A REVIEW ON STABILITY INDICATING HPLC METHOD DEVELOPMENT

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ABSTRACT

This article discusses strategies and issues pertinent to a review on Stability indicating HPLC method development. High performance liquid chromatography (HPLC) is an essential analytical tool in assessing drug product stability. HPLC methods should be able to separate, detect and quantify the various drug-related impurities that may be introduced during synthesis. It further understands the chemistry of the drug substance and drug product and facilitates the development of stability indicating analytical methodology. Various chromatographic factors were evaluated in order to optimize the detection of all potentially relevant degradants. The method should be carefully examined for its ability to distinguish the primary drug components from the impurities. New chemical entities and drug products must undergo forced degradation studies which would be helpful in developing and demonstrating the specificity of such stability indicating methods. At every stage of drug development practical recommendations are provided which will help to avoid failures.

KEY WORDS: HPLC, Forced degradation, stability indicating method.

INTRODUCTION

High Performance Liquid Chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantify the compounds that are present in any sample that can be dissolved in a liquid.
High performance liquid chromatography (HPLC) is the most accurate analytical methods widely used for the quantitative as well as qualitative analysis of drug product and used for determining drug product stability.[1,11]

**PRINCIPLE**

Adsorption chromatography: When the stationary phase is a solid and mobile phase is a liquid or gaseous phase, it is called as adsorption chromatography.

Examples: Thin layer chromatography, column chromatography, Gas-solid chromatography.

Partition chromatography: When then stationary phase and mobile phase are liquid, it is called partition chromatography.

Examples: Paper partition chromatography, Gas-liquid chromatography.[1]

**IMPORTANCE**

HPLC is a versatile, reproducible chromatographic technique for the estimation of drug products. It has wide applications in different fields in term of quantitative and qualitative estimation of active molecules. HPLC has many applications in the field of pharmaceutical, environmental, clinical, forensic and in food and flavor analysis.[11]

**METHOD DEVELOPMENT**

Before starting the method development, various physiochemical properties like pKa value, log P, solubility and absorption maximum of the drug must be known, for it lays a foundation for HPLC method development. Log P and solubility helps select mobile phase and sample solvent while pKa value is important as it helps determine the pH of the buffer and pH related changes in retention occur at pH values within ±2 of pKa value.[17]

Reverse phase column is a preferred choice to start the separation of sample components as the degradation is carried out in aqueous solution. Methanol, water and acetonitrile can be used as mobile phase in various ratios for the initial stages of separation. Selection between methanol and acetonitrile for organic phase is based on the solubility and properties of the analyte. Initially the water: organic phase ratio can be kept at 50:50 and suitable modifications can be made as trials proceed to obtain a good separation of peaks. Latter buffer can be added if it is required to obtain better peak separation and peak symmetry. Variation in column temperature affects the selectivity of the method as analytes respond differently to temperature changes. A temperature in the range of 30–40°C is suitable to
obtain good reproducibility. Also a sufficient run time after the drug peak is to be allowed to obtain the degradants peak eluting after the drug peak. During the method development it may happen that the drug peak may hide an impurity or degradant peak that co-elutes with the drug. This requires peak purity analysis which determines the specificity of the method. Direct analysis can be done online by using photodiode array (PDA) detection. PDA provides information of the homogeneity of the spectral peak but it is not applicable for the degradants that have the similar UV spectrum to the drug. Indirect method involves change in the chromatographic conditions like mobile phase ratio, column, etc. which will affect the peak separation. The spectrum of altered chromatographic condition is then compared with the original spectra. If the degradant peaks and area percentage of the drug peak remain same, then it can be confirmed that the drug peak is homogeneous. The degradant that co-elutes with the drug would be acceptable if it is not found to be formed in accelerated and long term storage conditions. The method is then optimized for separating closely eluting peaks by changing flow rate, injection volume, column type and mobile phase ratio.\textsuperscript{13,17,18,19}

**IMPORTANCE OF STABILITY INDICATING HPLC METHOD**

Quality control has become a stringent aspect of pharmaceutical manufacture to minimize batch-to-batch variation and ensure quality. Today, stability is the main and most significant quality requirement for a pharmaceutical product. Stable preparations have a direct emphasis on the quality of the product, assuring its precise delivery. Also the shelf life period of the drug formulation is dependent on the analytical studies at normal and stressed conditions. The ICH drug stability testing guideline Q1A (R2) emphasizes that the analysis of samples of active pharmaceutical ingredients, which are subjected to stress conditions, should be carried out, to establish their inherent stability characteristics, thereby leading to identification of the degradation products through the use of validated stability-indicating analytical methods. Stability-indicating-assay-methods (SIAMs) are specific ones, which evaluate the drug in the presence of its degradation products, excipients, and additives.\textsuperscript{2,19,20}

The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors, such as temperature, humidity, and light, and to establish a retest period for the drug substance or a shelf life for the drug product and recommended storage conditions.

The choice of test conditions defined in this guidance is based on an analysis of the effects of climatic conditions in the three regions of the EU, Japan, and the United States. The mean
Kinetic temperature in any part of the world can be derived from climatic data, and the world can be divided into four climatic zones, I-IV. This guidance addresses climatic zones I and II. The principle has been established that stability information generated in any one of the three regions of the EU, Japan, and the United States would be mutually acceptable to the other two regions, provided the information is consistent with this guidance and the labeling is in accord with national/regional requirements. A stability indicating method (SIM) is an analytical procedure used to quantitate the decrease in the amount of the active pharmaceutical ingredient (API) in drug product due to degradation. According to an FDA guidance document, a stability-indicating method is a validated quantitative with time. A stability-indicating method accurately measures the changes in active ingredients concentration without interference from other degradation products, impurities and excipients. Stress testing is carried out to demonstrate specificity of the developed method to measure the changes in concentration of drug substance when little information is available about potential degradation product. The development of a suitable stability indicating method provides a back ground for the pre-formulation studies, stability studies and the development of proper storage requirements.\cite{2,3,18,19}

The FDA and ICH guidances state the requirement of stability testing data to understand how the quality of a drug substance and drug product degradation studies are a regulatory requirement and scientific necessity during drug development, it is not considered as a requirement for formal stability program. It has become mandatory to perform stability studies of new drug moiety before filing in registration dossier. The stability studies include long term studies (12 months) and accelerated (6 months). But (6 months) can be performed at conditions milder than that used in accelerated studies. So the study of degradation products like separation, identification and quantitation would take even more time. As compared to stability studies, forced degradation studies help in generating degradants in much shorter span of time, mostly a few weeks.\cite{33,34}

**STEPS AND PROCEDURE IN STABILITY INDICATING HPLC METHOD**

There is no “one sets fits all” formula for developing stability indicating analytical HPLC method.

**Analytes**

For a related substance method, determining the “significant and relevant” related substances is very critical. Significant degradation products observed during testing should be
investigated in the method development. Based on the current ICH guidelines on specifications, the related substances method for active pharmaceutical ingredients (API) should focus on both the API degradation products and synthetic impurities, while the same method for drug products should focus only on the degradation products. In general practice, unless there is any special toxicology concerns, related substances below the limit of quantitation (LOQ) should not be reported and therefore should not be investigated. In this stage, relevant related substances should be separated into 2 groups

- Significant related substances: Linearity, accuracy and response factors should be established for the significant related substances during the method validation. To limit the workload during method development, usually 3 or less significant related substances should be selected in a method.

- Other related substances: These are potential degradation products that are not significant in amount. The developed HPLC conditions only need to provide good resolution for these related substances to show that they do not exist in significant levels.

Resolution
A stability indicating method must resolve all significant degradation products from each other. Typically the minimum requirement for baseline resolution is 1.5. This limit is valid only for 2 Gaussian-shape peaks of equal size. In actual method development, $R_s = 2.0$ should be used as a minimum to account for day to day variability, non-ideal peak shapes and differences in peak sizes.

Limit of Quantitation (LOQ)
The desired method LOQ is related to the ICH reporting limits. If the corresponding ICH reporting limit is 0.1%, the method LOQ should be 0.05% or less to ensure the results are accurate up to one decimal place. However, it is of little value to develop a method with an LOQ much below this level in standard practice because when the method is too sensitive, method precision and accuracy are compromised.

Precision, Accuracy
Expectations for precision and accuracy should be determined on a case by case basis. For a typical related substance method, the RSD of 6 replicates should be less than 10%. Accuracy should be within 70% to 130% of theory at the LOQ level.
Analysis time
A run time of about 5-10 minutes per injection is sufficient in most routine related substance analyses. Unless the method is intended to support a high-volume assay, shortening the run time further is not recommended as it may compromise the method performance in other aspects (e.g., specificity, precision and accuracy).

Adaptability for Automation
For methods that are likely to be used in a high sample volume application, it is very important for the method to be “automatable”. The manual sample preparation procedure should be easy to perform. This will ensure the sample preparation can be automated in common sample preparation workstations.[9,10]

The Steps Involved
Understand the chemistry/physicochemical properties of the drug: Knowledge of the physicochemical properties of the API and the formulations is essential in helping to frame the development of the method. Information about dissociation constants and partition coefficients, fluorescent properties (if any), chromatographic behavior, Spectro-photometric properties, oxidation-reduction potentials are useful in setting up preliminary experimental condition and also helpful in selecting the condition of stress studies or possibly in proposing degradation mechanism. Dissociation constant and partition coefficients can be used to develop an efficient sample extraction scheme and determine the optimum pH in mobile phase to achieve good separation. The data on fluorescence, spectrophotometric, chromatographic and oxidation-reduction properties can be used to determine the best means of measuring and quantifying the analyte of interest. Compatibility studies are done to assess the stability when mixed with excipients and lubricants as well as to determine any interaction between the drug and raw materials.[6]

Initial HPLC conditions
Official methods published in the united states pharmacopeia (USP) are considered validated and used for stability testing purposes. Proper column and mobile phase selection is very critical. Computer assisted method development can be very useful in developing the preliminary HPLC conditions quickly.[22,23]
Sample preparation for method development: Stability indicating method is developed by stressing the API under conditions exceeding those normally used for accelerated stability testing. Forced degradation also referred as SIMS, also can be used to provide information about degradation pathways and products that could form during storage and help facilitate formulation development, manufacturing, and packaging. The goal of these studies is to degrade the API 5-10%. Each forced degradation sample should be analyzed by using the preliminary HPLC conditions with suitable detector, most preferably PDA detector.\textsuperscript{[24,25]}

Developing separation-stability indicating chromatography conditions: The initial chromatographic conditions for a SIM of a new entity, most important is to make sure that degradants are in solution, separated, and detected. To this effect, a diluents of 1:1 water: organic solvent is a good starting point as it will increase the likelihood of solubility of most related materials and ensure proper disintegration of solid dosage forms. The second step is to obtain separation conditions that allow the determination of as many distinct peaks as possible from the set of test samples. The most common separation variables include solvent type, mobile phase PH, column type and temperature.\textsuperscript{[14]}

Isocratic or Gradient Mode
It is usually preferred to work in isocratic conditions, whereby the mobile phase composition remains constant. The system and column are equilibrated all the time and does not suffer from fast chemical changes. Selection of isocratic or gradient mode depends on the number of active compounds to be resolved or separated. To select which mode is adequate, an initial gradient run is performed, and the ratio between the total gradient time and difference in gradient time between the first and the last components are calculated. The calculated ratio is $<0.25$, isocratic is adequate; when the ratio is $>0.25$, gradient would be beneficial (Snyder-HPLC., 1988). Generally, Isocratic mode is used for product release and gradient mode for stability assessment because the isocratic method has generally a say less than 15 minutes, and no degradation product would be monitored, assuming that none are formed initially. With time the degradation products are formed and must be monitored, which requires a gradient method to resolve completely the mixture. The gradient method, then, would be the stability or regulatory method.

Solvent type
Solvent type (methanol, acetonitrile and tetrahydro-furan) will affect selectivity. The choice between methanol and acetonitrile may be dependent on the solubility of the analyte as well
as the buffer used. Tetrahydrofuran is least polar among these three solvent, often responsible for large changes in selectivity and is also incompatible with the low wavelength detection required for most pharmaceutical compounds.\[14,15,16\]

**Mobile phase pH**

Most pharmaceutical compounds contain ionisable functionalities such as amino, pyridine and carboxylic acid. Introduction of new packing material that are stable over a wide range of pH up to pH 12 allow for a broader applicability of a mobile phase pH as a retention/selectivity adjustment parameter. When the sample is eluted with a mobile phase of 100% (organic), there is no separation, as the sample is eluted in the void volume. This is because the sample is not retained; but retention is observed when the mobile phase solvent strength is decreased to allow equilibrium competition of the solute molecules between the bonded phase and the mobile phase. When the separation is complex, that is, many components are to be separated, and when the solvent strength is decreased and there is still no resolution between two close peaks, another organic solvent of a different polarity or even a mixture of two organics may need to be tried to effect separation. Additionally, mobile phase optimization can be enhanced in combination with bonded phase optimization (i.e., substituting C18/C8 with cyano or phenyl). A goal for the band spacing of a solute (K’) should be in the range of 4 to 9 and a run time of about 15 minutes or 20 minutes at most for most routine product release or stability runs.\[5\]

**Role of the column and column temperature**

The heart of a HPLC system is the column. Changing a column will have the greatest effect on the resolution of analytes during method development. Choosing the best column for application requires consideration of stationary phase chemistry, retention capacity, particle size, and column dimensions. The three main components of an HPLC column are the hardware (column housing), the matrix, and the stationary phase. Generally, modern RP-HPLC columns are made by packing the column housing with spherical silica gel beads which are coated with the hydrophobic stationary phase.

Identifying the best stationary phase for separation is the most critical step of column selection, and decision should be based on sample solubility and the chemical differences among the compounds of interest. There are several types of matrices for support of the stationary phase, including silica, polymers, alumina, and zirconium. Silica is the most common matrix. Silica matrices are robust, easily derivatized, manufactured to consistent conditions, and easy to replace.
sphere size, and does not tend to compress under pressure. Silica is chemically stable to most organic solvents and to low pH systems. In recent years, silica supported columns have been developed for use at high pH. The nature of the stationary phase will determine whether a column can be used for normal phase or reverse phase chromatography. Common stationary phases are C4 (butyl), C8 (MOS), C18 (ODS), nitrile (cyanopropyl), and phenyl (phenylpropyl) columns. In general, longer alkyl chains, higher phase loading, and higher carbon loads provide greater retention of non-polar analytes. Selectivity is most influenced by the amount of accessible surface area of the derivatized silica gel particles and the carbon load. Thus it is often a benefit to not only have columns with different stationary phases, but columns with the same phase from different manufacturers.

Commonly used reverse phase columns and their uses are Propyl (C3), Butyl (C4), and Pentyl (C5) phases are useful for ion-pairing chromatography (C4) (vide infra) and peptides with hydrophobic residues, and other large molecules. C3–C5 columns generally retain non-polar solutes more poorly when compared to C8 or C18 phases. Examples include Zorbax SB-C3, YMC-Pack C4, and Luna C5. These columns are generally less stable to hydrolysis than columns with longer alkyl chains. Octyl (C8, MOS) phases have wide applicability. This phase is less retentive than the C18 phases, but is still quite useful for pharmaceuticals, nucleosides, and steroids. Standard C18 Columns and similar stationary phases will undergo phase collapse at highly aqueous mobile phases, typically at less than 5-10% organic composition; this will decrease analyte-stationary phase interaction. Collapsed phases are also difficult to re-equilibrate. To prevent phase collapse, C18 columns with a polar group embedded in the alkyl chain have been developed to help solvate the hydrophobic chain in >90% aqueous mobile phases. Examples include Zorbax SB-Aq, Synergi Hydro-RP and YMC-Pack ODS-Aq.²⁶

Column Temperature

Column temperature control is important for long-term method reproducibility as temperature can affect selectivity. A target temperature in the range of 30-40°C is normally sufficient for good reproducibility. Use of elevated temperatures can bring benefits to HPLC, particularly in instances where columns are stable over an extended temperature range. First, operating at a temperature higher than ambient reduces the viscosity of the mobile phase and thus overall backpressure on the column. Lower system pressures allow for faster flow rates and thus faster analyses. The temperature may also effect the selectivity patterns because analytes will
respond dissimilarly to different temperatures. Finally, use of a column oven eliminates variability due to normal fluctuations in the air temperature surrounding the column.

While temperature is a variable that can affect selectivity $\alpha$, its effect is relatively small. Also the $k'$ generally decreases with an increase in temperature for neutral compounds but less dramatically for partially ionized analytes. Some effect when there is a significant difference in size and shape. Overall, it is better to use solvent strength to control selectivity than to use temperature; its effect is much more dramatic. An increase of 1°C will decrease the $k'$ by 1 to 2%, a both ionic and neutral samples are reported to show significant changes in with a temperature changes. Possible temperature fluctuations during method development and validation, it is recommended that the column be thermo stated to control the temperature.[17]

**Peak Purity**

An essential requisite of a separation analysis is the ability to verify the purity of the separated species, that is, to ensure that no co eluting or co migrating impurity contributes to the peak response. Peak purity (or peak homogeneity) analysis of the main peak, to assess for the presence of impurities under the main peak, is an essential part of the validation of a SIM. Direct evaluation can be performed in-line by employing PDA detection, LC-MS or LC-NMR. Indirect evaluation of peak purity can be accomplished by changing one or more chromatographic parameters (column, mobile phase, gradient composition, etc.) that will significantly impact the separation selectivity. The resulting impurity profile is then compared against that of the original method. If the number of degradant peaks is the same in both separations, and if the percent of the main component is the same in both separations, then there can be reasonable confidence that all the degradants have been resolved from the main component. Automated versions of this approach have been successfully utilized in a multi-dimensional screening with instrumentation capable of systematically evaluating several different columns and eluents for impurity analysis.[27,28,29,30]

**Method Optimization**

The experimental conditions should be optimized to get desired seperations and sensitivity after getting appropriate separations. The optimization of mobile phase parameters is always considered first as this is much easier and convenient than stationary phase optimization. Primary control variables (factors) in the optimization of liquid chromatography (LC) methods are the different components of the mobile phase determining acidity, solvent strength, gradient, flow rate, temperature, sample amounts, injection volume, and diluents
solvent type. This is used to find the desired balance between resolution and analysis time after satisfactory selectivity has been achieved. The parameters involved include column dimensions, column-packing particle size and flow rate. These parameters may be changed without affecting capacity factors or selectivity.[4]

Validation of Analytical methods

Methods should be validated according to the ICH guidelines on validation of analytical methods. The degree of analytical validation performed should reflect the purpose of the analysis and the stage of the API production process. In validation, accuracy, precision, specificity, linearity, range, detection limit, quantitation limit, ruggedness, and robustness of the method are done. It is necessary to isolate, identify, characterize, and qualify the degradation products if they are above the identification threshold (usually 0.1%). Analytical method validation is now required by regulatory authorities for marketing authorizations and guidelines have been published. It is important to isolate analytical method validation from the selection and development of the method.

Method selection is the first step in establishing an analytical method and consideration must be given to what is to be measured, and with what accuracy and precision. Method validation must have a written and approved protocol prior to use.[7]

Method development and validation can be simultaneous, but they are two different processes, both downstream of method selection. Analytical methods used in quality control should ensure an acceptable degree of confidence that results of the analyses of raw materials, excipients, intermediates, bulk products or finished products are viable. Before a test procedure is validated, the criteria to be used must be determined.

Analytical methods should be used within good manufacturing practice (GMP) and good laboratory practice (GLP) environments, and must be developed using the protocols set out in the International Conference on Harmonization (ICH) guidelines (Q2A and Q2B, 2000 and Monika Bakshi et al., 2002). The US Food and Drug Administration (FDA) John W Dolan et al 2002 and Smela et al., 2005) and US Pharmacopoeia (USP) (Huynh Ba et al., 2009) both refer to ICH guidelines.[13]
Forced Degradation Studies in Stability-Indicating Method Development

Forced degradation is a process that involves degradation of drug products and drug substances at conditions more severe than accelerated conditions and thus generates degradation products that can be studied to determine the stability of the drug. The ICH guidelines state that stress testing is intended to identify the likely degradation products which further helps in the determination of the intrinsic stability of the drug molecule and establishing degradation pathways, and to validate the stability indicating procedures used. Chemical stability of pharmaceutical molecules is a matter of great concern as it affects the safety and efficacy of the drug product. The study of degradation products like separation, identification and quantitation would take even more time. As compared to stability studies, forced degradation studies help in generating degradants in much shorter span of time, mostly a few weeks. The samples generated from forced degradation can be used to develop the stability indicating method which can be applied later for the analysis of samples generated from accelerated and long term stability studies.\textsuperscript{[12]}

Objectives of Forced degradation studies

Forced degradation studies are carried out to achieve the following purposes:

a) To establish degradation pathways of drug substances and drug products.

b) To differentiate degradation products that are related to drug products from those that are generated from non-drug product in a formulation.

c) To elucidate the structure of degradation products.

d) To determine the intrinsic stability of the drug substance in formulation.

e) To reveal the degradation mechanisms such as hydrolysis, oxidation, thermolysis or photolysis of the drug substance and drug product.

f) To establish stability indicating nature of the developed method.

g) To understand the chemical properties of drug molecules.

h) To generate more stable formulations.

i) To produce a degradation profile similar to that of what would be observed in a formal stability study under ICH conditions.

j) To solve stability-related problems.\textsuperscript{[13,31,32]}

Limits of Degradation

The question of how much degradation is sufficient has been the topic of many discussions amongst pharmaceutical scientists. Degradation of drug substances between 5% and 20% has
been accepted as reasonable for validation of chromatographic assays. It is not necessary that forced degradation would result in a degradation product. The study can be terminated if no degradation is seen after the drug substance or drug product has been exposed to stress conditions than those conditions mentioned in an accelerated stability protocol. This is indicative of the stability of the molecule under test. Over stressing a sample may lead to the formation of a secondary degradation product that would not be seen in formal shelf-life stability studies and under-stressing may not generate sufficient degradation products.\(^{(2,33)}\)

**Strategy of selection for degradation conditions**

Forced degradation is carried out to produce representative samples for developing stability-indicating methods for drug substances and drug products. The choice of stress conditions should be consistent with the product's decomposition under normal manufacturing, storage, and use conditions which are specific in each case. A minimal list of stress factors suggested for forced degradation studies include acid and base hydrolysis, thermal degradation, photolysis and oxidation. The initial trial should have the aim to come upon the conditions that degrade the drug by approximately 10%.\(^{(34)}\)

Some conditions mostly used for forced degradation studies are presented in Table 1.

![Diagram showing different forced degradation processes for drug substance and drug product](image)

**Figure 1. An Illustrative Diagram Showing the Different Forced Degradation to be used for Drug Substance and Drug Product**

The primary degradants and their secondary degradation products can be distinguished by testing at early time points and thus help in a better degradation pathway determination. Studies should be repeated when formulations or methods change because the change may lead to the production of new degradation products.
Selection of Drug concentration
The recommended concentration of the drug is that the studies should be initiated at 1 mg/mL. By using this concentration, it is usually possible to get even minor decomposition products in the range of detection. It is suggested that some degradation studies should also be done at a concentration which the drug is expected to be present in the final formulations.[2,18]

Degradation conditions
Typical degradation studies include four main degradation mechanisms: heat, hydrolytic, oxidative, and photolytic degradation. Selecting suitable reagents such as the concentration of acid, base, or oxidizing agent and varying the conditions (e.g., temperature) and length of exposure the preferred level of degradation can be achieved.

Table 1. Conditions Generally Employed for Forced degradation

<table>
<thead>
<tr>
<th>Degradation Type</th>
<th>Experimental Condition</th>
<th>Storage Condition</th>
<th>Sampling Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrolysis</td>
<td>Control API (no acid or base)</td>
<td>40°C, 60°C</td>
<td>1, 3, 5 days</td>
</tr>
<tr>
<td></td>
<td>0.1N HCL</td>
<td>40°C, 60°C</td>
<td>1, 3, 5 days</td>
</tr>
<tr>
<td></td>
<td>0.1N NaOH</td>
<td>40°C, 60°C</td>
<td>1, 3, 5 days</td>
</tr>
<tr>
<td></td>
<td>Acid Control (no API)</td>
<td>40°C, 60°C</td>
<td>1, 3, 5 days</td>
</tr>
<tr>
<td></td>
<td>Base Control (no API)</td>
<td>40°C, 60°C</td>
<td>1, 3, 5 days</td>
</tr>
<tr>
<td></td>
<td>pH 2, 4, 6, 8</td>
<td>40°C, 60°C</td>
<td>1, 3, 5 days</td>
</tr>
<tr>
<td></td>
<td>3% H₂O₂</td>
<td>25°C, 60°C</td>
<td>1, 3, 5 days</td>
</tr>
<tr>
<td>Oxidation</td>
<td>Peroxide Control</td>
<td>25°C, 60°C</td>
<td>1, 3, 5 days</td>
</tr>
<tr>
<td></td>
<td>Azobisisobutyronitrile</td>
<td>40°C, 60°C</td>
<td>1, 3, 5 days</td>
</tr>
<tr>
<td></td>
<td>AIBN Control</td>
<td>40°C, 60°C</td>
<td>1, 3, 5 days</td>
</tr>
<tr>
<td>Photolytic</td>
<td>Light, 1XICH</td>
<td>NA</td>
<td>1, 3, 5 days</td>
</tr>
<tr>
<td></td>
<td>Light, 2XICH</td>
<td>NA</td>
<td>1, 3, 5 days</td>
</tr>
<tr>
<td></td>
<td>Light Control</td>
<td>NA</td>
<td>1, 3, 5 days</td>
</tr>
<tr>
<td>Thermal</td>
<td>Heat chamber</td>
<td>60°C</td>
<td>1, 3, 5 days</td>
</tr>
<tr>
<td></td>
<td>Heat chamber</td>
<td>60°C, 75% RH</td>
<td>1, 3, 5 days</td>
</tr>
<tr>
<td></td>
<td>Heat chamber</td>
<td>80°C</td>
<td>1, 3, 5 days</td>
</tr>
<tr>
<td></td>
<td>Heat chamber</td>
<td>80°C, 75% RH</td>
<td>1, 3, 5 days</td>
</tr>
<tr>
<td></td>
<td>Heat control</td>
<td>Room Temp.</td>
<td>1, 3, 5 days</td>
</tr>
</tbody>
</table>

Hydrolytic conditions
Hydrolysis is one of the most common degradation chemical reaction over a wide range of pH. Hydrolysis is a chemical process that includes decomposition of a chemical compound by reaction with water. Hydrolytic study under acid or basic conditions involve catalysis of ionizable functional groups present in the molecule. Acid or base stress testing involves forced degradation of a drug substance by exposure to acidic or basic conditions which generates primary degradants in desirable range. The selection of the type and concentrations of acid or base depends on the stability of the drug substance. Hydrochloric acid or sulfuric acids (0.1–1 M) for acid hydrolysis and sodium hydroxide or potassium hydroxide (0.1–1 M)
for base hydrolysis are suggested as suitable reagents. If the compounds for stress testing are poorly soluble in water, then co-solvents can be used to dissolve them in HCl or NaOH. The selection of co-solvent is based on the drug substance structure. Stress testing trial is normally started at room temperature and if there is no degradation, elevated temperatures (50–70°C) are applied. Stress testing should not exceed more than 7 days. The degraded sample is then neutralized using suitable acid, base or buffer, to avoid further decomposition.[2,18,35]

**Oxidation conditions**

Hydrogen peroxide is widely used for the oxidation of drug substances in forced degradation studies but other oxidizing agents such as metal ions, oxygen, and radical initiators (e.g., azobisisobutyronitrile, AIBN) can also be used. Selection of an oxidizing agent, its concentration, and conditions depends on the drug substance. It is reported that subjecting the solutions to 0.1–3% hydrogen peroxide at neutral pH and room temperature for seven days or up to a maximum of 20% degradation could potentially generate the relevant degradation products. The oxidative degradation of drug substance involves an electron transfer mechanism to form reactive anions and cations. Amines, sulfides and phenols are susceptible to electron transfer oxidation to give N-oxides, hydroxylamine, sulfones and sulfoxide. The functional group with labile hydrogen like benzylic carbon, allylic carbon, and tertiary carbon or α-positions with respect to hetero atom is susceptible to oxidation to form hydro peroxides, hydroxide or ketone.[18,35,36]

**Thermal conditions**

Thermal degradation (e.g., dry heat and wet heat) should be carried out at more strenuous conditions than the recommended ICH, Q1A accelerated testing conditions. Samples of solid-state drug substances and drug products should be exposed to dry or wet heat, while liquid drug products should be exposed to dry heat. Studies may be conducted at higher temperatures for a shorter period. Effect of temperature on thermal degradation of a substance is studied through the Arrhenius equation

\[ k = Ae^{-\frac{Ea}{RT}} \]

Where \( k \) is specific reaction rate, \( A \) is frequency factor, \( Ea \) is energy of activation, \( R \) is gas constant (1.987 cal/deg mole) and \( T \) is absolute temperature. Thermal degradation study is carried out at 40–80°C.[2,18,35,36]
Photolytic conditions: The photo stability testing of drug substances must be evaluated to demonstrate that a light exposure does not result in unacceptable change. Photo stability studies are performed to generate primary degradants of drug substance by exposure to UV or fluorescent conditions. Samples of drug substance and solid/liquid drug products should be exposed to a minimum of 1.2 million lux hours. The most commonly accepted wavelength of light is in the range of 300–800 nm to cause the photolytic degradation. The maximum illumination recommended is 6 million lux hours. Light stress conditions can induce photo oxidation by free radical mechanism.[2,36]

CONCLUSION
Stability-indicating method is an analytical procedure that is capable of discriminating between the major active (intact) pharmaceutical ingredients (API) from any degradation (decomposition) product(s) formed under defined storage conditions during the stability evaluation period.

Forced degradation studies are indispensable in the development of stability-indicating and degradant-monitoring methods as part of a validation protocol. Forced degradation studies also provide invaluable insight in investigating degradation products. The use of properly designed and executed forced degradation study will generate a representative sample that will in turn help to develop stability-indicating HPLC method.

Chromatographic factors should be evaluated to optimize the stability indicating HPLC method for detection of all potentially relevant degradants. An appropriate sample solvent and mobile phase must be found that afford suitable stability and compatibility with the component of interest, as well as the impurities and degradants. Therefore, resulting stability indicating HPLC is truly fit for finding the degradants and impurities in pharmaceutical products.

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