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DEVELOPMENT, VALIDATION AND STABILITY INDICATING HPLC METHOD: AN OVERVIEW

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Abstract:
The objective of this overview is coverage of development of RP-HPLC method, its validation and evaluation of stability indicating method. .Here, short comings of reported HPLC methods with respect to regulatory aspects was highlighted. Stability-indicating assay method (SIAM) was used to differentiate the API from its potential decomposition product. Regulatory guidance status in ICH Q1A (R2), ICH Q3B (R2), Q6A, and FDA 21 CFR section 211 requires validated stability indicating methods. Force degradation of drug is required to demonstrate the specificity when developing SIAMs. For this purpose, the stability indicating methods was adopted. Force degradation of drug standard and drug product is carried out under different conditions to determine the analytical method was robust Here, the separation of all degradation products, establishment of mass balance, stress testing of drug product, development of SIAMs for combination products, etc. was also addressed. Systematic approaches for the development of stability indicating methods are discussed.

Keywords: *HPLC; Stability Indicating Method; Analytical Development; Validation.*

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INTRODUCTION:

The analytical stability-indicating method development was employed for the analysis of stability and estimation of drug in pharmaceutical industry. With aid of International Conference on Harmonisation (ICH) guidelines, the requirement of establishment of stability-indicating assay method (SIAM) has become more visibly mandated studies are regulated by many regulatory guidelines. The ICH guidelines explicitly involve conduct of forced decomposition studies under a variety of conditions, like pH, light, humidity, oxidation, dry heat, etc. and separation of drug from degradation products. Forced degradation studies was also recognised as stress testing, stress studies, stress decomposition studies, forced decomposition studies, etc. The method is expected to agree analysis of individual degradation products.

A review of literature revelations a large number of method regulatory guidelines for stress test in reported over the period of last 4 periods under the nomenclature 'stability-indicating'. However, the reported methods fall meets the current regulatory requirements. These studies are regulated by many regulatory guidelines but the ICH guideline is the integral part of stability testing. [1]

The determination of this write-up was a systematic approach for the development of validated SIAMs that should meet the present ICH and regulatory requirements. This discussion was touched a several critical issues, such as the extent of separation of degradation products, establishment of mass balance, etc. These are important with respect to the development of stability-indicating assays. Some other aspects like suitability of pharmacopoeia methods for the resolution and the role of SIAMs in stability evaluation of biological/biotechnological materials and products are also delved upon. [2]

REGULATORY STATUS OF STABILITY-INDICATING METHODS:

The ICH guidelines for stability indicating evaluation is based on the real time analysis of the belongings of climatic circumstances in the three zones of the Japan, EC and USA. As these guidelines reflect the present inspectional tendencies, they carry de facto force of regulation. The International Conference on Harmonization (ICH) guideline is the integral part of stability testing. Q1A on Stability Testing of New Drug Substances and new drug Products [3] accentuates that the testing of those which are susceptible to change during storage and are likely to influence quality, safety and/or efficacy by validated stability-indicating testing methods. It is also declared that stress testing at limits of pH and degradation condition like oxidative and photolytic conditions should be carried out on the drug substance. According to ICH guidelineQ3B, the 'Impurities in New Drug Products 'emphasizes on providing document evidence of analytical procedures. These are validated and evaluation of quantitation of degradation stability-indicating products [4]. It is also required that analytical methods should be validated to demonstrate that impurities exclusive to the new drug substance do not interfere with or are separated from specified and unspecified degradation products in the drug product. The ICH guideline Q6A, which delivers note for guidance on the requirement of stability-indicating assays under Universal Tests Criteria for both drug substances and drug products.

ICH guidelines provide an elaborate exact definition of a stability-indicating method. The stability-indicating guideline are provided by United States-Food and Drug Administration (US-FDA) in the middle of 1987 [5] and the current guideline of FDA in 1998. [6]. Studies of methods Stability-indicating according to 1987 guideline were defined as the 'quantitative analytical methods that are based on the characteristic structural, chemical or biological properties of each active ingredient of a stability-indicating drug product. 'This definition in the draft guideline stability-indicating of 1998 reads definition as 'validated quantitative analytical methods that can detect the changes with time in the chemical, physical, or microbiological properties of the drug substance and stability drug product, and that are specific so that the contents of active ingredient, degradation products, and other components of interest can be accurately measured without interference'. The main changes carried in the stability-indicating guideline are (1) the requirement of analysis of degradation products and other components, separately from the active ingredients(s). (2) Introduction of the requirement of validation.

The requirement is listed in European Committee for Proprietary Medicinal Products and Canadian Therapeutic Products Directorate's guidelines on stability testing of well-established or existing drug substances and products [1–7]. Even the **United States Pharmacopoeia (USP)**needs a requirement under 'Stability Studies in Manufacturing' that samples of the products should be assayed for potency by the use of a stability-indicating assay method. [2]. The requirement in such explicit manner is, however, absent in any other pharmacopoeias. Current ICH guideline on Good Manufacturing Practices (GMP) for Active Pharmaceutical

Ingredients (Q7A), which is under adoption by WHO, also clearly mentions that the test procedures used in stability testing should be validated and be stability-indicating [8].

REVIEW OF THE FEW LITERATURES ON STABILITY-INDICATING ASSAYS:

The review of literature survey was found to be complete with development of stability-indicating assays of exact drugs. A general review was published as early as 1971. It gave general principles and discussed the developed methods till date [9]. Kumar and Sunder also discussed the perspective of stability-indicating testing actions. Subsequently, How and Chen reviewed stability-indicating Reversed Phase High-Performance Liquid Chromatography (RP-HPLC) assay methods reported till 1996 [10, 11]. A compilation of stability-indicating assays for various drugs was published in 1999 by Xu and Trissel [12]. A more recent publication is in the form of a chapter in the book 'Drug Stability: Principles and Practices' by Christensen and Rhodes [13], which provides general discussion on RP-HPLC method development and validation, with emphasis on stability-indicating assays. Finally, critical articles on this topic, which encompasses current ICH requirements and discusses several critical issues, is still elusive.

TECHNIQUES USED FOR THE DEVELOPMENT OF SIAMS:

There are several techniques found in literature reports such as titrimetric, spectrophotometric and chromatographic techniques.

Titrimetric and spectrophotometric

The objective of these methods usually for the analysis of the drug of interest alone in the matrix of excipients, additives, degradation products, impurities,etc., and also other drugs in case of the mixture products. Their benefit is the low-cost and easiness, however sometimes they are not sensitive. Due to limitation of specificity, there are hardly any reports these days on their use for the assay of stability samples. However, a few reports connecting derivative spectroscopy has some use in stability indicating assay. These methods are used in quantitative determination of analyte e.g., stress study of carbachol under alkali condition done by IR spectrophotometer [14-15]

Chromatographic

The separation of multiple components during analysis of stability samples, chromatographic methods have taken precedence ended the conservative 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 amounts of degradation products produced. Different chromatographic technology is used in SIAM like thin layer chromatography systems that have been used are thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), gas chromatography (GC), RP-HPLC and newer technique like capillary electrophoresis (CE).

TLC is a simple technique that has been used in the historical for evolving SIAMs [16-17]. Its disadvantages, such as variability and non-quantitative nature, edge its use as a basic technique for development. However, it is very much used, especially during original degradation and stress studies to study the number of degradation products shaped, to identify the products formed through similar studies using principles and even for isolation where preparative TLC is employed [18].

A large number of publications have reviewed for use of HPTLC for stability-indicating method development [19, 20, 21, 22]. This technique overcomes the weaknesses of TLC, and is reliable, fast and accurate for quantitative drug analysis. Moreover, several samples can be run simultaneously using a small quantity of mobile phase, thus minimizing analysis time and cost per analysis.

Gas Chromatography (GC) is common techniques for separating and analysing component that can be vaporised without decomposition. Therefore, there are very few reports available on the use of GC for the resolution of establishment of SIAMs [23].

In comparison, RP-HPLC has been very widely employed. It has increased popularity in stability studies due to its reverse phase high-resolution capacity, sensitivity and specificity. Non-volatile, thermally unbalanced or polar/ionic compounds can also be analysed by this technique. Therefore, most of the SIAMs have been established using RP-HPLC.

STEPS INVOLVED DURING THE DEVELOPMENT OF STABILITY–INDICATING ANALYTICAL METHODS (SIAMS):

The analytical method used to notice a trace level or residual levels of the API present into the degradation or designing of its synthesis route. As per the FDA regulations, a SIAM is defined as a completely validated method that accurately and precisely measures API free from potential interferences like degrades, by products, intermediates, and excipients;

and the FDA recommend that all assay content methodologies for stability indicating studies. It is expected that by following the steps, one should be in a position to develop a SIAM that would meet the regulatory requirements. Our discussion is typically oriented towards development of SIAMs by RP-HPLC, as it is found that 85–90% of the methods found in literature [24].

There are three constituents necessary for implementing a SIAM.

1. Sample generation, 2. Method development, 3. Method validation

Step-1 Sample generation

Forced degradation of the active substance in both solution medium and in solid-state to form most realistic storage conditions. Which is in turn used to develop the SIAM? In simple terms, the goal of the SIAM is to baseline resolution of all the resulting products [25].

Step-2 Method development

Several parameters must be evaluated and optimized during the analytical method development process. Proper development of a method, as well as optimization and troubleshooting, requires an understanding of the influence that each of these parameters plays in the overall process [26]. The following parameters are to be evaluated critically in developing a robust analytical method.

A) Literature collection (analytical journal, patents and innovator etc.) B) Chemical structure (synthesis route), C) Diluent selection (Suitable sample media), D) Selection of stationary phase, E) Detector selectionF) Mobile phase selection, G) Flow rate and Column temperature H) Degradation studies

A) Literature collection (analytical journal, patents and innovator etc.)

Thorough literature search to be done like chromatography journals, USP, EP, IP, patents, innovator etc., before initiating the method development activity for same or similar type of drug molecules. This should be the pre-requisite need when assignment of project on establishment of a stability-indicating LC method development. Collected information (if available) on solubility profile (solubility of drug in different solvents, different mobile phase (Solvent-A and Solvent-B) and at different pH conditions), analytical profile (physico-chemical properties, example pH, PKa, melting point, degradation pathways, etc.) and stress and stability profile (sensitivity of the drug to light, heat, hygroscopic study etc.) [27].

B) Chemical structure (synthesis route)

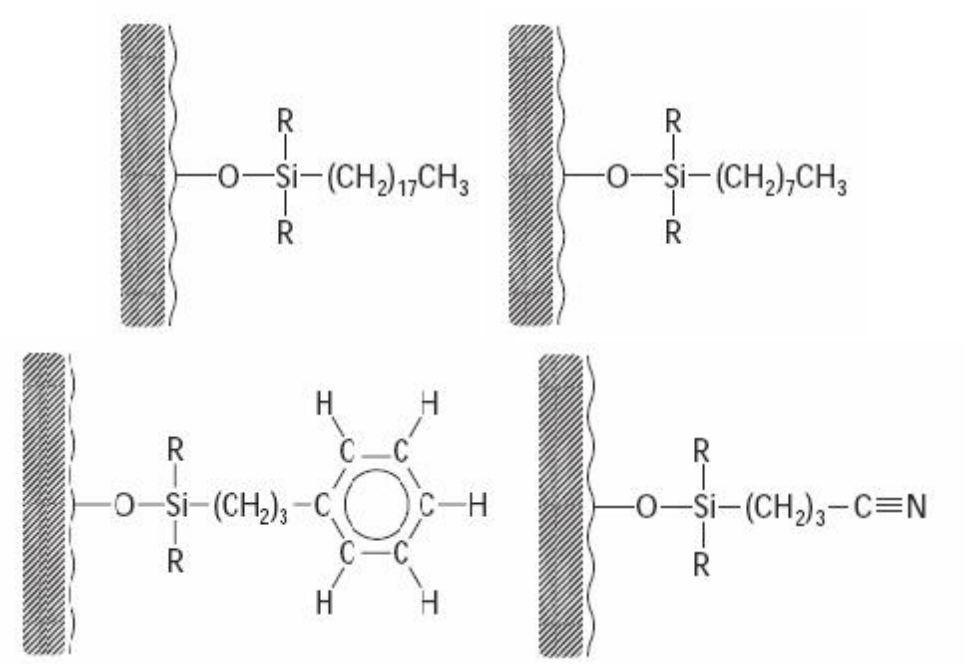
Collected the synthetic route from raw material to finished dosage forms (structures) of the molecule and the impurities likely to be present, starting material, by-product, analogues and intermediates in the reaction and degradation products. Identify the closely related structures. And method design is to be made to get the best resolution between the closely related compounds. Compare the structures of impurities, starting material, by-product, intermediates and degradation products with the structure of drug substances and arrive at the polarity whether they are less polar or more polar than the compound of interest. [28]

C) Diluent selection (Suitable sample media)

It is suitable to check first in mobile phase (solvent-A or solvent-B), select a diluent in which impurities, starting material, by-product, intermediates and degradation products and the analyte are soluble. All the analytes should be completely soluble and solution should be clear. Solution should not be hazy. Diluents should be compatible with the mobile phase to get the good symmetrical peak shape. [29]

D) Selection of stationary phase

Bonding phase can be chosen based on the polarity of the molecule and its by-products. For RP-LC, a wide variety of columns are available covering a wide range of polarity by cross-linking the Si-OH groups with alkyl chains like C8, C18 and nitrile groups (CN), phenyl groups (-C6H6), different embedment (hybridized groups), Pie-Pie stationary phase and amino groups (-NH2) etc.**[Fig. 1**].



Silica based columns with different cross linking's in the increasing
Order of polarity is as follows:
---------Non-polar--------moderately (mid) polar--------Polar--------
C18 < C8 < C6<C4<C2 < Phenyl < Amino <Cyano< Silica

E) Detector selection

DAD is useful for initial method development based on the chromospheres (wavelength maximum) present in the compounds to be separated. The initial wavelength analysing the UV spectra of the compounds using UV-VIS Spectrophotometer was selected. If the compounds are not having chromospheres, choose other detectors like RI, ELSD/CCAD. In recent DAD technology is a powerful tool for evaluating specificity. DAD can collect spectra across a range of wavelengths at each data point collected across a peak, and through software data processes involving multidimensional vector algebra; they compare each of the spectra to determine peak purity [30].

F) Mobile phase selection

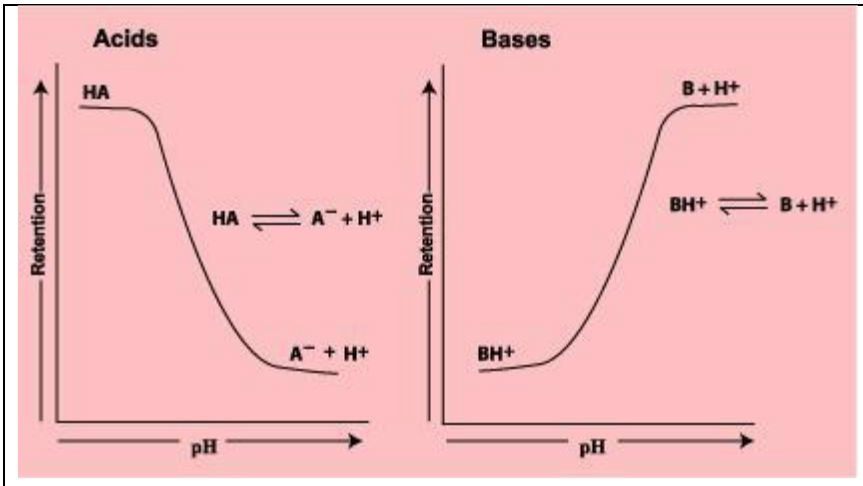
In RP-HPLC/UPLC, the retention of analytes is related to their hydrophobic nature. The more hydrophobic the nature of analyte, the longer it is retained. When an analyte is ionized, it becomes less hydrophobic and, therefore, its retention time decreases. Acids lost aproton and become ionized when pH increases and sources gain a proton and become ionized when pH decreases is shown in the fig reversedare below. Therefore, when separating mixtures of impurities containing acids and/or bases by phase RP-HPLC/UPLC, it is necessary to control the pH of the mobile phase using a suitable buffer in order to achieve reproducible and repeatable results [Fig.2].

Buffer imparts continuous ionic strength to the mobile phase. Therefore, it is always better to use buffer in aqueous portion of the mobile phase for reverse phase chromatography, buffering increases the ruggedness of the method. Most commonly used buffers were tabulated [31].

G) Flow rate and Column temperature

For RP-HPLC initial flow rate between 1.0 ml/min and 1.5 ml/min; for UPLC flow rate between 0.2 ml/min and 0.5 ml/min. Column temperature as ambient (25–40°C) is preferable [32].

Fig. 2.Effect of pH on the Retention of Acids and Bases



As acids lose a proton and become ionized (with increasingpH), their retention decreases. As bases gain a proton and become ionized (with decreasing pH) their retention decreases.

H) Degradation studies

The degradation products samples generated in the stressed samples are termed as "potential" degradation products samples that may or may not be formed under relevant storage conditions. Below are the major forced degradation studies. A general protocol of degradation conditions used for drug substance and drug product is shown in **[Fig.3].**

1) Acid degradation 2) Base degradation 3) Oxidative degradation

4) Thermolytic degradation 5) Photolytic degradation

A review of various literature reports shows that very few methods that are titled or claimed to be stability-indicating fit into the current definition of a stability-indicating assay in correct sense. While the current requirement is of subjecting the drug substance to variety of stress conditions and then separation of drug from all degradation products, many studies have just shown the separation of drug from known synthetic impurities and/or potential degradation products without subjecting it to any type of stress. There are also reports in which drug has been disintegrated by exposing it to (Table 1) conditions among acidic, neutral or alkaline hydrolysis, photolysis, oxidation and thermal stress. Thus very few studies are truly stability-indicating, where drug has been exposed to all types of stress conditions and challenges have been made to separate the drug from degradation products and the latter among themselves. Different approaches have been employed in these cases, in absence of any defined requirements. There are some reports where directly the formulation, instead of the drug substance, has been subjected to stress studies for establishment of the stability-indicating behaviour (Table 2). A few report exit even on combination of drug. Representative and do not mean comprehensive coverage of all literature reports.

Fig. 3 an illustrative flowchart describing various stresses conditions used for degradation of drug substance and drug product.

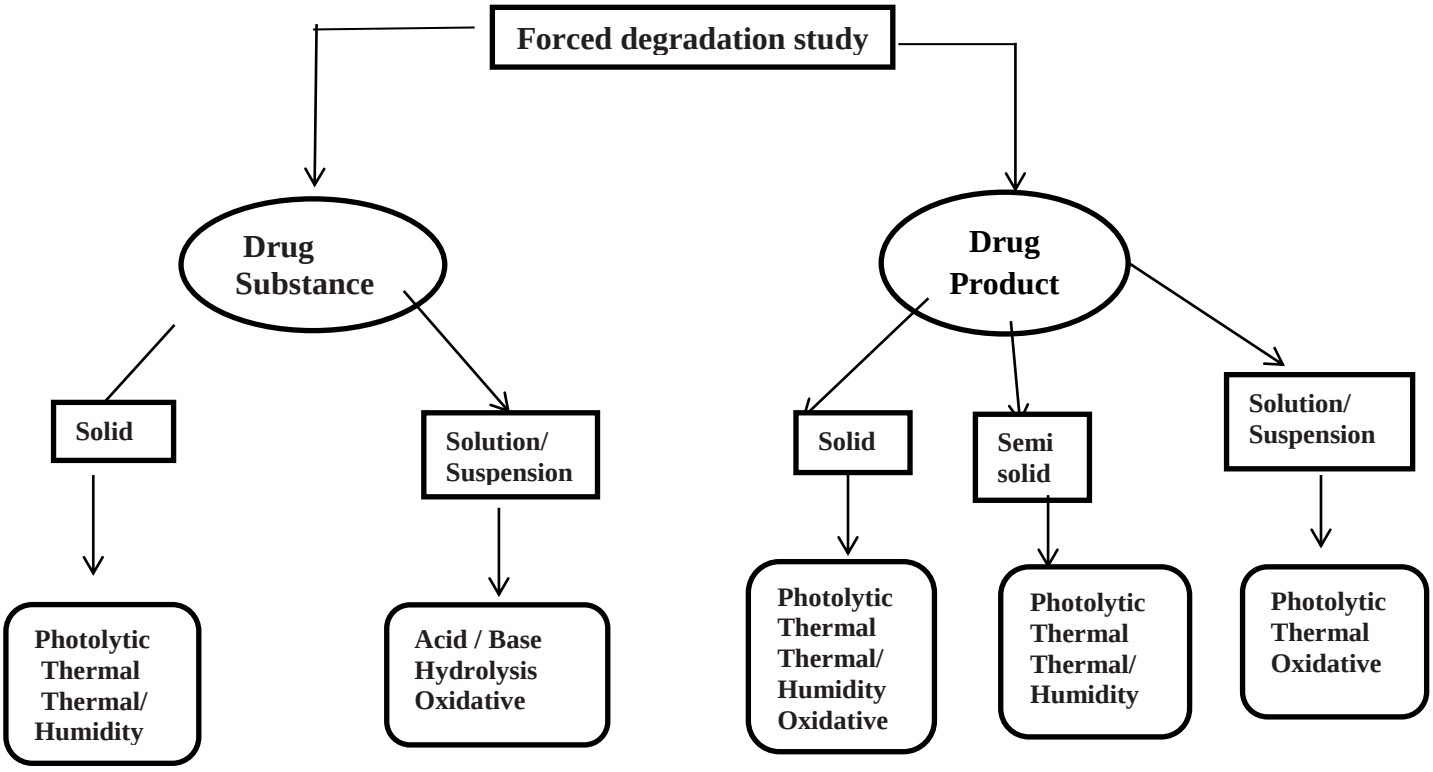


Table 1: Conditions mostly used for forced degradation studies

Degradation type	Experimental conditions	Storage conditions	Sampling time (days)
Hydrolysis	Control API (no acid or base) 0.1 M HCl 0.1 M NaOH Acid control (no API) Base control (no API) pH: 2,4,6,8	40 °C, 60 °C 40 °C, 60 °C 40 °C, 60 °C 40 °C, 60 °C 40 °C, 60 °C 40 °C, 60 °C	1,3,5 1,3,5 1,3,5 1,3,5 1,3,5 1,3,5
Oxidation	3% H2O2 Peroxide control Azobisisobutyronitrile (AIBN) AIBN control	25 °C, 60 °C 25 °C, 60 °C 40 °C, 60 °C 40 °C, 60 °C	1,3,5 1,3,5 1,3,5 1,3,5
Photolytic	Light 1_ ICH Light 3_ ICH Light control	NA NA NA	1,3,5 1,3,5 1,3,5
Thermal	Heat chamber Heat chamber Heat chamber Heat chamber Heat control	60 °C 60 °C/75% RH 80 °C 80 °C/75% RH Room temp.	1,3,5 1,3,5 1,3,5 1,3,5 1,3,5

Table 2: Selected reports of 'stability-indicating' methods where five (and additional) stress conditions have been employed.

Stress conditions	Drug	Methodology
Acid, alkali, oxidation, dry heat, light	Sodium levothyroxine Enalapril maleate	RP-HPLC RP-HPLC
Acid, alkali, oxidation, dry heat, light(separation from synthetic impurities also seen)	Sildenafil citrate	RP-HPLC
Acid, neutral, alkali, oxidation, light	Nicardipine hydrochloride	RP-HPLC
Acid, alkali, oxidation, dry heat, wet heat, light dry, light wet	Paroxetine	RP-HPLC
Acid, alkali, oxidation, dry heat, light, reduction	Cyproterone acetate	RP-HPLC
Acid, alkali, light, oxidation, dry heat, moisture, sonication	Buspirone hydrochloride	RP-HPLC

5.3 Steps-3 Method Validations

Appropriate validation of analytical methods is very much important for pharmaceutical analysis when ensurance of the continuing efficacy and safety of each batch manufactured relies only on the determination of quality from quality control releases. The ability to control this quality is dependent upon the capability of the designed analytical method, as applied under distinct circumstances and at an established required level of detectability, to give a consistent, reproducible and demonstration of all deviation from acceptance criteria. Validation is the process of providing documented evidence that something does what it is intended to do [33-34]

The USP has available specific guidelines for method validation for compound evaluation. USP/ICH defines eight analytical performance parameters for validation (Table. 3)

Table 3 : Validation Parameters

Analytical Performance characteristics	Assay Category I	Assay Category II		Assay Category III	Assay Category IV
		Quantitative	Limit tests		
Accuracy	Yes	Yes	*	*	No
Precision	Yes	Yes	No	Yes	No
Specificity	Yes	Yes	No	*	Yes
Limit of Detection	No	No	Yes	*	No
Limit of Quantification	No	Yes	Yes	*	No
Linearity	Yes	Yes	No	*	No
Range	Yes	Yes	*	*	No

May be necessary, subject on the nature of the exact test

A)Precision, B) Accuracy, C) Limit of Detection, D) Limit of Quantification,
E) Specificity, F) Linearity and Range, G) Ruggedness, H) Robustness.

A) Precision

Precision is the measure of the degree of repeatability of a specified analytical method below usual operation and is normally expressed as the percent relative standard deviation for a statistically significant number of samples Single-laboratory validation precision should include an intra-day (repeatability) and inter-day component.

According to the ICH, precision should be performed at three different levels: repeatability, reproducibility and intermediate precision. Repeatability refers to the results of the method operating finished a short time interval under the same conditions. It should be the determined from a minimum of nine determinations covering the specified range of the procedure. Intermediate precision refers to the results from within-lab variations due to casual events such as differences in experimental periods, analysts, equipment, and so forth. Reproducibility refers to the results of collaborative samples Single-laboratory validation precision should include an intra-day (repeatability) and inter-day studies between laboratories.

B) Accuracy

Accuracy is the measure of the exactness of the specified analytical method developed. The accuracy of an analytical method may be defined as the nearness of the test results obtained by the method to the true value. Accuracy may often express as percent recovery by the assay of a known amount of analyte added/spiked.

C) Limit of Detection (LOD)

The LOD is defined as the lowest concentration of an analyte in a sample that can be detected, but not necessarily determined in a quantitative fashion, using a specific method under the required experimental conditions. It is expressed as a concentration at a specifiedS/N ratio of 2:1 or 3:1 is generally accepted.

D) Selectivity and Specificity

The terms specificity and selectivity are normally used interchangeably. The term specific refers to a method those products a response for a single analyte only, while the term selective refers to a method that provides responses for a number of chemical entities that may or may not be illustrious from each other. If the response is notable from all other responses, the method is said to be selective. Since there are very a small number of methods that respond to only one analyte, the term selectivity is usually more appropriate. Specificity is the ability to measure specifically and accurately the analyte of interest in the presence of other components/impurities that may be expected to be present in the sample matrix. Specificity is measured and recognized in a separation by the resolution of each impurity, plate count (efficiency) and tailing factor. Specificity can also be evaluated with modern DAD's that compare spectra collected across a peak mathematically as a sign of peak homogeneity.

E) Ruggedness

Ruggedness, according to the USP, is the degree of reproducibility of the results obtained under a variety of normal test conditions, expressed as % relative standard deviation (RSD). These conditions include differences in analysts, instruments, laboratories, reagents and experimental periods. In the guideline on definitions and terminology, the ICH does not address ruggedness specifically. This apparent omission is really a matter of semantics, however, as ICH chooses instead to cover the topic of ruggedness as part of precision.

F) Robustness

Robustness is the capacity of a method to remain unchanged by small deliberate variations in method parameters. The robustness of a method is evaluated by varying method parameters such as flow rate, percent organic solvent, pH, column oven temperature and determining the effect(if any) on the results of the method. As documented in the ICH guidelines, robustness should be measured early in the development of a method. The variations such as stability of analytical solutions, different equipment and different analysts should be studied.

ROLE OF MASS BALANCE DURING SIAM DEVELOPMENT:

In mass balance estimates, the loss of main API substance or the quantity of analyte left over is estimated from an API assay. The calculated increase in degradation impurities is quantification by RS by RP-HPLC or UPLC analytical method. The basic formula for estimating mass balance is to establish the degradation pathway using degradation methodology and then reconcile the calculated loss in the main drug with the total of converted drug products. The mass balance is a process of adding together the assay value and levels of degradation products to see how closely these add up to 100% of the initial value, with due consideration of the margin of analytical mistake. This is the definition of mass balance given in the ICH drug stability guideline[35]. The mass balance is very carefully linked to development of a SIAM as it acts as an approach to establish its validity. The balance would not be achieved unless all degradation products are

separated well. If a few specified and stable degradation products are designed, which can be separated easily and for which the standards are available, then the establishment of mass balance becomes an easy affair. By the use of the standards, one can easily determine the exact response factors and hence the levels of the products. However, there might be many situations where the mass balance may be difficult to establish. This can happen due to one or more of the following situations [36].

COMMERCIAL AVAILABILITY OF STANDARDS OF DEGRADATION PRODUCTS:

The success of establishment of validated SIAM and the mass balance depends on the availability of standards of degradation products. Therefore, a brief discussion on the commercial sources from where one can obtain them would be applicable here. The standards for old and established degradation products controlled by pharmacopoeial monographs. This can be procured from the individual pharmacopoeial authorities (www.pheur.org; www.usp.org; www. promochem.com). Also, there are other national and international organizations which provides help from a comprehensive list of globally available standards published annually by the WHO (WHO/EDM/QSM/2001.2). Apart from this, there are independent agencies that also supply these standards, and information on such sources can be accessed through internet, using search engines like, Altavista, Yahoo, Google, etc. It may be related to add here that the author's lab at NIPER specializes in separation, synthesis and supply of degradation products and those interested can check the institute's web site (www.niper.nic.in) for an updated list [37].

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