1.0 INTRODUCTION

1.1 Preamble

1.1a Introduction

Bioanalytical chemistry is the determination of drug substances in biological fluids like plasma, serum in terms of qualitative and quantitative analysis. It plays important role in evaluation of pharmacokinetic parameters which required for bioavailability and bioequivalence studies. The validation of developed method required to assure common level of quality therefore use of validated methods increased in current practice.

Analytical methods plays important role in research and development of new product. The quality assurance and quality control are the main control parameters used in method development and validation. The chromatographic and spectrometric or spectroscopic methods are selected on the basis of their characteristics, features along with deficiencies. The selected method for determination must be gone through every stage of all validation parameters. Investigation of each stage must be determine in same procedural variables, same environment and same matrix which can affect analyte evaluation in biological matrix started from clinical sample collection to sample analysis.

Method validation will be start after confirmation of complete method development. Full method validation will be start after promising results of method development because the method developer doesn’t have idea about the actual method conditions during method validation.

Method development mainly involves evaluation of different conditions as per analyte nature and optimization of those conditions accordingly. The important stages in method development are preparation of sample, separation of prepared sample by using chromatography, detection of separated sample by using suitable detection method. To start method development of newer analyte extensive literature survey required. After literature survey the primary importance given to summarized import points and determines the concept of future work. Literature survey information will be used to select instrument that is comfortable for analysis. This includes analytical
column, high performance liquid chromatography system and detector system like Mass spectrometry. Another parameter is internal standard, select suitable internal standard as per analyte parameters. Also select suitable extraction method which will give high recovery, accuracy and precision along with economical as industrial prospective.

Two factors mainly consider for determination of quality are recovery and standardization of method. Recovery of analyte into biological matrix is refers to response for total amount of analyte in the contained sample. Comparison between matrix content and pure solvent is nothing but relative recovery and the absolute recovery is nothing but true test of recovery.

Another important parameter is selection of internals standard. Generally it consider on the basis of molecule nature and suitability of chromatographic methods. The coefficient of the analyte and the internal standard are very similar is the main assumption consider for selection of internal standard. Now a day’s structural isotope analogue of targeted analyte used as internal standard. The structural and isotopic analogue of analyte is added to the biological sample prior to sample pretreatment to calculate the area ratio between analyte and internal standard is back calculated against standard curve which determine the concentration of drug.

The selection of suitable instrument and there make is an important issue during method development. The difference between different makes of instrument is not surprising and should be considering for method development.

Limit of detection and calibration curve are the most important parameters and it’s necessary to determined prior to method development as it is platform for future method development work.

As per US pharmacopeia limit of detection is nothing but lowest concentration of an analyte in a sample that can be detected but not necessarily quantitated and lowest limit of qualification is nothing but a sample can be determined with acceptable precision and accuracy and under the stated operation condition of the method. These parameters associated with signal to noise ratio. The signal is measured in blank plasma and measured from base line to peak of apex and divided by peak to peak
noise.

The calibration curve is determined between lowest concentration of analyte to the highest required concentration as per reported Cmax concentration. The minimum six concentrations are required to define relationship between analyte response and concentration and it should be reproducible at all stages of method. The more concentration may be required in case of non linear relationship. The most appropriate weighing factor is $1/x$, $1/x^2$ commonly observed. Among the seven non zero standards at least five should be pass the criteria.

**Introduction to HPLC method for analysis of drugs**

HPLC method having several advantages like easy automation, rapidity, specificity, accuracy and precision therefore HPLC selected for analysis of most of the drugs. HPLC method involves good extraction as well as isolation procedures. Following are the advantages of HPLC:

- Speed
- Greater sensitivity by using different instruments
- Improved resolution by using different stationary phases
- Reusable columns for different drug analysis.
- Ideally use for low volatility substances
- Easy to handling and maintenance
- Good sample recovery
- Less time and less labor requires.
- Precise and reproducible
- Integrator calculations are done by instrument itself.
- On a larger scale suitable for preparative liquid chromatography

### Types of HPLC

- Depending on relative polarity it mainly divided into two modes of the two phases.
  - a) Normal phase chromatography
  - b) Reversed-phase chromatography
• In normal-phase chromatography the stationary phase is polar in nature and the mobile phase is non polar. Due to this principle polar samples are thus retained on the polar surface.

• Reverse-phase chromatography is the completely inverse of this. The stationary phase is nonpolar in, while the mobile phase is a polar.

HPLC equipment diagram is given in Figure 1.

![HPLC equipment diagram](image)

**Figure 1: A schematic diagram of HPLC equipment**

Various components of HPLC are described below\(^{19,20}\)

**a) Solvent delivery system**

- **Pumps**

  It is most important components of HPLC, as its performance directly relate with retention time, detector sensitivity and reproducibility.

  Three main types of pumps are:

  - Displacement pump:
- Reciprocating pump:
- Pneumatic or constant pressure pump:

b) Sample injection system

The design characteristics divided HPLC injectors into four types.

- **Type 1 Injectors**
  In this type completely filled sample loop used to determine the injected volume. These injectors are simple, reliable devices having six-port rotary valves for injections. A syringe is used for pushing sucking and filling of excess sample into sample loop. These injectors are highly precise injections because injected volume determine by the loop volume.

- **Type 2 Injectors**
  In this type of injectors micro-syringe is used to transfer sample into the loop. This syringe determines injected volume, as its sample size is always smaller than the loop volume. Also the sample is not trapped or wasted, but the comparative precision is lesser than type 1.

- **Type 3 Injectors**
  This type of injectors used for both complete and partial filling methods. Sample trapping is major drawback of this type. The syringe inserted into needle port and it dispensed the contents after that sample loop is loaded. After syringe left from port then the valve is switched. In the switching process the loop inserted into the stream without exposing the high pressure to the syringe. Some sample remains trapped into the connecting passage of the injector after changing of syringe position. The drawbacks of this trapping method are wastage of sample, error in trapped volume and injector flushing required after each injection.

- **Type 4 Injectors**
  In it both methods used but it does not trap the sample. It is similar with type 3 injector but it not contains a connecting passage between syringe needle tip and sample loop. Therefore no trapping of sample and it results no sample waste, no
error in syringe reading and not required flushing between injections, except in trace analysis.

c) Chromatographic column

Basically column is made up of heavy glass or stainless steel tubing for standing in high pressure. The usual column length is 10 to 30 cm long and 4 to 10 mm inside diameter which contains stationary phase of 25 µm or less particle diameter. The 5 mm columns gives good results than other columns because of compromise between efficiency, sample capacity and the amount of packing and solvent required.

Column packing

The small, rigid particles with narrow particle size distribution column packing used in modern type HPLC. The column packing mainly divided into following three types.

- Porous, polymeric beds
- Porous layer beds
- Totally Porous silica particles

In recent years, above column packing's widely used in analytical HPLC. The dry packed column particles having diameter more than 20 µm. In slurry packed column particles of diameter less than 20 µm used. In this particles are suspended on a suitable solvent and the obtained slurry is driven into the column under suitable pressure.

d) Detectors

Min function of detector is to monitor mobile phase which merges from column in HPLC. Different types of detectors are given in Table 1.
### Table 1: Types of detectors

<table>
<thead>
<tr>
<th>Detector</th>
<th>Analytes</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-visible</td>
<td>Any with chromophores</td>
<td>It’s having degree of selectivity and mostly use in different HPLC applications.</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>Fluorescent compounds</td>
<td>It is highly selective and sensitive. Used in derivatization of compounds.</td>
</tr>
<tr>
<td>Refractive Index (RI)</td>
<td>Compounds with a different RI to the mobile phase</td>
<td>It is universal detector but less sensitive.</td>
</tr>
<tr>
<td>Electrochemical</td>
<td>Readily oxidized or reduced compounds, especially biological samples</td>
<td>Very selective and sensitive</td>
</tr>
<tr>
<td>Evaporative Light Scattering (ELSD)</td>
<td>Virtually all compounds</td>
<td>It is universal detector with highly sensitivity but not selective.</td>
</tr>
<tr>
<td>Mass Spectrometer (MS)</td>
<td>Broad range of compounds</td>
<td>It is a powerful second dimensional analytical tool and highly sensitive. Different modes available. Required trained operators.</td>
</tr>
</tbody>
</table>

**e) Recorder**

Recorder is a electromechanical instrument which transforms the chromatographic signal into a graphical record.
Introduction of mass spectrometry\textsuperscript{21,22}

Mass spectrometry (MS) has an important place amongst the various spectrometric techniques for molecular analysis. The phenomenon of deflection of ions in electric or magnetic field first was proposed by Wien in 1898. A mass spectrometer for general use however available after 1930 only. It is an important spectrometric technique for molecular mass analysis.

Mass spectrometry is an analytical technique involves the study in the gas phase of ionized molecules with the aim of one or more of the following:

- Determination of Molecular weight.
- Characterization of structure.
- Study of Gas phase reactivity.
- Qualitative and quantitative analysis of components in a mixture.

The basic principle of Mass spectrometry is production and separation or filtration of ions on the basis of their mass-to-charge (m/z) ratio and detected. The detected mass responses were resulting mass spectrum which is a plot of the (relative) abundance of the generated ions as a function of m/z ratio. Thus MS is a very sensitive, highly selective and quantitative analytical technique. Sample size is usually in microgram to the nanogram range and fragmentation patterns are highly reproducible even for multi-component mixtures.

Presently the mass spectrometer is highly developed and completely computerized instrument. It consisted into basic:

- Introduction of sample,
- Ionization of samples,
- Analysis of samples,
- Detection of ion,
- Handling of data.

Sample introduction systems comprise controlled leaks, through which a sample vapor is introduced from a reservoir, various direct insertion probes for the
introduction of solids and low-volatility liquid and combination with various chromatographic techniques.

The analyte ionization can be performed by different ways, the available ionization techniques can be classified following groups.

- Electron Ionization (EI)
- Chemical Ionization (CI)
- Electrospray ionization (ESI)
- Matrix-assisted Laser Desorption Ionization (MALDI)
- Field desorption (FD)
- Fast Atom Bombardment (FAB)

Proton transfer, charge exchange, electrophilic addition and anion abstraction reaction used to produce positively charged ions, \( M^+ \) or \( MH^+ \). Negative ions are produce by proton transfer or abstraction and anion attachment. After the generation of ions, analysis of produced ions is done according to their \( m/z \) ratio in time or space with the mass analyzer. This means that a singly charged molecule with molecular mass of 400 will give a peak at \( m/z \) 400, while a molecule carrying 40 charges and a molecular mass of 16,000 will also give a peak at 400 \( m/z \).

Five types of mass analyzers are currently available

- Quadrupole mass filter
- Quadrupole ion trap
- Time-of-flight
- Fourier-transform ion-cyclotron resonance instruments

In practice, most interfaces for coupling of LC with MS have been developed on quadrupole instrument. The detection of ions after the mass analysis is mostly performed by using electron multiplier. The signal of the electron multiplier is fed to multichannel analyzer to perform ion counting. In most modern instruments a high-voltage conversion dynode is used in both positive-ion and negative-ion detection. A mass spectrometer produces an enormous amount of data. The most important type of
output are mass spectrum, mass chromatogram and total ion chromatogram. The mass spectrum is a plot of relative intensity as a function of the m/z. It contains a base peak, which is the peak with highest intensity used to normalize the mass spectrum. The mass spectrum is also contains fragment peaks, isotope peak and background peak.

- **Tandem mass spectrometry (MS/MS)**

All the mass spectrum gives information about molecular weight, but it does not provide structure information for the component of interest. To avoid this limitation took the form of the development of tandem mass spectrometric methods (MS/MS). Tandem mass spectrometry divided into two stages, first to select the parent ion from all other ions produced in the ion source and second to analyze the daughter ions after collisions. In this technique ions are separated, identify and fragmented in a single instrument. It is a fast collision event, where ion translational energy is converted into ion internal energy to obtain an ion in the excited state, and slow unimolecular decomposition. The obtained MS/MS spectrum is consist selected product and precursor ions. MS/MS can be done in a number of single-stage instruments and a variety of two-stage instruments. Triple quadrupole mass analyzer instrument is the most widely used MS/MS configuration as compare to other type of mass analyzer. TheRecently different types of hybrid mass analyzers are developed for specific application for e.g quadrupole-linear trap, quadrupole-time-of-flight. Triple quadrupole instruments consisted of first and third quadrupole while the second quadrupole is act as collision cell in the RF only mode. Different scan modes are available like product ion scan mode - for structure elucidation, parent ion scan mode and neutral scan mode - for screening, selected ion monitoring (SIM) and selected reaction monitoring (SRM) scan mode for quantitative analysis.

**Introduction of liquid chromatography-tandem mass spectrometry (LC-MS/MS)**

By putting a separating chemistry from an HPLC in front of a specialized detector such as the mass spectrometer, one has a true multidimensional analytical technique that raises the level of the confidence of what you are looking at. Combining HPLC and MS offer the possibility of taking advantage of both HPLC as separation technique and MS as sensitive detection and identification technique. This coupled
with the need for more sensitive and specific detector for HPLC has generated considerable interest in the development of routine liquid chromatography - mass spectrometry (LC-MS)\textsuperscript{24}. The mass spectrometer can be considered as a universal, selective and specific detector depending on selective mode of operation. An HPLC followed by mass spectrometric detection enables the quantification of therapeutically relevant drug concentration (µg/ml or ng/ml range) in small volume of biological samples and determinations of the chemical structures of biotransformation. The principle advantage of HPLC separation followed by the MS detection is that analyte molecular weight is identified by both chromatographic retention time and by molecular weight & fragmentation pattern.

The coupling between LC & MS has not been straightforward since the normal operation condition of both HPLC and MS are different high pressure/ high vacuum, low temperature/ high temperature, liquid phase/ gas phase, high flow/ low flow to achieve and to cope with these problem different LC-MS interfaces have been developed. The constant amount of liquid introduced into ion source of a MS prevented the widespread development and use of LC-MS. But now many of the difficulties associated with it have been solved and there are now several interface designs and LC-MS is in routine use in analytical laboratories worldwide\textsuperscript{25}.

The diversity of available interfaces: capillary inlet, Pneumatic nebulizer interfaces, direct liquid interface, thermo spray, Continuous flow fast atom bombardment (FAB), particle beam and atmospheric pressure ionization (API)\textsuperscript{26-28}. Currently used API based LC/MS interfaces i.e. electro spray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the most widely approaches\textsuperscript{29}, while other has limited extent. More analysis of LC-MS performed today by using API interfacing. These interfaces in combination with tandem mass spectrometry of single stage and double stage fragmentation instrument enables a LC-MS/MS technique. ESI is technique in which direct ionization from solution, due to this advantage its use as an LC-MS/MS interface. Extensions of the ESI interface led to miniaturized formats, micro electrospray and nano electrospray.

The major application area of LC-MS is in the pharmaceutical field, where LC-MS used from drug development to formulation development. LC-MS/MS is widely applied for drug development as well as routine analysis. In drug discovery activities
like lead identification, lead optimization, in vitro and in vivo drug screening, preclinical activities like identification of metabolite, impurity screening and degradant screening, clinical activities like quantitative bioanalysis and metabolite identification application of LC-MS and LC-MS/MS enhanced extensively.

**Instrumentation for LC-MS/MS**

![Picture of triple quadrupole instrument](image)

**Figure 2: Schematic diagram of LC-MS/MS using triple quadrupole instrument**
The mass spectrometer consists of an ionization source, ion guides, triple-stage mass analyzer, and ion detection system. The ion guides, mass analyzer, ion detection system, and part of the ionization source are enclosed in a vacuum manifold. Ionization mode used for to ionize the sample is referred. In the mass ionization source ions produced and transmitted produced ions by the ion guides into the mass analyzer. In mass analyzer transmitted mass analyzer separated according to their mass to charge ratio. In the lenses polarity of the potentials applied in the ionization source and ion guides determines the positively and negatively charged ions are transferred to the mass analyzer. It can analyze positively or negatively charged ions.

In the ionization source produced ions are filtered according to their mass to charge ratios (mass analyzed) by the triple stage mass analyzer. When the system is operated as a conventional mass spectrometer with one stage of mass analysis, the ionized sample subjected to mass analysis. The resulting mass selected ions are then transmitted to the ion detection system.

When the system is operated as a tandem mass spectrometer, as before, the sample is ionized in the ion source and is mass analyzed by the first rod. In this case, the selected ions mass exiting the first rod and allowed to collide with second rod with inert gas and produce fragment for new ion products. The fragmented secondary ion
products then analyze in third rod for mass analysis the detection of selected ions is done. In this mode, two stages of analysis are performed.

Each sequence of single or triple stage mass analysis of the ions is called a scan. To solve complex analytical problem instrument should have ability to vary different scan mode and scan type, different ionization and polarity modes, flexible to different users.

**Principle of chromatography**

Chromatography is the science, which studies the separation of molecules based on difference in their structure and/or composition. The HPLC is versatile techniques for separation and determination of mixture compounds. The distribution and equilibrium of analytes between mobile phase and stationary phase. HPLC is based on the mechanism of adsorption, mass distribution, ion exchange & stereo chemical interaction.

**1.1b Problems in hand**

- Identifying newer drugs
- Method development of newer drugs
- Development of chromatographic parameters
- Development of Mass parameters
- Method validation of developed drugs.

**1.1c Importance & Scopes**

**Need for bio-analytical method**

A bioanalytical method defines the determination or quantification of analyte or drugs and their metabolites in biological matrices such as plasma, urine, and serum. For reliability it must be validated. Bioanalytical method overall procedures involved collection of sample at clinical phase, processing of collected sample, storage of
collected sample, and analysis of collected biological matrix for determination of drugs\(^8\). Bioanalytical method validation involves following major activities such as selectivity, accuracy, precision, recovery, sensitivity, and stability\(^{11}\). The selective and sensitive bioanalytical methods require for the creation of accurate pharmacokinetics, bioavailability, and bioequivalence of drugs data. Validated Bioanalytical method is used for quantitative determination of drugs and their metabolites in biological fluids \(^{12,13}\). These studies generally support regulatory filings.

**Need for analytical method\(^{14}\)**

Following are the major reasons for the development of newer bioanalytical methods:

- In literature method for determination of drug is not available.
- Unavailability of method in biological matrix.
- In official pharmacopeias drug or drug combination method not available.
- Due to the interference observed by the formulation excipients, as per formulation analytical methods may not be available for the drug.
- Due to different drug in combination with other drugs, analytical method may not be available.
- The available analytical method may require expensive reagents and solvents.

**1.1 Organization**

**1.2a Where work is carried out**

The work will be carried out in Wockhardt Research Center, Aurangabad, Maharashtra.

**1.2 b Products**

- Febuxostat
- Clebopride
- Darifenacin
- Cycloserine
- Carbocisteine
1.2c Processes

Validation of analytical or bio-analytical methods

Bioanalytical methods must be validated if the method development results support for new drug development or new formulation of exciting analyte. Validation required defining performance of developed method and reliability of obtained results. If the bioanalytical developed method will be used for bioequivalence application then it must be validated to ensure minimum requirement of validation experiments along with satisfactory results.

Types of Method Validation

1) Full Validation

A full validation is required

- Fresh method development,
- New drug entity
- Addition of metabolite in exciting

2) Partial Validation

It is performed if validated bioanalytical methods have been modified.

Typical situations for a partial validation are:

- Method transfers between laboratories and analysts,
- Instrument and/or software platform changes,
- Changes in species within the same matrix,
- Changes in matrix within the same species,
- Change in analytical methodology, and
- Change in sample processing procedures.
3) **Cross Validation**

Cross validations are necessary when two or more bioanalytical methods are used to generate data within the same study.

A cross validation should be also considered when

- Sample analyses within a single study are conducted in more than one laboratory,
- Data generated using different analytical techniques in different studies are included in a regulatory submission.

As per the United States Pharmacopoeia (USP), validation of an analytical method is the process by which it is established, by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical application and performance characteristics are expressed in terms of analytical parameters\(^\text{39}\).

The following parameters are required for the bioanalytical method validation as per the regulatory guideline\(^\text{40}\).

- Selectivity
- Sensitivity
- Calibration range
- Linearity
- Carryover check
- Accuracy
- Precision
- Extraction efficiency (Recovery)
- Dilution integrity
- Reproducibility
- Stability studies
(i) **System suitability**

System suitability was performed separately before start of every new batch. Six injections of aqueous equivalent MQC solution mixed with the ISTD working solution were performed. The mean, S.D, and % CV for the peak area ratio and for the retention time of analyte and ISTD were calculated.

**Acceptance criteria**

- % CV for peak area ratio should be $\leq 5.0$
- % CV for retention time for both the analyte and ISTD should be $\leq 5.0\%$.

(ii) **Selectivity and specificity**

Selectivity is ability of developed method to produce a response for targeted molecule which distinguishing from interferences like endogenous and exogenous sources. Endogenous sources are analyte metabolite, degradation products, chemicals occurring in biological fluids, co-administered drugs and exogenous sources dirty lab wares, impurities in reagents, chemicals\(^{10}\).

This was determining by checking six different lots of buffered plasma for any interference at the retention time of the drug and internal standard by using the selected method conditions. The blank plasma was processed and run for 45 min to observe any interfering peaks.

**Acceptance criteria**

- Any interfering peak if found should not be present within $\pm 10\%$ of the retention times of drug and ISTD.
- If interference observed at the retention times of drug and ISTD, it should be $\leq 20\%$ and $\leq 5\%$ of response of the mean extracted LLOQ and mean extracted ISTD respectively at the concentration to be used in study.
- A minimum of 75% plasma lots used for the specificity should meet the above criteria.
(iii) **Sensitivity**

Sensitivity is the specific response which depends on the concentrations to be measured in biological matrix for the specific molecule. It can be expressed as slope of linear of the linear regression calibration curve and it measured at the time as the linearity test. This was determined by comparing extracted blank plasma with extracted LLOQ samples (5 replicates) with ISTD using the proposed bioanalytical method conditions.

**Acceptance criteria**

Lowest concentration in calibration curve is accepted as LLOQ if

- % deviation from the nominal concentration is within ± 20%
- % CV of the calculated concentration is ≤ 20%.

(iv) **Linearity (Calibration curve)**

It included a blank sample, a zero sample and eight non zero samples covering the calibration curve range were processed and analyzed.

**Acceptance criteria**

- A correlation coefficient (r²) of the calibration curve must be ≥ 0.9800.
- The back-calculated concentration of the lower calibration standard (CC-1) must be within 80-120% of its nominal concentration & of all other calibration standards must be within 85-115% of their nominal concentration.
- The curve must contain at least 66.66% (6 out of 9) of the calibration standards for evaluation of curve fitting.
- Both the lowest CC standard (CC-1) and highest CC standard (CC-9) should pass to accept the calibration curve.

The weighing factor selected was \[(1/x^2)\] where x: analyte concentration] linear regression. It was used to determine slopes, intercepts, and correlation coefficients. And its results used for calculation of concentration.
(v) **Accuracy and precision**

The deviation of the mean value from the nominal value (relative error) serves as the measure of accuracy. Precision is the agreement between replicate measurements of the same sample\(^\text{3}\).

Precision is subdivided into

- Within batch precision (intra batch)
- Between batch precision (inter batch)
- CC standards and QC samples were prepared according to the proposed method. The within batch precision and between batch precision was assessed by analyzing four validation batches.

Each batch consisted of

1. A blank solution
2. A reference standard solution (system suitability)
3. A blank matrix (blank sample)
4. A blank matrix with IS (zero sample)
5. Spiked calibration standards (1 set of 8 non zero concentrations)
6. LLOQ (6 samples)
7. Low QC (6 samples)
8. Middle QC (6 samples)
9. High QC (6 samples)

The mean concentrations, standard deviation, precision and accuracy at QC’s concentration levels were determined.
Acceptance criteria

- Precision: The between and within batch % CV at each QC level should not exceed 15% except for the LLOQ is 20%.
- Accuracy: The between and within batch mean concentration at each QC level should not exceed 15% of the nominal concentration value except for the LLOQ is 20%.

(vi) Recovery

Absolute recovery is the measured response of processed spiked sample expressed as percentage response of pure standard, which has not subjected to pretreatment and indicates whether the method provides, a response for the entire amount present in the sample.

Recovery for drug and IS was performed by comparing extracted samples area at three different QC’s concentrations with un-extracted standards area that represents 100% recovery. % recoveries for both drug and IS were calculated.

Acceptance criteria

- Recovery of analyte and IS should be consistent, precise and reproducible.
- Variability within areas at each QC levels for analyte should be within CV of 15.00%.
- Variability of the analyte recoveries across all the three QC level should be within CV of 20.00%.
- Variability within areas of IS found with each extracted QC levels should be within CV of 15.00%, while the variability of internal standard area found between all the QC level should be within CV of 20.00%.

(vii) Dilution integrity

Three times of ULOQ drug stock solution was spiked in blank plasma. Dilute the achieved concentration with blank plasma to get 1/5 and 1/10 concentrations of the spiked sample or as per requirement. Calibration standards and six aliquots each of diluted samples were analyzed as per the method described in sample preparation.
Acceptance criteria

- Precision and accuracy of the dilution integrity of the QC’s should be \( \leq 15\% \) and within \( \pm 15\% \) of the nominal concentration respectively.

(viii) Stability Tests

Stability test is done to prove that the difference between concentration of analyte at the time of analysis to the concentration of the time of sampling\(^2\). It is an important aspect in bioanalytical method validation which referred in subject sample analysis.

1. Stock solution stability

It was performed at 2 to 8ºC for minimum 6 days. It was assessed by comparing freshly prepared samples of analyte and IS with that of stability samples at MQC level by performing five injections of each. The % mean change of analyte and IS after evaluated period was calculated.

Acceptance criteria

- The % Mean Change of analyte and ISTD to be within \( \pm 10\% \).

2. Bench top stability

Six replicates of lowest and highest QC’s in biological matrix were withdrawn and kept unprocessed for minimum 7.0 hrs (stability samples) at room temperature. Samples were processed as per validated method. Accuracy and precision were calculated. By using freshly spiked calibration curve and LQC, HQC level with comparing LQC, HQC level of comparison samples, stability was evaluated.

Acceptance criteria

- The back-calculated concentrations of all LQC and HQC samples must be within 85.00 - 115.00% of their theoretical concentration
- At least 67.00% QC samples must fall within above-mentioned criteria at each LQC and HQC levels.
- The % mean change of febuxostat to be within \( \pm 15\% \).
3. Post-preparative (In-Injector) stability

To check injector stability of the processed samples, the QC samples were processed according to the proposed method and placed in the auto sampler. After it stability samples injected for analysis into the HPLC system. By using freshly spiked calibration curve and LQC, HQC level with comparing LQC, HQC level of comparison samples, stability was evaluated.

Acceptance criteria

- The back-calculated concentrations of all LQC and HQC samples must be within 85.00-115.00% of their nominal concentration.
- At least 67.00% of quality control samples must fall within above mentioned criteria at each QC levels
- The % Mean change must be within ±15.00%.

4. Freeze and thaw stability

Stability of both the drug and IS after freeze and thaw cycles was determined at 6 replicates of the LQC and HQC concentrations. The QC samples were frozen at −70 °C for 24 hrs and then thawed at room temperature. After thawing, the samples were frozen again at the −70 °C for 12-24 hrs and then withdrawn for thawing. Minimum three times freeze-thaw cycle was repeated and then processed. The concentration of freeze-thaw stability samples was calculated and stability was assessed by using freshly spiked calibration curve and LQC, HQC level with comparing LQC, HQC level of comparison samples, stability was evaluated. Comparison and stability samples were processed together and analyzed in single run.

Acceptance criteria

- The back-calculated concentrations of all LQC and HQC samples must be within 85-115% of their nominal concentration.
- At least 67% of QC samples must fall within above mentioned criteria at each QC levels.
- The % Mean Change of drug to be within ±15%
5. Re-injection Reproducibility

Re-Inject the CC standards and QC’s levels from one of the pass activity to check the reproducibility for reinjection.

Acceptance Criteria

- For the CC 75% and minimum of 6 standards must be within 85-115% of their nominal concentration except LLOQ should be ± 20% of nominal value
- Minimum 67% QC's at each level must be within 85-115% of their nominal concentration.

(ix) Matrix effect

Process blank sample from at least 6 lots of plasma as per the method being validated (test samples) except the addition of analyte and IS which will be done after processing. Prepare an LQC level aqueous solution of analyte with internal standard in reconstitution solvent (reference solutions) and inject 6 replicates. Spike the test samples with analyte (AQS LQC) and IS injected before the LC-MS/MS. Analyze both the test and the reference samples using LC-MS/MS. Compare the peak area in reconstitution solvent (reference solutions) with that of "samples spiked after sample processing" (test samples). Calculate matrix effect and matrix factor individually for analyte and internal standard.

Acceptance Criteria

- Matrix factor should be within 0.85-1.15 and the %CV should be: 15%.
- Matrix effect should be between 85 -115%
- If more than one analyte for quantification then each analyte, internal standard should be tested.
- If the above criteria are not met, repeat this experiment with the same or different matrix lots.
Calculation of the sample concentration

The concentration of the drug and IS were calculated from the area ratio v/s spiked plasma concentration regression equations with reciprocate of the drug concentration, as a weighting factor \((1/\text{concentration})^2\), i.e. \(1/X^2\):

\[
y = mx + c
\]

Where,

\(y\) = peak area ratio of drug to IS

\(m\) = slope of the calibration curve

\(x\) = concentration of drug

For calculating accuracy, precision, recovery and % change the following formulae were used:

- **Precision**

  \[
  \% \text{ CV} = \frac{\text{Standard deviation} \times 100}{\text{Mean}}
  \]

- **Accuracy**

  \[
  \% \text{ Nominal} = \frac{\text{Concentration found} \times 100}{\text{Nominal concentration}}
  \]

- **Recovery**

  \[
  \% \text{ Recovery} = \frac{\text{Extracted peak area} \times 100}{\text{Unextracted peak area}}
  \]

- **Mean percent of change of stability samples**

  \[
  \frac{\text{(Mean conc. of stability samples - Mean conc. of comparison samples) \times 100}}{\text{Mean conc. of comparison samples}}
  \]
Mean Percent of change of stock solutions

\[
\frac{\text{Mean response of stability samples} - \text{Mean response of comparison sample}}{\text{Mean peak response of comparison samples}} \times 100
\]

During the HPLC Method Development, different conditions are to be optimized like sample preparation, column, detection, mobile phase etc. By using control biological matrix samples bioanalytical method validation is done and drugs quantified accordingly.

Typical parameters used in analytical validation are:

- Specificity
- Linearity
- Accuracy
- Precision
- Limit of Detection
- Limit of Quantitation
- Robustness
- Ruggedness

The different parameters of analytical method development are discussed above as per ICH guideline\(^{40}\).

1.2d Profiles

The organization having bioanalytical laboratory which approved by various regulatory authorities like USFDA, MHRA.

1.3 Problems

1.3a Introduction

Method development and design of separation method on LC-MS/MS\(^{30}\)

Method development plays a great role in concluding for any analytical method. In quantitative bioanalysis method development can broadly divided into three parts
optimization of chromatography conditions, mass spectrometry parameters and sample preparation. Depending upon the physical and chemical properties of drug method development begins i.e. selection of column, mobile phase, pH of mobile phase, flow rates for setting chromatography. The choice of ionization technique is depend on bioanalytical results with pretreated samples. LC-MS/MS tuning parameters and scan modes are decided by direct infusion of drug solution, depending on the sensitivity and specificity needed. The based on the intended use of method the lower limit of quantification and upper limit of quantification is set. Some important elements of method development are sample pretreatment, chromatography, choice between ESI and APCI, Internal standard and mass spectrometry.

1.3b Description

Following are the main area in which problem arises:

- Sample pretreatment
- Chromatography optimization
- Mass spectrometry optimization
- Selection of internal standard.

1.3c Details

Sample pretreatment

Sample pretreatment is a procedure in which analyte transfer for analysis. In general three major things in sample pretreatment consider they are extraction of analyte for bioanalysis using LC-MS, preconcentration of the analyte for improving the lower limits of quantitation and converting the analyte in suitable solvent composition. Most commonly used extraction techniques for sample pretreatment in bioanalysis include protein precipitation, liquid-liquid extraction and solid phase extraction.

- **Protein precipitation (PPT)**

It is a technique used to separate the drug from the biological matrices by means of precipitating the proteins using additive. The most selective organic solvents used in protein precipitation are acetonitrile, methanol, trichloroacetic acid, trifluoroacetic
acid and perchloric acid. In the protein precipitation firstly additive is added into sample. Then sample process for mixing and centrifugation. After it supernatant in final mixture is separated and injected into the HPLC and LC-MS system. Protein precipitation is a fast method to produce pretested anlyte samples. Protein precipitation is very easy and fast sample pretreatment method.

- **Liquid-liquid extraction (LLE)**

It is a technique based on partitioning coefficient of analytes between two immiscible liquid phases. It is mainly used for removal of nonvolatile liquids also simple, fast and efficient method. The immiscible organic solvents used in this method are methyl t-butyl ether, dichloromethane, ethyl acetate and hexane. It takes advantage of the relative solubility of the solutes in immiscible solvents. The sequence for acid and basic drugs is “sample pH adjustment (acid or base), extraction, pH adjustment (base or acid) and back extraction” offers an effective generic approach. Liquid-liquid extraction is labour intensive and time consuming as it involves drying followed by reconstitution to meet the desired quantification limits. LLE is relatively specific and selective method.

- **Solid phase extraction (SPE)**

Solid phase extraction is most preferable extraction method for bioanalysis. In this technique concentrate analyte obtained by removal of interfering endogenous material for HPLC and LC/MS analysis of biological samples. Solid phase extraction can be performed on 96 well plated automated systems. The general procedure firstly SPE cartridges activated by using respective solvent then biological sample loaded into the cartridges then washed out the cartridge and finely remove the targeted analyte into suitable solvent. The final obtained analyte again concentrate by using evaporation step. Other solid-liquid extraction techniques include solid phase micro extraction, accelerated fluid extraction, supercritical fluid extraction and microwave-assisted extraction. SPE is a universal technique, which can be applied to polar, non-polar, acidic, basic or neutral drugs.

Sample preparation (extraction method) techniques are selected on the basis of drug nature i.e. for polar compound one should select PPT and SPE, non-polar compound
one should select LLE and SPE and for moderately polar and moderately non-polar compounds all the three techniques can be applied. In general the analytes can best extract when they are in non-polar or non-ionic stage. The recovery of analytes can be optimizing by addition of acid, base or a buffer, depending upon the pKa value of drug.

**Chromatography optimization**

Separation targeted molecule from related substance and metabolite is main purpose of chromatography in bioanalytical methods. In bioanalytical most preferable chromatographic technique is reverse phase chromatography. Depending on the type of molecule column selection is made. Modified silica, polymer or hybrid silica based bonded phase material columns e.g. Octadecyl (C$_{18}$) or Octyl (C$_{8}$). Shorter narrow bore columns e.g. 30-50 mm length, and 3-5 µm ID packing materials are used. As LC-MS/MS is an ionization technique, the pH of mobile phase is an important factor for ionization of analyte. The acid analyte gets ionized in acidic pH higher than its pKa. It results in less interaction with stationary phase, low retention time and low run time. The phenomenon is vies-a-versa for base analytes. The mobile phase is optimized with volatile buffer and organic modifiers to get maximum ionization and peak symmetry for analyte of interest. Clear the sample extract lesser is the chromatography optimization.

**Mass spectrometry optimization**

According analyte thermally labile, ESI or APCI are preferably used for quantification. The reliability of information obtained by MS relies on proper tuning of the instrument for the analytes. Tuning determines the ion transmission i.e. the sensitivity of the instrument. The ion spray voltage, molecular ions, tube lens offset, collision energy, collision gas pressure and fragments to be monitored need to optimize for better sensitivity and reproducibility. SRM mode of analysis provides ultimate selectivity with a particular m/z of precursor and product ions of the analytes. Any matrix interference can be eliminated with enhanced resolution possibilities of instruments.
Selection of internal standard

In order to compensate the extraction and detection losses during the analysis that is due to presence of endogenous matrix components, an internal standard and chemical compound analog to analyte should be used. A proper selection of internal standard is important parameter to obtain results with good accuracy and precision. The co-elution of analogue internal standard and analyte provides best results. The ion enhancement and suppression effects of target analytes and analogue must be evaluated using the blank extracted matrix with choice of extraction.

In general on the basis of logical trial extraction method is finalized to have a specific, sensitive and fast method of extraction. The optimized chromatography conditions, mass spectrometry parameters and sample preparation should result in best possible selectivity, sensitivity and recovery.

1.3d History

Method Development and Design of Separation Method on HPLC

Analytical method development requires knowledge about the nature of drug, molecular weight, polarity, solubility ionic character of drug. On the basis of all this parameters we can develop method for particular drug into multi component dosage form. Method development never provide exact results at the initiation of development, it is a trial and error basis activity. The most problematic phase is selection of mobile phase and selection of analytical column. The most preferable type is reverse phase chromatography because of most of the compounds are hydrophilic, polar groups and water soluble in nature. Method development and design of separation method depends on selection of best mobile phase, detector, column length and diameter, buffer, pH of buffer, type of stationary phase etc.

a) The Best Mobile Phase

The basic principle of reverse-phase chromatography is mobile phase is polar in nature and stationary phase is non polar in nature. Mobile phase generally made up of two or more solvents with additives and buffers. The initial approach is selection appropriate analytical column with proper mobile phase. The separation molecule and
optimize the retention of molecule is the second stage after selection of mobile phase. Separation is depending on different degrees of hydrophobicity of solute. The rate of elution of molecule in the mobile phase is control by the organic modifier. The rate of elution is increased by reducing the polarity. Small change into the mobile phase composition changes different parameters like elution rate, flow rate. The simple alteration of mobile phase composition or rate of flow allows the rate of the elution of solutes to be adjusted to an optimum value and permits the wide scope separation of the chemical types. First isocratic run followed by gradient run is preferred.

Since the mobile phase governs solute-stationary phase interaction, its choice is critical.

- Practical considerations dictate that it should not degrade the equipment or the column packing. For this reason, strong acids, bases and halide solutions should be avoided.
- Chemical purity of solvents is an important factor. Since large volumes of solvent are pumped through the column, trace impurities can easily concentrate in column and eventually be detrimental to the results. HPLC grade solvents are recommended.
- Volatility should be considered if sample recovery is required.
- Viscosity should be less than 0.5 centipoises, otherwise higher pump pressures are required and mass transfer between solvent and stationary phase will be reduced.
- Only volatile buffers should be used in LC/MS.

b) The Best Detector

The next consideration should be the choice of detector. There is little use in running a separation if detector one uses cannot “see” all the components of interest, or conversely, if it “sees” too much. UV-visible detectors are the most popular as they can detect a broad range of compounds and have a fair degree of selectivity for some analytes. Unfortunately UV-visible detectors are not universal detectors so it is worthwhile to see the chemical nature of the molecule, if it has suitable chromophores, such as aromatic rings, for UV-visible detection.
c) **The Best Column Length**

Many chromatographers make the mistake of simply using what is available. Often this is a $250 \times 4.6$ mm $C_{18}$ column. Due high plate count and wide selectivity of this column, it resolves many of the molecules therefore commonly used in most of the laboratories. While many reverse phase separations can be carried out on such column, its high resolving capabilities are often unnecessary, as illustrated in Figure 4. Method development can be started with shorter columns; 150, 100 or even 50 mm long. This is simply because they have proportionally shorter run times.

![Figure 4: Effect of Column length](image)

**d) The Best Stationary Phase**

The efficiency of development in method can be help by selecting appropriate stationary phase. For example, reverse phase chromatography the $C_8$ phase column can be provide better time than $C_{18}$, because it not retain molecules as strongly as the
C_{18} phase. Normal phase chromatography mainly prefers cyano (nitrile) phases columns because of the versatile nature.

**e) The Best Internal Diameter**

The optimization of mobile phase and elution rate can be minimized if run time is minimized and for minimization of run time selection of shorter column with appropriate phase necessary. Consideration of column internal diameter may be advantageous for method development. Most preferable column internal diameter is 4.6 mm in analytical laboratories because this require 75% of the solvent flow, this results 25% solvent saving analytical column life and it is more significant if a routine method.

**f) Gradient Programming**

Gradient programming of mobile phase is considered as fastest and easiest way for method development. In it firstly start with weak solvent and end with suitable solvent strength. Gradient programming start with very fast gradient and after it optimize as per proper separation with adjusting mobile phase combination. Different solvent and buffers may be used for optimization of mobile phase during method development. In HPLC systems different results expected in different system with same gradient therefore method requires validating into different laboratories, isocratic method recommended. The optimization of mobile phase is directly proportional to improvement of separation of molecule. In it different factors consider for solvent selection.

**g) Retention**

The solvent strength increases which can strongly impact on retention of analyte. The reverse phase chromatography point of view a higher percentage of organic solvent requires in mobile phase. The organic solvent helps to retain the molecule with fast rate.
h) Poor Separation

Generally analyte elute with its impurities and metabolites. To separate all impurities and metabolites along with molecules, different solvent strength uses from lower strength to higher strength so far best separation may be determined. During method development different types of organic solvent is used for better separation e.g. select acetonitrile instead of methanol. By changing pH of buffers may impact on separation of molecule with considering column recommended pH. After achieving optimum condition of separation, improve resolution by changing column length, particle size to increases the analytical column efficiency e.g, in reversed phase analysis started with C8 100mm column will changes with C18 for better resolution may be observe. Along with this select shorter column in this stage for saving time of analysis.

i) Peak Shape

The peak shape is most important factor in chromatographic separation in method development. In this mainly problem creates for basic nature compounds which analyzed by reverse phase chromatography. The high purity silica phase like Wakosil II is preferred for minimization of potential problems. Due to use of modern phase secondary interactions expected minimal. Generally buffers with proper pH selected for sharp peak shape. After getting proper sharp peak any problem remain continue then use organic modifier such as triethylamine but this type of modifier not necessary in modern silica phases such as Wakosil. The auto sampler temperature impact on peak shape is most important consideration during method development. The auto sampler constant temperature maximizes the reproducibility of method. The recommended temperature for better peak shape and separation is 35 to 40°C.

j) Buffer selection

In reverse phase HPLC, according to hydrophobicity of molecules the retention takes place. If hydrophobicity is longer, retention of molecule is longer. When ionization of molecule takes place proportionally hydrophobicity reduces and it result decreasing retention of molecule. In reverse phase HPLC the mobile phase contains acid and bases therefore controlling pH of mobile phase is necessary for better reproducible results.
When separating acids and bases a buffered mobile phase is recommended to maintain consistent retention and selectivity. A buffered mobile phase, by definition, resists changes in pH so that the analytes and silica will be consistently ionized, resulting in reproducible chromatography. In case of neutral samples, buffers or additives are not required in mobile phase. Generally addition of buffer in mobile phase requires for acids and bases samples. Less acidic reverse phase columns and amine additives in mobile phase are beneficial for basic or cationic samples. When \( pK_a \) of the buffer is equal to pH of molecule it results optimum buffering capacity of molecule.

Buffers play an additional role in the reproducibility of a separation. The buffer salts reduce peak tailing for basic compounds by effectively masking silanols. They also reduce potential ion-exchange interactions with unprotonated silanols (Figure 5). To be most effective, 10 - 50 mM is better buffer concentration range is recommended for most basic compounds.

![Peaking Interactions](image)

**Figure 5: Peak Tailing Interaction**
Table 2: HPLC buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>P^Ka (25°C)</th>
<th>Maximum Buffer Range</th>
<th>UV Cutoff (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate, p^K_1 H_2PO_4</td>
<td>2.1</td>
<td>1.1-3.1</td>
<td>&lt; 200</td>
</tr>
<tr>
<td>Phosphate, p^K_2 HPO_4</td>
<td>7.2</td>
<td>6.2-8.2</td>
<td>&lt; 200</td>
</tr>
<tr>
<td>Phosphate, p^K_3 PO_4^-</td>
<td>12.3</td>
<td>11.3-13.3</td>
<td>&lt; 200</td>
</tr>
<tr>
<td>Citrate, p^K_1 C_3H_5O(COOH)_2(COOH)</td>
<td>3.1</td>
<td>2.1-4.1</td>
<td>230</td>
</tr>
<tr>
<td>Citrate, p^K_2 C_3H_5O(COOH)_2(4COO)</td>
<td>4.7</td>
<td>3.7-5.7</td>
<td>230</td>
</tr>
<tr>
<td>Citrate, p^K_3 C_3H_5O(COO)</td>
<td>6.4</td>
<td>4.4-6.4</td>
<td>230</td>
</tr>
<tr>
<td>Carbonate, p^K_1 HCO_3^-</td>
<td>6.1</td>
<td>5.1-7.1</td>
<td>&lt; 200</td>
</tr>
<tr>
<td>Carbonate, p^K_2 CO_3^-</td>
<td>10.3</td>
<td>9.3-11.3</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>Formate</td>
<td>3.8</td>
<td>2.8-4.8</td>
<td>210</td>
</tr>
<tr>
<td>Acetate</td>
<td>4.8</td>
<td>3.8-5.8</td>
<td>210</td>
</tr>
<tr>
<td>Ammonia</td>
<td>9.3</td>
<td>8.3-10.3</td>
<td>200</td>
</tr>
<tr>
<td>Borate</td>
<td>9.2</td>
<td>8.2-10.2</td>
<td>N/A</td>
</tr>
</tbody>
</table>

k) Selection of pH

The pH range 1 to 8 mostly used in reversed-phase chromatography can be divided into low pH (1 to 4) and intermediate pH (4 to 8) ranges. Each range has a number of advantages. Low pH has the advantage of creating an environment in which method ruggedness is increases with decreases of peak tailing. For this reason, operating at low pH is recommended.

If pH of mobile phase is more than 7 then dissolution of silica takes place which directly shorten lifetime of columns containing silica-based stationary phases.

The pKa value [acid dissociation (ionization) constant] for a compound is the pH at which equal concentrations of the acidic and basic forms of the molecule are present.
in aqueous solutions. Analytes may sometimes appear as broad or tailing peaks when the mobile phase pH is at, or near, their pKa values. The difference between pH and pka of analyte is 1, it consider as more rugged mobile phase. This shifts the equilibrium so that 99% of the sample will be in one form. The result is consistent chromatography.

The pH of mobile phase changes for basic and acidic compounds then the changes observed in retention and selectivity of molecules. This is often a result of different interactions between the column and the analytes when the ionization of these compounds changes. It is important to evaluate these changes when a method is developed in order to select the mobile phase pH that provides the most reproducible results.

**System Suitability Tests for Chromatographic Methods**

System suitability ensure whether system working properly before or during the analysis of unknown samples. In chromatography parameters like resolution, plate count, tailing factors and reproducibility were consider in specific predefined set of injections. In bioanalytical application % RSD, retention time and area for six repetitions are generally determined and compared with acceptance criteria of particular runs. The system suitability sample is contains mixture of analyte, metabolite and internal standard. The general parameters measure in system suitability and their criteria given in Table 3.

**Definition**

The main purpose of system suitability is testing of complete system which includes instrumental parameter, reagents used in analysis, analytical column and performance of analyst.

The USP Chromatography General Chapter states:

“System suitability tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The test is based on
the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such.”

Table 3: System suitability parameters and recommendations

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capacity Factor (k’)</td>
<td>The peak should be well resolved from other peaks and the void volume, generally k’ &gt; 2.0</td>
</tr>
<tr>
<td>Repeatability</td>
<td>RSD ≤ 1% for N &gt; 5 is desirable</td>
</tr>
<tr>
<td>Relative Retention</td>
<td>Not essential as long as the resolution is stated</td>
</tr>
<tr>
<td>Resolution (R_s)</td>
<td>R_s of &gt; 2.0 between the peak of interest and the closest eluting potential interferents (impurity, excipient, degrade product etc.)</td>
</tr>
<tr>
<td>Tailing Factor (T)</td>
<td>T of ≤ 2</td>
</tr>
<tr>
<td>Theoretical Plates (N)</td>
<td>In general should be &gt; 2000</td>
</tr>
</tbody>
</table>

1.3e Criticality

The parameters that are affected by the changes in chromatographic conditions are:

a. Resolution (R_s)

Resolution defines the relation between separation power of complete chromatographic system to the particular component of given mixture.

The ratio of two peak maxima distance is the resolution (R_s) of two neighboring peaks. Also defines the difference between the retention times of two different solutes divided by their average peak width. The ideal value for separation of baseline is 1.5.

The resolution is calculated as follows,

\[ R_s = \frac{R_{t2} - R_{t1}}{0.5 \left( W_1 + W_2 \right)} \]

Where,
Rt₁ and Rt₂ = Retention times of two components

W₁ and W₂ = Peak width of two components.

The basic parameters impact on resolution of chromatographic resolution are given below

- Capacity factor
- Selectivity
- Column efficiency

b. Capacity Factor (k’)

The ratio between the dead volume and reduced retention volume is called as Capacity factor.

Capacity factor is denoting as k’. It is defined as the ratio of the total number of molecules of solute in the stationary phase to the total number of molecules of the same in the mobile phase. During isocratic separation the sample molecule gets separated by analytical column is nothing but the measurement of Capacity factor. 2 to 10 is the ideal value for capacity factor (k’). Capacity factor can be calculated by using following formula

\[ k' = \frac{V_1 - V_0}{V_0} \]

Where,

\[ V_1 = \text{Retention volume at the apex of the peak (solute)} \]

\[ V_0 = \text{Void volume of the system.} \]

If the solvent strength get changed then the values of capacity factor (k’) of individual molecule bands increases or decreases respectively. In water / organic mobile phase of reversed phase chromatography, the volume of organic solvent is increases then organic strength of chromatography increases. Ideally, If 10 % organic volume is increases it will decrease capacity factor of the bands 2 to 3 factor.
Adjusting capacity factor (k’)

The capacity factor with the range 2 to 10 is considered as good isocratic method. The lower value of capacity factor may give inadequate resolution and higher values represents excessively broad peak along with unacceptable longer run time.

The shifting in capacity observed with analyst as well column test solution, then chances of problem due to analytical column change, change in temperature or mobile phase composition. If the shift occurs continuously over series of run then it’s true.

Capacity factor (k’) values are sensitive to following parameters:

- Solvent strength of mobile phase
- Composition of mobile phase
- Purity of solvent
- Temperature of column
- Column chemistry with material used
- Sample mixture.

c. Selectivity (α)

The selectivity is also called as separation factor) and it denoted as α. Selectivity measures difference between two different components in a mixture. It is ratio between capacity factors of both peaks and ratio between two adjusted retention times. Selectivity represents the separation power of particular adsorbent to the mixture of these particular components. The dependent parameters of selectivity are as follows:

- Nature of components in mixture
- Type of eluent
- Composition of eluent
- Surface chemistry of absorbent.

Column efficiency is independent factor. Suppose selectivity of two different components is equal to then there is no way for separation of components by improving the column efficiency. The reference value of selectivity (α) is 2.
It can be determined by using formula given below,

\[ \alpha = \frac{V_2 - V_1}{V_1 - V_0} = \frac{k_1'}{k_2'} \]

Where,

\[ V_0 = \text{The void volume of the column,} \]
\[ V_1 \text{ and } V_2 = \text{The retention volumes of the second and the first peak respectively.} \]

➢ Adjusting selectivity (\( \alpha \))

This approach is similar with the capacity factor when trouble shooting changes in selectivity. The corrective action depends on problem associated with mobile phase and analytical column, when selectivity is affected. Comparison of results obtained with test solution and response at the time of new column is necessary.

Selectivity (\( \alpha \)) values are sensitive to following parameters:

- Changes in mobile phase composition specifically pH change
- Purity of sample and solvents
- Temperature of analytical column.

d. Column Efficiency (\( N \))

Column efficiency is also called as band broadening and it denoted by N. The total no of theoretical plates per meter of a column is called as column efficiency. The measurement of peak band spreading is done by column efficiency. If theoretical plates are higher in number then it indicates system performance and good column similar to band spread. The reference value for column efficiency is 5000 to 100000.

Column efficiency is determined by using the following formula,

\[ N = \frac{16 \text{ Rt}^2}{W^2} \]

Where,

\[ \text{Rt} = \text{Retention time} \]
W = Peak width.

- **Column efficiency may be decline due to**
  - Usage and history of the analytical column.
  - Extra column band broadening
  - Improper detector setting
  - Change in flow rate and solvent viscosity.

If peak width and peak shape affected for all peaks then it recognize the problems in separation due to column efficiency.

- **Methods of measuring column efficiency (N)**

In order with sensitivity to abnormal peak shape for measurement with calculation of column methods are given below:

- Asymmetry based in terms of tailing or fronting
- 5 sigma
- 4 sigma
- Tangent
- sigma
- ½ height
- sigma

Select method as per operating requirements which suits for calculation. It is difficult that continues usage of same method will give executed reproducibly.

### e. Peak asymmetry factor ($A_s$)

Peak asymmetry factor is denoted by $A_s$, peak symmetry can be used as a criteria of column performance. The peak asymmetry factor can be calculated as the peak half width (b) of a peak at 10% of the peak height is divided by the corresponding front half width (a).

The formula for asymmetry factor calculation given below:
A_s = b / a

The ideal asymmetry factor ranges from 0.9 to 1.1 indicates well packed column.
1.4 AIM AND PLAN OF WORK

OBJECTIVE OF THE RESEARCH WORK

1) To develop bioanalytical methods for pharmaceutical industry by using novel techniques for quantification of drug molecules such as liquid chromatography–tandem mass spectrometry. To handle various challenges, novel analytical techniques with automated high-throughput techniques uses. By using this approach in short time more experiments were performed with increased data quality.

2) The developed bioanalytical method should be fully validated for quantifying drug molecules fast, selective and reproducible. Validation experiments included study of matrix effect, anticoagulant effect from different biological matrixes lots, precision and accuracy, selectivity/specificity, sensitivity metabolite interferences and various stability tests such as freeze thaw stability, stock solution stability, and bench top stability.

3) Febuxostat is categorised as a xanthine oxidase inhibitor. On comprehensive literature review of febuxostat, it was observed that there was not a single analytical or bioanalytical method reported for estimation of febuxostat. No stability indicating assay method is published to reveal specificity and degradation study. Due to less number of availability of bioanalytical method until now for determination of febuxostat from biological matrix form made it worthwhile objective to develop a precise method using LC-MS/MS for the determination of febuxostat in human plasma form according to regulatory guidelines.

4) Clebopride is a substituted benzamide with prokinetic and antiemetic properties. On exhaustive literature review of clebopride, it was observed that there was few bioanalytical method reported for quantification of clebopride from plasma, which was found time consuming and costly. Therefore, the aim of present work was to develop simple isocratic bioanalytical method to estimate clebopride from human plasma with due consideration of accuracy, sensitivity, rapidity, economy, selectivity, stability according to regulatory guidelines.

5) Darifenacin is antimuscarinics drug. On complete literature review of darifenacin,
it was observed that there was few bioanalytical method reported for determination of
darifenacin from human, which was found time consuming and costly. Therefore, the
aim of present work is to develop simple isocratic bioanalytical method for
determination of darifenacin from human plasma with due consideration of accuracy,
sensitivity, rapidity, economy, selectivity, stability according to regulatory
guidelines.

6) Cycloserine is anti tubercular antibiotic. On comprehensive literature review of
cycloserine, it was observed that there was few bioanalytical method reported for
quantification of cycloserine from plasma, which was found time consuming and
costly. The non-availability of bioanalytical method until now for determination of
cycloserine from biological matrix form made it worthwhile aim to establish a
precise method using tandem mass spectrometry with HPLC (LC/MS-MS) for the
quantitation of cycloserine in plasma form according to regulatory guidelines.

7) Carbocisteine is mucoactive drug. On exhaustive literature review of
carbocisteine, it was observed that there was few bioanalytical method reported for
quantification of carbocisteine from plasma, which was found time consuming and
costly. The non-availability of bioanalytical method until now for determination of
carbocisteine from biological matrix form made it worthwhile aim to found a precise
method using tandem mass spectrometry with HPLC (LC/MS-MS) for the
quantitation of carbocisteine in plasma form according to regulatory guidelines.

WORK PLAN AND METHODOLOGY

1 Literature review
To perform literature survey of pharmaceutical drugs physicochemical properties,
dosage forms, dose strengths and pharmacokinetic profile of interested drug
molecules. Also search method for pharmaceutical drugs in published journals
regulatory guidelines.

2 Solubility of drug
Determine the solubility of pharmaceutical drugs in different solvent, pH and pKa of
the selected drug molecules.
3 Instrumental parameter optimization
During the development of analytical method different instrumental parameters will be optimized. Information on Drug molecular weight, physicochemical properties etc. Mass tuning of molecule will be done for determination of Q1 and Q3 values for drug and internal standard.

4 Optimization of Chromatography
Chromatographic procedure includes selection of Column, compositions of solvents for separation and mobile phase, flow rate.

5 Optimization of Extraction procedures
Separation of drug from biological matrix by using extraction techniques like
  ➢ Precipitation method
  ➢ Liquid-Liquid Extraction
  ➢ Solid phase extraction

6 Validation of the developed methods
Different regulatory systems are designed with different requirements of bioanalytical method validation (BMV) e.g. USFDA, ANVISA, WHO, MHRA. Bioanalytical validation parameters are as given as selectivity, sensitivity, calibration range, linearity, carryover check, accuracy, precision, extraction efficiency (Recovery), dilution integrity and reproducibility.

The developed method may be employed for further research in pharmaceutical field. They should be greatly useful for routine drug analysis of same or any of the pharmacological class.