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Review Article

STABILITY INDICATING HPLC METHOD DEVELOPMENT – A REVIEW

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ABSTRACT
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 degradants that can form on storage or manufacturing, plus detect and quantify any drug-related impurities that may be introduced during synthesis. This article discusses the strategies and the issues pertinent to designing stability-indicating HPLC methods for drug substances. It furthers understanding of the chemistry of the drug substance and drug product and facilitates the development of stability indicating analytical methodology. A number of key chromatographic factors were evaluated in order to optimize the detection of all potentially relevant degradants. An appropriate sample solvent and mobile phase must be found that affords suitable stability and compatibility with the component of interest as well as the potential impurities and degradants. The method should be carefully examined for its ability to distinguish primary degradants from secondary degradants. Forced degradation studies of new chemical entities and drug products are essential to help develop and demonstrate the specificity of such stability-indicating methods. Practical recommendations are provided for developing forced degradation protocols at every stage of drug development and avoiding common pitfalls that may confuse data interpretation.
KEY WORDS: HPLC, Forced degradation, Stability indicating method

INTRODUCTION

Stability testing of drug substance requires an accurate analytical method that quantitates active pharmaceutical ingredients (API) without interference from degradation products, process impurities and other potential impurities. With the advent of International Conference on Harmonization (ICH) guidelines, the requirement of establishment of stability-indicating assay method (SIAM) has become more clearly mandated. The guidelines explicitly require conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, dry heat, etc. and separation of drug from degradation products.

Stability-indicating method

A stability-indicating assay is a validated quantitative analytical procedure that can detect the changes with time in the pertinent properties of the drug substance and drug product. A stability-indicating assay accurately measures the active ingredients, without interference from degradation products, process impurities, excipients, or other potential impurities.[1]

Forced degradation plays an important role in the development of stability indicating analytical methodology. In addition to demonstrating specificity, forced degradation studies can be used to determine the degradation pathways and degradation products of the APIs that could form during storage, and facilitate formulation development, manufacturing, and packaging. Procedures for the preparation of specific degradation products needed for method validation often emerge from these studies.

STABILITY INDICATING METHOD DEVELOPMENT STRATEGIES

There is no "one set fits all" formula for developing stability indicating analytical method. Before beginning with actual experimentation it would be advantageous to view method development from a broader perspective. Bakshi and Singh[2] reviewed and discussed some critical issues about developing stability indicating methods. Dolan[3] made comments and suggestions on stability indicating assays. Smela[4] discussed from regulatory point of view about stability indicating analytical methods. The method development process can be visualized from a high-level process map perspective better to define the general steps encountered to achieving the end product, stability –indicating method. Figure 1 shows a scheme of stability-indicating HPLC method development strategy.[5] The following is a discussion of a general idea for designing stability indicating analytical method.

Step I - Understand the chemistry/ Physicochemical properties of drug

Knowledge of the physicochemical properties of the API and the formulations is essential in helping to frame the development of the method. Information on various properties has been collected either through systematic program of generating the appropriate information in support of drug discovery or from a search of the literature, company drug profiles, spectral libraries, and reports. Information about dissociation constants, partition coefficients, fluorescent properties (if any), chromatographic behavior, Spectrophotometric 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.[6]

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. Structure of the analyte, especially functional group will indicate the potential active sites for degradation and the susceptibility of the drug to hydrolysis, oxidation, thermal degradation, etc. is determined. Compatibility studies are performed to assess the stability of the when mixed with common excipients and lubricants as well as to determine any interaction between the drug and the (inactive) raw materials.[7] Sometimes this physicochemical information may not be known or available, so that an initial separation would have to be tried, based on prior experience, in order to determine a course of action for subsequent experimentation.

Step II – Set up Preliminary HPLC condition

Preliminary experimental conditions may be adapted from official or unofficial methods and from literature as a starting point. Official methods published in the United States Pharmacopeia (USP) are considered validated and can be used for stability testing if it is proved stability indicating and suitable for intended purposes. New methods have to be created if there are no suitable methods available. Establishing experimental conditions should be based on the properties of API and impurities if known. Proper column and mobile phase selection is very critical. Copious information about various HPLC columns is available nowadays and it is possible to select a right column for any kind of API.[7] One of the very useful sources of information about column is the catalogs from vendors. Get appropriate separation conditions by selecting columns and mobile phase combinations. Computer assisted method development can be very helpful in developing the preliminary HPLC conditions quickly. Since the objective at this stage is to quickly develop HPLC conditions for subsequent method development experiments, scientists should focus on the separation of the significant related substances instead of trying to achieve good resolution for all related substances. A proper experimental condition at the beginning will save a lot of time in subsequent development stage.[8]

Step III – Preparation of samples required for method development

SIMs is developed routinely by stressing the API under conditions exceeding those normally used for accelerated stability testing. In addition to demonstrating specificity in SIMs, stress testing, also referred to as forced degradation, 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. It is hard to get actual representative samples in the early stage of development. Stressing the API generates the sample that contains the products most likely to form under most realistic storage conditions, which is in turn used to develop the SIM.[9]Generally, the goal of these studies is to degrade the API 5-10 %.Perform forced degradation study through thermolysis, hydrolysis, oxidation, photolysis, and or combination conditions. Each forced degradation sample should be analyzed by using the preliminary HPLC conditions with suitable detector, most preferably PDA detector. While the typical dosage form-solid (tablet/capsule), semisolid (ointment /cream), or solution (cough syrup/ophthalmic solution)-utilizes a solid-phase extraction (SPE) for sample preparation, especially for biosamples and as an alternative to liquid-liquid extractions in many U.S. Environmental Protection Agency (EPA) methods.[10]

Step IV – Developing Separation – Stability-Indicating Chromatography Conditions

In selecting 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.[11]

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.[12]

- **Isocratic or Gradient Mode**

Selection of isocratic or gradient mode depends on the number of active components to be resolved or separated. In deciding whether a gradient would be required or whether isocratic mode would be adequate, an initial gradient run is performed, and the ratio between the total gradient time and the difference in gradient time between the first and last components are calculate. The calculated ratio is <0.25, isocratic is adequate; when the ratio is >0.25, gradient would be beneficial[13] as shown in figure 2.

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 tetrahydrofuran) 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.[5, 12]

- **Mobile phase pH**

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. The three main components of an HPLC column are the hardware (column housing), the matrix, and the stationary phase. Generally, modern reverse phase HPLC columns are made by packing the column housing with spherical silica gel beads which are coated with the hydrophobic stationary phase. The stationary phase is introduced to the matrix by reacting a chlorosilane with the hydroxyl groups present on the silica gel surface. In general, the nature of stationary phase has the greatest effect on capacity factor, selectivity, efficiency and elution. There are several types of matrices for support of the stationary phase, including silica, polymers, alumina, and zirconium. Silica is the most common matrix for HPLC columns. Silica matrices are robust, easily derivatized, manufactured to consistent sphere size, and does not tend to compress under pressure. Silica is chemically stable to most organic solvents and to low pH systems. One short coming of a silica solid support is that it will dissolve above pH 7. In recent years, silica supported columns have been developed for use at high PH. The nature, shape and particle size of the silica support effects separation. Smaller particle results in a greater number of theoretical plates, or increased separation efficiency. However, the use of smaller particles also results in increased backpressure during chromatography and the column more easily becomes plugged. For this reason 5 Å columns are more frequently used than 3 Å columns in development work. Narrower particle size distribution of the silica particles also results in better resolution. Hence, similar phase columns from different manufacturers or different lots of columns from the same manufacture may have very different separation properties due to differing methods of matrix preparation. The nature of the stationary phase will determine whether a column can be used for normal phase or reverse phase chromatography. Normal phase chromatography utilizes a polar stationary phase and a non-polar mobile phase. Generally, more polar compounds elute later than non-polar compounds. Types of columns suitable for normal phase chromatography include underivatized silica, nitrile, amino (or amino propyl), glycerol and nitro columns. Chiral separation is usually performed under normal phase conditions. Since highly polar and ionic compounds are retained on normal phase columns, a guard column or silica gel sample purification should be used to extend the column life. In reverse phase chromatography the stationary phase is non-polar and the mobile phase is polar, causing polar

peaks to generally elute earlier than non-polar peaks. To create a stationary phase for reverse phase chromatography on silica support, the free silanols are reacted with a chlorosilane with hydrophobic functionality to introduce the non-polar surface. Due to steric constraints, only about 1/3 of the surface silanols are derivatized. The remaining free silanols can interact with analytes, causing peak tailing. Typically, after the derivitization of a column with the desired stationary phase, the column is further reacted with chlorotrimethylsilane to end cap the remaining freesilanols and improve the column efficiency. Common stationary phases are C4 (butyl), C8 (MOS), C18 (ODS), nitrile (cyanopropyl), and phenyl (phenyl propyl) 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 listed below.

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. C_3–C_5 columns generally retain non-polar solutes more poorly when compared to C8 or C18 phases. Examples include Zorbax SB-C3, YMC-Pack C_4, 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 C18phases, but is still quite useful for pharmaceuticals, nucleosides, and steroids. Octyl columns are also useful for peptides, peptide mapping and small hydrophilic proteins when bonded to 300 Å silica particles. Examples include Zorbax SB-C8, Luna C8, and YMC-Pack-MOS.

Octadecyl (C18, ODS) columns are the most widely used and tend to be the most retentive for non-polar analytes. This phase is useful in ion-pairing chromatography and has wide applicability (same as C8 in addition to vitamins, fatty acids, environmental compounds).Examples include Zorbax SB-C18, YMC-Pack ODS and Luna C18.

Xterra RP-C18 and Zorbax Extend-C18 columns have been formulated to tolerate high pH systems (pH >7, normally up to pH 11). Varying the pH can dramatically affect selectivity and resolution of polar analytes, especially for ionizable compounds.

Phenyl (Ph) columns offer unique selectivity from the alkyl phases and are generally less retentive than C8 or C18 phases. Phenyl columns are commonly used to resolve aromatic compounds. Examples include Zorbax SB-Phenyl, YMC-Pack Phenyl and Luna Phenyl-Hexyl.

Nitrile (CN or cyano) columns are polar and can be used for both reverse and normal phase applications. This phase is often used to increase retention of polar analytes. The nitrile derivatization allows for rapid column equilibration. Examples include Zorbax SB-CN, Luna-CN, and YMC-Pack CN.

Standard C_{18} 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, C_{18} 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 andYMC-Pack ODS-Aq.[14]

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 temperature can be advantageous for several reasons. First, operating at a temperature higher than ambient reduces the viscosity of the mobile phase and thus the overall backpressure on the column. Lower system pressures allow for faster flow rates and thus faster analyses. The temperature may also affect 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 α, 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 shape and size. 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 a with temperature changes. Possible temperature fluctuations during method

development and validation, it is recommended that the column be thermo stated to control the temperature.[15]

- **Peak Purity**

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[16], LC-MS[17], or LC-NMR. However, PDA only works well for degradants that have a different UV spectrum from that of the drug. LC-MS evaluation will not work if the degradant has the same molecular weight, as is the case for diastereomers, or if the ionization of the degradant is suppressed by the co-eluting API.

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 area 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[18, 19, 20]. Other approaches use alternate separation techniques such as thin-layer chromatography (TLC), normal-phase-HPLC, capillary electrophoresis (CE), or supercritical fluid chromatography (SFC), with similar goals as explained in general terms by Lee Polite in a chapter on liquid chromatography[21].

Step V – Method Optimization

The experimental conditions should be optimized to get desired separations and sensitivity after getting appropriate separations. Stability-indicating assay experimental conditions will be achieved through planned/systematic examination on parameters including pH (if ionic), mobile phase components and ratio, gradient, flow rate, temperature, sample amounts, injection volume, and diluents solvent type.[8]

Step VI – Validation of analytical method

The methods have to be validated according to USP /ICH guidelines to show accuracy, precision, specificity, linearity, range, detection limit, quantitation limit, ruggedness, and robustness of the method. Validation protocol should be written and acceptance criteria should be defined. It is necessary to isolate, identify, characterize, and qualify the degradation products if they are above the identification threshold (usually 0.1%).[22, 23]

A variety of techniques are available to identify and characterize impurities and degradation products such as HPLC with PDA (Photodiode Array) Detector, IR (Infrared) Spectrometry, elemental analysis, MS (Mass Spectrometry), NMR (Nuclear Magnetic Resonance), GC/MS, LC/MS, LC/MS/MS, LC/NMR, etc. Method development and validation are cyclic activities. If new problems are encountered for the method during validation or the results are failed to meet acceptance criteria, the method should be modified and be revalidated until the method is suitable for intended purposes.

FORCED DEGRADATION STUDIES IN STABILITY-INDICATING METHOD DEVELOPMENT

Forced degradation studies typically involve the exposure of representative samples of the drug substance or drug product to the relevant stress conditions of light, heat, humidity, acid/base hydrolysis, and oxidation. These experiments play an important role in the drug development process to facilitate: stability indicating method development, drug formulation design, selection of storage conditions and packaging, better understanding of the potential liabilities of the drug molecule chemistry, and the resolution of stability related problems.[9,24-26]Forced degradation on the drug substance and product will (in addition to establishing specificity) also provide the following information: (1) Determination of degradation pathways of drug substances and drug products;(2) Discernment of degradation products in formulations that are related to drug substances versus those that are related to non-drug substances(e.g., excipients); (3) Structure elucidation of degradation products; (4) Determination of the intrinsic stability of a drug substance molecule in solution and solid state;(5) reveal the thermolytic, hydrolytic, oxidative, and photolytic degradation mechanism of the drug substance and drug product.[27]

According to the ICH and FDA guidance documents, Forced degradation study is conducted to fulfill three main purposes: to provide a stability assessment of the drug substance or the drug product; to elucidate the possible degradation pathways of the drug substance or the active pharmaceutical ingredient in the drug product; and to investigate the stability-indicating power of the analytical procedures applied for the drug substance and the drug product. Although the FDA guidance[28] and ICH guidelines[23] provide useful definitions and general comments about forced degradation studies, their direction concerning the scope, timing, and best practices is very general and lacking in details.

This article includes a general study protocol, experimental design, and specific test condition and time line for conducting the studies relative to the stage of drug development.

Experimental Design to Forced Degradation Studies

Study protocol

A general protocol for conducting forced degradation studies, shown in figure 3[27] is arranged according to the type of test material (drug substance, drug product) and the type of degradation (hydrolysis, oxidation, etc.)

Condition for stress Testing

The initial experiments should be focused on determining the conditions that degrade the drug by approximately 10%. The different stress conditions and exposure time generally employed for forced degradation are summarized in Table 1.

The concentration of drug in the stressed sample solution may affect the target level of degradation that is ultimately achieved. A more dilute sample concentration generally yields more extensive degradation than does a more concentrated solution, as exemplified in Figure 4 Therefore, lowering the drug concentration may help to increase degradation when necessary.

Timeline for conducting studies

ICH guidelines make no mention of any regulatory requirement for forced degradation studies at Phase I or Phase II of development. There are good reasons for initiating forced degradation studies on drug substances at Phase I. The most important reason is to support the development of a preliminary method that would be highly discriminating due to its ability to detect most if not all of the potential degradation products. Such a method would have stability-indicating power and would require only minimal validation at this stage. Forced degradation studies on drug substance and drug product should be completed prior to registration stability studies and it would be useful to have identified major degradants by that time. [29, 30]

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. 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 SIM-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 SIM - HPLC is truly fit for finding the degradants and impurities in pharmaceutical products.

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Table 1: Conditions generally employed for forced degradation

CONDITION GENERALLY EMPLOYED FOR DEGRADATION			
Degradation Type	Experimental Condition	Storage Condition	Sampling Time
Hydrolysis	Control API (no acid or base)	40 °C, 60 °C	1,3,5 days
	0.1N HCL	40 °C, 60 °C	1,3,5 days
	0.1 N NAOH	40 °C, 60 °C	1,3,5 days
	Acid Control(no API)	40 °C, 60 °C	1,3,5 days
	Base Control(no API)	40 °C, 60 °C	1,3,5 days
	pH: 2,4,6.8	40 °C, 60 °C	1,3,5 days
Oxidative	3% H_2O_2	25 °C, 40 °C	1,3,5 days
	Peroxide Control	25 °C, 40 °C	1,3,5 days
	Azobisisobutyronitrile (AIBN)	40 °C, 60 °C	1,3,5 days
	AIBN control	40 °C, 60 °C	1,3,5 days
Photolytic	Light, 1 X ICH	NA	1,3,5 days
	Light, 3 X ICH	NA	1,3,5 days
	Light Control	NA	1,3,5 days
Thermal	Heat Chamber	60 °C	1,3,5 days
	Heat Chamber	60 °C/75% RH	1,3,5 days
	Heat Chamber	80 °C	1,3,5 days
	Heat Chamber	80 °C/ 75% RH	1,3,5 days
	Heat Control	Room Temp.	1,3,5 days

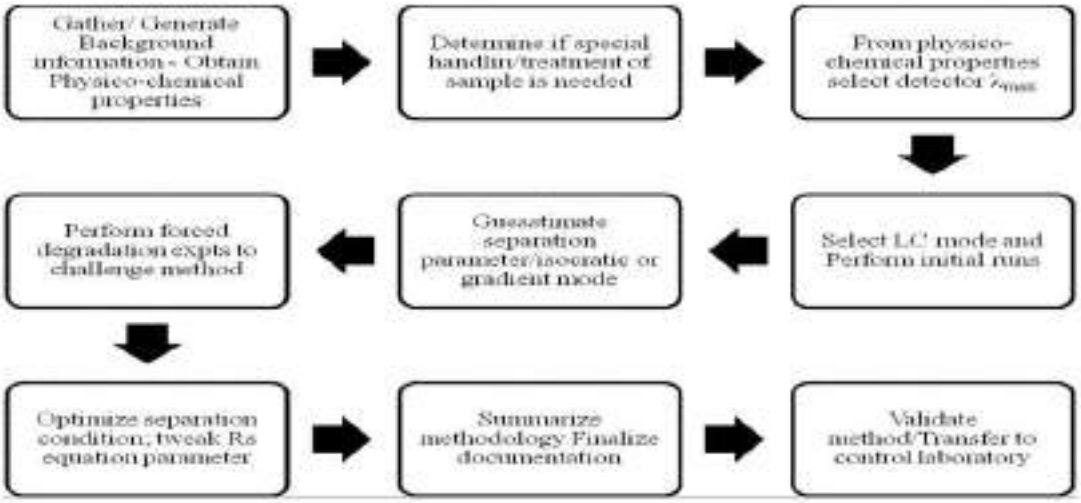
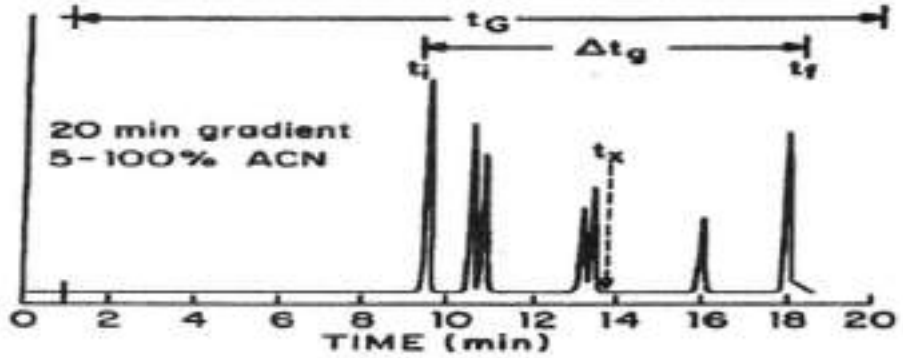


Figure 1: Overview of the Method Development Process



$$t_x = (t_f + t_i)/2$$

$\Delta t_g/t_G > 0.25 \longrightarrow$ gradient

$\Delta t_g/t_G < 0.25 \longrightarrow$ isocratic

$$\Delta t_g/t_G = \frac{18.0-9.5}{20} = 0.425$$

$\therefore$ gradient required

Figure 2: Isocratic or Gradient?

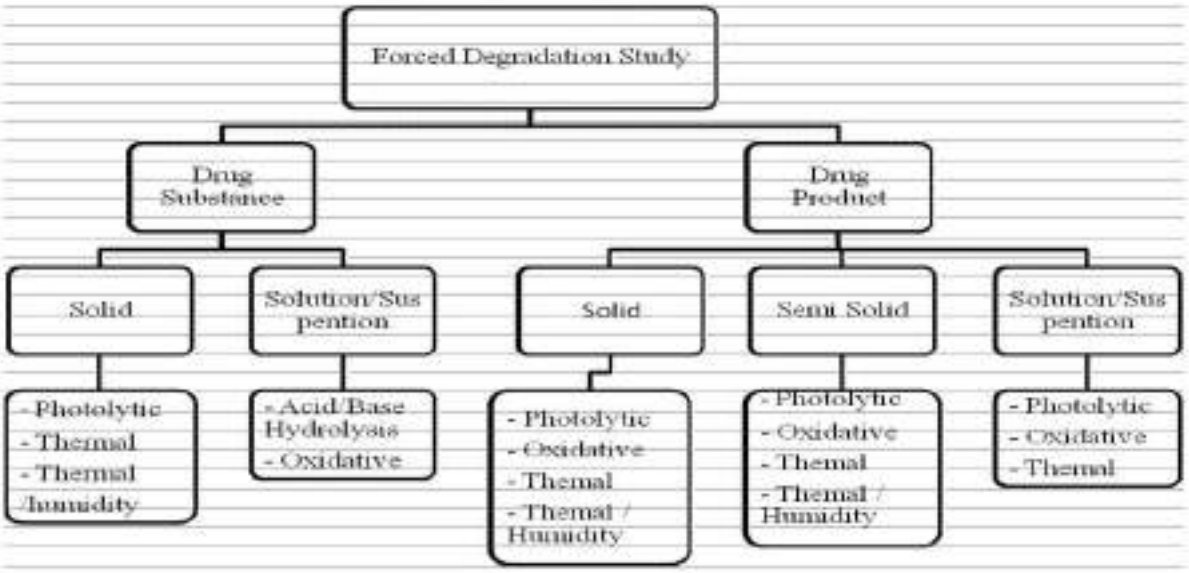


Figure 3: An illustrative diagram showing the different forced degradation conditions to be used for drug substance and drug product

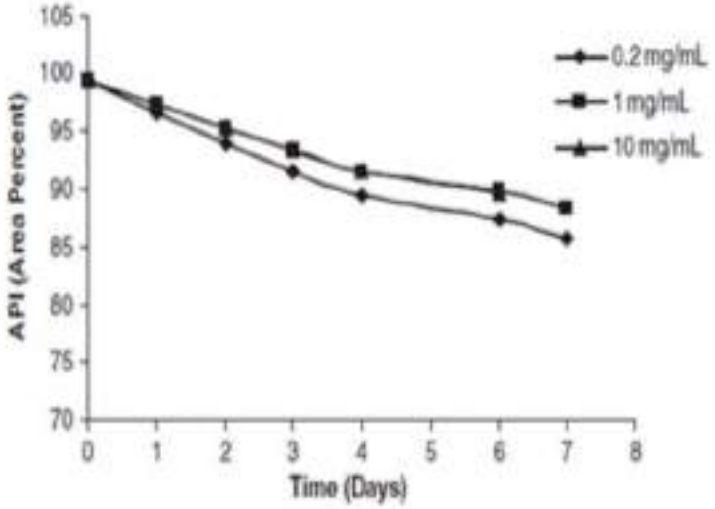


Figure 4: Thermal hydrolysis profile of an API (Structure not shown) at 70^{0}C: degradation vs. time at three sample concentrations