1.0 **Introduction and importance of stability–indicating analytical method development**

1.1 **Stability requirements during development of a drug**

Stability indicating methods are developed to supervise the stability of drug substance and pharmaceutical dosage forms for the duration of the early phase of medicine development, and once the medicine is entered into the marketed, for the continuing product stability studies which must be performed as per ICH or regulatory guidelines. The reason of stability studies testing to give evidence on how the quality of drug differs with moment under the influence of a multiplicity of ecological factors such as humidity, temperature and light, enables suggested storage conditions, re-analysis intervals and shelf life (live) to be recognized. The development of these methods for pharmaceutical dosage forms and API can be come near from quite a few possibilities. Methods can be developed which determine the impurities of drug left over.

The ICH guideline primarily addresses the in sequence necessary in Registration Applications for new drug products. This instruction does not at present look for to wrap the information necessary for abbreviated, variations, clinical trail new applications, etc. The preference of test conditions distinct in the guideline is based on the real time analysis of the belongings of climatic circumstances in the three zones of the Japan, EC and USA. The represent or average kinetic temperature in any zone or region of the globe can be consequential from climatic information.
In order to the stability of the drug is an essential part of the organized approach to stability assessment as shelf life. The goal of stability program depends on the stage of development of the drug product. At very beginning of product development, it is essential to recognize the inherent firmness of the medicine and its interaction with the proposed excipients. At this stage the effect of pH, moisture, air (oxygen), and light on the stability of the drug substance also storage produced. The accelerated testing on drug substance and drug product provides the in sequence to the inherent stability of the bulk drug molecule formation and may establish the likely degradation pathways. The formulation group also has the responsibility for recommending to the toxicology group about stability of drug substance in the medium used in the animal trials. On the analytical side, the analytical research group supports the pre-formulation stability programs, which are ultimately responsible for developing and validating the stability-indicating related substance and assays that will be included in New Drug Application (NDA).

In the pre-clinical formulation stage, the selection of a stable drug product formula is the primary goal. The temporary preclinical formula is included in the Investigation new drug application (IND). The goal of the stability program in the clinical trial stage is to ascertain that the drug product batches tested in the clinical trials are stable, and these data will be subsequently included in the NDA. At the NDA approval stage, the
validated stability-indicating analytical method will be transferred to the quality control department, to ascertain that it works well in the hands of those who have to monitor the stability of the marketed drug product/drug substance. The marketed product stability program fulfills the commitment of part of the NDA and also ensures that the marketed products are stable (potent) until the expiry date stamped on the product label. Usually, the first three marketed batches and at least one batch per year are subjected to stability program.

1.2 Shelf life of drug and its importance

New drug applications need to submit scientific data that guarantee the stability of the product over a specified time period when maintained under specific storage conditions. If you see at the tag on recommendation or OTC drug product, we would see a use before or expiry date\(^1\). Prior to this date, the medicine should remain fully efficient under typical storage circumstances. The product shelf life is established using consistent storage circumstances of restricted room temperature and relative humidity, which could be changeable into acknowledged drug self-life. The performance of a drug when given as a tablet, capsule, syrup, or injectables (injections) depends not only on the content of the dissolution, disintegration, hardness, and so on. All of these aspects are therefore the part of stability program \(^2\)-\(^3\).
The ICH was organized in order to humanize stability–testing requirements for new drug applications with in the European Union (EU), the United States and Japan. ICH guidelines of the permanence testing of new drug substances (NDS) and products and for UV /photo stability permanence testing of NDS and products were officially adopted in October 1993 and November 1996, respectively 4-6.

1.3 Stability testing of new drug substances and drug products

1.3.1 Drug substance

The initial stability studies performance for the drug substances shows that it could be stay surrounded by specification for the duration of the re-analysis phase. Long term could be 12 months, and 40°C ± 2°C/75% (Acc) testing are performed on as a minimum three different lots or batches. The lots could be produced by pilot scale (CPP) or commercial manufacturing lots, but must have to use the same synthetic process and a routine of manufacture that of simulates the end process to be used at built-up commercial scale-up. In adding together, underneath stability on developmental or lab–scale produced lots could be produced at premature stages.

At the time of regulatory authority submission to Drug Master Filing (DMF), at least of 12 months at long term (25°C ± 2°C/60%) and 6 months at accelerated (40°C ± 2°C/75%) is necessary. If major changes are observed at the established conditions, supplementary testing at a middle condition, like 30°C ± 2 °C /60% could be establish.
The storage conditions range can be based on the permanence information and used in agreement with the countrywide or local necessities. Exact labeling requirements could be declared, mainly for drugs should not be in freezer.

1.3.2 Drug Product

The permanence of stability agenda for the drug dosage forms can be based on the information of the API and knowledge from developmental lab studies. Unless in particular noted in this division, the necessities for API also are valid to drug products. Accelerated (acc) data and long-term (LT) stability data would be shared on three commercial batches of the same formulation and its dosage forms in the containers and closure planned for marketing or selling to user end.

1.4 Stress testing for development of stability–indicating analytical methods (SIAMs)

Stress testing of the API can help recognize the probable degradation impurities, which should help, know the degradation pathway and the fundamental stability performance of the drugs and validate the permanence of stability indicating power or fitness of the analytical methodologies used for the test. The character of the stress testing will be based on the individual API and drug product concerned.

In generally for stress testing should be analysis carried out by final frozen synthesis process produced single batch or lot of API. It should exposure to temperature that is less than (<20ºC -30ºC) melting point of
drug substance, if exceeds more than melting range or high temperature the drug may be melted. Humidity is also applied about 75% relative humidity or grater like up to 90% relative humidity where appropriate, oxidation hydrolysis and photolysis (UV) on the API. The study should also assess the propensity of the API to hydrolysis crossways broad series of pH values when in solution form or in suspension form. UV/Photo stability performance should be a fundamental division of stress testing. The standard circumstances for UV stability performance were described in ICH Q1B and recent updated stability protocols from the new explanation in ICH Q1A.

1.5. Techniques engaged in literature data for the development of SIAMs

1.5.1 Titrimetric and spectroscopic

In titrimetric methodologies, typically the goal is the analysis API substance unaccompanied in the environment of compounds or residual organic impurities, stress sensitive bi-products and other analog of impurities. Their benefit in terms of cost is very low and effortlessness, and more over in few cases that may not be responsive or sensitive. Due to limiting property of titrimetric analysis, so that current days we may not seen any more on the usage for the assay of stability samples.
1.5.2. Chromatographic

Recent days the current guidelines and regulatory requirements are very stringent in terms of estimation and quantification of residual impurities in any of synthetic route or process, due to this reason the separation of all compounds in single run or during analysis, the LC methods was taken preference over regular conservative mode methods of analysis. The advantage of LC methods is that the developed analytical methods were posses greater selectivity, sensitivity, accurate, precise and robust. In this category the various kinds of LC methods that have been used are TLC, HP-TLC, GC, HPLC, UPLC and CE.

In comparison, HPLC and UPLC recently have been generally working. Due to the specificity, sensitivity and greater resolution between all pairs of compounds therefore it has increased recognition in stability indicting methodologies. We can find the solution or analyzing these type analysis i.e non-volatiles, thermal sensitive, high polar nature of products by using above new and conventional techniques. Therefore, all most all methods were of the SIAMs was developed by using UPLC and HPLC.

The control of drug substances impurities is currently a serious subject to the pharmaceutical industry. The bi-products in pharmaceuticals are the unwanted chemicals that leftovers at residual levels with the APIs, develop during the synthesis route and upon preparation of drug products. The attendance of these residual impurities may influence the safety and efficacy of bulk products. The
composition of impurities allows one to draw conclusions regarding the manufacturing of the drug products and its adulteration, which is becoming pervasive in all countries of the world therefore, it is strictly to firmly manage the safety of products and to determine the content of impurities at all stages of production from raw materials to finished medicinal forms 7-11.

1.5.3 Importance of impurity evaluation in pharmaceutical industry

Pharmaceutical industry is emergent day by day with the aim to develop new drugs extracted from synthetically or natural products produced chemical substances. But one thing always remains important that the product/substances should be the best pure as possible. Therefore, purity (assay) has been always considered as an essential factor to ensuring drug quality and its safety.

To purify a substance and remove the excess impurities one should first identify that whether they are actually present and what their nature is. In the past, this was not always done, because for non-availability of advanced techniques. But presently drug analysis and pharmaceutical impurities are the subjects of continuous review in the public concern. The ICH guidelines achieved an enormous deal in harmonizing the definitions of the impurities in new drug substances 12-13.
1.5.4 The role of chromatography in impurity evaluation

The applications of chromatography have grown explosively in the last fifty years, owing not only to the development of several new types of chromatographic techniques introduced or available in the market, but also to the growing need by scientists for better methods for characterizing complex molecules.

Indeed the greatest advantage of the chromatographic method over any other analytical procedure is the ability of separating specific analytes, a feature that appeals to all branches of science, which enables to discover and analyze unknown elements and chemical compounds.

1.6. Separation Mechanisms

A useful classification of the different kinds of LC techniques is based on the type of distribution or equilibrium that is responsible for the separation. The widespread interactions mechanism encountered in LC methods are classified as adsorption, partition, ion-exchange, gel permeation or size exclusion and chiral interaction. In practice, the most LC separations are the outcome of mixed mechanisms.

1.6.1. Adsorption

When the column stationary phase in HPLC/UPLC is a solid, the type of equilibrium between this phase and the liquid mobile phase is termed ‘adsorption’.
1.6.2. Partition

The equilibrium between the mobile phase (MP) and a column stationary phase comprising of either a liquid adsorbed on a solid or an organic species bonded to a solid is described as ‘partition’. The following table depicts the typical classification of chromatographic methods (Table 1.6.T1).

**Table 1.6.T1: Classification of chromatographic methods**

<table>
<thead>
<tr>
<th>Type of stationery phase</th>
<th>Type of mobile phase</th>
<th>Apparatus for stationary phase</th>
<th>Type of chromatography</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adsorption chromatography</strong></td>
<td>Gas</td>
<td>Column</td>
<td>Gas solid chromatography (GSC or GC)</td>
</tr>
<tr>
<td>Competition between a solid adsorbent and the mobile phase</td>
<td>Liquid</td>
<td>Column</td>
<td>Liquid column chromatography (LC), HPLC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Planar layer</td>
<td>Thin layer chromatography (TLC), paper chromatography (PC)</td>
</tr>
<tr>
<td><strong>Partition chromatography</strong></td>
<td>Gas</td>
<td>Column</td>
<td>Gas liquid chromatography (GLC or GC)</td>
</tr>
<tr>
<td>Competition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromatography</td>
<td>Phase 1</td>
<td>Phase 2</td>
<td>Technique</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>--------------------------------</td>
<td>------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Liquid Liquid chromatography (LLC)</td>
<td>Liquid Column HPLC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ion exchange chromatography</td>
<td>Liquid Column IEC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Permeation chromatography</td>
<td>Liquid Column Gel permeation</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All the above types of chromatographic techniques the most widely used technique in the bulk drug and pharmaceutical applications for impurity evaluation is high performance liquid chromatography, which was discussed further elaborately $^{14-17}$. 
1.6.3 High performance liquid chromatography

In HPLC/UPLC, separations are achieved by adsorption, partition or ion exchange depending on the stationary phase. The schematic diagram of HPLC is shown in Fig.1.6.F1.

**Fig. 1.6.F1: Schematic diagram of HPLC**

[Diagram of HPLC equipment]

The various components that are present in HPLC equipment are,

The operation has specific procedure and it depends on various factors. They are pumps, injector and detectors.

**1.6.3.1 Pumps**

The pump is one of the most important components in HPLC/UPLC, since it performance directly affects retention time, reproducibility and detector sensitivity.
Various types of pumps are used in HPLC, they are:

1.6.3.2 Reciprocating Piston Pumps

Consist of a small motor driven piston, which moves rapidly back and forth in a hydraulic chamber that may vary from 35-400 µl in volume. On the backstroke, the separation column valve is closed, and the piston pulls in solvent from the mobile phase reservoir. On the forward stroke, the pump pushes solvent out to the column from the mobile phase. This type of pump system is considerably smoother because one pump is filling while the other is in the delivery cycle.

1.6.3.3 Syringe Type Pumps

They are most suitable for small-bore columns because this pump delivers only a finite volume of mobile phase before it has to be refilled. These pumps have a capacity volume between 250 to 500 ml. The pump operates by a motorized lead screw that delivers mobile phase to the column at a steady rate. The rate of solvent delivery is restricted or controlled by changing the voltage on the motor.

1.6.3.4 Constant Pressure Pumps

The mobile phase is driven through the column with the use of low pressure from a gas cylinder to generate high liquid pressures. The valve arrangement allows the rapid refill of the solvent chamber whose capacity is about 70ml. This indicates continuous mobile phase flow rates.
1.6.3.5 Injector

Samples are injected into the HPLC/UPLC via an injection port. The injection port of an HPLC/UPLC commonly consists of an injection valve and the sample loop. The sample is typically dissolved in the mobile phase or in any of suitable solvents before injection into the sample loop. The sample solution is then drawn into a syringe and injected into the loop via the injection valve. A rotation of the valve rotor closes the valve and opens the loop in order to inject the sample solution into the stream of the mobile phase. Loop volumes can range between 200 µl to over 1.0 µl. In modern HPLC/UPLC systems, the sample injection is typically automated.

1.6.3.6 Column

LC columns are stainless steel tubes, typically of 5-30 cm in length and 1.7-5mm inner diameter. Guard columns, which are placed before an analytical column to trap junk and make longer the lifetime of the analytical columns, are 3-10 cm long. The stationary phase or packing is retained at each end by thin stainless steel frits with a mesh of 2µm or less. The most popular material is octadecyl-silica, which contains C\textsubscript{18} chains, but materials with C\textsubscript{2}, C\textsubscript{6}, C\textsubscript{8} and C\textsubscript{22} chains are also available\textsuperscript{18}. 

1.6.3.7 Detectors

Detector for HPLC/UPLC is the component that emits a response due to eluting sample compound and subsequently signals a peak on the chromatogram. It is positioned immediately posterior to the column stationary phase in order to detect the compounds as they elute from the column. The bandwidth and height of the peaks may usually be adjusted using the common and fine-tuning controls and the detection and sensitivity parameters may also be controlled.

Regardless of the principle of operation, an ideal LC detector should have the following properties

(i) Low drift and noise level (particularly crucial in trace analysis),
(ii) High sensitivity, (iii) Fast response, (iv) Wide linear dynamic range (this simplifies quantitation), (v) Low dead volume (minimal peak broadening),
(vi) Cell design, which eliminates remixing of the separated bands, (vii) Insensitivity to changes in type of solvent, flow rate, and temperature, (viii)
Operational simplicity and reliability, (ix) It should be tunable so that detection can be optimized for different compounds and (x) It should be non-destructive, many types of detectors can be used with HPLC/UPLC.

### 1.6.3.7.2 Ultra violet (UV) detectors

For the greatest sensitivity wavelength maxima should be used, which detects all sample components that contain chromophores. The schematic diagram of UV detector is shown in Fig.1.6.F3.

**Fig. 1.6.F3: The schematic diagram of UV detector**

![Diagram of UV detector](image.png)

**Fixed wavelength:** Measures at one wavelength, usually 254 nm.

**Variable wavelength:** Measures one wavelength (example: 210) at a time, but can detect over a wide range of wavelengths i.e 190 to 400 nm.

**Diode array:** Measures a spectrum of wavelengths simultaneously.

The schematic diagram of Diode array detector is shown in Fig.1.6.F4.
1.6.3.7.3 **Fluorescent detectors**

They measure the ability of a compound to absorb then re-emit light at given wavelengths. The excitation is set to the utmost value then the emission is scanned to locate the emission intensity. Selection of the initial system could, therefore, be based on assessment of the nature of test sample and drug substances.

1.6.3.7.4 **Radio chemical detectors**

Involves use of radio labeled material usually tritium (³H) or carbon-14 (¹⁴C). It operates by detection of fluorescence along with beta-particle ionization.

1.6.3.7.5 **Electrochemical detectors**

Used in analysis of compounds that undergoes oxidation or reduction reactions. They measure the difference in electrical potential when the sample passes between the electrodes.
Table 1.6.T2: HPLC detector comparison

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Refractive index</th>
<th>UV/Vis</th>
<th>Fluorescence</th>
<th>Electrochemical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection sensitivity (g)</td>
<td>$10^{-6}$</td>
<td>$10^{-9}$</td>
<td>$10^{-12}$</td>
<td>$10^{-12}$</td>
</tr>
<tr>
<td>Linear range</td>
<td>$10^4$</td>
<td>$10^5$</td>
<td>$10^3$</td>
<td>$10^8$</td>
</tr>
<tr>
<td>Flow sensitivity</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Temperature sensitivity</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Gradient Composition</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Range of application</td>
<td>Universal</td>
<td>Selective</td>
<td>Very selective</td>
<td>Very selective</td>
</tr>
<tr>
<td>Peak shape</td>
<td>Positive or Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
</tbody>
</table>

1.6.4 Applications of HPLC/UPLC \(^{19-23}\)

1.6.4.1 Chemical separation

This can be accomplished using HPLC/UPLC by utilizing the fact that, certain compounds have different migration rates due to different forces occurs given a particular column and mobile phase. The extent or degree of separation is mostly determined by the choice of column stationary phase and its mobile phases.

1.6.4.2 Identification

For this purpose a clean peak of known sample assay has to be observed from the chromatogram by using qualified reference standard or working standards. Selection of column, mobile phase and flow rate matter to certain level in this process by comparing with reference compound does identification and it can be assured by combining two or more detection methods.
1.6.4.3 Quantification

It is the analyte confirmation by using the known reference standards. Quantification of known and unknown areas with respect to the principal peak by various methods like internal standard method, area normalization method, and external standard method.

1.7 Steps involved during the development of stability-indicating analytical methods (SIAMs)

A SIAM is an estimative analytical method used to detect a trace level amount or residual levels of the API present due to 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 degradants, bi-products, intermediates, and excipients and the FDA recommend that all assay content methodologies for stability studies be stability-indicating. There are three components necessary for implementing a SIAM.


1.7.1 Step-1: Sample generation

Forcibly degrading the active substance in both that is solution medium and in solid-state form generates the test sample that contains the products most likely to form under 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.
1.7.2 Step-2: Analytical method development approaches

Many parameters must be evaluated and optimized during the 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. 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 selection

F) Mobile phase selection, G) Flow rate and Column temperature

H) Degradation studies

1.7.2.1 Literature collection

Thorough literature search to be carried out 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 first element whenever assignment takes up the 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 towards light, heat, hygroscopic study etc).

1.7.2.2 Chemical structure
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.

1.7.2.3 Diluent selection

It is advisable 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 obtain the good symmetrical peak shape.

1.7.2.4 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. [Refer Fig 1.7.F1].
Fig 1.7.F1: Different alkyl chains attached to Si-OH

Silica based columns with different crosslinkings in the increasing order of polarity are as follows:

\[ \text{\textla} \text{Non-polar} \text{\textla} \text{moderately (mid) polar} \text{\textla} \text{Polar} \text{\textra} \]

\[ C_{18} < C_8 < C_6 < C_4 < C_2 < \text{Phenyl} < \text{Amino} < \text{Cyano} < \text{Silica} \]

1.7.2.4.1 Particle shape

Particles are either spherical or irregular [Fig.1.7.F2]. Irregular particles have in generally higher surface areas and higher carbon loads. Spherical particles provide higher efficiency, better column stability, fast stabilization and lower back-pressures compared to irregularly shaped particles.

Fig. 1.7.F2: The shapes of spherical and irregular particles
1.7.2.4.2 Particle size

Particle size for HPLC column packing refers to the average diameter of the packing particles [Fig.1.7.F3]. Particle size affects the back-pressure and the separation efficiency of the column. The column back-pressure and column efficiency (performance) are inversely proportional to the square of the particle diameter. As the particle size \( d \) is lower, the column back-pressure and efficiency increase. The particle diameter range is about 1.8–20 µm (sub-2 micron columns for especially UPLC purpose). Smaller particles offer higher efficiency. Fast, high-resolution separations can be achieved with smaller particles packed in short (5-50 mm) length columns.

Fig.1.7.F3: Different particle sizes of HPLC column packing

1.7.2.4.3 Surface area
The surface area is the sum of particle outer surface and interior pore surface in square meters per gram [Fig.1.7.F4]. A high surface area generally provides high retention times (RT), capacity and USP resolution (Rs) for separating complex, multi component samples. The physical structure of the particle substrate determines the surface area of the stuffing material in LC column stationary phases. A packing material with a thin pore size will have a big surface area, and vice versa. Surface area is determined by pore size. Pore size and surface area are inversely related. High surface area materials offer greater capacity and greatest (high) analyte retention times. Low surface area packing offer shorter equilibration time and are often used for large molecular weight molecules.

**Fig.1.7.F4: The schematic diagram of surface area**

1.7.2.4.4 Pore size

The pore size of a packing material represents the average size of the pores within each particle [Fig.1.7.F5]. Generally pore size of 150 Å or less is chosen for samples with molecular weights less than 2000 and a pore size of 300 Å or greater for samples with molecular weights greater than 2000. In generally the range in value from 60 Å to 10,000 Å. Larger
pores allow larger solute molecules to be retained through maximum exposure to the surface area of the particles.

**Fig. 1.7.F5: A representative diagram of pore size**

![Diagram of pore size](image)

1.7.2.4.5 Carbon load

The carbon load is the amount of bonded phase attached to the base material, expressed as the percentage of carbon [Fig.1.7.F6]. High carbon loads generally offer greater resolution and greater retention times for hydrophobic samples. Low carbon loads shorten run times and often show different selectivity.

**Fig. 1.7.F6: A representative diagram for carbon load**

![Diagram for carbon load](image)

Most of the columns are limited between pH 2 and 8. Recently many of column manufactures are offering a wide range of pH scale i.e around 1.0 to 11 [Fig. 1.7.F7].

**Fig. 1.7.F7: Diagram for understanding the pH limitations**
1.7.2.4.7 Effect of variables on column efficiency

A mathematical rough calculation of the behavior of chromatographic column efficiency is obtained from the Van Deemter equation.

$$H = A + \frac{B}{u} + Cu,$$

Where $H$ is the plate height, $u$ is the linear velocity of the mobile phase, $A$ is the eddy diffusion term, $B$ is the longitudinal diffusion coefficient, and $C$ is the coefficient of the mass transfer term. The minor the value of $H$, the more efficient the column.

The eddy diffusion term $A$, indicates the multitude of pathways by which a component finds its way through the column. The longitudinal diffusion term, $B/u$, indicates a band-broadening process that is inversely related to the mobile phase velocity. The mass transfer term, $Cu$, indicates the time available for equilibrium of an analyte to be established between the mobile and stationary phases [Fig.1.7.F8].
1.7.2.4.8 Detector selection

DAD is useful for initial method development based on the chromophores (wavelength maximum) present in the compounds to be separated. Select the initial wavelength analyzing the UV spectra of the compounds using UV-VIS Spectrophotometer. If the compounds are not having chromophores, 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.

1.7.2.4.9 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 a proton and become ionized when pH increases and bases gain a proton and become ionized when pH decreases is shown in the figure below. Therefore, when separating mixtures of impurities containing acids...
and/or bases by reversed phase 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.1.7.F9].

**Fig. 1.7.F9: Effect of pH on the Retention of Acids and Bases**

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

1.7.2.4.9.1 Fixing of the pH

The pH of the Buffer in the mobile phase is selected based on the pKa of the analyte and some times need to be consider for impurities, intermediate, raw materials and bi-products also, which is based on the structure of the molecule [Table 1.7.T2]. For most practical purposes and for most robust methods, the pH of the mobile phase can be ± 1 units from the pKa of analytes of interest.

**Table 1.7.T1: pKa values for different functional groups**
When the pH of mobile phase is near the pKa value of the analytes, even a small change in pH will have a major impact on the resolution and peak or zone distortion [Fig.1.7.F10 & Fig.1.7.F11].

**Fig.1.7.F10: pH variation study (Representative chromatogram on pH impact)**

<table>
<thead>
<tr>
<th></th>
<th>pKa Acid</th>
<th>pKa Base</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfonic acid ---SO₂H</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Amino acid, ---C(NH₂)---COOH</td>
<td>2 - 4</td>
<td>9 - 12</td>
</tr>
<tr>
<td>Carboxylic acid, ---COOH</td>
<td>4 - 5</td>
<td>1 - 2</td>
</tr>
<tr>
<td>Thiol, ---SH</td>
<td>10 - 11</td>
<td>1 - 2</td>
</tr>
<tr>
<td>Purine</td>
<td>2 - 4</td>
<td>3 - 7</td>
</tr>
<tr>
<td>Phenol, ---OH</td>
<td>10 - 12</td>
<td>9</td>
</tr>
<tr>
<td>Pyrazine</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Sulfoxide, ---SO</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Thiazole, ---N2, Pyridine</td>
<td>1 - 3</td>
<td>9 - 11</td>
</tr>
<tr>
<td>Amino, ---NH2, ---N2, Pyridine</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Imidazole</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>Piperazine</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

Note a: Aliph, Aliphatic Substituent [e.g., acetic acid for ---COOH]
Note b: Arom, Aromatic Substituent [e.g., benzoic acid for ---COOH]

When the pH of mobile phase is near the pKa value of the analytes, even a small change in pH will have a major impact on the resolution and peak or zone distortion [Fig.1.7.F10 & Fig.1.7.F11].

**Fig.1.7.F11: Retention time Vs resolution graph**
1.7.2.4.9.2 Acidic compounds

Preferable to use acidic mobile phase as the compound will be in unionized form and will retain more.

1.7.2.4.9.3 Basic compounds

In acidic mobile phase compound will be ionized and will elute early; peak shapes will be better. In basic mobile phase compound will be unionized and will retain more but peaks may tail due to active silanols via secondary interaction occurs in the stionationary phase at basic pH.

1.7.2.4.9.4 Neutral Compounds

Neutral mobile phase is suitable, with increasing pH acids losses a proton and become ionized. When acids are ionized, it becomes less hydrophobic and more hydrophilic resulting in shorten retention times. With decreasing pH bases gain a proton and become ionized. When bases are ionized, it becomes less hydrophobic and more hydrophilic resulting in again shorten retention times [Fig.1.7.F12].

**Fig.1.7.F12: Representative Graph of retention factor Vs pH**
Mobile phase pH should be selected so that it is at least ±1.0 pH units from the analyte pKa value. This assures that the analytes are either 100% ionized or 100% non-ionized and helps in controlling peak shape and the run-run reproducibility.

1.7.2.4.10 Fixing of the Buffer

Buffer imparts constant 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 in Table 1.7.T3.

Table 1.7.T2: Commonly used buffers for reversed Phase HPLC

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pKa</th>
<th>Buffer Range</th>
<th>UV Cutoff (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate</td>
<td>2.1</td>
<td>1.1 – 3.1</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>6.2 – 8.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12.9</td>
<td>11.3 – 12.3</td>
<td></td>
</tr>
<tr>
<td>Formic acid*</td>
<td>3.8</td>
<td>2.3 – 4.8</td>
<td>210</td>
</tr>
<tr>
<td>Acetic acid*</td>
<td>4.8</td>
<td>3.8 – 5.8</td>
<td>210</td>
</tr>
<tr>
<td>Citrate</td>
<td>3.1</td>
<td>2.1 – 4.1</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>4.7</td>
<td>3.7 – 5.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.4</td>
<td>4.4 – 6.4</td>
<td></td>
</tr>
<tr>
<td>Tria</td>
<td>8.3</td>
<td>7.9 – 8.9</td>
<td>255</td>
</tr>
<tr>
<td>Triethylamine*</td>
<td>11.0</td>
<td>10.0 – 12.0</td>
<td>200</td>
</tr>
<tr>
<td>Pyrrolidine</td>
<td>11.3</td>
<td>10.3 – 12.3</td>
<td>200</td>
</tr>
</tbody>
</table>

* Volatile buffers

1.7.2.4.11 Buffer concentration
The concentration of the mobile phase containing buffer usually has small effect on retention time in reversed phase HPLC, just as long as the buffer concentration is high enough to control pH. A buffer concentration in the range of 5 to 100 mM is adequate for most reversed phase applications. This concentration is also low enough to avoid problems with precipitation when significant amounts of organic modifiers (acetonitrile, methanol and tetrahydrofuran (THF)) are used in the mobile phase.

For ionic compounds which behave as highly polar and difficult to retain by reverse phase, ion pairing chromatography can be helpful. Negatively charged reagent such as alkyl sulfonic acid will be used to retain positively charged ionic bases. Positively charged reagent such as tetra butyl ammonium salts will be used to retain negatively charged ionic acids. Alkyl sulphonates are good first choice for basic compounds and Quaternary amines are useful for acidic compounds [Fig.1.7.F13].

**Fig.1.7.F13: The chemical structures of ion pair reagents**

1.7.2.4.12 Fixing of organic modifier
Acetonitrile and methanol are the first choice for organic modifiers. Acetonitrile is best among the two due to the low UV cut off and low viscosity. Methanol is a proton donor and acetonitrile is proton acceptor and so selectivity will be significantly different, IPA & THF are other alternate modifiers, but the THF mobile phases are not stable because of highly oxidisable nature. For acetonitrile mobile phases, to avoid pumping problems associated with 100% acetonitrile use always with about 5-10% aqueous portion otherwise pumps will be chocked. An example for the solvent strength shown in Fig.1.7.F14.

**Fig.1.7.F14: Representative chromatogram on different solvent strengths**

![Representative chromatogram on different solvent strengths](image)
1.7.2.5 Flow rate and column temperature

For 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.

1.7.2.6 Degradation studies

The degradation products generated in the stressed samples are termed as “potential” degradation products that may or may not be formed under relevant storage conditions. Below are the major forced degradation studies.

1). Acid degradation, 2). Base degradation, 3) Oxidative degradation
4). Thermolytic degradation, 5) Photolytic degradation

Step-3: 1.7.3 Analytical method validation process

Appropriate validation of analytical methods is very much important for pharmaceutical analysis when assurance 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 33-38.
The USP has available specific guidelines for method validation for compound evaluation. USP/ICH defines eight analytical performance parameters for validation (Table 1.7.T3).

**Table 1.7.T3: Validation parameters**

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

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


**1.7.3.1 Precision**

Precision is the measure of the degree of repeatability of a specified analytical method under usual operation and is normally expressed as the percent relative standard deviation for a statistically significant number of samples.

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 over a short time interval under the same conditions. It should be 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 random events such as differences in experimental periods, analysts, equipment, and so forth. Reproducibility refers to the results of collaborative studies among laboratories.

**1.7.3.2 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 closeness 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.

**1.7.3.3 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 specified S/N ratio of 2:1 or 3:1 is generally accepted.

**1.7.3.4 Limit of Quantification (LOQ)**

The LOQ is defined as the lowest concentration of an analyte in a test sample that may be determined with acceptable precision and accuracy under the stated experimental, operational conditions of the method. LOD, LOQ is expressed as a concentration, with the acceptable precision and accuracy of the measurement also reported. The S/N ratio between 9.5 and 10.4 is considered for the determine LOQ.

**1.7.3.5 Selectivity and Specificity**
The terms specificity and selectivity are normally used interchangeably. The term specific usually refers to a method that produces 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.

### 1.7.3.6 Linearity and Range

Linearity is the ability of the method to elicit test results that are directly proportional to analyte concentration within an agreed range. Linearity is generally reported as the conflict of the slope of the regression line. Range is the interval between the upper and lower levels of analyte that have been demonstrated to be determined with precision, accuracy, and linearity using the method. The range is usually expressed in the same units as the test results obtained by the method.

### 1.7.3.7 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.

### 1.7.3.8 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.

1.8 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, and the calculated increase in degradation impurities is quantification by RS by 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 39.

1.9 Scope and objective of investigate work

The current research exertion focuses on the new stability capable analytical method developments for a few of drug substances and their dosage forms as well its precursors. The research work comprises also the analytical method validation for the above stated or developed methods as in-line with current ICH and USP guidelines fitfulness and checked the fitness of developed methods to assess the stability studies power of drug substances (Table 1.9.T1).
Table 1.9.T1: The list of API and its procuresses

<table>
<thead>
<tr>
<th>S.No.</th>
<th>API/intermediate chemical names</th>
<th>Structure</th>
<th>Therapeutic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>2,3-Dichlorobenzoyl cyanide</td>
<td>![Structure Image]</td>
<td>2,3-DCBC is the Advanced intermediate of Lamtrigine drug substance (anti seizure activity of some anticonvulsants)</td>
</tr>
<tr>
<td>2.0</td>
<td>N-(1-oxopentyl)-N-[[2-(1H-tetrazol-5-yl) [1,1-biphenyl]-4-yl]methyl]-L-valine</td>
<td>![Structure Image]</td>
<td>treatment of hypertension</td>
</tr>
<tr>
<td>3.0</td>
<td>4-(5-cyclopentyloxy-carbonylamino-1-methyl-indol-3-yl-methyl) methoxy-N-o-tolylsulfonyl benzamide</td>
<td>![Structure Image]</td>
<td>Treatment of asthma</td>
</tr>
<tr>
<td>4.0</td>
<td>(2-methyl-4-(4-methyl-1piperazinyl)-10H-thieno[2,3b][1,5]benzodiazepine</td>
<td>![Structure Image]</td>
<td>atypical antipsychotic</td>
</tr>
</tbody>
</table>

New stability-indicating analytical UPLC and HPLC methods have been developed for above chosen API and its precursors.
References


7. CPMP Guideline on control of impurities of pharmacopoeial substances: compliance with the European pharmacopoeia general monograph “substances for pharmaceutical use” and general chapter “control of impurities in substances for pharmaceutical use”, 2004.


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