1.1 Introduction to impurity profile

In the field of pharmaceutical chemistry, impurities are considered to be extraneous materials present in the therapeutically active pharmaceutical compounds. They are expected to have unusually potent, toxic or unexpected pharmacological effects which are detrimental to human health. So the control of impurities even at low levels in the drug is of paramount importance in the field of pharmaceutical chemistry to assure the safety and efficacy of the drug. Therefore, an appropriate assessment and quantification of impurities in the drug is essential.

The impurity profile of a drug is defined as "A description of the identified and unidentified impurities present in a new drug product" [**1**]. It provides an account of impurities present in the drug. It not only provides maximum possible types of impurities but estimates the definite amount of various kinds of impurities present in the drug.

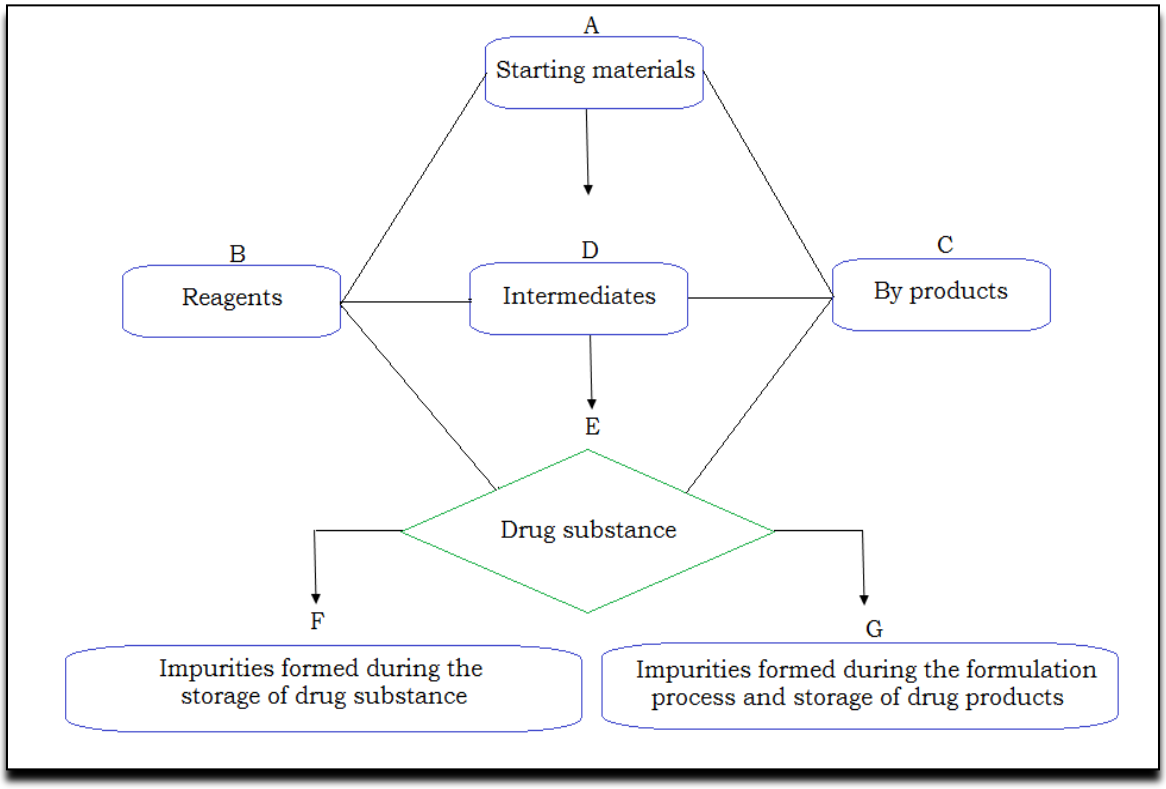
1.2 Sources of impurities:

The most possible source of impurities is the synthesis that involves various stages, i.e. from starting material to finished product through intermediate steps [**2**]. During this process, intermediates and by-products may be either carried into the final stages as impurities or transformed into a source of other impurities resulting from them.

The other sources of impurities are solvents and reagents that are being used during the synthesis. Impurities can also be formed due to the instability of the drug substance during storage and incompatibility

with the excepients in the drug product. The sources for the origin of impurities are schematically represented in **Fig. 1.1.**

Fig. 1.1 Schematic representation of origin of impurities



A: Raw materials at initial stage of synthesis.

B: Reagents that are used to move forward the reaction, eg. catalysts.

C: Impurities originated from side reactions during synthesis.

D: Compounds produced intermediately during the synthesis of required compound (Drug substance).

E: Therapeutically active compound.

F: Impurities originated from thermolytic, photolytic, hydrolytic degradation of drug substance.

G: Impurities formed due to excipient incompatibility, wet granulation, compression & Impurities under category F.

The formation of impurities in the drug product can be not only due to API or inactive ingredients but the formulation processes applied during the manufacturing of drug products i.e., water addition during the wet granulation process, solvent additions etc. In addition to this, the other source of impurities is the packaging materials used.

1.3 Classification of impurities

Impurities can be categorized into the following types [**3**].

1. Organic impurities: These impurities can be formed during the manufacturing process and storage of the API.
   a) Starting materials
   b) By-products
   c) Intermediates
   d) Degradation products
   e) Reagents, ligands, catalysts and solvents.
2. Inorganic impurities can be formed from the manufacturing process. They are generally known and identified compounds such as reagents, ligands, catalysts, heavy metals, other residual metals, inorganic salts and other materials like filter aids, carbon etc.
3. Inorganic or organic solvents are widely used as reaction vehicles during the manufacturing of drugs. These solvents remain as residuals in the finished products and are considered as impurities due to their toxicological effects.

4. Polymorphic forms and isomers are also considered to be impurities. So, in order to evaluate the quality of the drug we must quantify the contents of these two.

Apart from this, impurities can also be formed due to either intentional or inadvertent contamination. This extraneous contamination, of course, should not arise in drugs and is more properly addressed as GMP issues.

1.4 Regulatory perspective

Impurities in drug substances and drug products are key regulatory issues in the Office of Generic Drugs and have a significant impact on the approvability of drugs [**4**]. The International Conference on Harmonization (ICH) has formulated a workable guideline regarding the control of impurities [ICH Q3]. Regulatory guidelines also often highlight this importance while discussing the generic drug product development, and comparing impurity profiles with innovator products. ICH and FDA guidelines introduce the identification and qualification procedures for drugs and approaches to the establishment of acceptance criteria for both drug substances and drug products [**5-6**].

1.5 Analytical techniques

In order to study the impurity profiles of pharmaceutical compounds, suitable analytical techniques should be used. The Identification and quantification of impurities can be performed by various individual or a combination of analytical techniques. These techniques include TLC, LC,

GC, MS, NMR, IR, UV, LC-DAD, LC-DAD-MS, LC-MS, GC-MS, LC-NMR etc. The application of each analytical technique is briefly discussed in **Table 1.1.**

Table 1.1 Description of analytical techniques that are generally used for identification, impurity content and assay determinations.

Name of the technique	Tests can be performed	Remarks
1.Titrimetric/Potentiometric	Assay &Stoichiometric salt content	Non specific
2.Spectroscopy techniques		
a)UV spectroscopy	Assay& identification	Non specific
b)Mass spectroscopy		
c)NMR	Identification & Characterization(b to d)	Specific
d)IR		
e)AAS	Metallic impurities (e to g)	Specific
f)ICP		
g)ICP-MS		
3.Chromatographic techniques		
a)Thin layer chromatography		
b)Liquid chromatography		
c)Gas chromatography [a]	Impurity content (a to e)	Specific
d)Capillary electrophoresis		
e) Super critical fluid chromatography		

[a] Gas chromatography is mainly used for the determination of residual solvents.

The complexity of a chromatographic separation depends on the properties of the solutes in the mixture. The separation is even more complex when the physico-chemical properties of analytes are similar. One of the most challenging tasks throughout the pharmaceutical analyses is the separation of impurities in category 3 in table 1.1. This becomes even more complex in the following cases.

1. Structurally similar impurities

   Eg. Difference in double bond or methyl group

2. Separation of isomers (Eg. positional isomers)

4. Separation of chiral impurities (Eg. Enantiomers and diastereomers)

5. Presence of more number of impurities (Eg. Multi-component mixture)

The analysis pharmaceutical compounds entails more frequently, the task of separating complex mixtures that contains structurally similar solutes. The most suitable example in this context is the separation of the enantiomers of a chiral compound and positional isomers. These isomers differ solely in the spatial orientation or position of functional groups rendering their solute properties basically identical [**7-12**]. So, in this area a wide range of method development strategies is needed to be developed to achieve the successful separation.

In this perspective, LC and its hyphenated techniques (LCMS) have gained immense importance in pharmaceutical analyses [**13-17**].

Table 1.2 Classification of chromatographic techniques

General classification	Mobile phase	Stationary phase	Type of chromatographic process
1.Thin Layer Chromatography (TLC)	Liquid	Solid coated on two dimensional plane.	Adsorption and partition.
2.Liquid Chromatography(LC)	Liquid	Liquid	Partition of analyte between two immiscible liquids.
	Liquid	Bonded phase	Adsorption and partition of analyte between liquid mobile phase and solid stationary phase.
	Liquid	Ion exchange resin	Ion exchange.
	Liquid	Liquid in interstices of a polymeric solid	Partition/sieving(size exclusion)
3.Gas Chromatography(GC)	Gas	Solid	Adsorption
	Gas	Liquid film coated on solid support	Partition
4.Capillary Electrophoresis (CE)	Liquid	Fused silica capillary	Electro migration
5. Super Critical Fluid Chromatography (SFC)	Fluid	Bonded phase(solid)	Partition

1.5.1 Thin layer chromatography

Thin-layer chromatography is a two-dimensional chromatographic technique wherein the separation takes place by the distribution of solutes between solid stationary phase and liquid mobile phase. Generally, the stationary phase in TLC is polar adsorbent coated on a glass or alumina plate and the mobile phase is a single or mixture of solvents with non-polar and polar natures. TLC is a simple, fast and less expensive micro-scale technique that can be employed for

a) Identification of compounds

b) Determination of starting materials during the reaction

c) Determination of number of components in given mixture

Though TLC can resolve a wide range of compounds by using different sorbents and mobile phases, the problems mentioned below made this technique limited to a certain extent.

a) Limited Resolution

b) Limited detection

c) Difficulties in accurate quantifications

TLC combined with densitometric detection is a more sensitive method when compared to classical methods. The improved methodologies (HPTLC) involve the use of lower particle size and thin coated stationary phases to maximize the resolution.

1.5.2 High performance liquid chromatography

HPLC is basically an improved version of column chromatography. Instead of a solvent being allowed to pass through a column by means of gravitation, the solvent is pumped through the column under high pressures up to 5000 psi. Thus the separation on column takes place much faster and in a more reproducible manner. Applications of this technique are very effective and have been significantly expanded by the use of a variety of detectors such as UV, refractive index, fluorescence, electrochemical, MS, NMR etc.[**18-19**].

The LC-UV technique becomes more versatile in the field of pharmaceutical analysis due to the high sensitivity of UV detectors, availability of wide range stationary phases, speed of analysis and cost effectiveness. The reasons can also be explained by the fact that most of the pharmaceutical compounds are organic compounds and a majority of these compounds are UV active. Therefore, a wide range of samples can be analyzed by means of a UV detector. A variety of stationary phases from polar (silica) to non polar (C_{18}) are available to achieve the best possible separation in LC. The major areas of applications are listed below:

a) Separation

b) Identification

c) Quantification

d) Isolation

1.5.3 Gas chromatography (GC)

GC technique involves vaporization of sample and subsequent injection into the gas chromatographic column. The sample is passed through the column by means of gas flow. The mobile phase is an inert gas and the stationary phase is a liquid film coated on a support of fused silica or a packed sorbent. The sample in vapor form moves through the column by adsorption and partition phenomenon. The components in the sample mixture are separated by means of their individual affinity to involve in the adsorption and desorption processes. The separated components are eluted from the column and detected by a suitable detector. Various types of detectors are used in gas chromatography based on the sample property as listed below.

a) Flame ionization detector (FID)

b) Electron capture detector (ECD)

c) Thermal conductivity detector (TCD)

d) Thermionic detector using alkali metal salt (TIDA)

e) Photo ionization detector (PID)

The working principles of detectors in gas chromatography are discussed in the literature [**20**].

GC is an extremely useful technique for the quantification of thermally stable and volatile compounds. Excellent selectivity of various stationary phases [**21**] provides the best column separations. This

technique is most useful for studying the impurity profile of residual solvents in pharmaceutical compounds.

1.5.4 Capillary electrophoresis (CE)

Capillary electrophoresis involves the introduction of a solution containing a mixture of components into a narrow capillary zone and induces to move through the zone by means of applied potential. The components in the mixture pass through the capillary zone with different rates of velocities or migration based on the individual mobility of components under the influence of electric field. Thus the mixture of components is then separated into different discrete zones of individual components after a certain time period. Various modes of electrophoresis methods have been developed in combination with chromatography which are as follows [**22-23**].

a) Capillary zone electrophoresis
b) Capillary gel electrophoresis
c) Micellar eletrokinetic capillary chromatography
d) Capillary electro chromatography
e) Capillary isoelectric focusing
f) Capillary isotachophoresis

CE is a useful and an effective technique when very small amounts of test samples are available. The resolutions are much higher when compared to other techniques. But the major drawback in this technique is less reproducibility.

1.5.5 Supercritical fluid chromatography (SFC)

Supercritical fluid chromatography allows the separation and quantification of compounds that cannot be separated by either HPLC or by GC. It has common features for both HPLC and GC. A supercritical fluid like carbon dioxide having low viscosity and low diffusion co-efficients is used as the mobile phase. The bonded stationary phase that can withstand at high column pressures and temperature is used along with flame ionization detection [**24**].

Supercritical fluid chromatography offers the advantages of HPLC in terms of separation and of GC in terms of sensitivity, but in this case the volatility of sample is not of great importance. Since the SFC is usually operated in normal phase mode, the TLC and HPLC methods of normal phase mode can be directly adopted to this technique. The major applications of this technique include the following.

a) Extraction of samples
b) Orthogonal separations to RP-LC methods
c) Chiral separations

1.6 Chromatographic method development

The basic principle involved in chromatography is the separation of components or molecules by means of migration. Chromatographic separations are accomplished on the basis of different migration rates of the solutes. The fundamental principles are the same in thin layer chromatography and column liquid chromatography.

1.6.1 Importance of method development

In order to evaluate the quality of the drug in terms of purity, a direct assessment of impurity profile is required by means of suitable analytical testing. The existing analytical procedures established for the monitoring of heavy metals, inorganic residues, metallic impurities and residual solvents can be commonly used for a majority of the pharmaceutical compounds with minimum assessment of suitability, but analytical methods for monitoring related compounds and assay are specific to each pharmaceutical compound.

Impurity and assay determinations in the pharmaceutical compounds are of a complex nature in the field of pharmaceutical analysis because of the separation issues. In particular, while developing a sensitive, selective and specific analytical method, the objective becomes more complex. Thus the chromatographic method development has gained immense importance in the pharmaceutical field [**25-28**].

1.6.2 Recent advances in technology

Ultra Performance Liquid Chromatography (UPLC) is the contemporary technology that changed the direction of liquid chromatography in the recent past. The UPLC technique resembles the HPLC with a greater degree of speed, sensitivity and separation. The basic principles involved in this technique are discussed below.

Efficiency in liquid chromatography is a function of operating parameters. Column efficiency (or) Number of theoretical plates can be derived from the below equation.

$$N = \frac{Length\ of\ the\ column}{HETP} \qquad \text{Equation-(1)}$$

Where,

N = Number of theoretical plates (or) Column efficiency

HETP = height equivalent to a theoretical plate

Since the column efficiency (*N*) is inversely proportional to the "height equivalent to a theoretical plate (HETP)", increased chromatographic performance can be achieved with decreased HETP values [**29**]. The magnitude of HETP value for packed chromatographic columns can be derived from the Van Deemter Equation. This model further explains band broadening that describes the relationship between mobile phase linear velocity and the height equivalent of a theoretical plate (HETP).

$$H = Ad_p + \frac{BD_M}{u} + \frac{C\,d_p^2\,u}{D_M} \qquad \text{Equation-(2)}$$

Where

H = Height equivalent theoretical plate (HETP)

u = linear velocity of the mobile phase

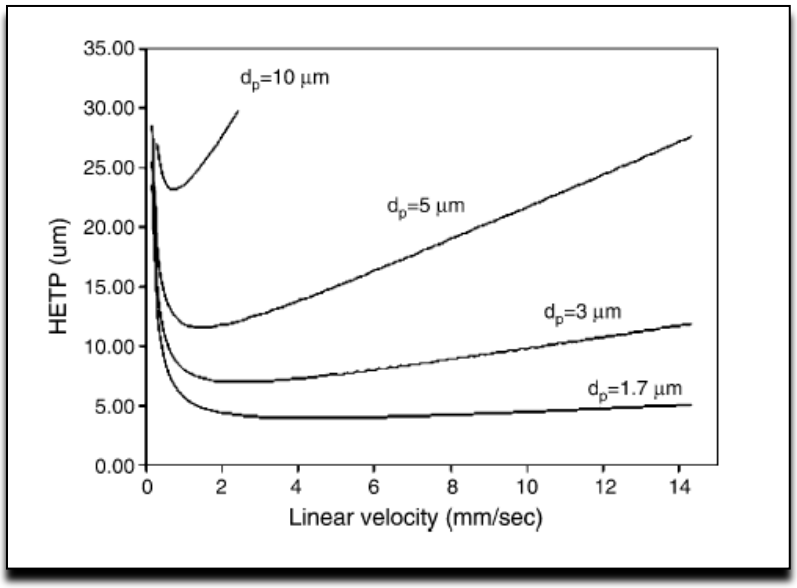
d_p = Particle size of the stationary phase

D_M = Analyte diffusion coefficient

A, B and *C* are constants

From the equation (2), HETP value changes with linear velocity (u) and particle size (d_p) of the stationary phase. As the particle size (d_p) is in numerator in the first and third terms of the equation (2), the lower values of HETP are possible only for low particle size stationary phases. The effect of linear velocity (u) on HETP at different particle diameters was reported by Van Deemter as shown in the **Fig. 1.2**.

Fig. 1.2 Van Deemter curves for different particle sizes (10, 5, 3, 1.7 μm).



From the curves cited above, it is clear that the HETP is reduced with the smaller particle size which gives rise to higher column efficiency, which implies that, minimum values of HETP are only possible at much lower values of linear velocities, that signifies the term 3 in the equation-(2). It is also known from the curves cited above that there is no significant negative effect on the column efficiency when the mobile phase flow rate increases for smaller particles [**30**]. But the third term of

Eq. (2) indicates that the squared values of particle size (dp^2) results in higher steepness for larger particles at higher linear velocities. It implies that, in order to have acceptable run times, columns packed with regular particle sizes (10-3μm) are often performed at higher linear velocities which do not offer maximum efficiency.

Based on discussion mentioned above, maximum efficiency can be seen with reduced particles at a range of linear velocities. Thus the columns packed with smaller particles offer potential benefits of separation even at elevated linear velocities. But the major concern with the use of smaller particle packing column is higher back pressures (>5000 psi), which lie beyond the operating pressure of HPLC systems. Therefore, to gain the complete benefits of smaller particles, systems that can withstand higher operating pressures are required.

Since there is a need to increase the column efficiency, much research work has been carried out using non porous silica materials to provide the best mechanical strengths [**31-36**].

Though non-porous 1.5 μm particles are efficient enough and commercially available, poor sample loading capacity and lesser retentions of analytes due to low surface area have become the major issues in the use of these columns.

Hence the need for increasing the best chromatographic performance and improved column chemistries that are of considerable interest from equipment manufacturers in elevated pressure HPLCs and new

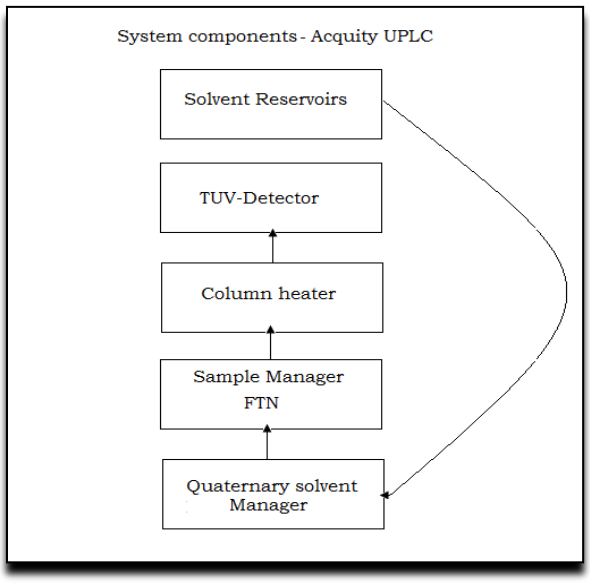
stationary phases of hybrid porous particle technology. In view of this, Waters Corporation has developed new equipment called UPLC and novel stationary phases of BEH technology to take advantage of improved chromatographic efficiency. These systems are now capable of generating column pressures up to 15000 psi. Thus significant improvements have taken place in the field of liquid chromatography to offer considerable benefits in regular analysis [**37-39**].

1.6.2.1 Instrumentation UPLC-H class

Typical UPLC-H class system key components include the following components [**40**].

1. Quaternary solvent manager
2. Sample Manager-Flow through needle
3. Column Heater
4. Detectors (Tunable UV/PDA/Fluorescent/ELSD/MS)

Fig. 1.3 Key components of Acquity UPLC, H-class system.



1.6.2.1.1 Quaternary solvent manager

Quaternary solvent manager (QSM) can pump the steady mobile phase flow rates up to 15000psi. It can also pump and degas four solvents simultaneously employing the gradient proportioning valve (GPC) thereby dynamically creating specified compositions.

1.6.2.1.2 Sample Manager-Flow through needle

The sample manager flow through needle (SM-FTN) uses a direct injection mechanism to inject the sample solutions from the vials onto the column. In-built sample loops permit up to 10 µL injection volume, whereas injection volumes greater than 10µL can be used by employing optional sample loops. Sample dilutions can be made using the auto dilutions option from the sample set method.

1.6.2.1.3 Column Heater

Variations in column temperature not only shifts the retention times but affects the peak shape of the analytes. The column heater provides consistent column temperature from 20°C to 90°C with ± 2°C temperature. The active column heater also heats the incoming mobile phase before it enters into the column. The in-built column oven compartment can accommodate the chromatographic columns up to 15 cm length. Column cooler is also available as optional with a different configuration that can cool the column up to 4°C.

1.6.2.1.4 Detectors

As smaller particle chemistries are used in UPLC, the resultant peaks (bands) are too narrow. The UPLC detectors are capable of collecting the data at faster rates. These detectors are specially made with reduced flow cell volumes and tubing volumes to control the band broadening.

Different types of detectors that can be used as individually or in combination are mentioned below.

a) UV visible/Photo diode array
b) Fluorescence
c) Evaporative light scattering detector
d) SQ or TQ mass detectors

The advancements in UV detector are discussed below.

A tunable ultraviolet/visible (TUV) detector assembled with new electronics (light guiding flow cell technology) and firmware to support Ethernet connections at the high data rates up to 80Hz resulting in low noise performance up to 6μAU is necessary for UPLC detection. Classical variable wavelength detectors (VWD) are concentration sensitive and make use of standard size flow cell, where as the tunable ultraviolet detectors (TUV) use the flow cell with reduced dimensions to maintain concentration and signal height. The use of smaller size classical flow cells may also reduce the path length but it leads to an increase in base line noise. So, if a classical HPLC flow cell is used, sensitivity would be compromised in UPLC. To avoid this problem, a new technology light

guided flow cell is used in Acquity TUV detector. Light guided flow cell offers efficient light transfer down to the flow cell.

1.7 Current challenges

1.7.1 Selectivity

Achieving the desired degree of separation is one of the prevalent problems encountered by the chromatographic researchers working in the field of pharmaceutical chemistry. Since a majority of the pharmaceutical compounds are synthesized through various steps, formation of structurally similar compounds, isomers and multiple number of impurities are most common in drug development. In addition to this, the separation of degradation impurities that are formed during the storage is also a general concern in developing stability indicating analytical methods. The quantification of such impurities in the finished products is of great importance in order to assure the safety and efficacy of the drug product. Development of analytical methods that demonstrates a greater degree of selectivity with reproducible separation is extremely important. But this becomes more complex while dealing with complex separation mixtures. The recent advances in analytical chemistry have some advantages to resolve such issues [**41**].

1.7.2 Sensitivity

Sensitivity of impurity to the detector up to the required level of concentration is of utmost importance, particularly while dealing with high dosage drugs. Impurities in dosage forms with higher maximum

daily doses must be controlled to the lowest extent. In addition, impurities with less chromophoric moieties in their chemical structure may also pose challenges while developing a chromatographic method [**42**].

The best possible approaches to enhance the sensitivity are,

1. The selection of a suitable detector
2. The selection of an appropriate detection wavelength
3. The selection of a suitable buffer and solvents that minimally absorb the UV light at selected wavelength.
4. Increasing sample concentration and sample load on the column.
5. Derivatization or alternative detection.

1.7.3 Complex separation

The separation of impurities is often intricate in the cases cited below:

1.7.3.1 Separation of Structurally similar compounds

The separation of impurities that shows similar retention behaviour under various chromatographic conditions is generally difficult. The reason for a similar retention behaviour is attributed to the similarities in the chemical structure. The chromatographic equilibrium constants for such impurities are the same or very close to each other.

Examples:

1. Compounds that differ in only one methyl group
2. Compounds that differ in only one double bond
3. Positional isomers

4. Geometrical isomers

5. Exo and endo isomers

6. Diastereomers

Separation of related compounds in Palonosetron hydrochloride represents the examples 2 and 6 whereas Zolpidem tartrate represents the example 3.

1.7.3.2 Separation of multi-component mixtures.

Multiple components in single mixture also pose many challenges in terms of separation and acceptable run time. This case becomes even more complex particularly while analyzing sample mixtures that contain components with a wide range of polarity.

Example:

Zolpidem tartarate and Darifenacin hydro bromide are the best examples.

1.7.3.3 Separation of enantiomeric compounds

Separation of chiral compounds has gained immense importance since many of the pharmaceutical compounds are chiral in nature. These isomers are proved to have same or different biological activity than the active compound. In this scenario, separation and quantification of chiral impurities in intermediates and subsequent finished stages are essential in order to understand the process capability and to control the isomer content in the final stage compound.

The separation of enatiomers and other chiral impurities is a difficult task because of their similarities in physical and chemical properties. The retention behaviour would be quite similar in some instances when chiral stationary phases are employed during the separation process. It becomes more challenging if more than one chiral center exists in the given molecule. It becomes even more complex while achieving the selectivity from penultimate stage chiral impurities. Though various chiral method development protocols have been developed, the use of chiral stationary phases with lower micron particles is not much reported in the literature. In order to separate the enantiomeric compounds, a minimum of three interaction differences are essential in the adsorption and partition equilibrium process of solutes with mobile and stationary phases. To achieve this, the first step is selection of suitable stationary phase bonded with appropriate chiral selector. In addition, to provide additional selectivity through greater surface area, the use of lower particle stationary phases are of great interest. Separation of palonosetron chiral impurities and its intermediate chiral impurities in single method are the best examples for this strategy.

1.7.4 Separation of degradation products

Development of stability indicating analytical methods is highly essential in order to effectively monitor the quality of the drug throughout the shelf life period. As discussed in the previous sections, pure drug compounds have a tendency to degrade over ageing process

because of the inherent chemical instability of the molecule. The separation of such degradation products may not be a significant problem but predicting such degradation products is quite important. Conducting forced degradation studies on the drug elucidate the degradation pathways of the molecule and also helps to understand the sensitivity and susceptibility of the drug towards the stress conditions applied. All the selected molecules are extensively studied in terms of degradation.

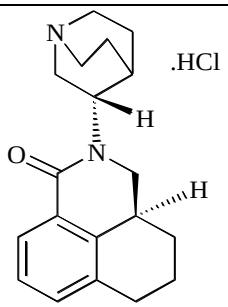
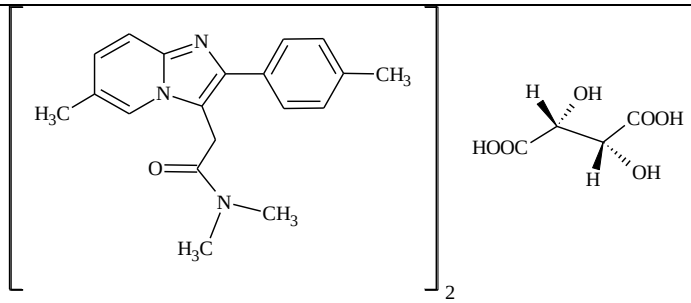
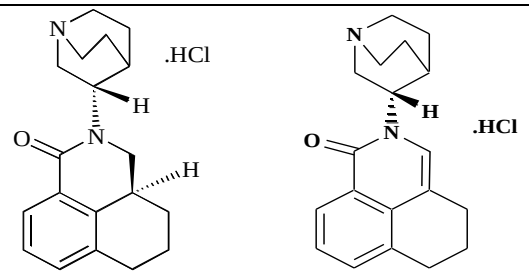
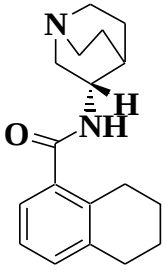
1.7.5 Time of analysis

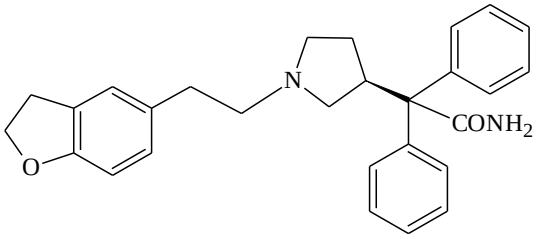
There is an increasing need for fast and ultra-fast methods with good efficiency and resolution for achieving separations in a few minutes [**43**]**.**

1.8 Objective and scope of the research work

The objective of the current research work is to develop new chromatographic methods for the molecules having complex separation mixtures. The present work involves in a systematic method development approach with new separation strategies. Molecules possessing the challenging separations are selected for the research work in order to prove the separation strategies (**Table.1.3**). The developed methods are completely validated as per the ICH guidelines. Experimental design concept is applied while optimizing the chromatographic conditions for some of the complex separation mixtures.

Table 1.3 Details of the drug substances and intermediates.

S.No	Structure & chemical names	Therapeutic Category
1.0	 (3aS)-2-[(S)-1-azabicyclo [2.2.2] oct-3-yl]-2, 3,3aS, 4, 5, 6-hexahydro-1H-benz [de] isoquinolin-1-one hydrochloride.	Antiemetic (Reduces the vomiting sensation during chemotherapy)
2.0	 N, N, 6-trimethyl-2-p-tolylimidazo [1, 2-a] pyridine-3-acetamide L-(+)-tartrate (2:1)	Anti insomnia (Reduces the sleep disorder)
3.0	 Compound-A Compound-B  Compound-C	Antiemetic (Reduces the vomiting sensation during themotherapy) Compound-B and Compound-C are intermediates of Compound-A

4.0	 (S)-2-[1-[2-(2, 3-Dihydro benzofuran-5-yl)ethyl]-3-pyrrolidinyl]-2, 2-diphenyl acetamide.	Antimuscarinic agent is used to treat urinary incontinence

A (3aS)-2-[(S)-1-Azabicyclo[2.2.2]oct-3-yl]- 2,3 ,3a,4,5,6,-hexahydro-1-oxo-1H-benz[de]isoquinoline hydrochloride.(PALO 3aS, 2S).

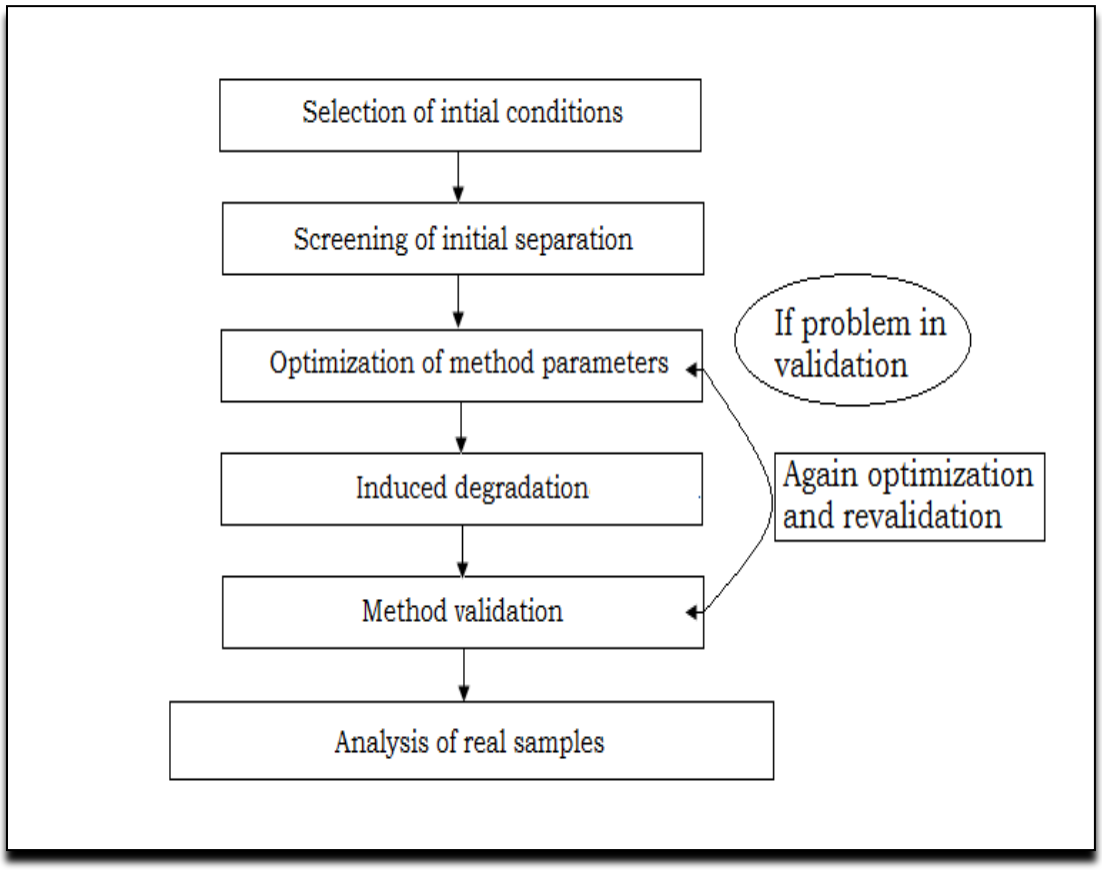
B 2-[(S)-1-azabicyclo[2.2.2]oct-3-yl]– 2,4,5,6,-tetrahydro-1H-benz[de]isoquinolin-1-one hydrochloride.

C N-[(S)-1-azabicyclo[2.2.2]oct-3-yl]-5,6,7,8-tetrahydro-1- naphthalenecarbox-amide.

1.9 Systematic method development approach

Development of a chromatographic method is often intricate and time consuming. The conventional trial and error approach typically involves choosing different solvents, their combinations, pH, buffer and column as an initial point followed by subsequent changes in the mobile phase composition until a satisfactory method is achieved. This approach is tedious and often does not arrive at the best method. A more systematic approach utilizes good experimental design and the work flow described below can be used to develop high quality RPLC methods efficiently and effectively [**44-45**].

Fig. 1.4 Schematic diagram of systematic method development



This method development work flow includes method scouting where selectivity factors such as buffer pH, organic modifier and column chemistry are evaluated to determine which experimental parameters are most effective in altering the selectivity to achieve the resolutions.

The final method may be validated to ascertain whether it meets the requirements for its intended use or not. The validation data should be summarized and evaluated against predetermined acceptance criteria. Any failures must be discussed and re-optimization followed by re-validation must be carried out based on the specific requirements.

1.9.1 Literature collection

Extensive literature search must be carried out before initiating the method development activity. The large part of literature collection towards this objective should include the physio-chemical properties, stability and degradation pathways of the molecule. This information is quite useful in the various stages of method development. It also helps to understand the molecule's intrinsic behavior from the stand point of stability. Survey of existing analytical methodologies and their applications is also imperative to evaluate further developments that need to be carried out for the projected research work.

1.9.2 Sources of literature

Pharmacopeia

- USP, EP, JP, BP and IP

Books

- Text books pertaining to analysis for Pharmaceutical compounds

Journals

- Chromatography-A, B, JPBA, Analytical chemistry,

  Chromatographia etc

Patents

- Innovators base (product) patent
- Information pertaining to analytical methods and synthesis.

To design a sound method development experiment, it is important to collect as much information about the chemical nature of the sample and analytes as possible.

1.9.3 Information required for the method development

- Sample solubility

  Whether compound is soluble in aqueous or non-aqueous media?

- Number of analytes

  How many components need to be separated?

- Chemical structures

  Information about possible impurities, structural isomers and degradation products.

- Functional groups

  Information about type of functional group (-COOH,-NH2,-CHO,-C=O etc)

- How the analytes are different?

  Is the sample regular or complex?

- Are they ionizible compounds?

  Acidic or basic or neutral?

- Acid dissociation constants

  pKa

- Partition coefficients

  Log P

1.9.4 Intended use of chromatographic method

Another consideration before developing a method is to define the goal which should be in consonance with the intended purpose of the method. A clear understanding of the intended use will help to define the performance parameters that must be considered in the method development and validation. For example, parameters mentioned below.

- Targeted Resolution
- Targeted Tailing factor
- Targeted Runtime

1.9.5 Selection of starting conditions

1.9.5.1 Detection

Selection of detection wavelength is an important parameter that significantly affects the method sensitivity. Thus the following points must be taken into consideration in selecting the wavelength.

- What type of detection is required or possible?
- Are the analytes UV-absorbing or not?
- What is the absorption maximum of analytes?
- What sensitivity levels of analytes are required?
- Would the placebo interfere with separation or detection?

1.9.5.2 Buffer

Addition of buffer to the mobile phase is strongly recommended while analyzing the ionic compounds. Buffer solutions provide constant ionic strength and controlled solution pH to the mobile phase that gives

consistent retention times of analyte peaks. The choice of buffer depends on the following characteristics.

1) Pka of the buffer

2) Buffer UV absorbance

3) Buffer solubility and stability

As the buffer ionization occurs at pKa±1.5, it, therefore, controls the mobile phase pH at pKa±1.0. This facilitates consistent retention times for the analyte peaks. In addition, the buffer should minimally absorb the UV light at the selected wavelength to minimize the background noise. The following table is quite useful in selecting the buffer.

Table 1.4 Details of various buffers used in RP-HPLC

S.No	Buffer	Pka	Buffer range	UV-cut off
1.	Trifluoro acetic acid	2.0	1.5 to 2.5	210nm
2.	phosphate[a]	2.1,7.2&12.3	6.2 to 8.4	<200nm
3.	Citrate[b]	3.1,4.4&5.4	2.1 to 6.4	230nm
4.	Formate[c]	3.8	2.8 to 4.8	210nm
5.	Acetate[d]	4.8	3.8 to 5.8	210nm
6.	Triethyl amines	11.0	10.0 to 12.0	<200nm

[a] salts of sodium or potassium

[b] salts of sodium or potassium

[c,d] salts of ammonium or potassium

1.9.6 Method optimization

In order to optimize the separation, many parameters have to be modified during the course of method development. The key parameters are listed below.

1.9.6.1 Selectivity

1.9.6.1.1 Buffer pH

Retention times of neutral molecules are not affected by buffer pH whereas retention times of ionic molecules are significantly affected at its pKa value. Therefore, acid dissociation constant (pKa value) of solutes is more important while fixing the mobile phase pH. It is a most effective way to alter the selectivity of the chromatographic method for ionic samples.

Acidic and basic analytes undergo complete ionization wherein the mobile phase pH and analyte pka are the same and they become much less hydrophobic. As a result, its retention time decreases significantly in reversed phase chromatography. When mobile phase pH is increased, acids lose a proton and thereby become ionized resulting in decreased retention time. In contrast, when mobile phase pH is decreased, bases gain a proton thereby becoming ionized resulting in decreased retention time. The typical retention behaviour of acid and bases with respect to mobile phase pH is graphically depicted below (**Fig. 1.5 to Fig. 1.6**).

Fig. 1.5 Typical retention behaviour of acidic compounds

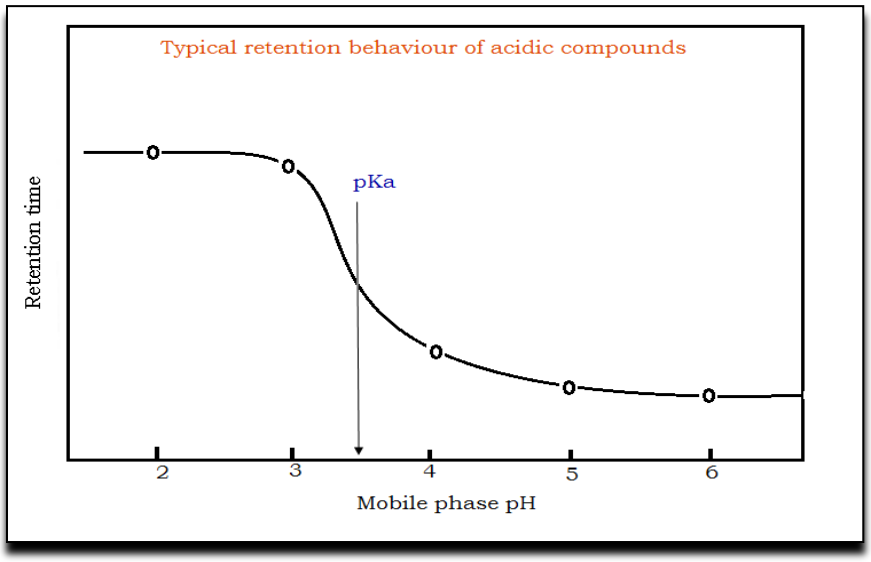
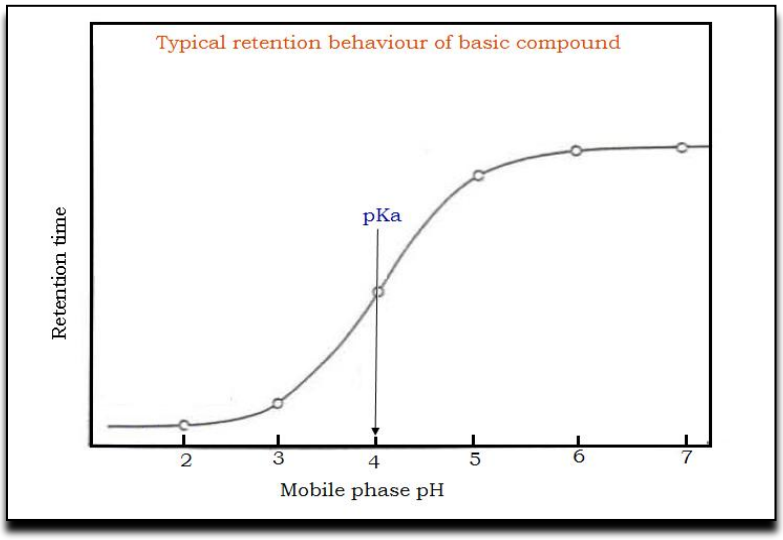
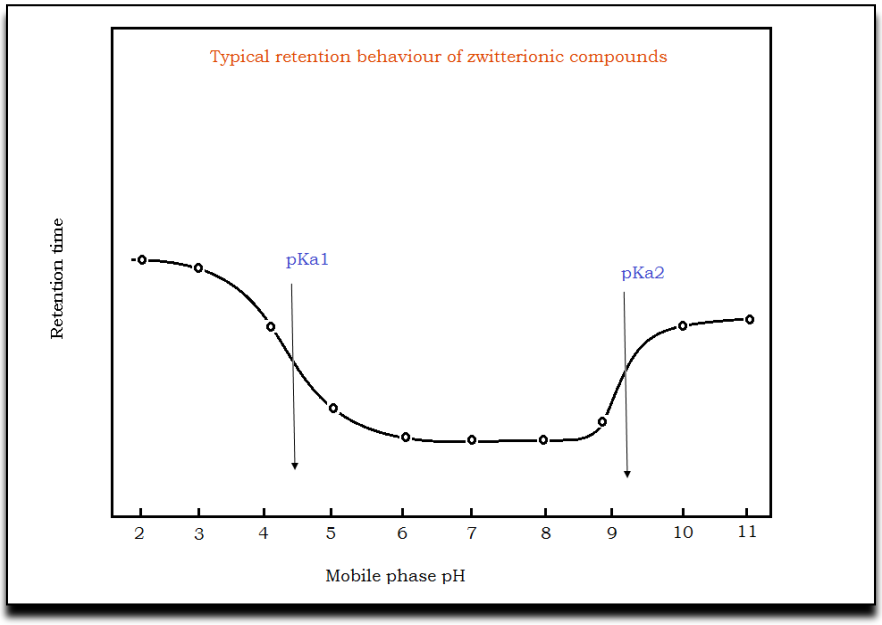


Fig. 1.6 Typical retention behaviour of basic compounds



Compounds having both acidic and basic functional groups in the structure exhibit amphoteric retention behavior. For instance, amino acids contain acidic (-COOH) group and basic (-NH_2) group in the same structure that shows zwitter ionic nature. The typical retention behaviour is shown in the following figure.

Fig. 1.7 Typical retention behaviour of zwitter ionic compound



When pH = pKa for a compound, then the concentration of ionized form and unionized form are equal. Near to the ionization point, the retentions are drastically changed with a slight variation of mobile phase pH. This effect will be active within the range pKa ±1.5 units. In order to obtain reproducible retention times, the mobile phase pH should be ± 1.5 units of the analyte pKa.

1.9.6.1.2 % Organic ratio

Altering the organic ratio in the mobile phase composition permits a significant control over both retention and band spacing. Concurrent changes in the percent organic ratio are required to alter selectivity while achieving the initial separation. Further, slight variations in percent

organic ratio often give rise to useful changes in the optimization stage.

1.9.6.1.3 Gradient steepness

Gradient elution mode is an alternative to isocratic mode of elution in order to achieve resolutions within acceptable runtimes. The steps involved in the optimization of a gradient program are outlined below [**46**].

1. Evaluation of initial and final percent of organic solvent.
2. Adjustment of gradient time & range to optimize the resolution.
3. Increase in gradient slope may improve resolution.
4. Decrease in gradient slope may reduce the sensitivity.

1.9.6.1.4 Column Temperature

Temperature variation often affects the chromatographic equilibrium process. If column temperature increases, mobile phase viscosity decreases. Consequently, it lowers column back pressures. In addition, temperature changes will alter the rate of analyte partition between the stationary phase and mobile phase thereby varying the analyte diffusivity. This affects the chromatographic efficiency in term 2 and term 3 of the equation (2) in section 1.6.2, which leads to changes in selectivity and retention behavior of the analytes [**47-48**].

1.9.6.1.5 Solvent type

The selection of solvent depends on the strength and UV absorbance of the solvent. The elution strength of the solvents that are used in reversed phase chromatography are Tetrahydrofuran > Acetonitrile >

Methanol. Generally, acetonitrile is the most preferred solvent in reversed phase chromatography because of its moderate eluting power and lower UV absorbance at 200nm to 400nm (UV cut off <200nm). The second alternative organic solvent is methanol. But relatively less eluting strength and significant UV absorbance up to 210nm make this solvent the second preferred one. However, a combination of these two solvents sometimes offers the best selectivity for certain analytes. Tetrahydrofuran (THF) is the next preferred solvent and usually the less desirable one because of its significant UV absorbance up to 250 nm. The chemical stability and more column equilibration times are the most common problems associated with this solvent. Despite these problems, tetrahydrofuran is useful due to its unique selectivity.

1.9.6.1.6 Stationary phase type

The selectivity of LC stationary phases lies in their physical and chemical properties. The chemical properties such as bonding chemistry, type of ligand, and silanol activity affect the retention and selectivity. On the other hand, the physical characters such as particle size, particle shape, specific surface area and pore size significantly influence the column efficiency.

1.9.6.1.6.1 Chemical properties

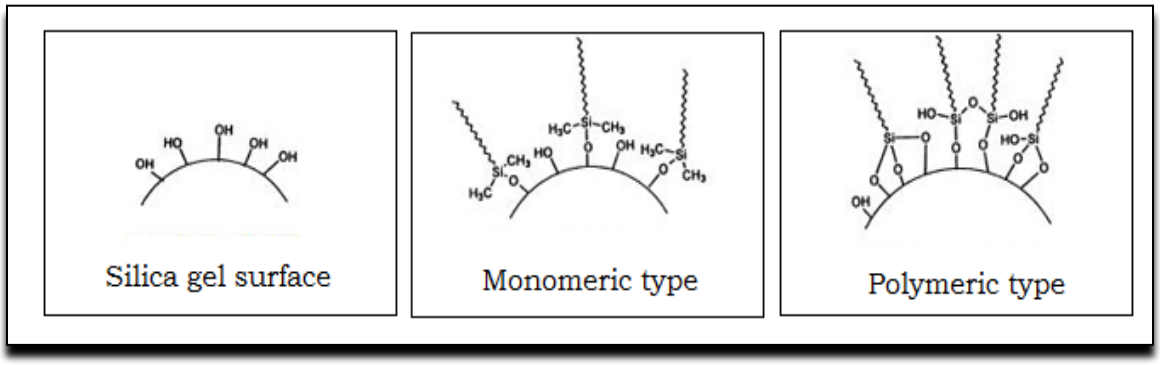
In bonded type stationary phases, silica-based stationary phases are most widely used in the chromatography. Silica-based reversed-phase

stationary phases are covalently bonded organosilanes or polymeric-coated organic layers over the silica surface [**49**].

The most frequently used method entails the reaction of monofuncticnal chlorodimethylsilanes with silanol functional groups. A variety of alkyl and substituted alkyl stationary phases on silica support are synthesized by this reaction. For example, n-octyl (C8) and n-octadecyl (C18) stationary phases. Stationary phases synthesized by this process often demonstrate the maximum efficiency due to the fast diffusion. But all the silanol groups present on the surface may not react with organosilanes owing to the steric factors. Moreover, the degree of reaction pertaining to silanol groups decreases if the length of the carbon chain increases. As a result, unreacted silanol group present in the stationary phase causes secondary interactions. So, various procedures are developed to reduce the residual silanol effects such as end capping.

While selecting a suitable stationary phase, it is important to understand the differences in the base particles that can provide various degrees of selectivity and retention times based on their chemical and physical properties [**50**].

Fig. 1.8 Chemical structures of bare silica, monomeric and polymeric coated stationary phases.

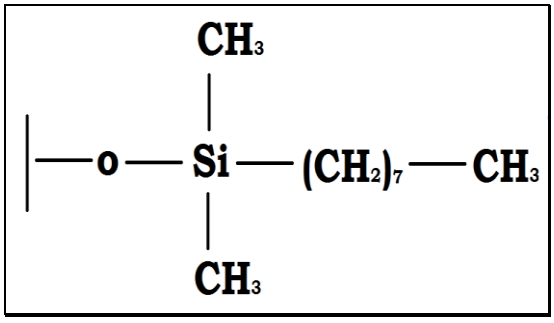


Secondary interactions can take place based on the number of residual silanol groups on the particle surface as well as the effectiveness of end-capping procedure. Additionally, differences in chemically-bonded stationary phases can offer significant variations in selectivity and retention times.

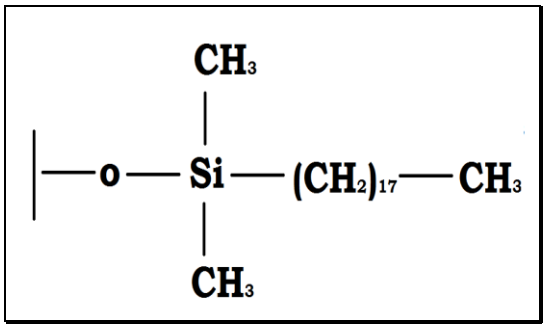
Fig. 1.9 Different types of stationary phases and chemical structures

Regular stationary phases

C8-Octyl

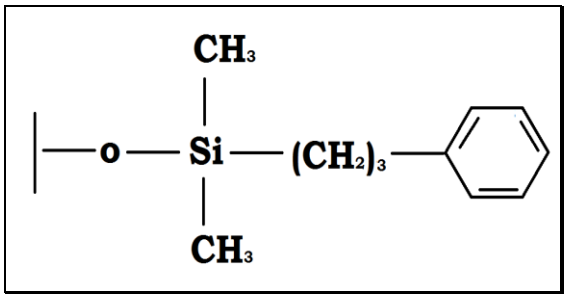


C18-Octadecyl

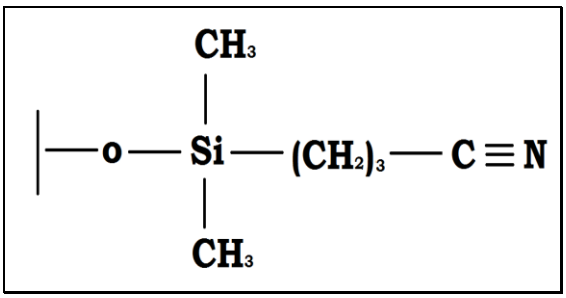


Regular stationary phases

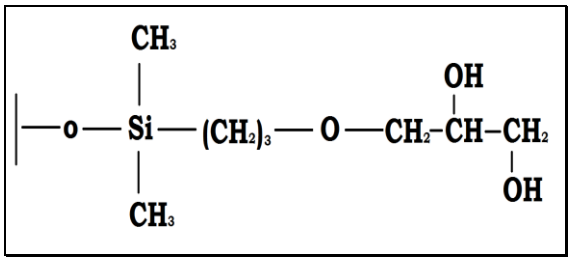
Phenyl



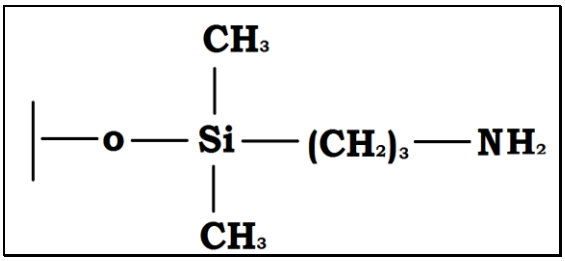
Cyano



Diol

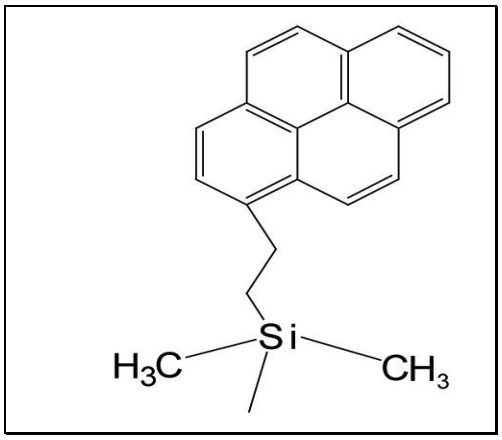


Amino

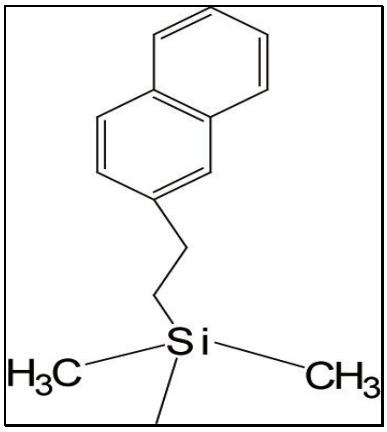


New stationary phases

Pyrenyl propyl phases

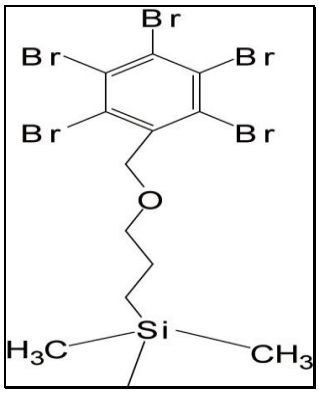


Naphthalethyl stationary phases

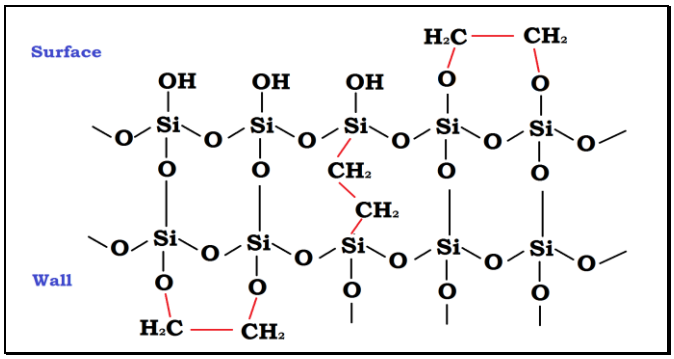


Pentabromobenzyl

Stationary Phases



HILIC Stationary Phases



Column screening through various mobile phase pHs and different solvent types provide the best column information that can offer comparably good separation. The differences in column chemistry are responsible for the changes that occur in retention time and selectivity. The key attributes of column chemistry are discussed below.

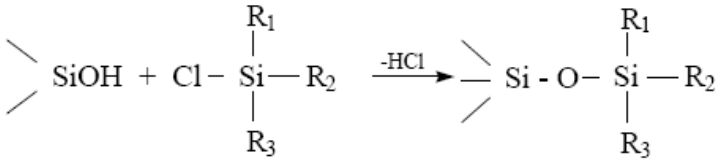
1.9.6.1.6.1.1 Hydrophobic nature

The degree of hydrophobic interactions for appropriate solutes increase with the increase of percent carbon load in organosilane bonded stationary phase resulting in increased retentions. The retention mechanism entails the partition of solute molecules between polar mobile phase and non-polar stationary phase (C8 or C18). In reversed phase chromatography, hydrophobic compounds retain more strongly whereas hydrophilic compounds retain less strongly. Compounds having intermediate polarity will retain moderately.

The greater the chain length (C18>C8>C4) the higher the solute retention times in bonded phases. But there is no significant difference between longer-chain packing columns such as C18 and C8 phases. Sample retention can be controlled to a significant extent by the selection of bonded phase type. The length of the packed columns also plays a major role in enhancing the selectivity and resolution. Various procedures are established to determine the hydrophobicity of the stationary phases [**51**]. Hydrophobicity of the stationary phase can be increased by adding ion pair reagent in the mobile phase. The ion pair is attracted to the stationary phase because of its hydrophobic alkyl group and the charge carried by the reagent. Thus the retention of the sample increases by way of ion exchange process and hydrophobic interactions.

1.9.6.1.6.1.2 Completeness of bonding and residual silanol activity

Completeness of bonding depends on the extent of the reaction cited below.



Owing to the steric factors, only fifty percent of the silanol groups can be bonded out of the originally available silanol groups from the bare silica. The reported values of bonded ligand densities are ~4μmol/m^2 and the unreacted silanol groups are ~4μmol/m^2 in RP stationary phases.

These unreacted silanol groups significantly affect retention and selectivity in reversed phase chromatography, particularly for polar and ionic compounds. Depending on the solute activity and the mobile phase pH, the residual silanol groups affect the retention behavior by means of hydrogen bonding, dipole interactions and ion exchange processes. These secondary interactions are generally undesired in reversed phase chromatography since they result in bad peak shape and inconsistent retention times for ionic compounds. Thus, along with the bonded ligands these un-reacted silanol groups largely influence the overall chromatographic characteristics of the reversed phase stationary phases.

1.9.6.1.6.1.3 End-capping

End-capping is the process of replacing residual silanol groups with trimethyl silyl groups. It minimizes the electro-static interactions between unreacted silanols and analytes in bonded stationary phases.

End capping reaction:

Ionic compounds, particularly basic compounds can interact with the residual silanols, which leads to increased retention, band broadening and tailing. It is generally desirable to minimize the resultant peak tailing in order to get reproducible peak areas. These acidic silanol interactions can be reduced by selecting a suitable end capped column.

1.9.6.1.6.2 Physical properties

1.9.6.1.6.2.1 Particle size and surface area

As the column efficiency is dependent on the particle size from the equation cited below, it is important to understand the influence of particle size on peak resolutions.

$$N = 3500\frac{L}{d_p}$$

Where,

L is the length of the column

d_p is the internal diameter of the particle

The particle size has a significant influence on peak separations. The lower the particle size, the higher the relative surface area per unit weight thereby facilitating more surfaces that offer more interactions with solute molecules resulting in better separation. On the other hand, smaller particles obstruct the mobile phase flow in the column resulting in higher back pressures. Thus, in order to obtain the separations at reasonably acceptable run times, particle size up to 3.0 μm only can be used in HPLC because of the pressure (up to 5000 psi) limitation. In order to avail the best column efficiencies of lower particle sizes (less than 2 μm) a better system (UPLC) that can withstand the high pressures (up to 15000 psi) is required. Particle diameter 5μm is usually considered in the initial stage of method development process. These particles provide moderately large values of chromatographic efficiencies for a

majority of the separations. Small columns of 3µm particles are useful to achieve fast separations.

1.9.6.1.6.2 .2 Particle shape

Size of the particles is usually measured employing methods that presume all the particles are spherical in shape. But it may not be the real case in the manufacture of silica or silica based bonded stationary phases. Particles with irregular shape may also exist up to a certain extent. The effect of particle shape on chromatography is discussed below.

1.9.6.1.6.2 .3 Irregular particles

Initially, column packings are made with irregular particle shapes. But their inherent problems have resulted in a shift to the use of homogeneous spherical particles in new generation stationary phases.

Fig. 1.10 Shape of the irregular particles:

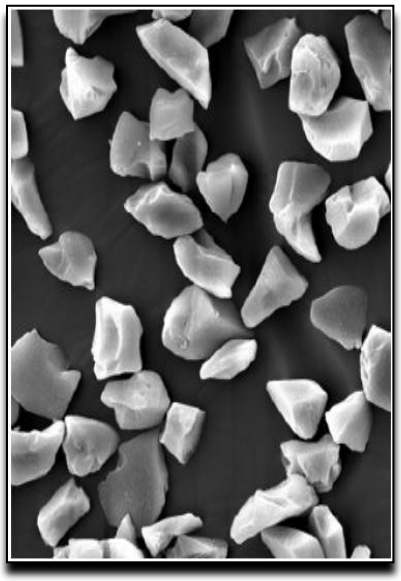
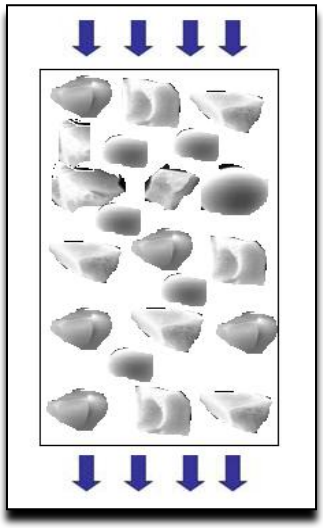


Fig. 1.11 Column packed with irregular particles that lack mechanical strength:



1.9.6.1.6.2 .4 Regular particles

In this case, the shape of the silica particles is completely spherical thereby enabling high column performance and low column back pressures. The major advantages of spherical particles are better column packing reproducibility and higher mechanical stability. These attributes offer reproducible separation when different lots of columns are used.

Fig. 1.12 Column packed with non uniform spherical particles

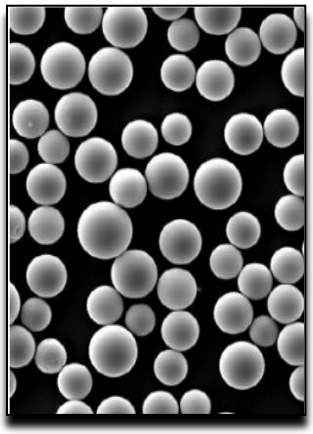
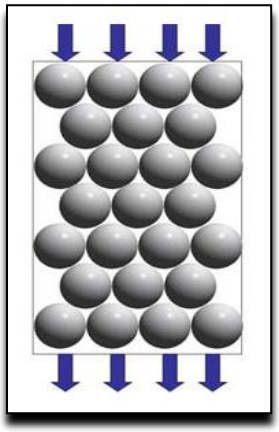


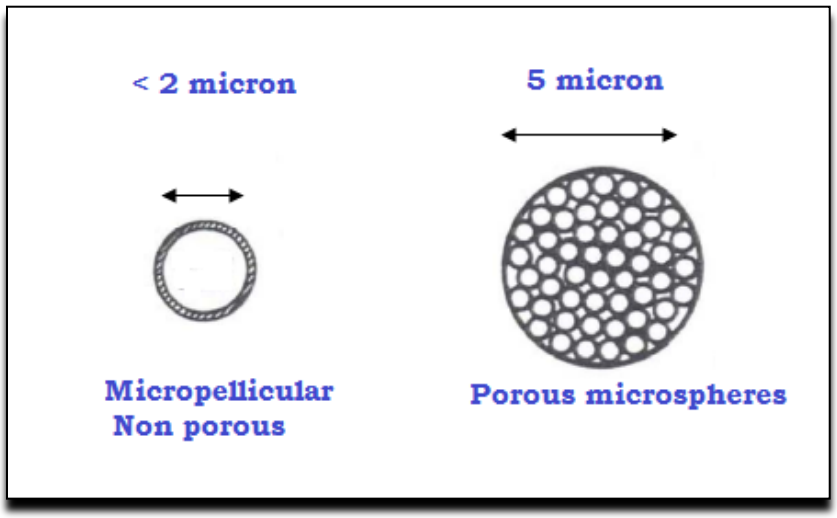
Fig. 1.13 Column packed with uniform spherical particles that offer consistent results:



1.9.6.1.6.2 .5 Pore size

Porosity and pore size are of great importance since these attributes determine the surface area. Porous silica particles have numerous advantages over non porous silica particles in terms of improved selectivity and higher sample load.

Fig. 1.14 Pictorial representation of porous and non porous particles



Particles with higher pore size (>300 Å) have lesser surface area about 100 m^2/g, compared with those containing 100 Å pores (ca. 170 m^2/g). Non porous materials have extremely (~20 folds) lower surface area than porous materials. Nevertheless, these particles have specific advantages as mentioned below.

- Exhibits fast mass transfer and shorter retentivity of the analytes.
- Packing with smaller particles has minimal void volumes which decreases the consumption of solvents.
- Eliminates the size exclusion effects.

Pore size less than 180 is generally recommended for the analysis of molecules having molecular weight 2000Da, whereas analytes having higher molecular weight (>2000Da) can be analyzed by using column packings with 300 Å or higher.

1.9.7 Computerized method optimization tools

A systematic approach with computerized method optimization is also one of the useful techniques that encompasses various advantages. DryLab® chromatography modeling software is one of such computer simulation tools that can be used to improve the efficiency and quality of method development efforts, saving time and money. It also helps understand the chromatographic process in a more efficient manner.

DryLab models chromatography variables in short a time and evaluates a much wider range of experimental conditions than would

ever be practical in the laboratory. It helps to assess the effects of pH, temperature and buffer concentration more quickly [**52-53**].

DryLab software includes LC modeling options for gradient conditions, isocratic %B, pH, ionic strength, additive/buffer concentration, normal phase, temperature, reversed-phase gradient /temperature etc.

1.9.8 Quality by Design (QbD) Approach

Chromatographic method development is often more complicated due to the presence of more impurities that trigger peak overlapping. Methods are generally developed by sequentially changing one variable at a time (OVAT) until a suitable method is achieved. The OVAT approach may produce an adequate method but offers limited understanding of method capabilities and method robustness [**54**]. In contrast, Quality by Design (QbD) is a systematic approach to the method development that demonstrates multidimensional combination, interaction of input variables and parameters using design of experiments (DOE) to obtain optimal conditions with the assurance of quality as defined in ICH-Q8 [**55**]. Furthermore, recent revisions in FDA's guidance suggested that the organizations should carry out design space studies to anticipate problematic parameters that negatively affect the quality. The agency wants these parameters to be alleviated and monitored with analytical testing by means of a statistical approach. Generally, robustness testing is carried out as per ICH at the end of method validation to assess the

effect of individual factor. This strategy is always difficult to predict the probable interactions between the factors. DOE, instead, creates design space (DS) where the robustness assessment is concomitantly performed during the optimization stage [**56**-**57**].

Three active pharmaceutical ingredients (API), their dosage forms and two key intermediates (chiral isomers) that involve separation of complex mixtures are selected for the present research work to develop new analytical methods for the determination of related compounds and degradation products. An extensive literature survey divulged that no stability indicating analytical methods were available for determining the impurities in the selected products (Table 1.3). Simple, selective and cost effective stability indicating analytical methods were developed by means of HPLC/UPLC. During this course of development, new stationary phases were employed. Additionally, computer method optimization strategy using computer simulated software such as DryLab® was also used. Furthermore, Quality by Design strategy was applied using Design Expert software to accomplish optimum separation between critical impurities.

1.10 Analytical method validation

Analytical method transfer is indirectly demonstrated by means of systematic method validation procedure. The objective of method validation procedure is to verify the method and to evaluate the allowable limits of variability in chromatographic conditions. It is necessary to have

a well defined method validation protocol that includes validation plan and acceptance criteria before starting the method validation process. ICH Q2 and USP general chapter on method validation procedure established the guidance on method validation activity [**58-60**].

A standard approach for the method validation is to identify the important parameters and carryout the required experiments for each parameter that provides useful information about the method capabilities. Subsequent method modifications and revalidations are allowed based on the requirement. The key parameters that need to carry out in the method validation activity includes specificity, accuracy, Precision, linearity range, limit of detection, limit of quantization, ruggedness, robustness [**61-64**].

1.10.1 Specificity

Specificity is defined as the ability to assess unequivocally the analyte in presence of components that may be expected to be present (ICH Q2A). In addition, ICH Q1A requires stability indicating analytical method to monitor the degradation impurities that are formed during the storage [**65**].

In pharmaceutical compounds the following are the possible components.

1. Impurities from synthetic process.
2. Degradation impurities.
3. Sample matrix components (Placebo).

The developed method for each analyte should be free of interference from other components. It can be proved in terms of resolution (Rs), selectivity (α) and peak homogeneity (Peak purity).

1.10.2 Accuracy

Accuracy of the analytical method is described as the degree of closeness of the experimental value to the true value. In a majority of the cases, the accuracy is determined by means of standard addition and recovery method and comparison with reference standard method.

In standard addition and recovery processes, the known quantities of analytes are spiked at various concentration levels to the sample matrix and then assay determinations are carried out. The actual concentration of the analyte in the unspiked sample would be analyzed simultaneously after which the recoveries are calculated mathematically using the below formula

$$\%\text{Recovery} = \frac{\text{Amount recovered x 100}}{\text{Amount added}}$$

1.10.3 Precision

Precision of an analytical method can be defined as "the degree of consistency among individual measurements when the method is applied repeatedly to multiple samplings of a homogeneous sample" [ICH Q2A].

An extensive definition drafted by the ICH Q2A categorizes the precision into three types:

1) Repeatability

2) Intermediate precision

3) Reproducibility

Repeatability is the precision of an analytical method under the same experimental conditions over short time intervals. Intermediate precision is the closeness of complete determinations when the method is applied several times within the same laboratory. For example, the variation should involve different days, equipments and chemists but multiple preparation of standards and samples. Reproducibility evaluates the inter laboratory precision, for example collaborative studies. The results are statistically represented in terms of %relative standard deviation or coefficient of variation or confidence intervals.

1.10.4 Linearity

Linearity of an analytical method is defined as a measure of how best a calibration plot of response vs. concentration approximates a straight line. Linearity can be evaluated by performing single determination at various concentration levels of the analyte. The obtained responses are then plotted against corresponding concentrations. The data are then treated statistically to determine the coefficient of determination (R^2) and correlation coefficient(r).

The numerical value of the slope and intercept will depend on the responses observed. The intercepts greater than 2% (relative to the 100% level response) are generally accepted for major component analysis. A linearity correlation coefficient(r) above 0.999 and coefficient of

determination (R^2) above 0.998 is acceptable for a majority of methods, especially for major components in assay methods.

1.10.5 Range

The range of the analytical method is defined as the lower and upper concentrations of the analyte where the accuracy, precision, and linearity lies within the acceptance criteria.

1.10.6 Sensitivity

Sensitivity is one of the important characteristics of an analytical method that determines the limit of detection (LOD) and limit of quantification (LOQ). The limit of detection (LOD) can be defined as the lowest amount of solute that provides a minimum detectable response.

The LOD is determined mostly based on a signal-to-noise (S/N) approach that should be typically in-between 2 and 3. The limit of quantification (LOQ) can be defined as the lowest amount of solute that gives a response which can be quantified with an acceptable degree of precision and accuracy. LOQ can be set at some arbitrarily defined level and should be typically in-between "S/N" ratio of 9.5 and 10.4.

1.10.7 Ruggedness

Ruggedness of analytical method is defined as the reproducibility of test results when the method is performed under the same experimental conditions. This includes different chemists, columns, laboratories, instruments, sources of chemicals and reagents. Ruggedness of analytical method may not be known when a method is initially

developed, but further it can be obtained during the subsequent use of the analytical method or can be determined in method validation part.

1.10.8 Robustness

ICH defines method robustness as a measure of its capacity to remain unaffected by small but deliberate variations in method parameters. A general approach is to systematically vary critical parameters in the chromatographic method and measure the degree of separation by means of system suitability.

Two approaches can be followed to determine the method robustness.

1) One Variable at a time (OVAT) approach.
2) Multi variate approach.

The latter approach has additional advantages of understanding the inherent capabilities of the method and achieving optimum method conditions.

1.10.9 Solution stability and Mobile phase stability

In order to obtain accurate and consistent results, standard solution, sample solution and mobile phase solutions must be certainly stable at least during the analysis time. For instance, the analysis of even one sample may require minimum chromatographic runs to ensure the system suitability, including standard solutions and resolution solutions. Thus, the observation minimum hours of solution stability is necessary for normal chromatographic run times. While analyzing more number of samples, overnight analysis is performed for better throughput. Such

practices need additional requirements for greater solution stability. Solution stability can be generally established up to 5 days by assaying stored solution against freshly prepared standard solution.

References

[1] Impurities in New Drug Substances, ICH Harmonised Tripartite Guideline Q3B (R2), Current Step 4 version, 2006, 6.

[2] Satinder Ahuja, Hand book of Isolation and characterization of impurities in pharmaceuticals, 2005, 8.

[3] Impurities in New Drug Substances, ICH Harmonised Tripartite Guideline Q3A (R2), Current Step 4 version, 2008,2.

[4] Arup K. Basak, Andre S. Raw, Ali H. Al Hakim, Scott Furness, Nashed I. Samaan, Devinder S. Gill, Hasmukh B. Patel, Roslyn F, Powers, Lawrence Yu, Pharmaceutical impurities: Regulatory perspective for Abbreviated New Drug Applications, Adv. Drug Delivery Rev, 59, 2007, 64-72.

[5] Radhika rajagopalan, Review of regulatory guidance on impurities, Separation science and technology, 5, 2004, 27-37.

[6] Sanjay B. Bari, Bharati R. Kadam, Yogini S. Jaiswal, Atul A. Shirkhedkar, Impurity profile: Significance in Active Pharmaceutical Ingredient, Eurasian J. Anal. Chem, 2, 2007, 32-53.

[7] Habilitationsschrift, Optimal Synthesis and Design of Advanced Chromatographic Process Concepts, chapter-1, 1.

[8] Ahuja S, Chiral Separations by Liquid Chromatography. Washington, DC, USA: American Chemical Society; 1991.

[9] Zief M, Crane L, editors. Chromatographic Chlral Separations. New York, USA: Marcel Dekker; 1987.

[10] Allenmark SG. Chromatographic Enantioseparation: Methods and Application. Chichester, West Sussex, UK: Ellis Horwood; 1989.

[11] Souter RW. Chromatographic Separation of Stereoisomers. Boca Raton, Florida, USA: CRC Press; 1985.

[12] Krstulovic AM, Chiral Separations by HPLC: Application to Pharmaceutical Compounds. Chichester, West Sussex, UK: Ellis Horwood; 1989.

[13] Sandor Gorog, The changing face of pharmaceutical analysis, *TrAC-Trend. Anal. Chem,* 26, 2007, 12-17.

[14] Görög S. Ultraviolet-Visible Spectrophotometry in Pharmaceuti-cal Analysis. Boca Raton, Florida, USA: CRC Press; 1995.

[15] Korfmacher WA, editor. Using Mass Spectrometry for Drug Metabolism Studies. Boca Raton,Florida, USA: CRC Press; 2005.

[16] Strasbourg. 6th ed. France: Council of Europe; 2008. European Pharmaceopoeia.

[17] Rossi DT, Sinz MW, editors. Mass Spectrometry in Drug Discovery. New York, USA: Marcel Dekker; 2002.

[18] S.Ahuja, N.Jespersen, Modern Instrumental Analysis, 47, 2006,

506-509.

[19] Dong, M. W, Modern HPLC for Practicing Scientists, 2006, 87-95.

[20] Jiri Sevcik, Detectors in Gas Chromatography, American publishing Elsevier Inc, 1975, 15-187.

[21] Colin Poole, Gas Chromatography, Elsevier Inc, 2012, 137-156.

[22] Sam Fong Yau Li, Capillary Electrophoresis: Principles, Practice, and Applications, Elsevier science publisher B.V, 1992, 1-11.

[23] Lunn G, Capillary Electrophoresis Methods for Pharmaceutical Analysis,New York, USA: Wiley; 1999.

[24] Erich Heftmann, Fundamentals and Techniques, Part 1, Elsevier science publisher B.V ,1992, A374-A420.

[25] Gorog S, Identification and Determination of Impurities in Drugs, Elsevier science publisher B.V, 2000. 4-5.

[26] Baertschi SW, Pharmaceutical Stress Testing: Predicting Drug Degradation, New York, USA, Taylor and Francis, 2005.

[27] Smith R, Webb M. Analysis of Drug Impurities, Oxford, Blackwell, 2007.

[28] Xu Q, Trissel L. Stability-indicating HPLC Methods for Drug Analysis. London, UK, Pharmaceutical Press, 2003.

[29] J.J. Van Deemter, F.J. Zuiderweg, A. Klingengerg,Longitudinal diffusion and resistance to mass transfer as causes of nonideality in chromatography, J. Chem. Eng.Sci. 5 (1956) 272.

[30] C.E. Meloan, Chemical separation principles, in: Techniques and Experiments, John Wiley & Sons, Canada, 1999.

[31] J.E. MacNair, K.D. Patel, J.W. Jorgenson, Anal. Chem, 71,1999, 700–708.

[32] N. Wu, J.A. Lippert, M.L. Lee, J. Chromatogr. A, 911 ,2001, 1–12

[33] J.E. MacNair, K.C. Lewis, J.W. Jorgenson, Anal. Chem, 69, 1997, 983–989.

[34] K. Lan, J.W. Jorgenson, Anal. Chem, 71, 1999, 709–714.

[35] Y. Xiang, B. Yan, C.V. McNeff, P.W. Carr, M.L. Lee, J. Chromatogr. A 1002 , 2003, 71–78.

[36] J.A. Lippert, B. Xin, N. Wu, M.L. Lee, J. Microcolumn Sep. 11,1997, 631–643.

[37] Lucie Novakova, Ludmila Matysova, Petr Solich, Advantages of application of UPLC in pharmaceutical analysis, Talanta, 68, 2006, 908–918.

[38] High throughput liquid chromatography with sub-2_m particles at high pressure and high temperature, Dao T.-T. Nguyen, Davy Guillarme, Sabine Heinisch, Marie-Pierre Barrioulet ,Jean-Louis Rocca, Serge Rudaz , Jean-Luc Veuthey, J. Chromatogr. A, 1167,2007, 76–84.

[39] Stephen A.C. Wren, Pierre Tchelitcheff, Use of ultra-performance liquid chromatography in pharmaceutical development, J. Chromatogr. A, 1119, 2006, 140–146.

[40] Acquity UPLC-H Class system guide, revision B, Waters corporation, 2010.

[41] Bernard A. Olsen,Bryan C. Castle, David P. Myers, Advances in HPLC technology for the determination of drug impurities, Volume 25, Issue 8, September 2006, Pages 796–805.

[42] W.B. Crummett, H.J. Cortes, T.G. Fawcett, G.J. Kallos, S.J. Martin, C.L. Putzig, J.C. Tou, V.T. Turkelson, L. Yurga, D. Zakett, Some industrial developments and applications of multidimensional techniques, Talanta 36, Issues 1–2, 1989, 63–87.

[43] Davy Guillarme, Dao T.T. Nguyen, Serge Rudaz, ean-Luc Veuthey. Method transfer for fast liquid chromatography in pharmaceutical analysis: Application to short columns packed with small particle. Part II: Gradient experiments, *Eur. J. Pharm. Biopharm,* 66, 2007, 475-482.

[44] L.Snyder, J.J. Kirkland, J. Glajch, Practical HPLC Method Development, Wiley-Interscience Publishers, New York, 1997.

[45] Lloyd R. Snyder, Joseph J. Kirkland, John W. Dolan, Introduction to Modern Liquid Chromatography, John wiley & sons inc, New

Jersy,2010.

[46] High-Performance Gradient Elution: **T**he Practical Application of the Linear-Solvent-Strength Model, John wiley & sons inc, New Jersy, 2007.

[47] John W. Dolan, Temperature selectivity in reversed-phase high performance liquid chromatography, Volume 965, Issues 1–2, 2 August 2002, Pages 195–20.

[48] Steffen Wiese, Thorsten Teutenberg, Torsten C. Schmidt, A general strategy for performing temperature-programming in high performance liquid chromatography-Further improvements in the accuracy of retention time predictions of segmented temperature gradients, 1222, 2012, 71–80.

[49] Henk A. Claessens ,Characterization of stationary phases for reversed-phase liquid chromatography ,Column Testing, Classification and Chemical Stability,1999.

[50] J.J. Kirkland, Development of some stationary phases for reversed-phase high-performance liquid chromatography, J. Chromatogr. A, 1060, 2004, 9–21.

[51] Geza Rippel, Edit Alattyani, Laszlo Szepesy, Characterization of stationary phases used in reversed-phase and hydrophobic interaction, chromatography , J. Chromatogr. A, Volume 668, Issue 2, 13 May 1994, 301–311.

[52] R.G. Lehmann, Development of a high-performance liquid chromatographic method for fluroxypyr heerbicide and metabolites using computer simulation with drylab software, J. Chromatogr. A, 1989, 581–584.

[53] D.J. Thompson, W.D. Ellenson, Computer-assisted development of a high-performance liquid chromatographic method for fractionating selected nitro derivatives of polyromatic hydrocarbons, J. Chromatogr. A, 485, 1989, 607–615.

[54] Bieke Dejaegher, Yvan Vander Heyden , Ruggedness and robustness testing, J. Chromatogr. A, 1158, 2007, 138–157.

[55] ICH Q8 (R2), Pharmaceutical Development, 2009.

[56] I. Molnár, H.J. Rieger,K.E. Monks,Aspects of the "DesignSpace" in high pressure liquid chromatography method development, J. Chromatogr. A 1217, 2010, 3193–3200.

[57] K. Monks,I. Molnár, H.J. Rieger, B. Bogáti, E. Szabó, Quality by Design: Multidimensional exploration of the designspace in high performance liquid chromatography method development for better robustness before validation, J. Chromatogr. A 1232, 2012, 218–230.

[58] Text on Validation of Analytical Procedures, ICHQ2A, 1994.

[59] Validation of Analytical Procedures: Methodology, ICH Q2B, 1997.

[60] Validation of compendial methods, The United States Pharmacopeia , 33st edn (2010), USP33 /NF28.

[61] Schwartz M, Krull IS. Analytical Regulatory and Validation Compliance. New York, USA: Marcel Dekker; 1997.

[62] Riley CM, Rosanske TW, Development and Validation of Analytical Methods. Oxford, UK, Pergamon, 1996.

[63] Miller JM, Crowther JB, editors. Analytical Chemistry in a GMP Environment. New York, USA, Wiley, 2000.

[64] Ermer J, Miller JH, editors. Method Validation in Pharmaceutical Analysis, Weinheim, Germany, Wiley-VCH Verlag, 2005.

[65] International Conference on Harmonization Tripartite Guideline: Stability Testing of New Drug Substances and Products, ICH Q1A, September, 1994.