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## Review on Analytical Method Development and Validation.

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#### ABSTRACT

Analytical methods development must be validated to provide reliable data for regulatory submissions. These methods are essential for a number of purposes, including testing for quality control release, testing of stability samples, testing of reference materials and to provide data to support specifications. Analytical method followed by process of establishing evidence that provides a high degree of assurance and is an important process in the drug discovery. Although the drug shows good potency, lack of validated analytical method will not allow the drug to enter into the market. This is to ensure the quality and safety of the drug. This review gives ideas about various methods to check the stability of drug and various validation parameters as per various regulatory authorities.

#### INTRODUCTION

The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. The objective of any analytical measurement is to obtain consistent, reliable and accurate data. Validated analytical methods play a major role in achieving this goal. The results from method validation can be used to judge the quality, reliability and consistency of analytical results, which is an integral part of any good analytical practice. Validation of analytical methods is also required by most regulations and quality standards that impact laboratories. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. There is a scope, therefore to develop newer analytical methods for such drugs. Analytical methods development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals. Pharmaceutical products formulated with more than one drug, typically referred to as combination products, are intended to meet previously unmet patients, need analytical method development and validation by combining the therapeutic effects of two or more drugs in one product. These combination products can present daunting challenges to the analytical chemist responsible for the development and validation of analytical methods. The official test methods that result from these processes are used by quality control laboratories to ensure the identity, purity, potency, and performance of drug products. Identification and quantification of impurities is a crucial task in pharmaceutical process development for quality and safety. Related components are the impurities in pharmaceuticals which are unwanted chemicals that remain with the active pharmaceutical ingredients (APIs), or develop during stability testing, or develop during formulation or upon aging of both API and formulated APIs to medicines. The presence of these unwanted chemicals even in small amounts may influence the efficacy and safety of the pharmaceutical products. Various analytical methodologies are employed for the determination of related components in pharmaceuticals. There is a great need for development of new analytical methods for quality evaluation of new emerging drugs.

### Basic criteria for new method development for drug analysis

- The drug or drug combination may not be official in any pharmacopoeias
- A proper analytical procedure for the drug may not be available in the literature due to patent regulations
- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients
- Analytical methods for the quantitation of the drug in biological fluids may not be available
- Analytical methods for a drug in combination with other drugs may not be available
- The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable [1].

### Method validation

The need to validate an analytical method is encountered by analysis in the pharmaceutical industry on an almost daily basis, because adequately validated methods are a necessity for approvable regulatory filings. What constitutes a validated method, however, is subject to analyst interpretation because there is no universally accepted industry practice for assay validation. Method validation has received considerable attention in literature and from industrial committees and regulatory agencies. The International Conference on Harmonization (ICH) of technical requirements for the registration of pharmaceuticals for human use has developed a consensus text on the validation of analytical procedures. The document includes definition of different validation parameters. The United States Environmental Protection Agency (US EPA), Resource Conservation and Recovery Act (RCRA), The American Association of Official Analytical Chemist (AOAC), United States Environmental Protection Agency (USP) and other scientific organizations provide methods that are validated through multi-laboratory studies [2]. The United States Food and Drug Administration (US FDA) has proposed guidelines on submitting sample and analytical data for methods validation. The United States Pharmacopoeia (USP) has published specific guidelines for method validation and compound evaluation [3].

The objective of validation of analytical procedures is to demonstrate that it is suitable for its intended purpose. The discussion of the validation of analytical procedures is directed to the four most common types [4].

- Identification tests.
- Quantitative tests for impurities content.
- Limit tests for the control of impurities.
- Quantitative tests of the active moiety in samples of drug substance or drug product or other selected components in the drug product.

### Methods need to be validation and revalidation [5].

- Before their introduction into routine use.
- Whenever the condition change for which the method has been validated e.g. instrument with different characteristics.
- Whenever the method is changed and the changes are outside the original scope of the method.

### Validation Parameters [6,7,8]

#### Specificity

Specificity is the ability of the method to measure the analyte in the presence of other relevant components those are expected to be present in a sample. Analytical techniques that can measure the analyte response in the presence of all potential sample components should be used for specificity validation. It is not always possible to demonstrate that a single analytical procedure is specific for a particular analyte. In this case a combination of two or more analytical procedures is recommended to achieve the necessary level of discrimination. A frequently used technique in pharmaceutical laboratories is high performance liquid chromatography (HPLC) and to some extent gas chromatography (GC). In practice, a test mixture is prepared that contains the analyte and all potential sample components. The

result is compared with the response of the analyte. In pharmaceutical test mixtures, components can come from synthesis intermediates, excipients and degradation products. Generation of degradation products can be accelerated by putting the sample under stress conditions, such as elevated temperature, humidity or light. Specificity in liquid chromatography is obtained by choosing optimal columns and setting chromatographic conditions, such as mobile phase composition, column temperature and detector wavelength. Besides chromatographic separation, the sample preparation step can also be optimized for best selectivity. It is a difficult task in chromatography to ascertain whether the peaks within a sample chromatogram are pure or consist of more than one compound. The analyst should know how many compounds are in the sample which is not always possible. Therefore, the target compound peak should be evaluated for purity.

### **Accuracy and Recovery**

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Thus, accuracy is a measure of the exactness of the analytical method. Accuracy can also be described as the extent to which test results generated by the method and the true value agree. The true value for accuracy assessment can be obtained in several ways. One alternative is to compare the results of the method with results from an established reference method. This approach assumes that the uncertainty of the reference method is known. Secondly, accuracy can be assessed by analyzing a sample with known concentrations (for example, a control sample or certified reference material) and comparing the measured value with the true value as supplied with the material. If certified reference materials or control samples are not available, a blank sample matrix of interest can be spiked with a known concentration by weight or volume. After extraction of the analyte from the matrix and injection into the analytical instrument, its recovery can be determined by comparing the response of the extract with the response of the reference material dissolved in a pure solvent. Because this accuracy assessment measures the effectiveness of sample preparation, care should be taken to mimic the actual sample preparation as closely as possible. If validated correctly, the recovery factor determined for different concentrations can be used to correct the final results. The concentration should cover the range of concern and should include concentrations close to the quantitation limit, one in the middle of the range and one at the high end of the calibration curve. Another approach is to use the critical decision value as the concentration point that must be the point of greatest accuracy. The ICH document on validation methodology recommends accuracy to be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range (for example, three concentrations with three replicates each). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value, together with the confidence intervals.

### **Precision**

The precision of an analytical procedure expresses the closeness of agreement between a series of measurement obtained from multiple sampling of the same homogeneous sample under the prescribed

### **Repeatability**

Repeatability expresses the precision under the same operating conditions over a small interval of time. Repeatability is also termed intra-assay precision.

### **Intermediate Precision**

Intermediate precision expresses within-laboratories variation: different days, different equipment, etc. Intermediate precision is determined by comparing the results of a method run within a single laboratory over a number of days. A method's intermediate precision may reflect discrepancies in results obtained from

- Different operators
- Inconsistent working practice
- Different instruments
- Standards and reagents from different suppliers
- Columns from different batches
- A combination

The objective of intermediate precision validation is to verify that in the same laboratory the method will provide the same results once the development phase is over.

### **Reproducibility**

Reproducibility expresses the precision between laboratories (collaborative studies usually applied to standardization of methodology). The objective of reproducibility is to verify that the method will provide the same results in different laboratories. The reproducibility of an analytical method is determined by analyzing aliquots from homogeneous lots in different laboratories with different analysts. In addition, typical variations of operational and environmental conditions that may differ from, but are still within, the specified parameters of the method are used. Validation of reproducibility is important if the method is to be used in different laboratories. Factors that can influence reproducibility include differences in room temperature and humidity, or equipment with different characteristics such as delay volume of an HPLC system, columns from different suppliers or different batches and operators with different experience and thoroughness.

### **Limit of Detection**

The detection limit of an individual analytical procedure is the lowest amount of analyte in the sample that can be detected but not quantified as an exact value.

The limit of detection (LOD) is the point at which a measured value is larger than the uncertainty associated with it. It is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified. The limit of detection is frequently confused with the sensitivity of the method.

The sensitivity of an analytical method is the capability of the method to discriminate small differences in concentration or mass of the test analyte. In practical terms, sensitivity is the slope of the calibration curve that is obtained by plotting the response against the analyte concentration or mass. In chromatography, the detection limit is the injected amount that results in a peak with a height at least two or three times as high as the baseline noise level. Besides this signal-to-noise method.

### **Quantitation Limit**

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in the sample that can be quantitatively determined with precision and accuracy. Quantitation limit is a parameter for quantitative assay of low level of compounds in sample matrices and is used particularly for the determination of impurities and/or degradation products. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities or degradation products. The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision. If the required precision of the method at the limit of quantitation has been specified, 5 or 6 samples with decreasing amounts of the analyte are injected six times. The amounts range from the known LOD as determined above to 20 times the LOD.

### **Linearity**

The linearity of an analytical procedure is the ability to obtain test results that are directly proportional to concentration of an analyte in the sample. Linearity may be demonstrated directly on the test substance (by dilution of a standard stock solution) or by separately weighing synthetic mixtures of the test product components. Linearity is determined by a series of five to six injections of five or more standards whose concentrations span 80–120 percent of the expected concentration range. The response should be directly proportional to the concentrations of the analytes or proportional by means of a well-defined mathematical calculation. A linear regression equation applied to the results should have an intercept not significantly different from zero. If a significant nonzero intercept is obtained, it should be demonstrated that this has no effect on the accuracy of the method. Frequently, the linearity is evaluated graphically, in addition to or as an alternative to mathematical evaluation. The evaluation is made by visually inspecting a plot of signal height or peak area as a function of analyte concentration. Because deviations from linearity are sometimes difficult to detect, two additional graphical procedures can be used. The first is to plot the deviations from the regression line versus the concentration.

If there is a linear relationship test results should be evaluated by appropriate statistical methods

- Correlation coefficient (r)
- Y-intercept
- Slope of regression line
- Residual sum of squares

Usual acceptance criteria for a linear calibration curve–  $r > 0.999$ ; y-intercept  $a < 0$  to 5% of target concentration RSD (w. r. t. calibration curve)  $< 1.5$ -2%.

### Range

The range of an analytical procedure is the interval between the upper and lower concentration of an analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable precision, accuracy and linearity. The range is normally expressed in the same units as the test results (for example percentage, parts per million) obtained by the analytical method.

- For Assay - 80 to 120% of test concentration
- Content uniformity - • 70 to 130% of test concentration
- Dissolution - Q-20% to 120%
- Impurities - reporting level – 120% of impurity specification limit
- Assay & Impurities - Reporting level to 120% of assay specific.

### Linearity is limited to 150% of shelf life specification of impurities

- Test concentration can be used to determine impurities
- To determine drug substance (assay) the test concentration must be diluted
- The range is 0 – ~ 150% of impurity specification

### Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its ability during normal range.

Robustness tests examine the effect that operational parameters have on the analysis results. For the determination of a method's robustness, a number of method parameters, such as pH, flow rate, column temperature, injection volume, detection wavelength or mobile phase composition, are varied within a realistic range, and the quantitative influence of the variables is determined. If the influence of the parameter is within a previously specified tolerance, the parameter is said to be within the method's robustness range. Obtaining data on these effects helps to assess whether a method needs to be revalidated when one or more parameters are changed, for example, to compensate for column performance over time. In the ICH document<sup>5</sup> it is recommended to consider the evaluation of a method's robustness during the development phase, and any results that are critical for the method should be documented.

### Ruggedness

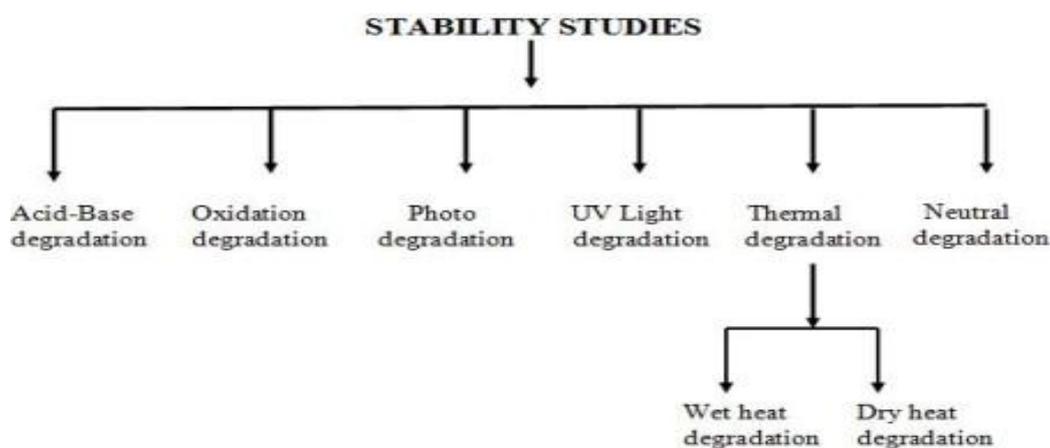
Ruggedness is the degree of reproducibility of results obtained under a variety of conditions, such as different laboratories, analysts, instruments, environmental conditions, operators and materials. Ruggedness is a measure of the reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst. Ruggedness is determined by the analysis of aliquots from homogeneous lots in different laboratories.

### Stability study of drug <sup>[9, 10,11,12]</sup>

Chemical compounds can decompose prior to chromatographic investigations, for example, during the preparation of the sample solutions, extraction, cleanup, phase transfer or storage of prepared vials (in refrigerators or in an automatic sampler). Under these circumstances, method development should investigate the stability of the analytes and standards. It is a measure of the bias in assay results

generated during a preselected time interval, for example, every hour up to 46 hours, using a single solution. Stability testing is important for estimating the allowed time span between sample collection and sample analysis. It is also important to evaluate an analytical method's ability to measure drug products in the presence of its degradation products. Experiments should be conducted under real sample storage conditions because the stability of drug substances is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system stability. The studies should evaluate the stability of the analytes during sample collection and handling after typical storage scenarios such as long term storage (when frozen at intended storage temperatures), short term storage (during a series of sample analyses at room temperature), and after freeze and thaw cycles. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling, storage and analysis. All stability determinations should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free matrix. Stock solutions of the analyte for stability evaluation are prepared in an appropriate solvent at known concentrations. The stability of the stock solutions of the drug and the internal standard should be evaluated at room temperature for at least six hours. After completion of the desired storage time, the stability is tested by comparing the instrument response with that of freshly prepared solutions. System stability is determined by replicate analysis of the sample solution and calculation of the RSD of the responses. System stability is considered appropriate when the RSD does not exceed more than 20 percent of the corresponding value of the short term system precision. If the value is higher on plotting the assay results as a function of time, the maximum duration of the sample solution usability can be calculated. To force degradation, ICH4 also recommends conducting stress studies, in conditions such as elevated temperature, humidity or light to demonstrate the specificity of the assay in presence of degradation products. The goal is to generate typical degradation products that may be expected. As a rule of thumb, stress conditions should be selected so that 5-20 percent of the drug substances are degraded. In addition, it is recommended to measure the stability under different freeze and thaw cycles, both short and long term. Below are example conditions suggested for bioanalytical studies. Exact conditions depend on application-specific storage conditions.

Figure 1: Flow Chart of Stability



## Studies

### Acid and alkali hydrolysis <sup>[13,14,15]</sup>

The hydrolytic degradation of a new drug in acidic and alkaline condition can be studied by refluxing the drug in 0.1 N HCl / 0.1 N NaOH. If reasonable degradation is seen, testing can be stopped at this point. However in case no degradation is seen under these conditions the drug should be refluxed in acid/alkali of higher strength & for longer duration of time. Alternatively if total degradation is seen after subjecting the drugs to initial condition, acid/alkali strength can be decreased with decrease in reaction temperature.

### Oxidation

To test for oxidation, it is suggested to use hydrogen peroxide in the concentration range of 3 to 30 %. In some drugs extensive degradation is seen when exposed to 3% of hydrogen peroxide for very shorter time period at room temperature. In other cases exposure to high concentration of hydrogen

peroxide, even under extreme condition does not cause any significant degradation. The behavior is on expected lines, as some drugs are in fact oxidisable, while there are others that are not. The latter are not expected to show any change even in the presence of high dose of oxidizing agent.

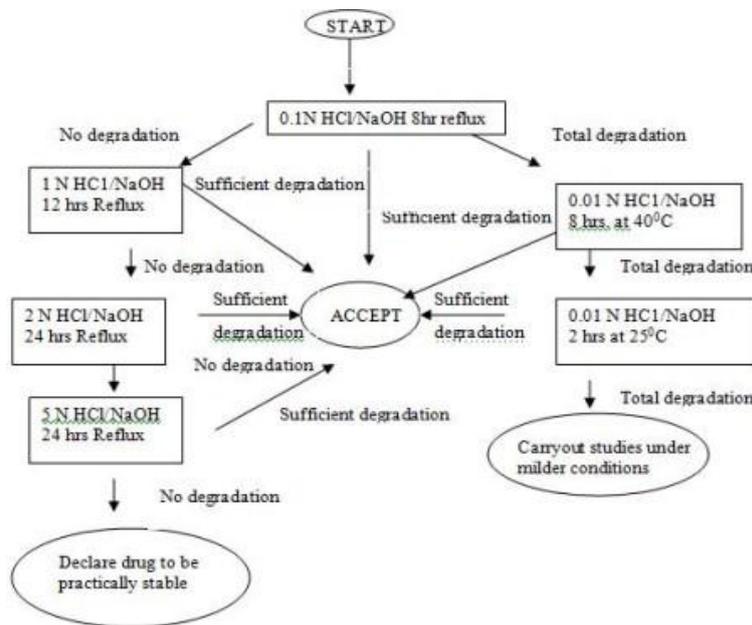


Figure 2: Flow chart of acid/alkali induced hydrolysis

### Photolytic degradation

**Sunlight:** The photolytic studies should cover the exposure of drug solution to sunlight. The drug solution should be exposed to sunlight for 4 days.

**UV light:** The drug solution should be exposed to UV radiation, in UV chamber for 4 days to study the photolytic stability of drug.

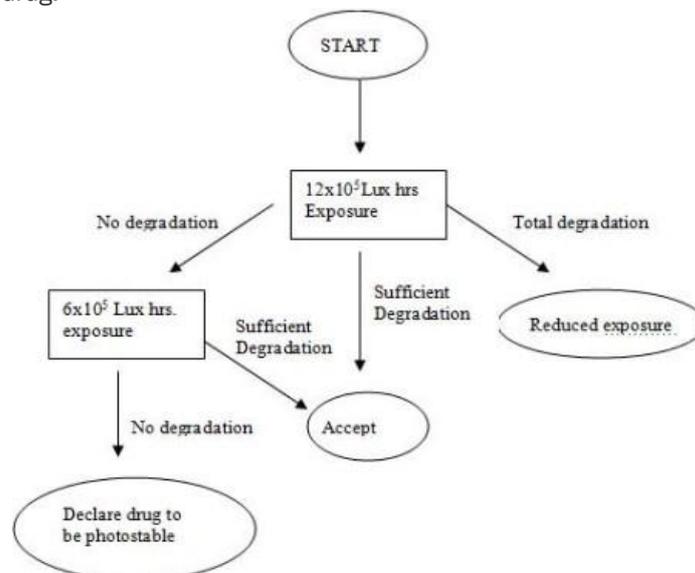


Figure 3: Flow chart of oxidation

### Neutral hydrolysis

Stress testing under neutral condition can be started by refluxing the drug in water for 12 hours. Refluxing time should be increased or decreased as per the degradation obtained in 12 hours.

**Dry heat:** Heating the drug powder at high temperature in oven can carry out stress testing for dry heat degradation. The heating time can be increased up to 12 hrs and above if there is no sufficient degradation seen in initial studies.

**Wet heat:** Wet heat degradation can be studied by refluxing the drug solution for several hrs.

#### Handling the reaction samples for chromatographic studies <sup>[16,17,18,19]</sup>

Another practical aspect of stress testing that generates enquiries from practitioners is one that concern the best way to handle samples containing high concentrations of acid, alkali or oxidizing agent for HPLC. One approach is to dilute the sample enough so that the concentration of reagent falls within the acceptable range. For HPLC the dilution can be performed in the mobile phase, where as for TLC a suitable solvent such as methanol or ethanol can be used. The second approach involves neutralization of acid and alkali solutions to tolerable pH. Dilution is often easier than neutralization. The problems with neutralizations are that it is difficult to perform in a quantitative manner and moreover it generally leads to precipitation of the dissolved ingredients of the sample. That can be controlled by filtration of that sample by syringe filter.

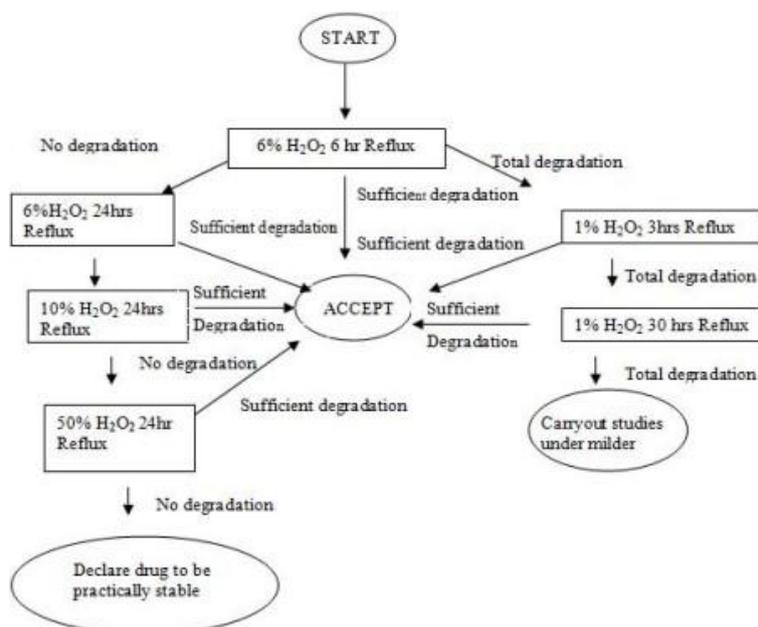


Figure 4: Flow chart of Photo stability

#### Freeze and Thaw Stability

Analyte stability should be determined after three freeze and thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature.

#### Short-Term Temperature Stability

Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed.

#### Long-Term Stability

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples. The concentrations of all the stability samples should be compared to the mean of back-

calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing.

### Stability of Processed Samples

The stabilities of processed samples, including the resident time in the autosampler, should be determined. The stability of the drug and the internal standard should be assessed over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards.

### Documentation <sup>[20]</sup>

The validation of an analytical method should be established and verified by laboratory studies, and documentation of successful completion of such studies should be provided in the assay validation report. General and specific SOPs and good record keeping are an essential part of a validated analytical method. The data generated for bio analytical method establishment and the QCs should be documented and available for data audit and inspection. Documentation for submission to the agency should include:

- Summary information
- Method development and establishment
- Bio analytical reports of the application of methods to routine sample analysis
- Other information applicable to method development and establishment and/or to routine sample analysis.

### Documentation for method establishment <sup>[21,22]</sup>

Documentation for method development and establishment should include:

- An operational description of the analytical method.
- Evidence of purity and identity of drug standards, metabolite standards, and internal standards used in validation experiments.
- A description of stability studies and supporting data.
- A description of experiments conducted to determine accuracy, precision, recovery, selectivity, limit of quantification, calibration curve (equations and weighing functions used, if any) and relevant data obtained from these studies.
- Documentation of intra- and inter-assay precision and accuracy.

### Application of analytical method development <sup>[23]</sup>

- Assays of all samples of an analyte in a biological matrix should be completed within the time period for which stability data are available. In general, biological samples are analyzed with a single determination without duplicate or replicate analysis if the assay method has acceptable variability as defined by validation data. The following recommendations should be noted in applying a bio-analytical method to routine drug analysis
- Response Function: Typically, the same curve fitting, weighing, and goodness, of fit determined during restudy validation should be used for the standard curve within the study. Response function is determined by appropriate statistical tests based on actual standard points during each run in the validation. Changes in the response function relationship between pre-study validation and routine run validation indicate potential problems.
- The QC samples should be used to accept or reject the run. These QC samples are matrix spiked with analyte.
- System Suitability
- Based on the analyte and technique, a specific SOP (or sample) should be identified to ensure optimum operation of the system used.

### CONCLUSION

Analytical methodology provides to an analyst the required data for a given analytical problem, sensitivity, accuracy, range of analysis, precision i.e. the minimum requirements which essentially are the specifications of the method for the intended purpose to be able to analyse the desired analyte in different matrices with surety and certainty. Analytical methods need to be validated before their introduction into

routine use; whenever the conditions change for which the method has been validated (e.g., an instrument with different characteristics or samples with a different matrix); and whenever the method is changed, the change is outside the original scope of the method. The stability indicating assays have been developed for a large number of drugs but most of them fail to meet current regulatory requirements of separation and analysis of individual degradation products. So the discussion provided would be general and of wide use. Nowadays, it is a mandatory requirement in various pharmacopoeias to know the impurities present in API's. Isolation and characterization of impurities are required for acquiring and evaluating data that establishes biological safety which reveals the need and scope of impurity profiling of drugs in pharmaceutical research. The aim of this article is to provide a simple way to use approaches with a correct scientific background to improve the quality of the bioanalytical method development and validation. Applications of bio analytical method in routine drug analysis are also taken into consideration in this article. Method development involves a series of simple steps. All the conditions are optimized as required for the purpose of the separation and the method is validated using ICH guidelines. The validated method and data can then be documented.

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