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COMPLETE PRACTICAL WAY OF STABILITY INDICATING METHOD DEVELOPMENT AND VALIDATION BY HPLC FOR DRUG SUBSTANCE AS WELL AS THE PRODUCT: A REVIEW

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ABSTRACT
Analytical method should be developed as stability indicating and validate to provide reliable data for regulatory submissions. Analytical method is establishing evidence that provides a high degree of assurance and is an important process in the drug discovery. Even though the drug shows good potency, lack of a validated analytical method will not allow the drug to enter into regular analysis as well as in the market. This review covers the importance/practical way of development, forced degradation and validates stability indicating analytical method and their strategies along with brief knowledge of analytical chromatographic parameters needs to be optimized of an effective method development for drug substance as well as product.

KEYWORDS: Analytical method validation, force degradation, drug substance, drug product, chromatographic parameters.

1. INTRODUCTION
Stability indicating analytical method (SIAM) is a method that is employed for the analysis of stability samples in pharmaceutical industry with assurance. With the advent of the International Conference on Harmonization (ICH) guidelines, the requirement of the establishment of 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. SIAM accurately measures the active ingredients without interference from degradation products, process impurities, excipients or other potential impurities. Some additional aspects like suitability of pharmacopoeia methods for the purpose and the role of SIAM in stability evaluation of biological/biotechnological substances and products are also developed upon.[1] The aim of any analytical measurement is to obtain consistent, reliable and accurate data 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 pharmaceutical products.[2]

As per the literature there are so many reviews were explained about the drug substance and some lack of method development on drug product. The purpose of this write-up is to indicate a systematic approach for the development of SIAM that should meet the current ICH and regulatory requirements for drug substance as well as product and also involves the understanding of chemistry of drug substance and facilitates the development of analytical method confidently.

2. TYPES OF HPLC METHODS
2.1. Reverse Phase HPLC
In reverse phase mode, the stationary phase is non polar and the mobile phase is polar in nature. Reversed phase chromatography has found both analytical and preparative applications in the area of biochemical separation and purification. Molecules that possess some degree of hydrophobic character can be separated by reversed phase chromatography with excellent recovery and resolution. It is the first choice for most samples especially neutral or non ionized compounds, that dissolve in water organic mixtures.[3,4]

2.2. Normal Phase HPLC
In normal phase mode, the stationary phase is polar and the mobile phase is non polar in nature. Here non polar compounds travel faster and are eluted fast. Polar compounds are retained for longer times because of their affinity towards stationary phase. Generally most of the

molecules are polar in nature and hence normal phase mode of applications is rare in reality.[3,4] There are some reasons mentioned below to select particularly the normal phase HPLC.

● Resolution of strongly retained hydrophobic molecules.
● Isomer separations.
● Sample injection solvents that are non polar and/or not water miscible.
● Recovery in non polar solvents.[3]

3. SIGNIFICANCE/NEED OF STABILITY INDICATING ANALYTICAL METHOD

A stability indicating method is a validated quantitative analytical procedure that can detect the changes with time in the properties of drug substances and drug product in the presence of temperature and humidity. Forced degradation is having a significant role in the development of stability indicating analytical method. A degradation product is a molecule arising out of a change in the active ingredient as a result of processing or storage (e.g. oxidation, hydrolysis). Compounds that are constituted from a reaction of the active ingredient with an excipients or container closure component are considered degradation products. Forced degradation studies help to determine the degradation pathway and degradation products of the APIs that could form during storage and help formulation development, manufacturing and packaging.[5] Critical issues related to development of SIAM, such as separation of all degradation products, establishment of mass balance, stress testing of formulations, development of stability indicating analytical methods for combination products, etc are also addressed.[6] Generally any drug product require to contain complete information of the drug substance including its physical and chemical characteristics , stability studies and such specification and analytical methods are essential to make sure the quality, purity, strength, identity, and bioavailability of the drug product along with the stability data with proposed expiration dates.[7]

4. PRACTICAL APPROCH TO METHOD DEVELOPMENT

There are many steps involve developing the method of HPLC

- Physiochemical properties of drug
- Synthetic route (Scientific)
- Buffer selection
- Selection of detector
- Selection of column
- Selection of column temperature
- Viscosity of solvents
- Mobile phase selection
- pH of mobile phase
- Mode of separation
- Sample preparation
- UV cut-off
- Filter test
- Method optimization

4.1. Physiochemical properties of drug

These properties play an significant role in method development and like solubility, polarity, pKa and pH of the drug. pH and pKa plays a major role in the mobile phase to get symmetrical and sharp peaks in HPLC and it will use in the determination of quantitative results in terms of the limit of detection as well as the limit of quantization. Generally pH of the buffer is pKa±1 gives better separation as well as peak shape.

4.2. Synthetic route

Synthetic route of the product information is mandatory to develop the method for impurity profile and quantification of the product. Draw the chemical structure of the each stage reactants, products, bi-products, solvents, catalysts and carry over products. Inject all impurities on to the HPLC system to identify and quantify accordingly.

4.3. Buffer selection

Buffer capacity is able to unchanged the pH in the entire analysis. A buffer is partially neutralized acid which resists changes in pH. i) Buffering capacity increases as the molar concentration (molarity) of the buffer salt/acid solution increases. (ii) The closer the buffered pH is the pKa, the greater the buffering capacity. (iii) Buffering capacity is expressed as the molarity of Sodium Hydroxide required to increase pH by 1.0. Consideration of the effect of pH on analyte retention, type of buffer to use, and its concentration, solubility in the organic modifier and its effect on detection is important in reversed-phase chromatography (RPC) method development of ionic analytes. An improper choice of buffer, in terms of buffering species, ionic strength and pH, can result in poor or irreproducible retention and tailing in reverse-phase separation of polar and ionizable compounds.[3,8-10] Selection of buffer is depending on the desired pH and also close to pKa value of the molecule since the buffer controls pH best at their pKa. General way is to consider pH is pKa±1 and shown in table 1.[3,8,11]

Table 1. HPLC buffers, pKa values, useful pH ranges and UV cut off

Name of the Buffer	pKa	Useful pH Range	UV cut off
Ammonium acetate	4.8	3.8-5.8	205 nm(10mM)
	9.2	8.2-10.2	
Ammonium formate	3.8	2.8-4.8	
	9.2	8.2-10.2	
Ammonium hydroxide/ ammonia	9.2	8.2-10.2	

KH2PO4/K2PO4	7.2	6.2-8.2	
KH2PO4/ phosphoric acid	2.1	1.1-3.1	<200 nm (0.1%)
Potassium Acetate/ acetic acid	4.8	3.8-5.8	<200 nm (0.1%)
Potassium format / formic acid	3.8	2.8-4.8	210 nm
Trifluoroacetic acid	<2	1.5-2.5	210 nm (10mM)
Tri-K-Citrate/hydrochloric acid 1	3.1	2.1-4.1	210 nm (0.1%)
Tri-K-Citrate/hydrochloric acid 2	4.7	3.7-5.7	230 nm (10mM)
Tri-K-Citrate/hydrochloric acid 3	5.4	4.4-6.4	230 nm (10mM)

4.3.1. General precautions to consider while selecting buffer

- Phosphate buffer is more soluble in methanol/water than in acetonitrile/water of THF/water.
- Ammonium salts are more soluble in organic and water mobile phases.
- Triethylamine (TFA) can degrade with time, is volatile, absorbs at low wavelength.
- Microbial growth can quickly grow in buffered mobile phases that contain little or no organic modifier. This growth will accumulate on column inlets and can damage chromatographic performance.
- pH greater than 7 can cause phosphate buffer accelerate the dissolution of silica based columns.
- After buffers are prepared, they should be filtered through a 0.22 µm filter.
- Mobile should be degassed before keeping in with the HPLC.
- A "test tube test" should be conducted to determine if the buffer at the concentration it is prepared will precipitate in the column/system when it is exposed to the highest organic concentration in the gradient. The temperature should also be considered as well. Buffers generally will have a higher solubility at higher temperatures. The test tube test can be performed by preparing the mobile phase in a 10-mL test tube and then putting the test tube in the refrigerator and/or water bath (to mimic higher temperatures) to determine if any precipitation occurs.
- It is not necessary to fully suppress ionization for success with HPLC – 90% suppression is generally considered adequate when sufficient buffer capacity is employed in the mobile phase.
- The buffering capacity of any mobile phase is related to the prepared molarity and how close the desired eluent pH is to the pKa of the buffering ion.
- Chromatographers may also decide a non-buffered mobile phase for pH modification. It is common for acidic analytes to be chromatographed with simple acid solutions, where the concentration of acid is sufficient to create a much lower pH than needed.
- On the alkaline side, choices are limited. TEA is not freely water soluble and has a high pKa (11) and ammonia itself dissolves freely but also has a pKa too high for most columns.[3,8]

4.3.2. Buffer concentration

Buffer concentration generally 10-50 mM is tolerable for small molecules and not more than 50% of the organic modifier should be used with a buffer. This will also depend on the buffer as well as its concentration. It's important to think about the solvent choice and modifiers. The UV cut-off for 1% acetic acid is 230nm, with 0.1% TFA it is 205nm. Historically, many chromatographers have used phosphate buffers or have diluted phosphoric acid. Whether at acidic or neutral pH, it has excellent UV transparency. However, phosphate can have solubility problems and is non-volatile, and it is not appropriate for use with mass spectrometry as well. You should avoid using phosphate buffers at concentrations greater than ~25-50 mM, especially at high organic mobile phase concentrations where precipitation may occur.[1,8]

4.4. Selection of detector

Detector selection is a key and important role in HPLC. Selection of the detector is based on the nature of analytes, potential interference, limit of detection required, availability and/or cost of the detector set out in table 2. UV-Visible detector in frequently used as it offers low level impurity identification and quantitative analysis. PDA detector offers multiple wavelengths and also useful to determine peak purity. MS detectors are useful in determination of an unknown entity. Refractive index detector is a universal detector and use in exclusively in non chromophore groups containing molecules. Characteristics that are to be fulfilled by a detector to be used in HPLC determination are: (i) High sensitivity, facilitating trace analysis. (ii) Negligible baseline noise is to facilitate lower detection. (iii) Low drift and noise level. (iv) Wide linear dynamic range (this simplifies quantization). (v) Low dead volume (minimal peak broadening). (vi) Cell design that eliminates remixing of the separated bands. (vii) Insensitivity to changes in type of solvent, flow rate and temperature. (viii)Operational simplicity and reliability. (ix) Tunability so that detection can be optimized for different compounds.[3,8]

Table 2. Detector selection based on application and nature of compound

Type of Detector	Applications
UV-Detectors	Compound having chromophore.
Diode array detector (DAD, PDA)	A wide variety of substances can be detected that absorb light from 190 to 900 nm. Sensitivity depends strongly on the component. The spectrum can be confirmed for each component.
Electrochemical detectors:	For easily oxidizable compounds.
Fluorescence detectors	For compounds exhibits fluorescence properties.
Refractive Index (RI) detectors	Generally compounds which are not having chromophore and high sensitivity is not required.
Evaporative Light Scattering	These are superior to RI detectors and can be used for higher sensitivity with gradient elution.
Conductivity detector	Ionized components are detected. This detector is used mainly for ion chromatography.
Mass detector	To know the molecular weight of unknown compound.

4.5. Column selection

A column is the heart of the HPLC. Replacing the column will effect greater resolution during method development. Now a day's silica is the common matrix for HPLC columns. Silica support is robust, easily derevetized, consistent spear size while manufacturing, and is also chemically stable most of organic and buffers. Generally silica will work from pH range from 2 to 7. Now a day's high pH range silica based columns is designed to make better chromatographic range. In reverse phase chromatography, the stationary phase is non-polar and mobile phase is polar, causing polar peaks elute fast than non-polar peaks. Due to some steric effect of silanols 1/3 of surface silanols are derevetized and the free silanols can interact with analytes, result in peak tailing. And now a day's further derevetized with chlorotrimethylsilane to end capping the reaming silanols groups to increase column efficiency (end capping). Typical stationary phases are C4, C8, C18, Nitrile and phenyl columns. Longer alkyl chains, higher carbon loading, and higher carbon loading give greater retention of non-polar analytes. Propyl, butyl and pentyl are useful for ion-pairing chromatography. Phenyl columns offer unique selectivity from alkyl phases are generally less retentive than C8 and C18 phases, and is used for aromatic compounds separation. Nitrile columns are polar and can be used both normal and reverse phase applications and also used for increase of polar retention analytes. The type of column chosen depends on the compound and aim of analysis as showed in table 3.[3,8]

Table 3. Selection of column

Factors	Effect on column efficiency
Column length	Choose longer columns for enhanced resolution. Choose shorter column for shorter analysis time, lower back pressure and fast equilibration and less solvent consumption.
Column internal diameter	Choose wider diameter column for greater sample loading. Choose narrow column for more sensitive and reduce mobile phase consumption.
Particle shape	Choose spherical particles for lower back pressure, column stability and greater stability. Choose irregular particles when high surface area and high capacity is required
Particle size	Choose smaller particle (3-4µm) for complex mixture with structure compounds. Choose larger particle (5-10µm) for sample with structurally different compounds. Choose very large particle (15-20µm) for preparative separation.
Pore size	Choose a pore size of 150 or less for sample with molecular weight less than 2000. Choose a pore size of 300 or less for sample with molecular weight greater than 2000.
Surface area	Choose end capped packing to eliminate unpredictable secondary interaction with the base materials. Choose non-end capped phase for selected differences for polar compounds by controlling secondary interaction.
Carbon load	Choose high carbon loads for greater column capacities and resolution. Choose low carbon loads for fast analysis.
End capping	Choose higher end capping to get best column performance and life as well.

4.6. Selection of column temperature

General temperature ranges from 30-40°C and will effect on the selectivity. Higher than ambient temperature uses in lesser back pressure and faster elution. Capacity factor decrease by 1 to 2% with an increase in temperature of

1°C for neutral compounds and less for partial ionizable analytes.[3]

4.7. Viscosity of solvents

Solvent of lowest possible viscosity should be utilized to minimize separation time. An added advantage of low viscosity is that high efficiency theoretical plate (HETP) values are usually lower than with solvents of higher viscosity, because mass transfer is faster. Viscosity should be lower than 0.5 centipoise, otherwise high pump pressures are required and mass transfer between solvent and stationary phase will be reduced.[12]

4.8. Mobile Phase selection

Solvents like methanol, acetonitrile and tetrahydrofuran will influence the selectivity. The option between methanol and acetonitrile depends on solubility of analyte and buffer used. Example acetonitrile do not use more than 60% in phosphate buffer, because of precipitation will occur. Tetrahydrofuran is least polar among three and is commonly used for large changes in selectivity and is also incompatible with low wavelengths. The effect of organic and buffer mixing gives better resolution, selectivity and efficiency. If pKa of the compound and buffer is similar then splitting off peak may occur.[3,8] If sample is eluted in void volume by using 100% organic solvent, this is because the sample is not retained, but retention is observed when the mobile strength is decreased by adding buffer. The separation is still complex and not able to resolve the adjacent peaks, another organic solvent with different polarity will may helps to resolve successfully.

4.9. pH of Mobile phase

General way is to consider pH is pKa±1. Changing the pH can affect the selectivity and resolution of polar analytes, especially for ionizable compounds. The pH the mobile phase will not affect greatly on non ionizable analytes

4.10. Mode of separation

4.10.1. Isocratic separation

Concentration throughout the analysis will be same, but separation power is low and the compound is retained in the column more and also difficult to separate complex analytes as well.

4.10.2. Gradient separation

Concentration will vary from time to time and makes more separation power of even complex analytes called gradient separation. Generally gradient program is used for multi-component samples since it may not be possible to get all the compounds between capacity factor from 1 to 10 using isocratic way. Finally selection of isocratic or gradient depends on the majority of functional components to be resolved or separated. In order to decide whether gradient is required or not, run the gradient, calculate the total gradient time and the difference in gradient time between first and last components, the calculated ratio is > 0.25, isocratic in suitable; when the ratio is <0.25, gradient would be useful.[3, 8]

4.11. Sample preparation techniques

First of all the sample should be soluble and stable in the selection diluent. If this is not possible due to stability or solubility problems, formic acid, acetic acid or salt can be added to the sample to increase solubility. These additives do not usually affect the separation so long as the volume of the sample loaded is small compared to the column volume. Selection of diluent is based on the solubility of the analyte and it should be soluble in the diluents and do not react with any of the diluent as well as with mobile phase. Diluent match with mobile phase composition will give better peak shape, eliminates ghost peaks, base line noise and negative peaks as well. Sample preparation is an essential component of HPLC analysis, intended to provide a reproducible and homogenous solution that is suitable for injection onto the column.

4.11.1. For solid dosage forms

Extraction of the sample from solid dosage forms by using different diluents as well as increasing sonication time from 10 to 30 minutes to get read of 100%.

4.12. UV Cut-off

Whenever using UV detection it is necessary to select solvent wavelength that has no significant UV absorption at which measurements are to be taken. Using a solvent with high UV cut-off at the selected wavelength can result in increased noise level and a loss of sensitivity. General solvents and UV cut-off are mentioned in below table 4.

Table 4. Solvents and UV cut-off (nm)

Solvent	UV cut-off (nm)
Acetonitrile	190
Water	190
Hexane	195
Isopropyl Alcohol	205
Methanol	205
Trifluoroacetic Acid	210
Tetrahydrofuran	212
Dichloromethane	233
N,N-Dimethylformamide	268
Ethanol	210
Toluene	285
Diethyl ether	220
Cyclohexane	195

4.13. Filter test

Filter paper study can be carried out when the filtration is done during sample preparation. The effectiveness of the syringe filters is largely dependent on their ability to remove contaminants/insoluble compounds without leaching undesirable artifacts (i.e., extractable) into the filter. If any additional peaks are observed in the filter samples, then the diluents must be filtered to determine if a leachable compound is coming from the syringe filter

housing/filter. It involves analysis by filtering the sample solution through different types of filter paper to get the absorption value and also without filtration and with centrifugation. Inject both on to the HPLC system, the difference value should not more than 2%.

4.14. Method optimization

Finally the experimental conditions should be optimized to get desired separations and sensitivity. To achieve the best method by optimizing the parameters like pH, the mobile phase ratio, gradient, flow rate, temperature, sample amounts, injection volume and diluents. All of these parameters are chosen on the basis of trials and followed by considering the system suitability parameters. Typical parameters of system suitability are, the theoretical plates should be more than 2000, the tailing factor should be less than 2, resolution between two peaks should be more than 2, % relative standard deviation of the area of analyte peaks in standard chromatograms should not be more than 2.0 %. All the analytical methods effort finally goes to develop stability indicating HPLC methods and the final aim is to separate the main active drug, the reaction impurities, intermediate and any degrades.

5. STRESSED/FORCED DEGRADATION STUDIES

Forced degradation studies are carried out to achieve the following purposes

- To establish degradation pathways of drug substances and drug products.
- To differentiate degradation products that are related to drug products from those that are generated from non-drug product in a formulation.
- To elucidate the structure of degradation products.
- To determine the intrinsic stability of a drug substance in the formulation.
- To reveal the degradation mechanisms such as hydrolysis, oxidation, thermal, sunlight and photolysis of the drug substance and drug product.[13]
- To establish stability indicating nature of a developed method.
- To understand the chemical properties of drug molecules.
- To generate more stable formulations.
- To produce a degradation profile similar to that of what would be observed in a formal stability study under ICH conditions.
- To solve stability-related problems.[13]

5.1. Limits for degradation

Degradation of drug substances between 2% and 20% has been considered to be reasonable for validation of chromatographic methods.[14,15] Other ways of proving the method are stability indicating by spiking with a mixture of degradation products in to drug substance and monitoring stability of drug product.[16] It is not necessary that forced degradation would end with a degradation product and the process would end without degradation after exposed with stress conditions.[17] Over-stressing a sample is not recommended and may lead to the formation of a secondary degradation product that would not be seen in typical shelf-life stability.[18] Degradation product protocols for may differ for drug substance and drug product due to differences in matrices and concentrations. Maximum time period for stress testing in solution is 14 days and 24 hours for oxidative tests to produce stressed simples for development methods.[13, 19] Generally stress studies conducted in normal room temperature and if there is no degradation, higher temperatures from 50°C to 70°C are applicable and also should not be more than 7 days. Finally the degraded sample is neutralized with acid or base to avoid further decomposition.[13,20,21]

5.2. Hydraulic condition

It is a chemical process that includes decomposition of a chemical compound by reaction with acid, base and neutral conditions. The selection of the concentration range depends on the nature of the molecule and generally Hydrochloric acid or sulphuric acid from 0.1 to 1 N for acid hydrolysis and for base hydrolysis from 0.1 to 1 N sodium hydroxide. If substances are not soluble in acid or base, then co-solvents can be utilized to dissolve them in acid and base.

5.3. Oxidative conditions

General oxidative reagent is hydrogen peroxide and also such as metal ions, radical initiators, and oxygen used for forced degradation. The range of concentration is from 0.1 to 3% hydrogen peroxide at neutral pH and maximum degradation would be 20%.[20, 21]

5.4. Photolytic conditions

Photolytic testing of drug substances and products must be generated to demonstrate that light exposure does not affect the same. These studies are evaluated by exposure to UV or fluorescent conditions are minimum of 1.2 million lx h and 200 W h/m2 lights and a maximum of 6 million lx h and wavelength of range is from 300 to 800nm to expose photolytic degradation. This is a free radical mechanism and functional groups like carbonyl, nitro aromatic, N-oxide, alkenes, aryl chlorides, weak C–H and O–H bonds, sulfides and polyenes are likely to introduce drug photosensitivity.[20,21]

5.5. Thermal conditions

Thermal degradation studies should be carried in dry heat and wet heat and also conducted at higher temperatures for a shorter period.[21] Effect of temperature on thermal degradation of a substance is studied through the Arrhenius equation:

$k = Ae^{-Ea/RT}$

Where k is the specific reaction rate, A is the frequency factor, Ea is energy of activation, R is gas constant (1.987cal/deg mole) and T is absolute temperature.[11, 14, 22]Thermal degradation study is carried out at 40–80°C.

5.6. Peak purity study

An essential for separation analysis is the ability to test the purity of the separated species, i.e. to ensure that no co-eluting or co-migrating impurity contributes to the peak response. To assess for the presence of impurities under the main peak is an essential component of the validation of a SIAM. Direct evaluation can be performed in-line by employing PDA detection. Indirect evaluation of peak purity can be accomplished by changing one or more chromatographic parameters i.e. column, gradient composition, mobile phase 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 degradants peaks is the same in both separations, and if the per cent 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.[6] All requirements of the software are to be met while evaluating peak purity. The purity angle should be less than purity threshold. The peak should not have any flag in purity result table.

5.7. Mass balance evaluation

Mass balance correlates the measured loss of a parent drug to the measured increase in the amount of degradation products. Mass balance of all stressed samples shall be verified by calculating Mass balance: (% assay of stressed sample + % impurities) X 100/ % assay of unstressed sample and should not less than 99.8%.

6. ANALYTICAL METHOD VALIDATION

Validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose.[23] Steps to follow for validation are mentioned below.

- Perform pre-validation experiments.
- Develop a validation protocol, an operating procedure or a validation master plan for the validation.
- For a specific validation project define owners and responsibilities.
- Develop a validation project plan.
- Define the application, purpose and scope of the method.
- Define the performance parameters and acceptance criteria.
- Define validation experiments.
- Verify relevant performance characteristics of equipment.
- Qualify materials, e.g. standards and reagents for purity, accurate amounts and sufficient stability.
- Adjust method parameters or/and acceptance criteria if necessary.
- Perform full internal (and external) validation experiments.
- Develop SOPs for executing the method in the routine.
- Define criteria for revalidation.
- Define type and frequency of system suitability tests and/or analytical quality control (AQC) checks for the routine.
- Document validation experiments and results in the validation report.[22]

6.1. Pre-validation study

Before going for the actual validation process so called pre-validation needs to be done. In pre-validation study large numbers of replicate spiked matrix samples are analyzed in single batch before a method validation is started. The utility of this pre-validation approach is illustrated using actual laboratory data. The process of interpreting the results and drawing conclusions about assay viability is demonstrated. The resulting conclusions provide sufficient background information to indicate if an assay is ready to enter the validation process.[6]

6.2. Full validation Parameters

- Specificity and selectivity
- Precision
  - Repeatability
  - Intermediate Precision
  - Reproducibility
- Detection Limit
- Quantitation Limit
- Linearity
- Accuracy
- Range
- Robustness
- System suitability determination
- Solution stability studies

Table 5. Validation parameters as per ICH guidelines[23]

Characteristics	Identification	Test for impurities		Assay
		Quantitative	Quantitative	
Accuracy	-	+	-	+
Precision	-	+	+	+
Repeatability	-	+	-	+
Intermediate Precision	-	+ (1)	-	+ (1)
Specificity (2)	+	+	+	+
Detection Limit	-	- (3)	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

+ indicate this need to be evaluated, - indicate this need not to be evaluated.
(1) In cases where reproducibility (see glossary) has been performed, intermediate precision is not needed.
(2) Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s).
(3) May be needed in some cases.

The united state pharmacopoeia (USP) has classified these methods into four categories and also specifies which parameters to be considered for validation of different types of methods as shown in table 6.[24]

Category I: Analytical methods for quantitation of measurement of bulk drug substances or active ingredients including preservatives in finished pharmaceutical products.

Category II: Analytical methods for determination of impurities in bulk drugs or for the determination of degradation compounds in finished pharmaceutical products.

Category III: Analytical methods for the determination of performance characteristics (e.g. dissolution, drug release).

Category IV: Identification tests.[25]

Table 6: Validation parameters as per USP guidelines

Analytical performance Characteristics	Category I	Category II		Category III	Category IV
		Quantitative	Qualitative		
Accuracy	Yes	Yes	*	*	No
Precision	Yes	Yes	No	Yes	No
Specificity	Yes	Yes	Yes	*	Yes
Detection limit	No	No	Yes	*	No
Quantitation limit	No	Yes	No	*	No
Linearity	Yes	Yes	No	*	No
Range	Yes	Yes	*	*	No

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

6.2.1. Specificity and Selectivity

Selectivity of the analytical method is described as the degree to which a method can quantify the analyte in the presence of interference.[23, 26] The other components which may be present include impurities, degradants, matrix, etc. The term specificity and selectivity are often used interchangeably. The term specificity generally refers to a method that produces a response to a single analyte only, while the term selective refers to a method that provides responses to a number of chemical entities that may or may not be distinguished from each other. The IUPAC expressed the view that "Specificity is the ultimate of selectivity'. The IUPAC discourages use of the term specificity and instead encourages the use of the term selectivity.[25, 27] Specificity study of the chromatographic method is performed by the separation of the analyte from other potential components such as impurities, degradants or excipients etc., in addition forced degradation studies are carried out to challenge the method. The forced degradation studies are of particular importance when the impurities are not available. During forced degradation studies, the sample is subjected to the stressed conditions of light, heat, humidity, acid/base hydrolysis and oxidation. The selectivity of chromatographic methods may be assessed by examination of peak homogeneity or peak purity test.[25]

6.2.2. Precision

Precision of an analytical method expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.[23] Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. The precision data are generally expressed in the form of standard deviation, relative standard deviation and confidence intervals. To ensure precision of the method for major analytes, RSD should be less than 2%. For low level impurities, RSD of 5-10% is usually acceptable.[25,28]

6.2.2.1. Repeatability

It is the precision under the same operating conditions over a short interval of time. It is likewise termed as intra-assay precision. It is evaluated by making six sample determinations at 100% concentration or by preparing three samples at three concentrations in triplicates covering the specified range for the procedure. It involves repeating determination of the same sample.

6.2.2.2. Intermediate precision

Intermediate precision expresses within laboratories variation: different days, different analyst, different equipments, etc. It is the term synonymous with the term 'ruggedness', defined by USP. The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. To study intermediate precision, use of an experimental design is encouraged. The intermediate precision is generally studied by multiple preparations of sample and standard solution.

6.2.2.3. Reproducibility

It is the precision obtained by analysis between laboratories. It is generally assessed during collaborative studies at the time of technology or method transfer. It is evaluated by means of an inter-laboratory trial.

6.3. Limit of Detection

The limit of detection of an individual analytical procedure is the lowest amount of analyte in the sample which can be detected but not necessarily quantified as an exact value. The detection limit can be determined in different ways. The simplest approach is based on the signal to noise ratio. The signal to noise ratio is established by comparing measured signals from samples with known low concentration of analyte with those of blank samples. The concentration showing the signal to noise ratio between 3:1 or 2:1 is generally considered as acceptable detection limit. The other approach is based on the standard deviation of the response and the slope.

LOD =3.3 σ/s

Where, σ = the standard deviation of the response, S = the slope of the calibration curve.

The slope may be calculated from the calibration curve of the analyte. The σ can be estimated as the standard deviation of the blank. The value of σ can also be estimated based on the calibration curve. For this the specific calibration curve should be studied using sample containing analyte in the range of the detection limit. The residual standard deviation of a regression line or the standard deviation of the y-intercept of regression lines may be used as standard deviation. Another approach to the estimation of the detection limit is based on a visual evaluation. This method is applied to non-instrumental methods but may be applied to the instrumental methods also. The LOD is determined by the analysis of samples with known concentrations of analyte and by determining the minimum level at which the analyte can be reliably detected. The relevant chromatograms are sufficient for the justification of the detection limit.[16,17] [23,25]

6.4. Limit of Quantitation

The Quantitation limit of an individual analytical procedure is the lowest amount of analyte in the sample which can be quantitatively determined with suitable precision and accuracy. It is mainly affected by the detector sensitivity and accuracy of sample preparation. The Quantitation limit can be determined in the similar way as that of the detection limit. It is the concentration showing the signal to noise ratio of 10:1. Based on the standard deviation of the response and the slope it is calculated by the formula.

LOQ =10 σ/s

Where, σ = the standard deviation of the response, S = the slope of the calibration curve

The value of S and σ are estimated as for the detection limit.

The LOQ can also be established from the visual evaluation as the LOD. The analyte concentration should be quantifiable with acceptable accuracy and precision at LOQ level. Typical acceptance criteria for LOQ are mean recovery at this level between 80 – 120% with % relative standard deviation of 10%.[23, 25]

6.5. Linearity

Linearity of a method is its ability to obtain test results that are directly proportional to the sample concentration over a given range.[23] For HPLC methods, the linear relationship between detector response (peak area and height) and sample concentration is determined. General linearity ranges and acceptance criteria for various pharmaceutical methods are indicated in the table 7.[23,25]

Table 7. Linearity ranges and criteria for various pharmaceutical methods

Test	Linearity levels and ranges	Acceptance criteria
Assay	Five levels, 50-150% of label claim.	Correlation coefficient, $R^2 \geq 0.999$.
Dissolution	Five to eight levels, 10-150% of label claim.	% y intercept NMT 2.0% and $R^2 \geq 0.99$.
Related substances	Five levels, LOQ to 150% of specification with respect to sample concentration	% y intercept NMT 5.0% and $R^2 \geq 0.999$.

6.6. Accuracy

The accuracy of an analytical method expresses the closeness of agreement between the value accepted either as a conventional true value or an accepted reference value and the value found. The accuracy studies are usually performed by determining the recovery of the spiked sample of analyte into the matrix of the sample (a placebo) or by comparing the result to the results of a certified reference material of known purity. If the placebo of the sample is not provided, the technique of standard addition is used. In case of methods, for quantification of impurities, the sample with a known amount of impurities is assessed. Accuracy should be measured using minimum of nine determinations over a minimum of three concentration levels covering the specified range (for e.g., three concentrations/ three replicates each of the total analytical procedure). Accuracy should be recorded as percent recovery. The concentration should cover the range of concern. The expected recovery depends on the sample matrix, the sample processing procedure, and the analyte concentration. The reported assay limits for accuracy for drug substances and products are 98.0 – 102.0% and 95.0 – 105.0% respectively. For the impurity limits range from 80 - 120% of average recovery may be accepted.[23, 25,29]

6.7. Range

Range of an analytical method is the interval between the upper and lower concentration of analyte in the sample

(including these concentrations) for which it has been shown that the analytical procedure has a suitable level of precision, accuracy and linearity. The range is typically derived from the linearity studies and depends on the intended application of the procedure. The following minimum specified ranges should be examined.[24,25]

- For the assay method, normally covering from 80 to 120 % of the test concentration.
- For content uniformity, covering a minimum of 70 to 130 % of the test concentration, based on the nature of the dosage form.
- For dissolution testing, 20% over the specified range.
- For impurity determination, from reporting level of impurity to 150% of the specification.

The range of a method is confirmed when linearity, accuracy and precision criteria are fulfilled.[23,25,29]

6.8. 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 reliability during normal usage.[23] It is practically evaluated during method development stages. The aim of the robustness study is to identify the critical operating parameters for the successful implementation of the analytical method. These parameters should be adequately controlled and a precautionary statement included in the method documentation. In case of an HPLC method, robustness study involves method parameters change like pH (± 0.1), flow rate (± 0.1), column temperature (± 2°C), wavelength (± 2 nm) and least mobile phase composition (± 10 %). The system suitability parameters obtained for each condition are studied to check the parameter which significantly affects the method.[25]

6.8.1. System suitability

System suitability testing (SST) is an integral part of various analytical procedures. The tests are built on the concept that the equipment, analytical operations and samples are the integral part of the system that can be evaluated as such. System suitability test provides the added assurance that on a specific occasion the method is giving, accurate and precise results. System suitability test is run every time a method is used either before or during analysis. The results of each system suitability test are compared with defined acceptance criteria and if they pass, the method is deemed satisfactory on that occasion. In case of HPLC methods, system suitability tests ensure the adequate for performing the intended application on a daily basis. The limits which are taken into account in the SST parameters are listed table 8.[25]

Table 8. SST parameters

SST parameter	Limits
Resolution	≥2.0
Repeatability (RSD)	<1.0 % for five replicates <2.0 % for six replicates
Plate count (N)	>2000
Tailing factor (T)	≤2.0
Separation factor (α)	>1.0
Retention (capacity) factor (k)	≥ 2

6.9. Solution stability

In order to study the solution stability of the sample by analyzing at different intervals of 0, 1, 2, 3, 4, 5,6,7,8,9,10,12,14,16,18,20 and 24 hours at room temperature and also at refrigerator (5° C) condition. The acceptance criteria are based on relative difference between the initial value and the value at specified solution stability time pints. For drug substances and products difference should be less than or equal 2.0% and for, impurity determination, it should be less than or equal 10%.

7. REVALIDATION

The degree of revalidation required depends on the nature of the changes. Certain other changes may require validation as well like.[23]

- Changes in the synthetic route of the drug substance.
- Changes in the composition of the finished product.
- Changes in the analytical procedure.[23]
- New samples with new compounds or new matrices.
- New analysts with different skills.
- New instruments with different characteristics.
- New location with different environmental conditions.
- New chemicals and/or reference standards and
- Modification of analytical parameters.[22]

CONCLUSIONS

Analytical methods development plays essential roles in the drug discovery, development and manufacture of pharmaceuticals. Stability indicating method is an analytical procedure that is adequate to discriminate between the major active pharmaceutical ingredients from any degradation product formed under defines storage condition during the stability evaluation period. The use of properly planned and executed, forced degradation study will give a representative sample that will in turn help to develop stability indicating HPLC method. Chromatographic factor should be evaluated to optimize the SIAM-HPLC for detecting of all potentially

relevant degardants. Therefore, resulting in SIAM-HPLC is truly capable for finding the degardants and impurities in pharmaceutical products.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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