INTRODUCTION

Today safety and quality are the important aspect in drug industry and also to control these impurities as per regulatory guidelines. The unwanted chemicals that remains has an impurities in API, drug product, the impurities developed during stability due to degradation and produced during formulation. These impurities present in drug substance causes the safety concern and also impacts on the drug quality. Therefore it is essential to develop new analytical methods to identify and quantify these impurities for these drugs.

HPLC method development

The steps involve in method development are

Literature collection:

- Search for literature for molecules having similar structures in the public domain (pharmacopeia, forum-USP, EP, chromatography journals, patents, the internet, etc.).
- Check the suitability of the method to meet the requirements or modify the way to suit the needs.
- Based on the synthetic process, all possible impurities are to be considered as process impurities, degradents and intermediates.
- Each stage possible impurities, sample collection and standard.
- Useful literature search websites are the Rx list, Science Direct, Scirus, Scopus, Springerlink, Scifinder, Nature, and Dekker.

Chemical structure:

- Depending upon the functional group present in the molecule, the selection of mobile phase can be chosen (i.e acidic, basic and neutral). If the compound is acidic, an acidic mobile phase is preferable. For neutral compounds, a neutral mobile phase can be use (Snyder 2006).
Molecular weight:

If molecular mass is less than 500 depending upon the molecule nature and solubility can select the technique for acidic, basic, and non-ionic samples.

- Ion pair chromatography of ionic samples
- Ion exchange chromatography of ionic samples
- Normal phase chromatography for isomers, nonionic and chiral molecules
- Size exclusion chromatography of protein related samples.
- Capillary electrophoresis for redox samples.

pKa value of compounds:

- The polarity of the molecule can be presumed based on pH or pKa values the nature of the compounds.
- Selection of the proper buffer to separate reproducibly ionizable compounds. For ionizable compounds often leads to asymmetric peaks that are broad, tail, split or shoulder when selecting an improper pH (Vibha Gupta 2012).

Solubility:

- Check the solubility of all components in the solutions like mobile phase, mobile phase organic mixtures, water-organic mixtures, and mixtures of acids like phosphoric acid, trifluoroacetic acid, formic acid and acetic acid, etc (Skoog DA 1988).

Column selection:

- Out of various commercial columns available, a column can be chosen by considering the quality of the column with respect to the batch to batch and lot to lot reproducibility.
- In reverse phase analysis, the mobile phase is highly polar, and the column is non-polar. Under these conditions C8, C18, phenyl, cyano and amino columns can be used (Phani R.S.Ch 2012).
• Phenyl phases also are non-polar, retention is due to hydrophobic and \( \pi-\pi \) interactions. The total hydrophobic retention of it is similar to that of a C8 bonded phase, but unique selectivity results in its \( \pi \) orbital interaction with analyte electron deficient functional groups. Phenyl phases also are non-polar, retention is a joint mechanism of hydrophobic and \( \pi-\pi \) interactions (Prathap B 2013).

• Cyano phases have intermediate polarity. The retention is due to hydrophobic, dipole-dipole and \( \pi-\pi \) interactions. These stages are used for analyzing polar organic compounds, and they are versatile enough for use in both normal and reversed phase modes.
Amino phases are polar phases that can be utilized in both normal and ion exchange methods. Retention is due to dipole-dipole or acid-base interactions. Amino phases commonly are used for carbohydrate analysis; sometimes can be used for analyzing both organic and inorganic ions.

Column parameters like internal diameter, particle surface area, volume and size of pore also to be checked (Patel RM 2011).

- Particle shape: Particles may be spherical or irregular. High surface area and high carbon loading can be seen in irregular particles. High efficiency and column stability is provided by spherical particles.
- Particle size: Column packing refers to the average diameters of the packing particles. It affects the column pressure and the resolution efficiency. Efficiency and column back pressure can be increased by decreasing the particle size.

High surface areas provide greater retention capacity and resolution for separation complex multi-component samples. Pore size and surface area are inversely related. A packing material with a small pore size will have a large surface area and vice versa.
• Pore size: the pore size of a packing material indicate the average size of the pores within each particle. Large pores allow larger solute molecules to be retained through maximum exposure to the surface area of the particles.

• Pore volume: Mechanical strength indicates the strength of the pour volume. The small pore volume is stronger than the large pore volumes. The recommendation in HPLC for pour volume is 1.0 mL/g. If the size of the pour volume is more, it is generally used in exclusion chromatography for low pressure methods.

• Bonding type: Bonding type is attachment mode of each bonded phase strand to the base silica. Monomeric phases have single point attachments of bonded phase molecules and provide faster equilibration and higher column efficiency (Dorsey G 1994).

Fig.1.8

Polymeric phases have multiple point attachments of bonded phase molecules. Polymeric bonding offers increased column stability, particularly when used with highly aqueous mobile phases.

Fig.1.9
• Carbon load: the carbon load is the amount of bonded phase attached to the base material, expressed as the percentage of carbon. High carbon loads offer greater resolution and longer run times for hydrophobic samples, small carbon loads offer to shorten run times and often show different selectivity (Azim Md 2013).

Fig.1.10

• End capping: end capping is the capping of exposed silanols with short hydrocarbon chains after the primary bonding step, it reduces peak tailing of polar solutes that interact excessively with the otherwise exposed silanols. Nonend capped packings provide a different selectivity than that of end capped packing, especially for polar samples.

Fig.1.11
Detector selection: Suitable detection technique can be selected based on the structure and nature of the molecule. UV and Fluorescence detectors used for compounds having chromophores and fluorophores. Electrochemical detectors used for readily oxidizable compounds. RI detectors are universal and these detector can’t be suitable for gradient elution and used for non-chromosphere compounds. Whereas ELSD can be used in gradient programme for non-chromosphere compounds for high sensitivity.

Mobile phase selection: the separation can be achieved in reversed phase by changing the solvent (methanol, acetonitrile, THF, etc.), column (C8, C18, cyano, phenyl, amino, etc.) and buffer (phosphate, acetate, perchlorate, etc.). Column parameters like internal diameter, particle surface, pore size, and pore volume also to be checked. For ionic samples prepare the ion pair reagents like (sodium dodecyl sulfate, Sodium 1-Heptane sulfonate) of 0.001%-0.1% concentration to get the better separation in mobile phase. Tetra butyl ammonium hydrogen sulfate, tetramethyl ammonium bromide, sodium perchlorates, etc. can be used in the mobile phase for acidic samples. Water, methanol, acetonitrile, tetrahydrofuran and isopropyl alcohol solvent can be used for reverse phase analysis( Mccown SM 1986, Lindholm J 2004) . Organic modifiers like triethylamine, diethylamine, trifluoroacetic acid, etc. can be used.
During the separation of acids and bases by reverse phase HPLC, pH of the mobile phase needs to be controlled by appropriate buffer. For molecules that do not contain any ionic functional group, pH control is not required. Selection of buffer pH depends on the pKa of the functional group present in the molecule when the analyte is ionized the hydrophobicity decrease, and the retention will decrease (Kaushal C 2010).

![Fig.1.13](image1.png)

The resolution was impacted by pH. The acidic compound prefers acidic mobile phase due to unionized form and will be retained in the column maximum time. In acidic mobile phase base compounds elution will be fast with better peak shape (Chatwal GR 2004). In basic mobile phase, the presence of active silanols peaks may have tailing. For neutral compounds have better peak and resolution in mobile phase at neutral pH.

![Fig.1.14](image2.png)
In reverse phase HPLC, the retention of the analyte is related to their hydrophobicity. Higher is hydrophobic; the longer is the retention time. Mobile phase pH should be different from the pKa by at least ±1.5 units (Kaushal C 2010).

![Retention Factor versus pH for Acids, Bases, and Neutrals](image)

**Fig.1.15**

In mobile phase buffer imparts the ionic strength constant. In reverse phase chromatography it is better to use buffers in the aqueous portion of mobile phase (Ranjit Singh 2013). Ruggedness can be increased by the buffer. Buffer strength of about 10 to 25 mM for initial experiments is desirable.

**Figure 2. Effect of pH Control on Separation of Ionizable Compounds**

- **A:** pH 3.5, buffered
- **B:** pH 7.0, buffered
- **C:** pH 7.0, not buffered

**Sample:**
1. Benzoic Acid
2. Sorbic Acid

![Graph of pH Control on Separation of Ionizable Compounds](image)

**Fig.1.16**
The organic modifiers first choice is ACN and methanol, due to low UV cutoff and low viscosity of ACN is the best choice. The alternate choice for acetonitrile and methanol is isopropanol and tetrahydrofuran. Mobile phase of tetrahydrofuran is not stable (Scoott RPW 2003).

Degradation Studies: Preliminary degradation studies should be carried out after getting a satisfied set of analytical conditions. Forcibly degraded sample analyzed under physical and chemical degradation studies. APIs depending on the nature of the functional groups present in them are susceptible to degradation under various conditions such as moisture, acid hydrolysis, alkali hydrolysis, temperature, light, and oxidation (Allwood M 1986, Alsante KM 2003, Aneesh TP 2012, Ranjit Singh 2012, Reynolds DW 2002. The potential degradents are generated under stress condition, it may or may not generate in stability condition. (Baertschi SW 2006, Boccardi G 2005, Charde MS 2013, Gupta A 2011, ICH Q1B 2006, Kishore Kumar Hotha 2013, Klick S 2005, Kovarikova P 2004, Ngwa G 2010).
Method Validation

Method validation is the procedure of defining an analytical prerequisite and approving that the method under consideration has performance abilities constant with what the application needs. As per ICH “Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use” (FDA guidance for industry 2000).

Validation is necessary if:

- New method developed for specific delinquent
- Established method was brushed up to include enhancements or prolonged to a new problem
- Quality control lab shows a recognized method is varying with time.
- To determine the equivalency of two methods, e.g. a new method and a standard.

Furthermore, revalidation may be essential in the following conditions:

- Modification of the process adopted to produce the drug substances
- Alteration in the configuration or mixture ratio of components in the finished product.
- Any change in the analytical procedure and parameters.

The analytical methods need to be validated and checked for following parameters in harmony with ICH Harmonized Tripartite Guidelines

- System suitability Parameter test
- Specificity
- Method Precision
- Intermediate Precision
- Linearity
- Range
- Accuracy
- Limit of Detection
- Limit of Quantification
- Robustness
**Objective of research work:**

It is essential to maintain a high quality of drugs that are necessary to treat various diseases. There is a need to develop newer, efficient and economical analytical methods for the estimation of impurities in different drugs. Hence, an attempt has been made to quantify the impurities using HPLC technique. The Results of the Investigation are presented in the Thesis.

The author focused the work on the Development of New RP-HPLC methods for the Quantification of the impurities of chosen drugs. The Work also includes the Validation of the proposed method according to ICH guidelines. The List of the Drugs and their profile selected for the Research work are presented in Table 1.1 and Structures are shown in Table 1.2

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Drug Name</th>
<th>Therapeutic Category</th>
<th>IUPAC Name</th>
<th>Molecular formulae and mass</th>
</tr>
</thead>
</table>
| 1       | Dabigatran | Anticoagulant        | “(ethyl (Z)-3-(2-(((4-((N-((hexyloxy) carboximido)
phenyl) amino) methyl)-1-methyl-N-(pyridine-2-yl)-
1H-benzo[d] imidazole-5-carboxamido) propanoate
methanesulfonate)” | C$_{34}$H$_{41}$N$_{7}$O$_{5}$ 627.734 |
| 2       | Ezogabine  | Anticonvulsant       | “(ethyl N-[2-amino-4-[(4-fluorophenyl)
methy lamino] phenyl] carbamate” | C$_{16}$H$_{18}$FN$_{3}$O$_{2}$ 303.331 |
<p>| 3       | Ivacaftor  | Respiratory Agent    | “(N-(2, 4-DI-tert-buty l-5-hydroxyphenyl) -4-Oxo- | C$<em>{24}$H$</em>{28}$N$<em>{3}$O$</em>{3}$ 392.490 |</p>
<table>
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<tr>
<th></th>
<th>Drug Name</th>
<th>Category</th>
<th>Chemical Structure and Molecular Formula</th>
<th>Molecular Weight</th>
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<tbody>
<tr>
<td>4</td>
<td>Fingolimod</td>
<td>Immunomodulating Drug</td>
<td>“(2-amino-2-(4-octylphenethyl) propane-1,3-diol hydrochloride)”</td>
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<tr>
<td>5</td>
<td>Raltegravir</td>
<td>Antiretroviral Agent</td>
<td>“(N-[(4-Fluorophenyl)methyl]-1,6-dihydro-5hydroxy-1-methyl-2-[1-methyl-1-[[5-methyl-1,3,4-oxadiazol-2-yl] carbonyl] amino] ethyl] - 6-Oxo-4 pyrimidine carboxamide monopotassium salt)”</td>
<td>C_{20}H_{21}FN_{6}O_{5} 444.42</td>
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<tr>
<td>6</td>
<td>Varenicline</td>
<td>Smoking Cessation</td>
<td>“(7,8,9,10-Tetrahydro-6,10-methano-6H-pyrazino [2,3-h][3] benzazepine)”</td>
<td>C_{13}H_{13}N_{3} 211.267</td>
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