**Original Review Article****DOI - 10.26479/2018.0403.06****REVIEW: INNOVATIVE CHROMATOGRAPHIC METHOD FOR
DEVELOPMENT AND VALIDATION****D. K. Kadam^{*1}, R. B. Saudagar¹, S. K. Paliwal², S. Sharma²****1.KCT's R. G. Sapkal College of Pharmacy, Anjaneri, Dist. Nashik, Maharashtra, India****2.Banasthali Vidyapith, Banasthali, Dist. Tonk, Rajasthan, India**

ABSTRACT: The primary focus of this article is on general approaches and considerations toward development of chromatographic methods for separation, identification, and quantification of pharmaceutical compounds, which may be applied within the various functions in the drug development continuum. This paper also discusses the issues and parameters that must be considered in the validation of analytical methods. To make drugs serve their purpose various chemical and instrumental methods were developed at regular intervals which are involved in the estimation of drugs. These pharmaceuticals may develop impurities at various stages of their development, transportation and storage which makes the pharmaceutical risky to be administered thus they must be detected and quantitated. For this analytical instrumentation and methods play an important role. This review highlights the role of the analytical instrumentation and the analytical methods in assessing the quality of the drugs. The review highlights a variety of analytical techniques such as titrimetric, chromatographic, spectroscopic, electrophoretic, and electrochemical and their corresponding methods that have been applied in the analysis of pharmaceuticals.

KEYWORDS: Method Development, Drug analysis, Validation, ICH

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1. 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. 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 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 of 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.

METHOD VALIDATION

The need to validate an analytical or bio 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

Literature survey

When develop an HPLC/UPLC method, the first step is always to consult the chromatographic literature to find out if anyone else has done the analysis, and how they did it. This will at least give an idea of the conditions that are needed, and may save one having to do a great deal of experimental work.

1.PHARMACEUTICAL IMPURITIES

An impurity in a drug substance as defined by the International Conference on Harmonization (ICH) Guidelines [1] is any component of the drug substance that is not the chemical entity defined as the drug substance. Similarly, an impurity in a drug product is any component of the drug product that is not the chemical entity defined as the drug substance or excipients in the drug product [2]. The safety of a drug product is dependent not only on the toxicological properties of the active drug substance itself, but also on the impurities that it contains. Therefore, identification, quantification, and control of impurities in the drug substance and drug product, are an important part of drug development and regulatory assessment. ICH Q3A and Q3B address issues relevant to the regulation of impurities in the drug substance and drug product. While many of the concepts and principles outlined in these documents are applicable to Abbreviated New Drug Applications (ANDAs), certain additional or modified restraints need to be considered. When FDA receives an ANDA, a monograph defining certain key attributes of the drug substance and drug product is frequently available in the United States Pharmacopeia (USP). Sometimes, literature information on drug product impurities may also be available. These public standards and literature data play a significant role in the regulatory assessment process of an ANDA.

1.1Classification of impurities

The safety and quality of the drug substance and drug product in a generic product can be impacted by the presence of impurities. The nature and the quantity of these impurities is governed by a number of factors, including synthetic route of the drug substance, reaction conditions, quality of the starting material of the drug substance, reagents, solvents, purification steps, excipients, drug product manufacturing processes, packaging, and storage of the end product. Based on ICH Q3A [1], drug substance impurities can be classified into the following categories:

- Organic impurities (process- and drug-related)
- Inorganic impurities
- Residual solvents

Organic impurities can arise during the manufacturing process and/or storage of the drug substance. They can be identified or unidentified, volatile or non-volatile, and include:

- Starting materials
- By-products
- Intermediates
- Degradation products
- Reagents, ligands, and catalysts

Inorganic impurities can result from the manufacturing process. They are normally known and identified and include:

- Reagents, ligands and catalysts
- Heavy metals or other residual metals
- Inorganic salts
- Other materials (e.g., filter aids, charcoal)

Solvents are inorganic or organic liquids used as vehicles for the preparation of solutions or suspensions in the synthesis of the drug substance or the manufacture of the drug product. Since these are generally of known toxicity, the selection of appropriate limits for these solvents is easily accomplished (ICH Q3C [3] on residual solvents).

1.2 Control of impurities

A specification is defined as a list of tests, references to analytical procedures, and appropriate acceptance criteria that are numerical limits, ranges, or other criteria for the tests described [4]. It establishes the set of criteria to which a drug substance or drug product should conform to be considered acceptable for its intended use. “Conformance to specifications” means that the drug substance and/or drug product, when tested according to the listed analytical procedures, will meet the listed acceptance criteria [5]. Specifications are critical quality standards that are proposed and justified by the manufacturer and approved by regulatory authorities as conditions of approval.

1.3 Listing of impurities in drug substance specification

The specifications for a drug substance include a list of impurities. Stability studies, chemical development studies, routine batch analyses, and scientific appraisal of potential by-products from synthetic steps and degradation pathways, can be used to predict those impurities likely to occur in the drug substance. The drug substance specification includes, where applicable, a list of the following types of impurities:

- Organic impurities
- Each identified specified impurity
- Each specified unidentified impurity
- Any unspecified impurity with an acceptance criterion of not more than (\leq) in the identification threshold in Table 1.
- Total impurities

- Residual solvents
- Inorganic impurities

Table: 1 Drug substances impurities thresholds ICH Q3A(R2)

Maximum daily dose ^a	Reporting threshold ^{b, c}	Identification threshold ^c	Quantification threshold ^c
< 2 g/day	0.05 %	0.01 % or 1.0 mg/day intake (whichever is less)	0.15 % or 1.0 mg/day intake (whichever is less)
> 2 g/day	0.03 %	0.05 %	0.05 %

^a The amount of drug substance administered per day.

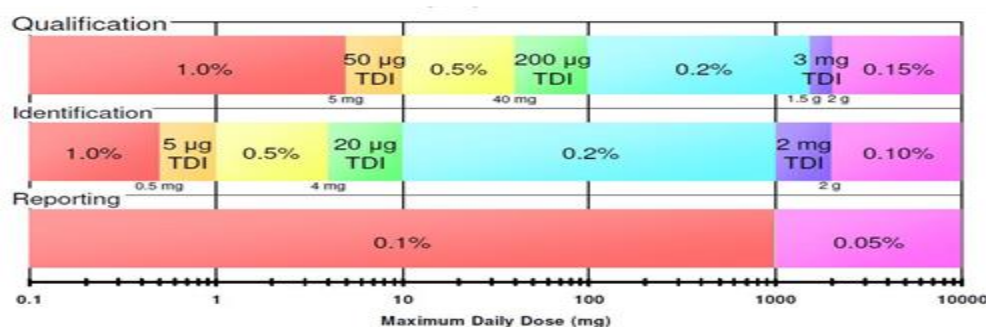
^b Higher reporting thresholds should be scientifically justified.

^c Lower thresholds can be appropriate if the impurities are unusually toxic.

1.4 Listing of impurities in drug product specification

The specification for a drug product should include a list of degradation products. Stability studies, chemical development studies, and routine batch analyses can be used to predict the degradation profile for the commercial product. The drug product specification includes, where applicable, types of degradation products are:

- Each specified identified degradation product
- Each specified unidentified degradation product
- Any unspecified degradation product with an acceptance criterion of not more than (\leq) the identification threshold in [Figure 1]
- Total degradation products Based on the maximum daily dose, impurities in new drug products should be controlled as shown in the Figure 1.

**Figure:1 ICH Q3B(R) Drug products impurity thresholds.**

2.REGULATORY STATUS OF STABILITY-INDICATING ASSAYS

The ICH guidelines have been incorporated as law in the EU, Japan and in the US, but in reality, besides these other countries are also using them. As these guidelines reflect the current inspectional tendencies, they carry the de facto force of regulation. The ICH guideline Q1A on Stability Testing

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of New Drug Substances and Products[6] emphasizes that the testing of those features which are susceptible to change during storage and are likely to influence quality, safety and/or efficacy must be done by validated stability-indicating testing methods. It is also mentioned that forced decomposition studies (stress testing) at temperatures in 10°C increments above the accelerated temperatures, extremes of pH and under oxidative and photolytic conditions should be carried out on the drug substance so as to establish the inherent stability characteristics and degradation pathways to support the suitability of the proposed analytical procedures. The ICH guideline Q3B entitled 'Impurities in New Drug Products' emphasizes on providing documented evidence that analytical procedures are validated and suitable for the detection and quantitation of degradation products [7]. It is also required that analytical methods should be validated to demonstrate that impurities unique 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 provides note for guidance on specifications [8], also mentions the requirement of stability indicating assays under Universal Tests/Criteria for both drug substances and drug products. The same is also a requirement in the guideline Q5C on Stability Testing of Biotechnological/ Biological Products [9]. Since there is no single assay or parameter that profiles the stability characteristics of such products, the onus has been put on the manufacturer to propose a stability indicating profile that provides assurance on detection of changes in identity, purity and potency of the product. Unfortunately, none of the ICH guidelines provides an exact definition of a stability-indicating method. Elaborate definitions of stability-indicating methodology are, however, provided in the United States-Food and Drug Administration (US-FDA) stability guideline of 1987 [10] and the draft guideline of 1998[11]. Stability-indicating methods 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 drug product and that will distinguish each active ingredient from its degradation products so that the active ingredient content can be accurately measured.' This definition in the draft guideline of 1998 reads as: "Validated quantitative analytical methods that can detect the changes with time in the chemical, physical, or microbiological properties of the drug substance and 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 major changes brought in the new guideline are with respect to (i) introduction of the requirement of validation, and (ii) the requirement of analysis of degradation products and other components, apart from the active ingredients. The requirement is also listed in World Health Organization (WHO), 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 [12-14]. Even the United States Pharmacopoeia (USP) has a

requirement listed under 'Stability Studies in Manufacturing', which states that samples of the products should be assayed for potency by the use of a stability-indicating assay [15]. The requirement in such explicit manner is, however, absent in other pharmacopoeias. Current ICH guideline on Good Manufacturing Practices 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 [16].

3.ROLE OF DEGRADANT PROFILING IN ACTIVE PHARMACEUTICAL INGREDIENTS AND DRUG PRODUCTS

3.1 Regulatory Requirements

From a regulatory perspective, forced degradation studies provide data to support the followings:

- Identification of possible degradants
- Degradation pathways and intrinsic stability of the drug molecule
- Validation of stability indicating analytical procedures.

Issues addressed in regulatory guidance include:

- Forced degradation studies are typically carried out using one batch of material.
- Forced degradation conditions are more severe than accelerated stability testing such as 50 °C; $\geq 75\%$ relative humidity; in excess of ICH light conditions; high and low pH, oxidation, etc.
- Photo stability should be an integral part of forced degradation study design [17].
- Degradation products that do not form in accelerated or long term stability may not have to be isolated or have their structure determined.
- Mass balance should be considered.

Issues not specifically addressed in regulatory guidance:

- Exact experimental conditions for forced degradation studies (temperatures, duration, extent of degradation, etc.) are not specified.
- Experimental design is left to the applicant's discretion. There is guidance available from the FDA as well as from private industry on regulatory requirements for IND and NDA filings [18].

3.2 Forced degradation timing and strategy

The requirements for forced degradation testing depend on project needs and the stage of development of the compound. For example, pre-clinical through phase-II project needs dictate intense method development [19, 20], and the rate of compound attrition is high. Therefore, when developing a rational study design, forced degradation deliverables should be focused on method development activities, and not isolation and identification of degradants. The focus of stress testing should be directed to characterization and elucidation of degradants.

Forced degradation process flow map is presented in Figure 2. CAMEO [Jorgensen WL *et al.*] [21]

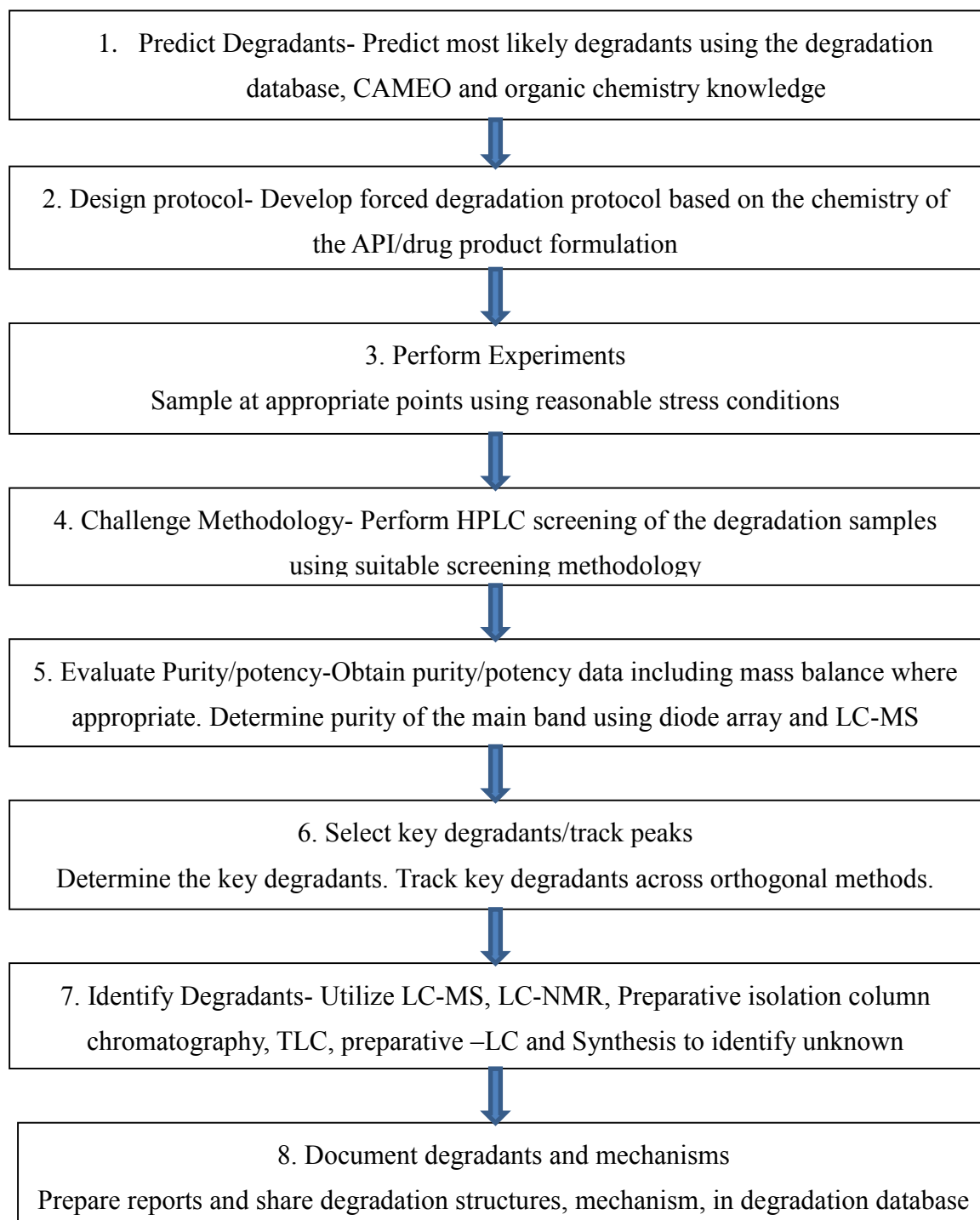


Figure: 2 Forced degradation process flow map-prediction to documentation in a structure searchable global degradation database

4.EXPERIMENTAL APPROACH TOOLS

Forced degradation studies of API and DP include appropriate solid state and solution state stress conditions (e.g. acid/base hydrolysis, heat, oxidation, and light exposure) in accordance with ICH guidelines [Figure 2, Steps 2 and 3: Design protocol and perform experiments] [17, 22]. Forced

4.1 Active pharmaceutical ingredient

The specified stress conditions should result in approximately 5-20% degradation of the API or represent a reasonable maximum condition achievable for the API. The specific conditions (intensity and duration) used will depend on the chemical characteristics of the API. The stressed sample should be compared to the unstressed sample (control) and the appropriate blank. A compound may not necessarily degrade under a given stress condition. No further stressing is advised in these cases [18].

4.2 Acid study

For a force degradation acid study for a particular API, the API is exposed to acidic conditions. The API (at a known concentration) is usually prepared in the sample preparation solvent, which gives 0.1-1 M acid solution of either hydrochloric acid or sulphuric acid. For certain APIs that are partially soluble or insoluble in the described acidic solution, addition of an appropriate co-solvent, or adjustment of solution pH in the acidic range may be required to achieve dissolution; or the APIs can be run as suspensions [18]. Special attention to the API structure should be paid when choosing the appropriate co-solvent (i.e. do not use alcohols for acidic conditions due to their reactivity). Dimethyl sulfoxide, acetic acid and propionic acid are useful under acidic conditions. Additionally, the sample may be heated for a defined time/temperature to accelerate degradation, depending on the API sensitivity to heat.

4.3 Base study

For a force degradation acid study for a particular API, the API is exposed to acidic conditions. The API (at a known concentration) is usually prepared in the sample preparation solvent, which gives 0.1-1 M base solution of either sodium hydroxide or potassium hydroxide or lithium hydroxide. For certain APIs which are partially soluble or insoluble in the described basic solution, addition of an appropriate co-solvent, or adjustment of solution pH may be required to achieve dissolution; or the APIs can be run as suspensions. Glyme and 1, 4-dioxane facilitates reactions in basic conditions [23]. Additionally, the sample may be heated for a defined time/temperature to accelerate degradation, depending on the API sensitivity to heat.

4.4 Oxidation study

Oxidation can be carried out under an oxygen atmosphere or in the presence of peroxides. The use of oxygen is a more realistic model. Free radical initiators may be used to accelerate oxidation. Generally, a free radical initiator and peroxide will produce all primary oxidation degradation products observed on real-time stability. Therefore, free radical and/or hydrogen peroxide conditions are strongly recommended at all stages of development. For peroxide conditions, hydrogen peroxide

reagent (up to 3% w/v) can be used. As previously indicated, the addition of an appropriate co-solvent may be necessary, depending on API solubility. Hydrogen peroxide stress testing can be useful in DP (drug product) studies where hydrogen peroxide is an impurity in excipients. Additionally, the sample may be heated for a defined time/ temperature to accelerate degradation, depending on the API sensitivity to heat.

4.5 Thermal/humidity study

Solid state stability can be evaluated utilizing accelerated storage temperatures in general greater than 50°C and 75% relative humidity. The duration of exposure is dependent on the API sensitivity. If the forced degradation thermal/humidity conditions produce a phase change, it is recommended to also run thermal/humidity conditions below the critical thermal/ humidity that produce the phase change.

4.6 Photo stability

Studies are performed in accordance with ICH photo stability guidelines[24]. Option 1 and/or Option 2 conditions can be used. According to the ICH guidelines, “the design of the forced degradation experiments is left to the applicant's discretion although the exposure levels should be justified. The recommended exposures for confirmatory stability studies are an overall illumination of not less than 1.2 million hours and an integrated near ultraviolet energy of not less than 200 W-h/m². For solution studies, acetonitrile is the co-solvent of choice. Methanol can produce more artificial degradation products from methoxy radicals produced from light exposure.

4.7 Drug product

Drug product (DP) degradation cannot be predicted solely from the stability studies of the API in the solid state or solution. The non-active pharmaceutical ingredients can also react with the API or catalyze degradation reactions. Impurities in the excipients can also lead to degradation in the DP not originally observed in the API. For DP formulations, heat, light, and humidity are often used. The DP stress conditions should result in approximately 5-20% degradation of the API or represent a reasonable maximum condition achievable for a given formulation. The specific conditions used will depend on the chemical characteristics of the DP. For a solid DP, the key experiments are thermal, humidity, photo stability and oxidation, if applicable. For solution formulations, key experiments are thermal, acid/ base hydrolysis, oxidation and photo stability. It is recommended to compare stressed samples with unstressed samples and an appropriate blank. For DP studies, the blank sample is an appropriate placebo. The stressed placebo sample will provide information about excipients compatibility.

5.STABILITY-INDICATING METHOD DEVELOPMENT

A stability-indicating method is defined as an analytical method that accurately quantitates the active ingredients without interference from degradation products, process impurities, excipients, or other

potential impurities. A method that accurately quantitates significant degradants may also be considered stability-indicating. A proactive approach to developing a stability indicating HPLC method should involve forced degradation at the early stages of development with the key degradation samples used in the method development process (Figure 2, Step 4: Challenge methodology). Forced degradation should be the first step in method development. If forced degradation studies are performed early, method development and identification of primary degradation products and unknown impurities can be run in parallel. Using this process, HPLC analytical assay, mechanisms of degradation, and the impurity/degradant information for filing can all be generated without delays in the project timeline.

5.1 Mass balance

Mass balance is defined in the 1999 ICH Guidelines as “adding together the assay value and levels of degradation products to see how closely these add up to 100 percent of the initial value, with due consideration of the margin of analytical error”. Assessment of mass balance may be informative in assuring that the chosen analytical strategy controls all significant degradants (Figure 2, Step 5: Evaluate purity/potency). The Guidelines recognize that it can be difficult to determine mass balance due to unknown analytical precision and differences in response factor. Additional guidance on helping the analyst obtain or approximate mass balance is given by Baertschi *et al.* [25].

5.2 Key degradation sample set

The key degradation-impurity sample set (Figure 2, Step 6: Select key degradants/track peaks) for a given compound is equal to the significant degradants plus process impurities which can include intermediates, starting materials, and by products. Process related impurities and known degradation products might be available as reference standards for use in method development. Unknown degradation products can also be critical in the development of a stability specific method.

Table: 2 Significance judgment guidelines for forced degradation studies

Condition	API		Drug product	
	Solid	Solution/ Suspension	Solid (tablets, capsules, blends)	Solution (IV, oral suspension)
Acid/base		+		O
Oxidative	O	+	+	+
Photo stability	+	O	+	+
Thermal	+		+	+
Thermal/humidity	+		+	

+...Recommended; O...Optional, suggested for some compounds

Forced degradation studies may generate complex mixtures of degradants, but method development should consider only significant degradants. Although project-specific factors may influence judgments of degradant significance, Table 2 describes guidelines that may generally be applied, considering the stage of development. A degradant in a degraded sample may be judged not significant and hence discounted, if it fails to exceed either threshold described.

5.3 Stereo chemical stability

Chiral APIs should be assessed for their stereo chemical stability during forced degradation studies on a case-by-case basis. If the degradation prediction suggests racemization to be likely by any condition, stereo chemical stability should be explored. APIs with one or two chiral centers should be analyzed with a chiral method. Based on predictive data and chemical knowledge, choose degradation conditions that are most likely to convert the molecule. If the chosen conditions do not invert or racemize the API, then chiral analysis does not need to be part of further forced degradation protocols. APIs with three or more chiral centers most likely convert to diastereoisomers and could be analyzed with a chiral method. Stereoisomers should be treated like any other API related impurity with respect to quantitation, identification and qualification thresholds, etc. [26].

5.4 Physiochemical stability

A polymorph appearing in the late stage of drug development may require reformulation, redevelopment of analytical method and change of manufacturing procedures. In addition to this, some physical form change only occurs in the solid state [27]. For these reasons, the solid form change should be monitored during forced degradation studies. In order to insure the solubility of the API, solvates and hydrates should be stressed in closed and open containers.

5.5 Identify degradants

Degradants structure elucidation is a collaborative effort involving the analytical chemist, process chemist and/or formulator, as well as the degradation, mass spectrometry and NMR experts [28]. Typically, the focus will be on collecting LC/MS data only through the Phase 1 clinical stage. At the Phase 2 clinical stage and beyond, more time is invested in isolation, synthesis and structural identity using NMR characterization of forced degradation products of concern.

6.METHOD DEVELOPMENT

Method development should be based on several considerations. It is preferable to have maximum sample information to make development fast and desired for intended analytical method application, physical and chemical properties are most preferable as primary information. Moreover, separation goal needs to define at beginning so; appropriate method can be developed for the purpose. An LC method development is very huge area for even pharmaceuticals with regulatory requirement of international standards. So, prior to method validation and usage at quality control

many aspects need to focus as per ICH guidelines. Method development can be based on a sample and goals as well as available resources for chromatography but few basic steps for method development are can be discussed as given below [29].

Steps in method development

1. Sample information, define separation goals
2. Sample pre-treatment, need of special HPLC procedure
3. Selection of detector and detector settings
4. Selection of LC method; preliminary run; estimate best separation conditions
5. Optimize separation conditions
6. Check for problems or requirement for special procedure
7. Method validation

Sample information

1. Number of compounds present
2. Chemical structure of compounds
3. Chemical nature
4. Molecular weight of compounds
5. pKa Value(s) of compounds
6. Sample solubility
7. Sample stability and storage
8. Concentration range of compounds in sample
9. UV spectra of compounds or properties for detection of compounds

Table: 3 Separation goals

Goal	Comment
Resolution	Precise and rugged quantitative analysis requires that resolution be greater than 1.5
Separation time	< 5-10 minutes is desirable for routine procedure (e.g. Dissolution profile)
Quantitation	< 2% RSD for assays
Pump pressure	< 150 Bar is desirable, < 200 bar is usually essential (For UPLC-Waters and RRLC-Agilent these values are 5 fold and 3 fold respectively)
Peak height	Narrow peaks are desirable for large signal/noise ratio
Solvent consumption	Minimum mobile phase use per run is desirable

Table: 4 Sample preparations

Select the optimum sample amount
Determine the diluents that gives the best; <ul style="list-style-type: none">-Solution stability-Solubility for sample or it's component (s)
Investigate the effect of diluents in terms of <ul style="list-style-type: none">-Organic and aqueous solvent ratio-pH-Extraction volume-Extraction procedure and time-Chromatographic changes like peak shape and resolution
Note: As a diluent mobile phase is preferable with consideration of above points for better baseline
Solution may require dilution or buffering
Determine sample concentration which gives LOQ below the identification threshold in case of related substances
Sample may require sample pre-treatment to remove interferences and/or protect the column and equipment
As a part of sample pre-treatment; filter compatibility study is required

6.1 Chromatographic detection

Before the first sample is injected during the HPLC/UPLC method development we must be reasonably sure that the detector selected will sense all sample components of interest. Normally variable wavelength UV detector is the first choice of the chromatographers, because of their convenience and applicability for most organic samples. UV spectra can be obtained by PDA detector. When the UV response of the sample is inadequate, other detector or derivative UPLC/HPLC method can be used.

6.2 Selection of LC method and mobile phase selection in partition chromatography

Chromatography requires a proper balance of the intermolecular forces between the analyte, the mobile phase, and the stationary phase for effective analysis. The important criteria to consider for method development are resolution, sensitivity, precision, accuracy, limit of detection, limit of quantitation, linearity, reproducibility, and time of analysis and robustness of the method. In all of these, the column quality plays an important role since the peak shape affects all criteria required for optimum separation. The factors that affect the column efficiency have already been described above. Column dimensions and particle size affect the speed of analysis, resolution, column backpressure, detection limit, and solvent consumption. UPLC methods have traditionally been

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developed using columns measuring 5, 7.5, 10 or 15 cm in length and 2.1 mm ID (Internal Diameter). Short columns of 2.5 cm or less in length and 1 or 2 mm ID (Internal Diameter) are now available; when packed with particles of size 1.7 micron or less, very high efficiency columns are obtained. The advantages of using shorter columns are lower backpressures, dramatic solvent savings, greater sensitivity, reduced analysis time, and applicability to small sample quantities - all achieved without compromising resolution. Using these columns, gradient methods may be used to achieve very rapid analyses of samples that contain a wide polarity range of analytes. The future of reversed-phase HPLC method development will involve a significant increase in the use of narrow-bore and micro-bore columns. Often in choosing a column for partition chromatography, the polarity of the stationary phase is matched roughly to that of the analytes in the sample; a mobile phase of different polarity is used for elution. The analytes must be soluble in the mobile phase and the solvent must be compatible with the analytical method. As a general guide, use normal phase chromatography for the separation of polar compounds and reversed-phase chromatography for components that are in the moderately polar to non-polar range. Normal phase chromatography commonly involves the use of silica, aminopropyl, diol, and cyanopropyl stationary phases. These columns may be used to separate polar compounds such as amines, anilines, nitro aromatics, phenols, and pesticides. Isocratic elution in reversed-phase chromatography is typically accomplished using a mobile phase mixture of water and another solvent of lower eluting strength (acetonitrile, methanol). In cases where the time of analysis is compromised or when the resolution is poor, gradient elution using 2 or 3 different solvents is recommended. The relative polarity of a solvent is a useful guide to solvent selection in partition chromatography. The relative polarities of the listed solvents may differ slightly depending on the literature source, since the scale used to measure polarity may be different. The following should suffice as a general reference for relative solvent polarity. Figure 3, 4 and 5 display typical chromatographic polarity ranges for mobile phase, sample analytes, and stationary phases, respectively.



Figure: 3 Mobile phase chromatographic polarity spectrum

Table: 5 List of solvent (based on polarity)

Sr. No.	Solvents
1	Fluoroalkanes (least polar)
2	Hexane
3	Isooctane
4	Carbon tetrachloride
5	Toluene
6	Diethyl ether (ether)
7	Chloroform
8	Methylene chloride
9	Tetrahydrofuran (THF)
10	Acetone
11	Ethyl acetate
12	Dioxane
13	Isopropanol
14	Ethanol
15	Acetic acid
16	Methanol
17	Acetonitrile
18	Water (most polar)

There is a strong dependence of the retention time on the mobile phase composition, and the retention parameter may be easily altered by variation of solvent polarity. This is the easiest way to improve chromatographic resolution of two overlapping species or to decrease overall separation time for components with widely differing retention values. A good starting point is a mixture of water and a polar organic solvent (methanol or acetonitrile). The effect of mobile phase polarity on elution time can be tested at a few different solvent proportions. If greater selectivity is required, a mobile phase comprising of 3-4 solvents may be used. Theoretical calculations have indicated that a mobile phase mixture of water, THF, methanol, and acetonitrile may be used to resolve most reversed-phase applications within a reasonable length of time. The various analytes to be separated may also be arranged based on the polarities of their functional groups. A general guide to relative solute polarity going from nonpolar to the most polar group is as shown in Figure 4.

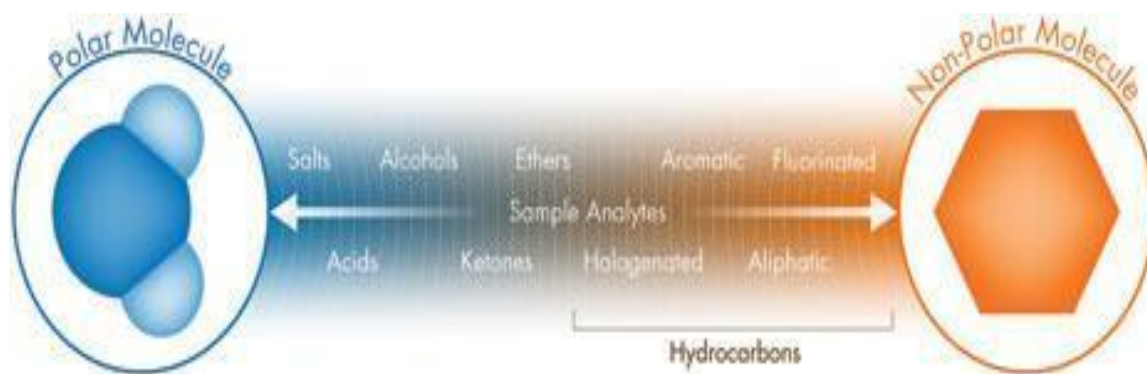


Figure: 4 Compound/Analyte chromatographic polarity spectrum.

Table: 6 List of functional group (based on polarity)

Sr. No.	Solvents
1	Hydrocarbons (least polar)
2	Ethers
3	Esters
4	Ketones
5	Aldehydes
6	Amides
7	Amines
8	Alcohols
9	Water (most polar)

6.3 Stationary phases

Many types of stationary phases available commercially with different column material chemistry start from C-18 for reversed phase to silica for Normal phase chromatography [Figure 5]. Chromatographers may need to consider many aspects before selecting a column.

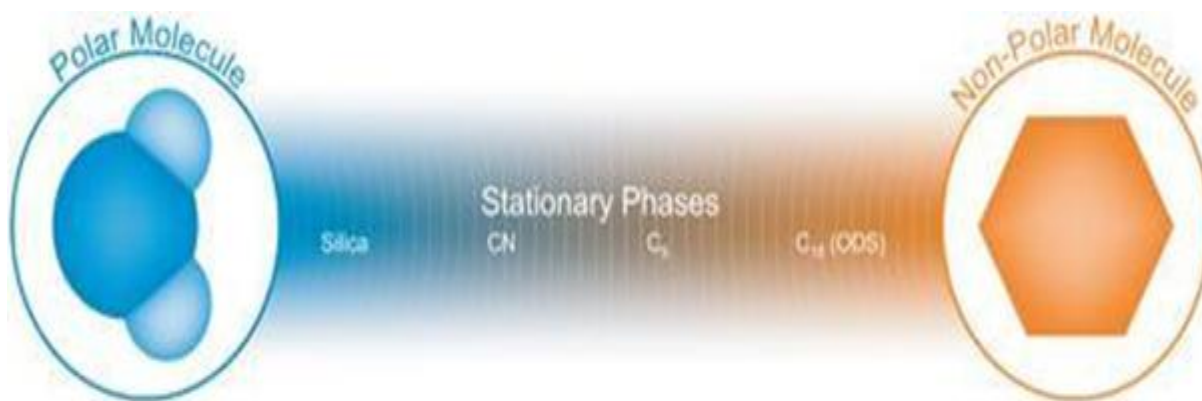


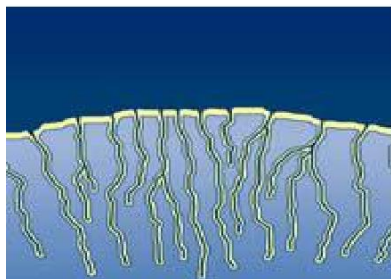
Figure: 5 Stationary phase particle chromatographic polarity spectrum**Table: 7 Types of column**

Type of Column	Remark
C-18 ("octadecyl", "ODS")	Rugged; retentive; widely used
C-8 ("octyl")	Similar to C18, but slightly less retentive
C-3 and C-4	Less retentive; less stable; used mainly for peptides and proteins
C-1 ("trimethylsilyl")	Least retentive; least stable
Phenyl and phenethyl	Moderately retentive; selectivity change
CN ("cyano")	Moderately retentive; also normal-phase
NH ₂ ("amino")	Weakly retentive; more often used for normal-phase; less stable
Polystyrene	Stable for 1 < pH < 13; good peak shape and lifetime ; selectivity change; selectivity change; often less efficient

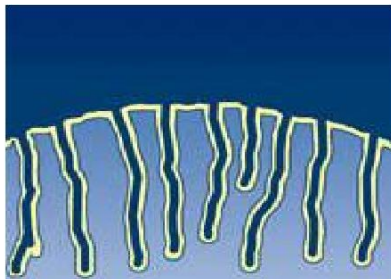
The most widely used HPLC/UPLC packings are the long-chain alkyls such as C18 or C8. These differ somewhat in their overall retentivity, although most separations can be carried out on either material. Shorter-chain alkyl packings are less retentive, but are also less stable. The silyl ether bonds are labile to hydrolysis at low pH. With long-chain packings, the hydrophobicity of the chain limits the rate of hydrolysis as well as protecting the underlying silica from dissolution in basic solution. Aromatic bonded phases typically have an overall retentivity comparable to that of C8 material, but with an added selectivity for samples which can differ in their interactions with aromatic groups. More polar groups, such as cyano or amino can also be bonded to silica to provide selectivity differences compared to the alkyl phases. The resulting packings are more commonly used for normal- phase than for reversed-phase LC. Finally, polystyrene based packings provide a viable alternative to silica for applications in which silanol interactions must be avoided altogether or for which high pH operation is required. Because such packings have no silanol groups, their selectivity can be quite different from that of silica-based materials. Beyond the very tenuous guidelines given above, there is no way to make sweeping generalizations concerning initial column choices for particular samples. Because selectivity is based on differences in molecular structure and depends on secondary interactions, the only effective way to establish suitability of a particular column for a particular sample is empirical and based on trial-and-error method. The most common type of HPLC/UPLC column is the C18 or C8 bonded phase silica. These provide a good compromise among retention, selectivity, lifetime, operating pressure, etc. In most applications, either a C8 or C18 will do equally well. There is often more selectivity difference between the “same” columns from different manufacturers than between “different” columns from the same source.

Silica columns are the most commonly used type because they tend to be more efficient and reproducible than their polymeric equivalents. The latter can be quite useful when an extended pH range is required, or when silanol interactions give rise to excessive tailing on silica based column.

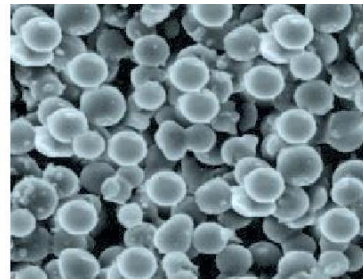
- Knowledge of the sample influences the choice of column bonded phase characteristics (column chemistry).



[A]



[B]



[C]

Figures 6:

[A] 60 Å Pore size provides maximum retention

[B] 100 Å Pore size provides moderate retention

[C] 3µm particle size provides faster separations

6.4 Choosing UPLC/HPLC mobile phase buffers

Buffers are used in HPLC mobile phase preparations in order to achieve reproducible chromatography. They are needed when an analyst is dealing with an ionizable sample species. In reversed phase chromatography samples are separated based on their hydrophobicity. The less polar a sample is the longer it is retained on the column. When an analyte is ionized it becomes more polar and subsequently less retained on the column. Acids become ionized as the pH increases, conversely bases become ionized as the pH decreases. To develop a rugged method buffers should be employed that are at least 2 pH units away from the analyte pKa. This is drawn from the Henderson-Hasselbach Equation:

$$\text{pH} = \text{pKa} + \log \left(\frac{[\text{A}^-]}{[\text{HA}]}\right)$$

Essentially, operating at a pH near to the pKa of the sample analyte means that it will be in a partially dissociated state, the analyte will partially in its weak acid or base form and partially in its conjugate form. This will cause peak distortion in the chromatography and poor peak reproducibility. Operating with a mobile phase at least 2 pH units away from the analyte pKa ensures that in excess of 99% of the sample will be in a single state. Once the proper pH range for the mobile phase is determined choosing the correct buffer can begin. Buffer capacity is optimized at or near a pH equal to the pKa of the buffer. As a rule of thumb, most buffers work suitably well within ± 1 pH unit of their pKa.

Table: 8 Buffers for reversed phase UPLC/HPLC.^[30]

Buffer	pKa	Buffer Range	UV Cutoff (nm)
Phosphate	pK1	2.1	1.1-3.1
	pK2	7.2	6.2-8.2
	pK3	12.3	11.3-13.3
Citrate	pK1	3.1	2.1-4.1
	pK2	4.7	3.7-5.7
	pK3	5.4	4.4-6.4
Formate		3.8	2.8-4.8
Acetate		4.8	3.8-5.8
Tri(hydroxymethyl) aminomethane		8.3	7.3-9.3
Borate		9.2	8.2-10.2
Triethylamine		10.8	9.8-11.8

Another consideration when choosing a buffer is the type of detector being used. Citrate may not be suitable for some UV/Vis applications due to its high UV cutoff limit. Likewise, if mass spectroscopy detection is used, a volatile buffer such as TEA or acetate should be employed while non-volatile buffers such as phosphate or citrate should be avoided. Once a buffer range and type are identified the proper concentration must be used. Ideally, unless using the buffer as ion-pair reagent, the buffer should have negligible effect on the overall separation and retention of the sample analytes. The concentration should be set just high enough to control the mobile phase pH. But still low enough to avoid possible precipitation of the buffer salts in the presence of organic solvents. Typically a buffer concentration in the range of 20-50mM is suitable. It is better to check the miscibility of an aqueous buffer solution in the highest concentration of organic mobile phase that will be present during the course of a gradient UPLC/HPLC run before putting it on chromatographic system. This can easily be checked by mixing the aqueous portion with the correct amount of organic in a beaker and observe the presence of any salting out. Also, recall that pH is only defined in an aqueous system. So, when adjusting the pH of the mobile phase, it must be done prior to the addition of the organic solvent. Proper selection and preparation of the mobile phase will help ensure good peak shape in a chromatogram. An understanding of pKa for an analyte and applying the recommendations outlined above will help in choosing and preparing the right buffer for the required application. Typically organic analytes are analyzed at a mobile phase pH either two units greater or less than pKa of the analyte to avoid any secondary equilibrium effects that might compromise the chromatography. Accounting for the pH shift of the mobile phase lead to faster method development, rugged methods and an accurate description of the analyte retention as a

function of pH at varying organic compositions. The pH of the mobile phase affects also the analyte UV response. Understanding the effects of charge delocalization and conjugation on the UV response will allow the chromatographer to choose the proper pH and wavelength of detection to obtain a method with high sensitivity. Reversed-phase UPLC/HPLC has become the dominant chromatography technique for separations and analysis. This is due to the subtle ways in which molecules interact with the reversed-phase chromatographic surface, offering the chromatographer remarkable control over the separation process through manipulation of the separation conditions. Optimizing separations may consist of enhancing resolution in order to better quantitate the key components or reducing analysis time in order to increase analytical throughput. In order to optimize a separation, the chromatographer needs to understand the underlying factors that affect resolution and analysis time.

6.5 Variables that affect plate number

Flow rate, column length, particle size, column quality and operating temperature, these variables also affect the run time and pressure. Because HPLC hardware is limited to operating pressures of about 5,000 psi, it is rarely feasible to generate extremely large column plate numbers. HPLC columns today provide 5,000 - 15,000 plates for well-behaved samples. This number has remained roughly constant for over twenty years, but the column length needed to achieve it has decreased by a factor of about 5, with a corresponding decrease in run time. A rough approximation of the plate number expected from a given column is estimated by the following expression:

$$N=3000* L / dp$$

Where, L is the column length in cm and dp is the packing particle size in μm .

The column in current routine practice consists of a 5- μm bonded-phase silica in a 10 or 15-cm column. 3- μm packings in shorter columns give faster analysis but are not as rugged as their 5- μm counterparts. Either configuration can generate the 10,000 or so plates required for general-purpose HPLC method development. Column dimensions can be arbitrarily divided into the following categories based on internal diameter (this terminology is not universal):

- A) Preparative ≥ 10 mm
- B) Analytical 3-5 mm
- C) Microbore ≤ 1 mm
- D) Semi-preparative 5-10 mm
- E) Narrow bore 2-3 mm

Standard analytical scale columns are typically available in 5, 10, 15, and 25-cm lengths. The chromatographic peaks are assumed to be symmetrical, that is chromatogram is Gaussian shape. In practice the chromatographic peaks, however, are rarely symmetrical. Although mathematically elegant general expressions for quantifying peak symmetry can be developed, practical difficulties

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 have led chromatographers to the “rough and ready” definition of asymmetry shown here. 10% of peak height is typically but not universally used for the measurement. The asymmetry factor generally increases as the measurement is made further down the peak. USP (Pharmacopeial) methods commonly specify a tailing factor of the peak measured by formula:

Tailing factor = $2CB/AB$

Tailing factor is measured at 5% of peak height (see Figure 7). Tailing factor at 5% and asymmetry factor at 10% give very roughly equivalent numbers overall, but may be quite different in specific cases (depending on the exact shape of the peak).

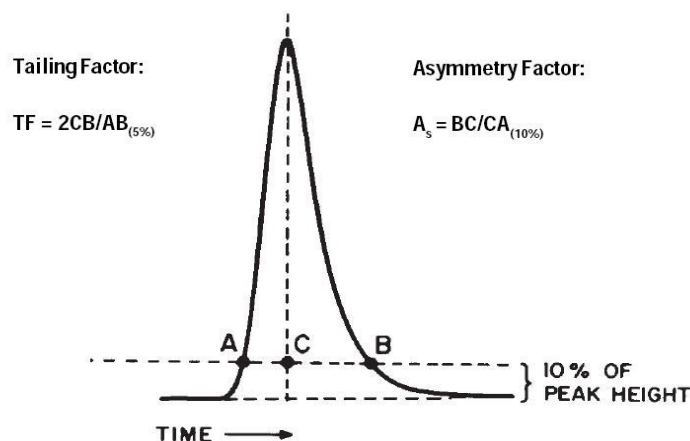


Figure: 7 Tailing and asymmetry factors

A bad column can lead to tailing bands in one of two ways. A plugged frit or a void will cause tailing for all bands in the chromatogram. A column packed with “acidic silica” particles will cause tailing of basic (amine) components of the sample. In the Figure 8, compound-3 is basic (dimethylaniline), and it is seen to tail in the top chromatogram, but not the bottom. The reason is that the top separation is carried out with an acidic silica packing. By far the major contributor to peak tailing is the existence of secondary retention effects. On silica based columns, these come primarily from interactions with underivatized silanol groups. Some silanols are quite acidic (pKa can range down to 3.5 or lower) which means that they can interact via ion exchange at most reasonable pH values. Even at low pH (or with neutral silanols), a sufficiently aggressive base can remove the proton to generate an ion-exchange interaction. Just to make life interesting, there is a non-bonding electron pair on the oxygen that can also interact with acids via hydrogen bonding. Actually, the problem is not interactions with silanols, but rather hindered interactions with silanols such that some sample molecules become strongly held via a two-point interaction (ion exchange + hydrophobic). The characteristics required for tailing are the existence of a low concentration of highly retentive active sites. As a result, these sites are quickly overloaded and attachment / release from these sites may be slow. Each of these latter effects can result in peak tailing. Unfortunately, theoretical predictions of mobile phase and stationary phase interactions with a given set of sample components are not

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 always accurate, but they do help to narrow down the choices for method development. The analyst must usually perform a series of trial-and-error experiments with different mobile phase compositions until a satisfactory separation is achieved [31, 32].

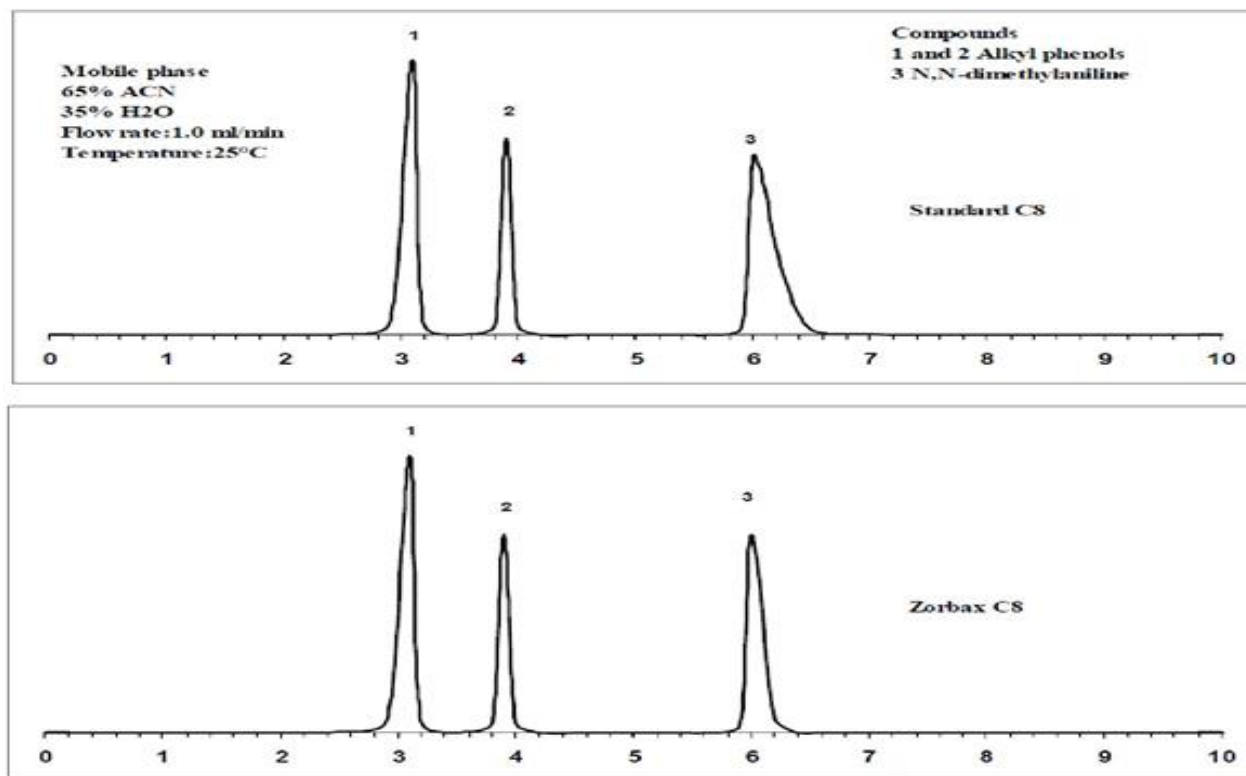


Figure: 8 Asymmetric chromatograms

7. METHOD VALIDATION

Once an analytical method is developed for its intended use, it must be validated. The extent of validation evolves with the drug development phase. Usually, a limited validation is carried out to support an Investigational New Drug (IND) application and a more extensive validation for New Drug Application (NDA) and Marketing Authorization Application (MAA). Typical parameters recommended by FDA, USP, and ICH are as follows [33]:

1. Specificity
2. Linearity & Range
3. Precision
 - (A) Method precision (Repeatability)
 - (B) Intermediate precision (Ruggedness)
4. Accuracy (Recovery)
5. Solution stability
6. Limit of Detection (LOD)
7. Limit of Quantification (LOQ)
8. Robustness

Method validation is vast area which includes many validation parameters with different approaches for different level of requirement based on intended use of analytical method, criticality and regulatory requirements. Validated method also can give the unpredicted or unknown problem during the course of routine usage, because validated method has also limited level of confidence, as method was validated for known or predicted variable parameters or every method can fail sooner or later[34]. But still after method development it needs to be validated as per requirement which gives certain level of confidence for its intended use. A common method validation protocol is followed for all the method developed during the research project (FDA, ICH Q2A & Q2B, 2005).

7.1 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. The relevant components might include impurities, degradants, matrix, etc. Lack of specificity of an individual procedure may be compensated by other supporting analytical procedure(s). Specificity can also be demonstrated by verification of the result with an independent analytical procedure. In the case of chromatographic separation, resolution factors should be obtained for critical separation. Tests for peak homogeneity, for example, by diode array detection (DAD) or mass spectrometry (MS) are recommended. The evaluation of the specificity of the method was determined against placebo. The interference of the excipients of the claimed placebo present in pharmaceutical dosage form is derived from placebo solution. Further the specificity of the method toward the drug is established by means of checking the interference of the degradation products in the drug quantification for assay during the forced degradation study. The peak purity of analyte peak was evaluated in each degraded sample with respect to total peak purity and three point peak purity. The peak purity value must be more than 0.999 (for Agilent system) or purity angle is less than threshold (for Waters system) in every case.

7.2 Linearity and Range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample. A linear relationship should be evaluated across the range of the analytical procedure. It is demonstrated directly on the drug substance by dilution of a standard stock solution of the drug product components, using the proposed procedure. For the establishment of linearity, minimum of five concentrations are recommended by ICH guideline. The value of correlation co-efficient (r^2) should fall around 0.99.

7.3 Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample. Precision may be considered at two levels: repeatability and intermediate precision. The

precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

Repeatability: Repeatability study is performed by preparing a minimum of 6 determinations at 100% of the test concentration and analyzed as per the respective methodology.

Intermediate Precision: The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The analyst should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts, equipment, etc. It is not considered necessary to study these effects individually. Here, intermediate precision of the method is checked by carrying out six independent assays of test sample preparation on the different day by another person under the same experimental condition and calculated the % RSD of assays.

7.4 Accuracy

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. The evaluation of accuracy has got very prime importance as it deliberately force the method to extract the drug and impurities at higher and lower level.

7.5 Solution stability

Drug stability in pharmaceutical formulations/active pharmaceutical ingredients is a function of storage conditions and chemical properties of the drug, preservative and its impurities. Condition used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. Stability data is required to show that the concentration and purity of analyte in the sample at the time of analysis corresponds to the concentration and purity of analyte at the time of sampling. Stability of sample solution was established by storage of sample solution at ambient temperature (25°C) for 24hrs.

7.6 Limit of detection

The limit of detection (LOD) for an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated as an exact value. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit. The limit of detection is evaluated by serial dilutions of analyte stock solution in order to obtain signal to noise ratios of 3:1.

7.7 Limit of quantitation

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy. The limit of

quantitation (LOQ) is a parameter of quantitative assays for low levels of compounds in sample matrices. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1. The limit of quantification was evaluated by serial dilutions of analyte stock solution in order to obtain signal to noise ratios of 10:1.

7.8 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.

In the case of liquid chromatography, examples of typical variations are:

- Influence of variations of pH in a mobile phase
- Influence of variations in mobile phase composition
- Different columns (different lots and/or suppliers)
- Temperature
- Flow rate

The factors chosen for all the drugs under investigation were the flow rate, mobile phase composition, pH of a mobile phase and using different lot of LC column. The observation shall be summarized and critical parameters shall be listed out in the validation report. System suitability parameter must be within the limit of acceptance criteria as mentioned in the method.

7.9 Force degradation studies:

These studies are undertaken to elucidate inherent stability characteristics. Such testing is part of the development strategy and is normally carried out under more severe condition than those used for accelerated stability studies. Force degradation of the drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used. The nature of the stress testing will depend on the individual drug substance and the type of drug product involved. Examining degradation products under stress conditions is useful in establishing degradation pathways and developing and validating suitable analytical procedures. So, as per the guidelines the stress studies for all the drug under investigation are done in the same conditions, the only difference is in temperature and the time required for each drug to degrade up to 5-20% level. Usually, the drugs are kept at solution and solid state stability in the following stability studies:

Solution state stability:

- Acidic hydrolysis
- Alkaline hydrolysis

- Hydrolytic
- Oxidative degradation

Solid state stability:

- Thermal degradation
- Photolytic degradation

7.10 Validation characteristics of the tests

Validation characteristics of the various types of tests are listed in Table 9.

Table: 9 Validation characteristics of the tests

Type of test or characteristics	Identification	Quantitative testing for impurities	Limit testing for impurities	Assay dissolution (measurement only) Content potency	Specific tests
Accuracy	-	+	-	+	+†
Precision (Repeatability)	-	+	-	+	+†
Precision (Intermediate precision)	+§	+‡	-	+‡	+†
Specificity	-	+	+	+	+†
Limit of Detection (LOD)	-	-#	+	-	-
Limit of Quantification (LOQ)	-	+	-	-	-
Linearity	-	+	-	+	-
Range	-	+	-	+	-
Robustness	-	+	-#	+	+†

- Signifies that this characteristic normally is not evaluated.

+ Signifies that this characteristic normally is evaluated.

† May not be needed in some cases.

‡ In cases where reproducibility has been performed, intermediate precision is not necessary.

§ Lack of specificity for an analytical procedure may be compensated for by the addition of second analytical procedure.

|| Lack of specificity for an assay for release may be compensated for by impurity testing.

May be needed in some cases.

7.11 Advantages of analytical method validation

The advantages of the analytical method validation are as follow:

- The biggest advantage of method validation is that it builds a degree of confidence, not only for the developer but also to the user.
- Although the validation exercise may appear costly and time consuming, it results inexpensive, eliminates frustrating repetitions and leads to better time management in the end.

Minor changes in the conditions such as reagent supplier or grade, analytical setup are unavoidable due to obvious reasons but the method validation absorbs the shock of such conditions and pays for more than invested on the process.

2. CONCLUSION

Analytical method validation and method transfer data playing a fundamental role in pharmaceutical industry for releasing the commercial batch and long term stability data therefore, the data must be produced to acceptable scientific standards. For this reason and the need to satisfy regulatory authority requirements, all analytical methods should be properly validated and documented. The aim of this article is to provide simple to use approaches with a correct scientific background to improve the quality of the analytical method development and validation process. Analytical methods should be used by following GMP and GLP guidelines and must be developed using the protocols and acceptance criteria set out in the ICH guidelines Q2(R1). Once the methods have been developed, qualified and validated the impact they have on out-of specification rates and process capability needs to be quantified and evaluated to determine its effectiveness for future use.

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