation of methods

When verifying a procedure, the ICH guidelines are adhered to. The accuracy, precision, linearity, Limit of Quantitation (LOQ), Limit of Detection (LOD), robustness, and ruggedness of each recently created novel technique are assessed. As per the guidelines provided by ICH, the Relative Standard deviation (RSD) value ought to be below 2%. Once it is determined that the degradants exceed the identification threshold (roughly 0.1%), they need to be separated, identified, and quantified. If the validation's acceptance requirements are not met, the method is updated and revalidated.

5. Relationship Between Stability and Forced Degradation

Forcible deterioration investigations yield more items than standard stability testing. It can be difficult to identify actual degradation products during stability testing because of their limited potential. From this perspective, studies on forced deterioration minimise this problem. Forced degradation analysis can also be used to study the proper storage conditions for specific medications. More significantly, forced degradation studies are useful in determining the mechanism of degradation of various pharmacological compounds [37].

6.Classification Of Chromatographic Techniques for Sim

Analytical techniques are used for stability indicating method development and validation. The classification by chromatographic techniques is provided in the below “figure 3”.

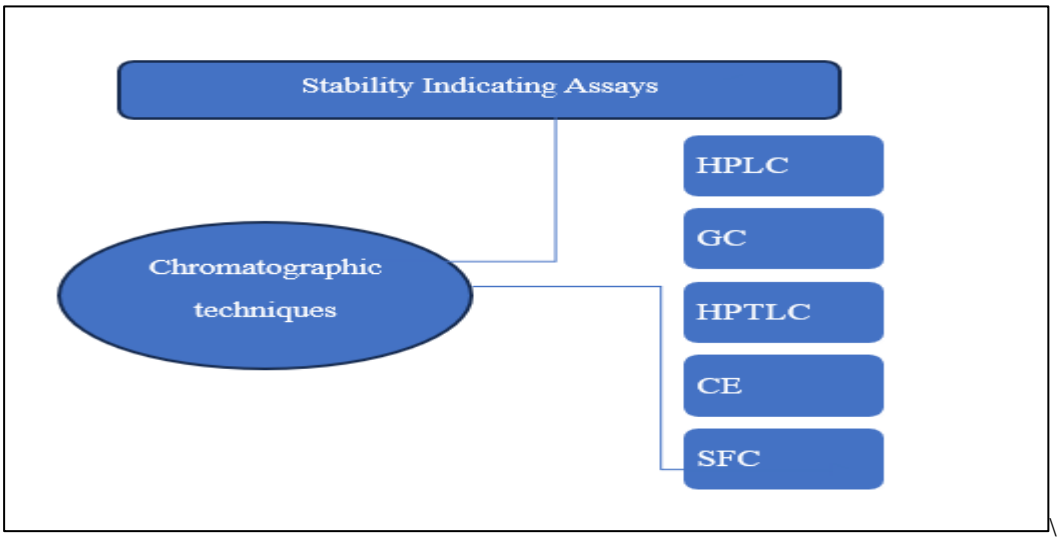

Fig.3: Classification of analytical Stability indicating Methods (SIM)

6.1. High performance liquid chromatography

Pharmaceutical analysis uses HPLC as its primary analytical method. Using a chromatographic column and an appropriate solvent to dissolve a solid or liquid sample, HPLC is performed. This system offers great recovery, high resolution, minimal sample

preparation, ease of use, and versatility [37]. Many different kinds of compounds, including those with varying polarity, molecular mass, volatility, and thermal sensitivity, can also be treated with this technique [38].

6.2. Gas chromatography

Compounds that can evaporate without breaking down can be separated and analysed using gases using gas chromatography (GC). A solvent is used to dissolve the sample before it is injected into the GC apparatus for analysis. Before the analytes are divided into stationary and mobile phases, the sample is evaporated. Analytes are transported through a heated column containing chemically inert gases, like nitrogen and helium, where they are separated and partitioned [39].

6.3. Capillary electrophoresis

High-performing separation techniques like capillary electrophoresis (CE) are used in narrow-bore capillaries under the influence of an external electric field[40]. This technique can be used with a variety of materials, including inorganic ions, chiral biomolecules, biotechnological materials, biopolymers, and clinical samples [41].

6.4. Supercritical fluid chromatography

Many different types of detectors can be connected to SFC, including Fourier Transformer Infrared, mass spectrometers, flame ionisation detectors (FID, FPD, and ECD), fluorescence emission spectrometers, and thermionic detectors. For SFC, FID and MS are frequently utilised [42].

Conclusion

An analytical technique called the stability-indicating method is used to determine the purity of a drug sample or drug substance. The major active (intact) pharmaceutical ingredients (API) can be distinguished from any degradation (decomposition) product(s) formed under specified storage conditions during the stability evaluation period using the stability-indicating method, an analytical procedure. As part of the validation protocol, forced degradation studies are essential to the development of stability indicating and degradant monitoring methods.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authorship contribution statement

Mukesh Patil: Supervision, Validation, Methodology, Investigation, Writing – original draft, Nanduri **Sri Sesha Sai Swaroop:** Conceptualization, Administration, **Ashish S Jain**: Funding, Data Curation.

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