Chapter - 1

Importance of

Organic Impurities Quantification in

Active Pharmaceutical Ingredients.
1.1 Introduction and importance of stability indicating analytical methods.

Stability indicating methods are quantitative test methods that can detect changes with time of drug substances and drug products. Information of type and amount of degradation products over time is important for safety of drugs. Therefore US FDA (United states food and drug administration) and other agencies, also such methods are to be well designed and validated.

A stability indicating analytical method (SIAM) is a validated analytical procedure that accurately and precisely measures active ingredients (drug substance or drug product) free from potential interferences like degradation products, process impurities, excipients, or other potential impurities, and the FDA recommends that all assay procedures for stability studies be stability indicating. It is recommended that forced degradation or chemical stress studies be carried out to determine if analytical methods are stability indicating prior to embarking on long term stability studies. SIAMs are routinely developed by stressing the API (Active pharmaceutical ingredients) under conditions exceeding those normally used for accelerated stability testing. In addition to demonstrating specificity in SIAMs, chemical stress testing, also referred to as forced degradation, can also be used to provide information about degradation pathways and products that could form during storage, and help facilitate formulation development, manufacturing and packaging. Stressing the API in both solutions and in solid state form generates the sample that contains the products most likely to form under most realistic storage conditions, that is in turn used to develop the stability indicating method (SIM). In simplest terms, the goal of the SIAM is to baseline resolve all of the resulting products (the API and all the degradation products) each from the other (i.e. no co-elutions). HPLC is a commonly used technique for SIAMs, primarily with UV detection.

Stability indicative analytical methods are specific so that the quantity of the active pharmaceutical ingredient, degradation products and other components of interest may be accurately measured without interference in the material being tested. A degradation product is a molecule resulting from a change in the active ingredient brought about over time as a result of processing or storage (e.g. oxidation,
hydrolysis). Compounds that are formed from a reaction of the active pharmaceutical ingredient with an excipient or container closure component are also considered degradation products. It is important to note that the necessity for stability indicating capability applies to the complete testing regiment used for a given material.

Analytical problems with co-elution of impurities/degradants with active ingredients or with each other may often be solved with the use of complementary methods. The ability to differentiate the active ingredient from closely related process and degradative impurities is usually the single most important requirement for stability indicating methodology.

Stability indicating analytical methods are developed to monitor the stability of pharmaceutical dosage forms during the investigational phase of drug development, and, once the drug is marketed, for the ongoing stability studies which must be conducted. 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, enables recommended storage conditions, re-test periods and shelf lives to be established. The development of these methods for pharmaceutical dosage forms can be approached from several avenues. Methods can be developed which measure the amount of drug remaining, the amount of drug lost (or the appearance of degradation products), or both.

Information on the stability of the drug substance is an integral part of the systematic approach to stability evaluation. The goal of stability program depends on the stage of development of the drug product. At very beginning of product development, it is necessary to understand the inherent stability of the drug substance and its interaction with the proposed excipients. At this stage the effect of pH, moisture, air (oxygen), and light on the stability of the drug substance also studied. The accelerated testing on drug substance and drug product provides the information to the intrinsic stability of the molecule/formulation and may establish the likely degradation pathways. The formulation group also has the responsibility for recommending to the toxicology group about the stability of drug substance in the vehicle used in the animal trials. On the analytical side, the analytical research group
supports the pre-formulation stability program, which is ultimately responsible for developing and validating the stability-indicating assays that will be included in New Drug Application [NDA].

In the preclinical formulation stage, the selection of a stable drug product formula is the primary goal. The temporary preclinical formula is included in the Investigational New Drug application [IND]. The goal of the stability program in the clinical trial stage is to ascertain that the drug product batches tested in the clinical trials are stable, and these data will be subsequently included in the NDA. At the NDA approval stage, the validated stability-indicating analytical method will be transferred to the quality control group, to ascertain that it works well in the hands of those who have to monitor the stability of the marketed product.

The marketed product stability program fulfills the commitment of part of the NDA and also ensures that the marketed drug products are stable (potent) until the expiry date stamped on the product label. Usually, the first three marketed batches and at least one batch per year are subjected to stability program.

1.2 Shelf life of drugs and its importance:

New drug applications need to submit scientific data that guarantee the stability of the product over a specified time period when maintained under specific storage conditions. If you look at the label on prescription or over-the-counter (OTC) pharmaceutical product, you will see a use before or expiry date \[1\]. Before this date, the product should remain fully effective under normal storage conditions. The product’s shelf life is determined using standardized storage conditions of controlled temperature and humidity, which can be translated into accepted product lifetimes. The performance of a drug when given as tablet, capsule, syrup, or injection depends not only on the content of the drug substance but also on its pharmaceutical properties such as dissolution, disintegration, hardness, and so on. All of these aspects are therefore the part of stability programs \[2,3\].

International conference on harmonization (ICH) of the technical requirements for the registration of pharmaceuticals for human use was organized in order to
harmonize stability-testing requirements for new drug applications within the European Union (EU), the United States, and Japan. ICH guidelines for stability testing of New Drug Substances and Products and for photo stability testing of New Drug Substances and Products were published in October 1993 and were made official in November 1996.

1.3 Stability testing of New Drug substances and Drug Products:

The expert Working Group of the International Conference on Harmonization of the Technical Requirements for Registration of Pharmaceuticals for Human Use developed a guideline on stability testing for a registration application within the European Union, Japan and the United States. The goal of the ICH stability guideline was to exemplify the core stability data package required for new drug substances and products in the European Union, Japan and the United States such that the data generated in any of the regions is mutually acceptable in the other two. The guideline applies to the information required for the registration applications of new molecular entities and drug products, but not to abbreviated or abridged applications, clinical trial applications, and so on. The test conditions were selected based on the climatic conditions in three areas so that test data provides evidence on the variation in quality with time under the influence of a variety of representative environmental factors. These data in turn allow recommended storage conditions and shelf lives to be established.

The primary stability studies for the drug substance show that it will remain within specification during the retest period. Long-term (12-month) and accelerated testing are performed on at least three batches, these batches can be manufactured at a minimum of pilot scale, but should use the same synthetic route and a method of manufacture that simulates the final process to be used at manufacturing scale. In addition, supporting stability on laboratory-scale batches may be submitted. The quality of the batches placed on stability should be representative of the quality of—

(a) Material used in preclinical and clinical studies

(b) Material to be made at a manufacturing scale.
The first three batches made post approval should also be placed on long-term stability using the product registration protocol. Testing should cover physical, chemical, and microbiological properties susceptible to change during storage and likely to affect product quality, safety, and/or efficacy, validated stability-indicating methods should be used. The number of replicates to be run depends on the results of validation studies, and limits should be derived from material used in preclinical and clinical studies, including both individual and total upper limits for impurities and degradation products. The length of the studies and the storage conditions should cover storage, shipment, and subsequent use, although use of the same conditions as for the drug product will facilitate comparative review and assessment. Other conditions should be included as scientifically justified. Temperature-sensitive drugs should be stored at the labeled long-term storage temperature, and accelerated testing should be conducted at 15°C above the designated long-term storage temperature with appropriate relative humidity conditions.

At the time of regulatory submission, a minimum of 12 months at 25°C ± 2°C/60% RH ± 5°C and 6 months at 40°C ± 2°C and 75% RH ± 5% is required. If significant changes are noted at the elevated temperature, additional testing at an intermediate condition, such as 30°C ± 2°C/60% RH ± 5%, should be conducted. The registration application should include a minimum of 6 months of data from a 12-month study at the intermediate condition. Significant change at 40°C and 75% RH is defined as failure to meet specification. Long-term stability testing should be continued to cover all retest periods. Normally, testing under long-term conditions is performed every 3 months for the first year, every 6 months for the second year, and then annually. Containers employed in the long-term stability study should be the same or simulate actual packaging used for storage and distribution.

Because long-term stability is used to establish appropriate retest periods, it should be noted that the degree of inter batch variability affects the confidence that a future batch will remain within specifications for the entire retest period. As a rule, determination of the time at which the 95% one-sided confidence limit for the mean degradation curve intersects the acceptable lower specification limit is acceptable, combining data into one overall estimate to account for variability. Before combining
the data, apply appropriate statistical tests (e.g., p test) to be sure it is allowable. If inappropriate to combine data, the retest period may depend on the minimum time a batch is actually measured to remain in specification. The nature of the degradation relationship determines the need for transformation of the data for linear regression analysis. This relationship can generally be fitted to a linear, quadratic, or cubic function on an arithmetic or logarithmic scale. Use statistical methods to test the goodness of fit of the data on all batches and combined batches, where appropriate, to the assumed degradation curve. If the data show little degradation or variability, a retest period can be justified without statistical analysis and a limited extrapolation of real-time data may be undertaken when supported by the accelerated data. Any extrapolation must be justified, because it assumes that the same mechanism of degradation will continue beyond the observed data; this evaluation should include assay, degradation products, and any other appropriate attributes.

The storage temperature range should be based on the stability data and used in accordance with the national or regional requirements. Specific labeling requirements should be stated, particularly for drugs that cannot freeze; terms such as ambient and room temperature are to be avoided.

The stability program for the drug product \(^{[4,5]}\) should be based on knowledge of the drug substance and experience from experimental and clinical formulations. Unless specifically noted in this section, the requirements for drug substances also apply to drug products. Accelerated and long-term data should be provided on three batches of the same formulation and dosage form in the containers and closure proposed for marketing. Data on laboratory-scale batches of drug product is not acceptable as primary stability data, but may be submitted as supportive information, as may data on associated formulations or packaging. If required, preservative efficacy testing and assays on stored samples should be performed to determine content and efficacy of antimicrobial preservatives. Differences between release and shelf-life specifications for antimicrobial preservatives should be supported by preservative efficacy testing. Limits for tests such as dissolution and particle size require reference to results of bioavailability and clinical batches.
Storage at high relative humidity is important for solid oral dosage forms, but is not necessary for products such as solutions, suspension, and so on, stored in containers designed to provide a permanent water barrier. Low relative humidity (10-20%) is appropriate for products of high water content stored in semi permeable containers. Testing of unprotected drug product can be a useful part of stress testing and package evaluation, as can studies in related packaging materials. If a product needs to be reconstituted or diluted, stability in the final form should also be addressed.

1.4 Stress testing route to the development of stability-indicating analytical methods (SIAMs):

Studies under taken to elucidate the intrinsic stability of the drug substance. Such testing is part of the development strategy and is normally carried out under more severe conditions than those used for accelerated testing. A more detailed description of stress testing is provided near the beginning of the ICH stability guideline, under the “Drug substance” heading:

“Stress testing of the drug substance can help identify the likely degradation products, which can turn help, establish the degradation pathways and the intrinsic stability of the molecules and validate the stability indicating power of the analytical procedures used”. The nature of the stress testing will depend on the individual drug substance and drug product involved.

Stress testing is likely to be carried out on single batch of drug substance. It should include the effect of temperatures (in 10° C increments (e.g., 50°C, 60°C etc.) above that for accelerated testing), humidity (e.g., 75% RH or greater) where appropriate, oxidation, and photolysis on the drug substance. The testing should also evaluate the susceptibility of the drug substance to hydrolysis across wide range of pH values when in solution or suspension. Photo stability testing should be an integral part of stress testing. The standard conditions for photo stability testing are described in ICH Q1B [5].
Examining the degradation products under stress conditions is useful in establishing the degradation pathways, developing, and validating suitable analytical procedures. However, it may not be necessary to examine specifically for certain degradation products if it has been demonstrated that they are not formed under accelerated or long term storage conditions.

Results from these studies will form an integral part of the information provided by the regulatory authorities. The description of stress testing was slightly modified in the revised stability guideline from the original description in ICH Q1A\(^4\). The original Q1A description contains this additional paragraph.

Stress testing is conducted to provide data on forced decomposition products and decomposition mechanisms for the drug substance. The severe conditions that may be encountered during distribution can be covered by stress testing of definitive batches of drug substance.

Studies undertaken to assess the effect of severe conditions on the drug product. Such studies include photo stability testing (ICH Q1B) and specific testing on certain products (e.g., metered dose inhalers, creams, emulsions, refrigerated aqueous liquid products).

From the ICH\(^6\) definition, it is now clear that there is now a (regulatory) differentiation between “accelerated testing” and “stress testing”. Stress testing is distinguished by both severity of the conditions and the focus or intent of the results. Stress testing, which is also often referred as “forced degradation,” is an investigation of the “intrinsic stability” characteristics of the molecule \(^7\) providing the foundation for developing and validating analytical methods and for developing stable formulations. Stress testing studies are intended to discover stability issues, and are therefore predictive in nature. Stress testing studies are not part of the “validated” formal stability program. Rather, pharmaceutical stress testing is a research investigation requiring a scientific expertise and judgment.
1.5 Techniques employed in literature reports for the development of SIAMs:

If one critically evaluates the literature reports, titrimetric, spectrophotometric and chromatographic techniques have been commonly employed in the analysis of stability samples.

1.5.1 Titrimetric and spectrophotometric

In these methods, usually the objective is the analysis of drug alone in the matrix of impurities, degradation products, impurities, etc., and also other drugs in case of the combination products. Their advantage is low cost and simplicity, though sometimes they are not sensitive. Due to limitation of specificity there are hardly any reports these days on the use for the assay of stability samples. However, a few reports involving derivative spectroscopy have been published lately.

1.5.2 Chromatographic methods

Because of very nature of requirement of separation of multiple components during analysis, chromatographic methods have taken precedence over conventional methods of analysis. Other than separation of multiple components, the advantage of chromatographic methods is that these possess greater accuracy and sensitivity for even small quantities of degradation products performed. Various chromatographic methods that have been used are thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), gas chromatography (GC), HPLC (High Performance Liquid Chromatography) and newer technique like RRLC (Rapid Resolution liquid chromatography).

In comparison, HPLC has been very widely employed. It has gained popularity in stability studies due to its high-resolution capacity, sensitivity and specificity. Non-volatile, thermally unstable or polar/ionic compounds can also be analyzed by this technique. Therefore, most of the SIAMs have been established using HPLC.
1.5.3 Steps involved during the development of stability-indicating analytical methods (SIAMs):

A SIAM is a quantitative analytical procedure used to detect a decrease in the amount of the active pharmaceutical ingredient (API) present due to degradation. According to FDA guidelines, a SIAM is defined as a validated analytical procedure that accurately and precisely measures active ingredients (drug substance or drug product) free from potential interferences like degradation products, process impurities, excipients, or other potential impurities, and the FDA recommends that all assay procedures for stability studies be stability-indicating. During stability studies, liquid chromatography (LC) is used routinely to separate and quantitate the analytes of interest. There are three components necessary for implementing a SIAM: sample generation, method development and method validation.

Step 1: Generation of the Sample:

Stressing the API in both solutions and in solid-state form generate the sample that contains the products most likely to form under most realistic storage conditions, which is in turn used to develop the SIAM. In simplest terms, the goal of the SIAM is to obtain baseline resolution of all the resulting products (the API and all the degradation products) with no co-elutions.

Table 1.5.3.T1 lists some common conditions used in conducting forced degradation studies for drug substances.

<table>
<thead>
<tr>
<th>Sample condition</th>
<th>Time / Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid / 60 - 70°C</td>
<td>7 – 10 days</td>
</tr>
<tr>
<td>Solid / 60 - 70°C / 75% RH</td>
<td>10 days</td>
</tr>
</tbody>
</table>
Table 1.5.3.T1(Contd.)

<table>
<thead>
<tr>
<th>Sample condition</th>
<th>Time / Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid / simulated sunlight</td>
<td>overall illumination of not less than 1.2 million lux hours and an integrated near</td>
</tr>
<tr>
<td></td>
<td>ultraviolet energy of not less than 200 watt hours/square meter</td>
</tr>
<tr>
<td>0.1 to 2 N HCl solutions either at RT or at 60 - 70°C</td>
<td>1 - 3 days</td>
</tr>
<tr>
<td>0.1 to 1 N NaOH solutions either at RT or at 60 - 70°C</td>
<td>1 - 3 days</td>
</tr>
<tr>
<td>Dilute hydrogen peroxide (0.1 to 6%) at RT or at 60 - 70°C</td>
<td>1 - 3 days</td>
</tr>
<tr>
<td>Solution in Water or at 60 - 70°C</td>
<td>1 - 3 days</td>
</tr>
</tbody>
</table>

Samples should be stored in appropriate vessels that allow sampling at timed intervals and that protect and preserve the integrity of the sample. Thermostated and humidity controlled ovens should also be employed. Generally, the goal of these studies is to degrade the API by 5-20%. Any more than this and relevant compounds can be destroyed, or irrelevant degradation products produced (for example, degradation products of the degradation products). Any less, and important products might be missed. Experience and data obtained from studies performed previously on related compounds also should be used when developing new protocols.

Step 2: Developing the LC Method:

After the sample is generated through the use of a properly designed and executed forced degradation, it can be used to develop the LC method. These days, LC method development often is performed on gradient systems capable of automated column and solvent switching, and temperature control. Systems and software that
automate the process, some with decision making built-in, also have been reported\textsuperscript{[13]}. Scouting experiments often are run, and then conditions are chosen for further optimization. Resolving power, specificity, and speed are key chromatographic method attributes to be kept in mind during method development\textsuperscript{[10,11]} However, excellent resources are available to anyone not already schooled in the art\textsuperscript{[14]}.

**Step 3: Evaluating Specificity during Method Development:**

Another key parameter to evaluate during method development is specificity. The United States Pharmacopoeia (USP\textsuperscript{[15]} and various International Conference on Harmonisation (ICH) guidelines\textsuperscript{[9]} define specificity as the ability of a method to assess unequivocally the analyte of interest in the presence of potential interferences\textsuperscript{[16,17]} In the past, it has been acceptable to evaluate resolution, peak shape, and tailing factors to measure and document specificity. However, starting with USP 24, and as a direct result of the ICH process, it was recommended that a peak purity test based upon photodiode-array (PDA) detection or mass spectrometry (MS) be used to demonstrate that a given peak was pure.

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’s response. The confirmation of peak purity should be performed before quantitative information from a chromatographic peak is used for further calculations. Neglecting peak purity confirmation means, in quality control, an impurity hidden under a peak could falsify the results or, in research analysis, important information might be lost or scientific observations rendered void should an impurity remain undiscovered.

Modern PDA technology is a powerful tool for evaluating specificity. PDA detectors can collect spectra across a range of wavelengths at each data point collected across a peak, and through software manipulations involving multidimensional vector algebra, they compare each of the spectra to determine peak purity. In this manner, PDA detectors today can distinguish minute spectral and chromatographic differences not readily observed by simple overlay comparisons\textsuperscript{[18-20]}.
To be successful, three components are required:

- A UV chromosphere, or some absorbance in the wavelength range selected\(^{[20]}\).
- Some degree of chromatographic resolution.
- Some degree of spectral difference.

**Peak purity determination** \(^{[21-26]}\): Spectra comparing peak spectra is probably the most popular method to discover an impurity. If a peak is pure all UV-visible spectra acquired during the peak’s elution or migration should be identical, allowing for amplitude differences due to concentration. The results obtained by comparison of these spectra against each other should be very close to a perfect 100% match. Significant deviations can be considered as an indication of impurity.

A valuable tool in peak purity analysis is the overlay of separation signals at different wavelengths to discover dissimilarities of peak profiles. The availability of spectral data in the three-dimensional matrix generated by the diode-array detector enables signals at any desired wavelength to be selected and reconstructed for peak purity determination after the analysis. A set of signals can be interpreted by the observer best when, before being displayed, it is normalized to maximum absorbance or to equal areas.

A good overlap, where peak shape and retention or migration time match, indicate a pure peak while a poor overlap indicates an impure peak, as demonstrated in figure 1.5.3.F1

![Normalized signals for pure and impure peaks](image)

**Figure 1.5.3.F1**: Normalized signals for pure and impure peaks
Peak purity Evaluation using similarity factor:

ChemStation’s special peak purity software routine does not only allow the display of spectra and their differences, it is also able to calculate a numerical value to characterize the degree of dissimilarity of the peak spectra, a so-called similarity factor, based on the match of the peak spectra to one another.

At the extremes, a similarity factor of 0 indicates no match and 1000 indicates identical spectra. Generally, values very close to the ideal similarity factor (greater than 995) indicate that the spectra are very similar, values lower than 990 but higher than 900 indicate some similarity and underlying data should be observed more carefully.

Examples for pure and impure peak spectra:

Example 1: Figure 1.5.3.F2 shows examples of similarity factors for similar, different, noisy and spectra with impurity. The slope of the regression lines represents the ratio of the concentration of the two spectra were demonstrated in Figure 1.5.3.F2

Figure 1.6.5.F2: Graphical display of similarity factor for different pairs of normalized spectra
**Example 2**: Figure 1.5.3.F3 shows the peak purity determination of an impure peak containing an impurity with a quite similar spectrum to that of the main compound. The overlay of the normalized spectra and the extracted signals indicate the presence of an impurity.

![Impure peak](image)

**Figure 1.5.3.F3: Impure peak**

**Example 3**: Figure 1.5.3.F4 shows the selected peak spectra. The signals overlap perfectly reaffirming the validity of the background correction. The similarity curve exhibits a profile very similar to and within the threshold curve limits and, the peak purity ratio is clear within the band.

![Pure peak](image)

**Figure 1.5.3.F4: Pure peak**
Step 3: Validation of SIAMs

Different methods have different requirements when it comes to validation. The USP recognizes four method categories and defines the analytical performance characteristics that must be measured to validate each method type:\[27]

- **Category 1**: Analytical methods for the quantification of major components of bulk drug substances or active ingredients.
- **Category 2**: Analytical methods for the determination of impurities in bulk drug substances or degradation compounds.
- **Category 3**: Analytical methods for the determination of performance characteristics.
- **Category 4**: Identification tests

Table 1.5.3.T2 summarizes each category and the analytical performance characteristics that must be investigated.

**Table 1.5.3.T2: Data elements required for assay validation (as per USP)**

<table>
<thead>
<tr>
<th>Analytical performance characteristics</th>
<th>Assay Category I</th>
<th>Assay Category II</th>
<th>Assay Category III</th>
<th>Assay Category IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy</td>
<td>Yes</td>
<td>Yes</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Precision</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Accuracy</td>
<td>Yes</td>
<td>Yes</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>
Table 1.5.3.T2: Data elements required for assay validation (as per USP): Contd.,

<table>
<thead>
<tr>
<th>Analytical performance characteristics</th>
<th>Assay Category I</th>
<th>Assay Category II</th>
<th>Assay Category III</th>
<th>Assay Category IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Quantitative</td>
<td>Limit tests</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precision</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Detection limit</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>*</td>
</tr>
<tr>
<td>Quantitation limit</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>*</td>
</tr>
<tr>
<td>Linearity</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>*</td>
</tr>
<tr>
<td>Range</td>
<td>Yes</td>
<td>Yes</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

*May be required, depending on the nature of the specific test.

SIAM falls into the quantitative division of Category I and II, and as such all analytical performance parameters must be determined, except for the limit of detection, limit of quantification would apply instead, because SIAMs need to be quantitative.

According to ICH guideline on validation of analytical methods. The objective of an analytical procedure is to demonstrate that it is suitable for its intended purpose. Stress testing methods are screening methods used to help in understanding the degradation chemistry of the drug and, therefore need not be (nor, in general, can they) validated to the extent of final control methods. The concepts in the ICH guideline on validation of analytical methods are a good starting point for validation of stress testing methods, however, the overall validation should be significantly
abbreviated when compared to the validation of final control methods, as stress
testing methods are investigational methods. Accuracy normally should not be a
problem with stress testing methods as long as the response of the detector is linear
and samples are completely dissolved prior to analysis. The specificity of the methods
can not be fully validated because one does not know all of the possible degradation
products during initial stress testing. Specificity can be addressed by using any known
impurities and degradation products produced in the method development samples.
Precision (repeatability) of the assay of the main component can be evaluated by
preparing a limited number of assay samples and using simple statistics to estimate
the standard deviation. Estimation of intermediate precision and reproducibility
should normally not be necessary for stress testing methods. Detection and
quantitation limits for degradation products can be determined by using parent
compound and assuming that the response of all degradation products will be similar.
Although there is no requirement to reach any specific detection limit, a reasonable is
0.1% since the aim of stress testing is to detect the major degradation products in
samples which is approximately 10–20% degraded. The linearity of the method
should be validated over ranges for both assay and impurity determination. A typical
assay range might be from 50% to 150% of nominal sample concentration, while a
typical range for impurity determination might cover a range from the quantitation
limit to a few percent. If one wishes to quantitate impurities vs. the parent peak, then
linearity (range) should be demonstrated from the quantitation limit to at least 120%
of nominal sample concentration.

One of the most important aspects of stress testing is the analysis of samples
using a suitable analytical method, which, in many cases, is reverse-phase LC. This
necessitates the development of an LC method capable of measuring both the loss of
parent compound as well the levels of degradation products or impurities formed in
stress conditions.
1.5.4 Role of Mass Balance during SIAM development:

Mass balance correlates the measured loss of a parent drug to the measured increase in the amount of degradation products. It is a good quality control check on analytical methods to show that all degradation products are adequately detected and do not interfere with quantitation of the parent drug (i.e., stability-indicating methods). Regulatory agencies use mass balance to assess the appropriateness of the analytical method as a stability-indicating method and determine whether all degradants have been accounted [28].

In mass balance calculations, the loss of parent drug or the amount of drug remaining is determined from a sample assay, and the measured increase in degradation products is determined by a related substances method. The fundamental approach for determining mass balance is to quantitate the decomposition peaks using degradation methods and then reconcile the measured loss in the parent drug with the amount of degradation products. If the loss in potency can be reasonably accounted for by the amount of degradants measured, then mass balance is achieved.

The assessment of degradation in pharmaceutical products involves two aspects of analytical measurement. Firstly, a specific or selective analytical method must be available for accurate assay of parent drug compound, in order to measure any loss. Second, methodology should be in place for quantification of the degradation products formed. Ideally, when degradation occurs, the measured amount of parent drug lost should correlate well with the measured increase in degradation products. This correlation is referred to as "mass balance". More recently, the International Conference on Harmonization (ICH) has provided definition of "mass balance; material balance" as "The process of adding together the assay value and levels of degradation products to see how closely these add up to 100% of initial value, with due consideration of the margin of analytical precision". The concept is useful scientific guide for evaluating data, but it is not achievable in all circumstances. The focus may instead be on assuring the specificity of the assay, the completeness of
the investigation of route of degradation, and the use, if necessary, of identified degradants as indicators of the extent of degradation via particular mechanism \textsuperscript{[29]}. The analyst must balance time and resource demands to provide the information necessary to understand degradation without going to extreme measures of quantify components of little interest.

Mass balance in pharmaceutical analysis is very important for several reasons. By demonstrating the degardative losses of parent drug correlate well with the measured increase in degradation products unaccounted for. Conversely, if one observes, for example, a 20% loss of parent drug but only measures a 5% increase in degradation products, it is likely that additional degradation products formed are not accurately determined by the given method(s). Because unknown degradation products could potentially be toxic or otherwise compromise the safety of drug, it is important to have methods that detect all major degradation products. Thus, safety is the major reason for the study of mass balance.

Mass balance is also useful in method validation \textsuperscript{[30]} In order to demonstrate that analytical methods are stability-indicating, unstressed and stressed materials are often compared. Any increase in degradation a product that correlates well with loss of parent drug, aids in demonstrating that the methods can accurately assess degradation \textsuperscript{[31]}.

\textbf{1.5.5 Application of SIAMs:}

Stability studies are used to establish the re-test period for the active ingredient – that is the length of time it can be stored and used without analyzing immediately before use and the shelf life of the finished product. The release and shelf life specifications for the product may differ to accommodate degradation of active ingredient or other acceptable changes, which may occur on storage. The International Conference on Harmonization (ICH) drug stability test guideline Q1A (R2) requires that analysis of stability samples should be done through the use of validated stability-
indicating analytical methods (SIAMs). Additional guidance is given only for photo stability testing. It also recommends carrying out the stress testing on drug substance to establish its inherent stability characteristics and to support the suitability of proposed analytical procedure. The validated SIAMs will be used extensively for testing the stability samples of both drug substance as well as drug product.

1.6. Scope and Objectives of research work:

The present research work focuses on the development of novel stability-indicating analytical methods for some active pharmaceutical ingredients using the latest and fast chromatographic technique Rapid resolution chromatography (RRLC) by developing the shorter elution of all the analytes, providing the stability indicative requirements of Analytical methods. The work also includes the validation of the developed methods as per ICH requirements and demonstrates the suitability of developed methods to assess the stability of active pharmaceutical ingredient (API). The list of active pharmaceutical compounds taken for research study was listed in the below Table 1.6.T1

<table>
<thead>
<tr>
<th>Name of the Drug</th>
<th>Therapeutic action</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stavudine</td>
<td>Antiretroviral; Reverse Transcriptase Inhibitor.</td>
<td></td>
</tr>
<tr>
<td>Nevirapine</td>
<td>Antiretroviral; Reverse Transcriptase Inhibitor.</td>
<td></td>
</tr>
</tbody>
</table>
Novel stability-indicating analytical methods were developed for above active pharmaceutical ingredients using RRLC technique. The developed methods can be successfully implemented during the quality monitoring, release, and also for the assessment of quality during its storage and stability.