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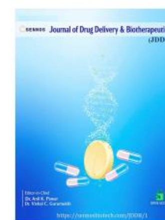

Review Article

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

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ABSTRACT

High-Performance Liquid Chromatography (HPLC) has emerged as a pivotal analytical technique for evaluating the stability of drug products. Renowned for its precision and accuracy, HPLC enables both qualitative and quantitative analyses, ensuring comprehensive assessment of pharmaceutical formulations. Among its applications, the development of stability-indicating methods holds particular significance, especially in identifying and separating drug-related impurities that arise during production or synthesis. Forced degradation studies play a central role in this process, as they provide valuable insights into the degradation pathways of drug substances and products under various stress conditions. These studies not only aid in understanding the chemical behavior of drugs but also assist in predicting their stability profiles, facilitating the design of robust formulations with prolonged shelf life. This review highlights the critical strategies and considerations in developing stability-indicating HPLC methods, emphasizing the importance of forced degradation in the pharmaceutical industry's quality control and regulatory compliance. By systematically addressing the degradation pathways and impurity profiles, HPLC ensures the reliability and efficacy of drug products throughout their lifecycle.

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

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1.**Introduction**

The development and validation of stability-indicating methods have become essential components of modern pharmaceutical analysis, particularly in ensuring the quality, safety, and efficacy of drug substances and products. Stability-indicating methods are specialized analytical techniques designed to detect and quantify active pharmaceutical ingredients (APIs) while simultaneously separating them from their degradation products, impurities, and other excipients. Among various analytical tools, chromatography, including high-performance liquid chromatography (HPLC), has emerged as the most effective and widely used technique for stability assessment due to its precision, accuracy, and versatility [1].

Forced degradation studies form the backbone of stability-indicating method development. These studies subject drugs to stress conditions such as light, heat, oxidation, and variations in pH, simulating conditions that might cause degradation during storage and use. The insights gained from such studies not only provide critical information about the degradation pathways but also guide the formulation of stable drug products and robust manufacturing processes [2].

This review focuses on the strategic aspects and regulatory requirements of developing stability-indicating methods using chromatographic techniques. It emphasizes the significance of forced degradation in identifying degradation products, understanding drug stability, and meeting regulatory guidelines such as those established by the International Council for Harmonisation (ICH) [3].

2. Strategies for Stability-Indicating Method Development

The development of stability-indicating methods (SIMs) requires a systematic approach to ensure accurate detection and quantification of active pharmaceutical ingredients (APIs) and their degradation products. Forced degradation is a critical process employed to simulate the stress conditions drugs might encounter during manufacturing, storage, and usage. The selection of stress conditions should correspond to the typical environmental challenges faced by the product. The key strategies for designing stability-indicating methods are outlined below [5]:

2.1 Understanding Physicochemical Properties of the Drug

An in-depth understanding of the physicochemical properties of the API and formulation is essential for method development. These properties can be analyzed through comprehensive literature reviews, drug profiles, spectral libraries, and scientific reports. Factors such as dissociation constants, oxidation-reduction potentials, and spectrophotometric characteristics play a crucial role in identifying optimal measurement techniques. Structural analysis, including functional groups and active sites, helps predict potential degradation products and sensitivity to oxidation, hydrolysis, and thermal stress. Compatibility studies are also crucial, as they assess the stability of the drug when mixed with common excipients, identifying potential interactions with inactive ingredients [6].

2.2 Setting Preliminary High-Performance Liquid Chromatography (HPLC) Conditions

The initial setup of HPLC conditions can be derived from existing official and unofficial sources, including published literature. When no established methods are available, new experimental conditions must be devised based on the properties of the API and impurities. Selecting an appropriate column and mobile phase is critical to ensuring successful separation and analysis. Properly designed initial conditions can significantly streamline subsequent developmental stages, saving time and resources [7].

2.3 Sample Preparation for Method Development

Forced degradation studies are vital in SIM development, as they expose the API to stress

conditions exceeding those used in accelerated stability testing. These studies help identify degradation pathways and products, providing valuable insights for optimizing formulation, manufacturing, and packaging processes. The objective is to degrade the API by 5–10% under controlled conditions, generating degradation products that may form during real storage conditions. Analytical tools like photodiode array (PDA) detectors are used to analyze stressed samples, ensuring the method's specificity and robustness in detecting degradation products [8].

2.4 Developing Separation-Stability Indicating Chromatographic Conditions

The development of chromatographic conditions for stability-indicating methods (SIMs) requires ensuring the complete dissolution of degradation products for efficient separation and detection. For this purpose, a diluent consisting of water and an organic phase in a 1:1 ratio is often utilized, as it increases the likelihood of solubilizing related substances while enabling proper disintegration of solid dosage forms. This approach facilitates the preparation of samples with degradants in solution form, enhancing their detectability during analysis [9].

The next step involves optimizing degradation conditions to resolve as many distinct peaks as possible from the test samples. Key variables for achieving successful separation include the type of solvent, mobile phase composition, pH, column type, and operational temperature [10].

2.4.1 Isocratic or Gradient Mode

Isocratic conditions, where the mobile phase composition remains constant throughout the chromatographic run, are often preferred due to their simplicity and equilibrium state. This minimizes abrupt chemical changes in the system or column, extending column life. However, the increasing complexity of pharmaceutical samples has led to the adoption of gradient mode in many cases. In gradient mode, the solvent strength increases progressively during the run, allowing the separation of multiple active components or degradants in a single analysis. The choice between isocratic and gradient modes depends on the number of analytes requiring resolution and their separation complexities [11].

2.4.2 Type of Solvent

The selection of the solvent is critical for separation, with its polarity playing a pivotal role in interaction with the sample. Matching the solvent to the most polar functional group of the analyte (e.g., alcohols for OH or amines for NH2) offers a starting point for separation. Adjustments can be made based on initial observations: if the sample elutes too quickly, a less polar solvent may be used, and if retention is too long, a more polar solvent or blend can be employed.

The choice of solvent impacts selectivity, with methanol, acetonitrile, and tetrahydrofuran being commonly used. Methanol and acetonitrile are typically preferred due to their compatibility with most pharmaceutical analytes and buffer systems. Tetrahydrofuran, though less polar, can significantly alter selectivity but may be unsuitable for low-wavelength detection required for many drugs [12,13].

2.4.3 Mobile Phase pH

The pH of the mobile phase is a crucial parameter in chromatographic method development, especially for pharmaceutical compounds with ionizable functional groups such as amino, pyridine, or carboxylic acid moieties. Advances in packing materials stable up to pH 12 have expanded the applicability of pH adjustments for retention and selectivity optimization. Separation challenges, such as elution of samples in void volumes, can be addressed by fine-tuning the solvent strength and utilizing bonded phase materials such as C18/C8, cyano, or phenyl. Adjusting the mobile phase pH influences the equilibrium between the analyte and stationary phase, thus enabling improved separation and resolution [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].

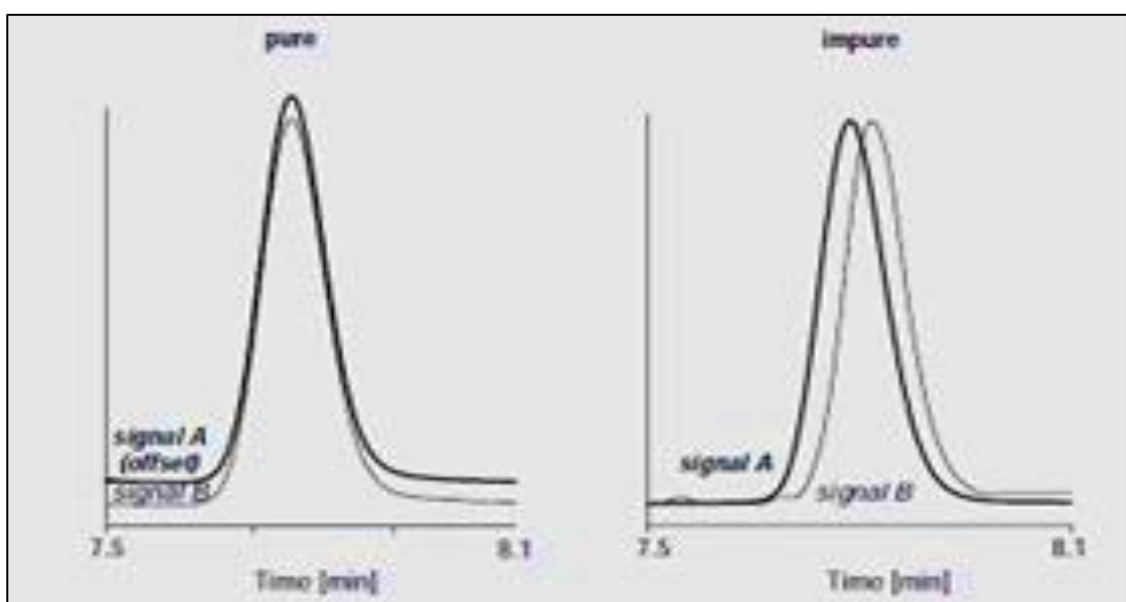

Fig.1: Signal normalisation for pure and impure peak

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 is a critical concern in pharmaceutical molecules as it directly impacts the efficacy and safety of medications. Regulatory guidelines established by the FDA and ICH mandate stability testing to evaluate how the quality of a drug substance or product changes over time under various environmental conditions. Such data are vital for determining appropriate formulation, packaging, storage conditions, and shelf life. Forced degradation studies, beyond establishing specificity, provide crucial insights, including the degradation pathways of drug substances and products, differentiation between drug-related and excipient-related degradation products, structural elucidation of degradation products, intrinsic stability in solid and solution states, and the mechanisms driving degradation, such as thermolysis, hydrolysis, oxidation, and photolysis [20][21].

3.1 Time to Perform Forced Degradation

Understanding the appropriate timing for forced degradation studies is essential in drug development. FDA guidelines recommend conducting these studies during Phase 3 of regulatory submission. However, initiating stress testing early, during the preclinical Phase 1 trials, is highly beneficial. Early stress testing allows sufficient time to identify degradation products, obtain structural information, and optimize stress conditions. Additionally, it informs improvements in the manufacturing process and the development of stability-indicating analytical methods. Stress studies are typically conducted under diverse conditions, such as varying pH levels, exposure to light and oxygen, high humidity, and elevated temperatures, often on a single batch. The results are compiled and reported annually [22][24].

3.2 Limits for Degradation

Determining acceptable limits for degradation has been a topic of significant discussion among pharmaceutical scientists. For chromatographic assay validation, a degradation range between 5% and 20% is generally accepted. For small molecules with stability limits of 90% of the label claim, a degradation level of approximately 10% is often considered ideal for analytical validation [24].

3.3 Strategy for Selection of Degradation Conditions

Forced degradation studies aim to create representative samples to develop stability-indicating

techniques. The selection of stress conditions should mimic the product's manufacturing, storage, and use environments. Minimum required stress factors include oxidation, photolysis, thermal degradation, acid and base hydrolysis, freeze-thaw cycles, and mechanical shear. Regulatory guidelines, such as ICH Q1B, provide general recommendations, such as using light sources emitting both visible and UV light (320–400 nm), but leave specifics like pH, temperature, and oxidizing agents to the applicant's discretion [25][27]. Initial degradation trials typically aim to achieve approximately 10% degradation. Testing under harsher conditions is often recommended, with degradation monitored at shorter intervals (e.g., 2, 5, 8, 24 hours) and elevated temperatures, such as 80°C or higher. Early-stage testing facilitates the identification of primary degradants and secondary degradation products, improving the understanding of degradation pathways. Alternative approaches involve starting degradation under milder conditions and escalating as necessary to account for drug lability [28].

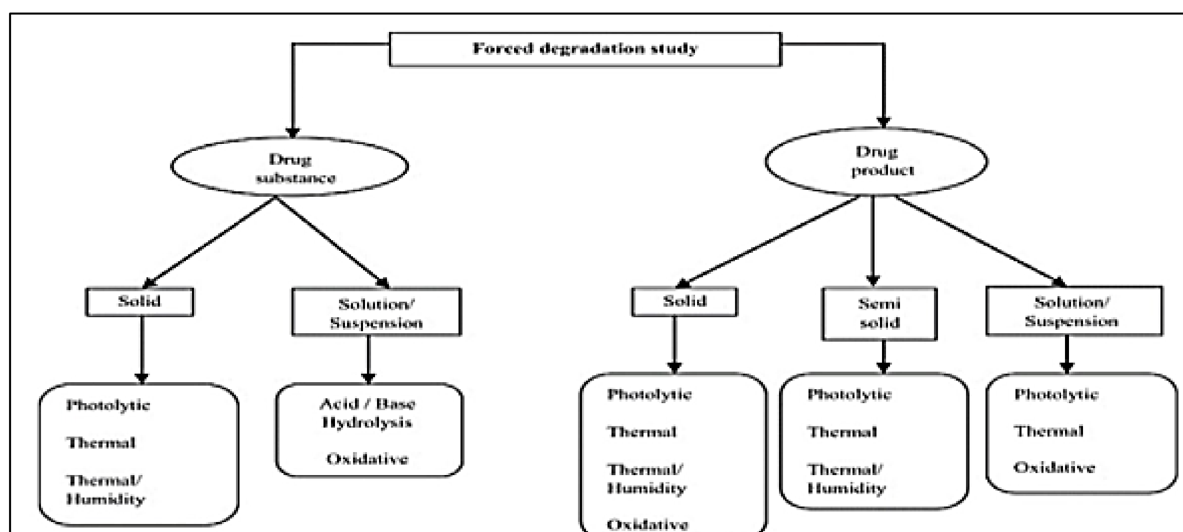

Fig. 2: General protocol for forced degradation conditions

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.

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

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".

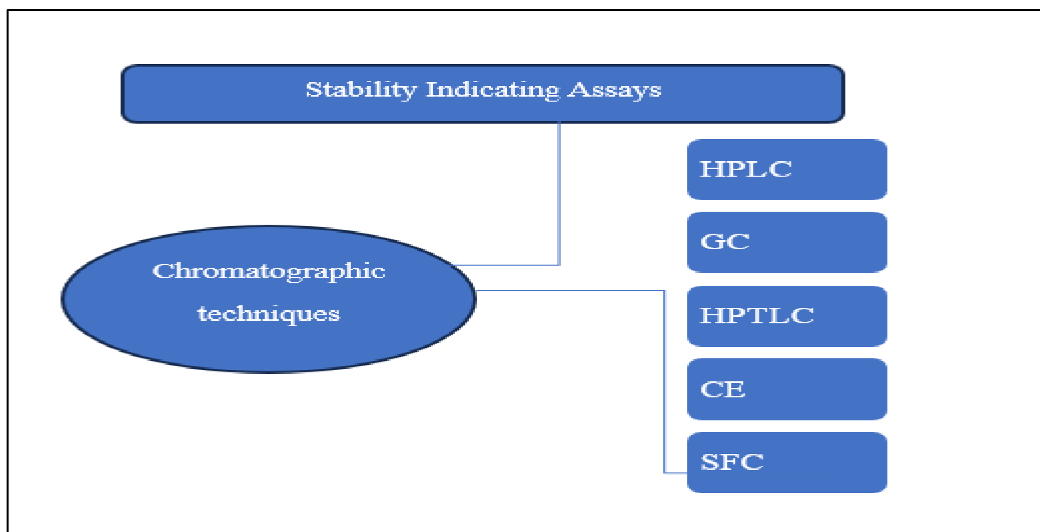

Fig.3: Classification of analytical Stability indicating Methods (SIM)

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 method.

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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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