INTRODUCTION:
The great innovation was discovered by Michael Tswett in 1906. He was working on separation of green pigment from green leaves of the plant. He used chalk column to determine this colour pigment so described term “chromatography” used by Tswett to describe the colour which comes down from column. Nowadays chromatography in industries is driven by chemistry as well as all types of instrumentation. Chemistry vastly used for developing instrumental method via their chemical appearance, physical properties. At the time of chromatographic development molecular structure, pH, pKa consideration in method determination. Chemistry of column also important in chromatography for method establishment.

Analytical chemistry is concerned with the theory and practice of methods used to determine the composition of matter. There are many new techniques available today for application to analytical problems, and for this reason the analytical chemist needs to have a good knowledge of a number of scientific fields. To analytical chemists, developing methods is the challenging part of research. Analytical methods are required for a variety of reasons during drug development projects.

Pharmaceutical industry is a growing industry and the growth is driven by the type of drug discovery and developments. Basic expectation of organization is to provide the high quality product with low cost. As the medicines are given to the patients for mitigation of symptoms or disease, this industry is controlled by the Government Authorities of respective countries e.g. Drug Controller General of India (DCGI), United State Food and Drug Administration (USFDA), Medicines and Health Care Product Regulating Agency (MHRA) etc. These regulatory authorities ensure that the Safety, Identity, Strength, Purity and Quality (SISPQ) are assured throughout the life cycle of the product. Quality of the drug products has become the focus of both industry and regulatory authorities. Earlier days regulatory authorities were evaluating the product quality based on the analytical testing performed on drug substance and drug product. Now the industry is undergoing a shift i.e. Evaluation of Product quality not only by testing final product but to ensure the SISPQ during all stages of manufacturing. However, still it is expected to ensure the product quality by performing the analytical testing before the product is released into market.

There are different analytical techniques like Chromatography (HPLC, GC, TLC etc), Potentiometer, XRD, DSC, dissolution etc. to evaluate the quality of drug product and
drug substance. The regulatory agency expects that the test methods used should be validated and documented to ensure the accuracy, sensitivity, specificity and reproducibility.

As the drug product and drug substance undergoes physical and chemical changes and generates degradation impurities, decrease in assay potency changes in dissolution profiles, etc. based on this each product has a specified shelf life based on the stability data generated for each product. In the literature, there is no method for the rapid separation of impurities of many bulk drugs i.e. active pharmaceutical ingredients (API’S) by using normal phase and reverse phase high performance liquid chromatography. Normal-phase chromatography is the most popular mode of liquid chromatography at present. Briefly, the major disadvantages of normal phase HPLC lie in the highly non-polar nature of the mobile phase, the possibility of column inactivation by water, contamination by polar compounds and lower potential in terms of selectivity . Normal-phase chromatography is the most popular mode of liquid chromatography at present. Briefly, the major disadvantages of normal phase HPLC lie in the highly non-polar nature of the mobile phase, the possibility of column inactivation by water, contamination by polar compounds and lower potential in terms of selectivity.

**PRINCIPLE OF HPLC:**

Chromatography is a separation process in which physical means are used to distribute sample components between a stationary and a moving as a mobile phase. The process occurs as a result of many sorption-desorption steps during the movement of sample components through the stationary phase. Separation is due to differences in the distribution coefficients of the sample components.

HPLC is defined as High Performance Liquid Chromatography or High Pressure Liquid Chromatography. In HPLC, separations are achieved by partition, adsorption or ion exchange depending on the stationary phase.

In chromatography there are different Methods available for separation of components are given as follows,

- **Type-1 – Adsorption-chromatography**
Discrption of liquid high pressure or performance chromatography

Separation of different compounds is nothing but chromatography, to separate out component from a mixture by using the combination of solvents with different types of liquid buffers. In liquid chromatography, the mobile phase is pumped through a column containing a stationary phase under high pressure. As a result of significant development during the past two decades, which have brought about significant improvements in instrumentation and column packing, HPLC has become useful not only in quantitative but also in qualitative types of analysis.

This ability to separate plays an important role in medicinal and biological chemistry. The technique offers less time for analysis with perfect accuracy and is useful in any determination of analysis. Modern HPLC techniques became available in the 1960s, but from the 1990s, HPLC became the most popular instrument for drug analysis, which is presently used in pharmaceutical research and development;

- Chromatography is useful in natural product and synthetic molecules.
- Metabolites characterization also carried out.
- Used to determine assay, related impurities in the sample.
- It is also in pharmacodynamic and different pharmacokinetic study.

Recent improvements in HPLC deal with:

- Changing particle size, packing material, different types of silica with lower size.
- Separation technique with high speed.
- HPLC assets with computer to operate automatically.
- I.e. hyphenated detection systems.

**BLOCK DIAGRAM OF HPLC:**
An HPLC system consists of a pumping unit, sample-injection unit, separation unit, detection unit, and data-processing unit. Each of these units is essential for performing the analysis.

There are mainly two types of chromatographic phases

A Normal Phase:

In this chromatography, the stationary phase is polar and the mobile phase is Non-polar.

B Reverse Phase:

In this chromatography, the stationary phase is non-polar and the mobile phase is polar.

Bonded stationary phases for HPLC

<table>
<thead>
<tr>
<th>Stationary Phase</th>
<th>Functional groups</th>
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<tbody>
<tr>
<td>Silica</td>
<td>Si-OH</td>
</tr>
<tr>
<td>C₁₈</td>
<td>Octadecyl</td>
</tr>
<tr>
<td>C₈</td>
<td>Octyl</td>
</tr>
<tr>
<td>C₆H₅</td>
<td>Phenyl</td>
</tr>
<tr>
<td>CN</td>
<td>Cyano</td>
</tr>
<tr>
<td>OH</td>
<td>Diol</td>
</tr>
<tr>
<td>SCX</td>
<td>Sulphonic acid</td>
</tr>
<tr>
<td>SAX</td>
<td>Quaternary ammonium</td>
</tr>
</tbody>
</table>
Mobile phase:

Mixtures of water or aqueous buffers and organic solvents are used to elute analytes from a reversed-phase column. The solvents must be miscible with water, and the most common organic solvents used are acetonitrile, methanol, and tetrahydrofuran (THF). The pH of the mobile phase can have an important role on the retention of an analyte and can change the selectivity of certain analytes. For normal phase, n hexane, isopropanol, ethanol etc are used as a solvent.

Columns and column efficiency:

Any inert non-polar substance that achieves sufficient packing can be used for reversed-phase chromatography. The most popular column is an octadecyl carbon chain (C18)-bonded silica (USP classification L1) with 297 columns commercially available. This is followed by C8-bonded silica (L7 - 166 columns), pure silica (L3 - 88 columns), cyano-bonded silica (L10 - 73 columns) and phenyl-bonded silica (L11 - 72 columns). Note that C18, C8 and phenyl are dedicated reversed-phase resins, while cyano columns can be used in a reversed-phase mode depending on analyte and mobile phase conditions. It should be noted at this point that not all C18 columns have identical retention properties. Surface functionalization of silica can be performed in a monomeric or a polymeric reaction with different short-chain organosilanes used in a second step to cover remaining silanol groups (end-capping). While the overall retention mechanism remains the same, subtle differences in the surface chemistries of different stationary phases will lead to changes in selectivity.

Modern columns have different polarity. PFP is pentafluorphenyl. CN is cyano. NH2 is amino. ODS is octadecylsilane or C18. ODCN is a mixed mode column consisting of C18 and nitrile. SCX is strong cationic exchange. SAX is strong anionic exchange.

HPLC pumps:

It is necessary to pump the eluent at a constant flow rate and pressure. Conventional, analytical HPLC pumps are the most common type, but semi-micro and a preparative pumps are also used depending on the range of the eluent flow rate required. The pump is selected to suit the
purpose of the analysis. Analyses were first performed using isocratic separations in which the eluent composition remains unchanged during the analysis. This technique is adequate for simple separations. When a sample contains many components, such as a sample for amino-acid analysis is analyzed, it is very difficult to separate all of the components effectively using only one eluent.

A gradient analysis allows the composition of the eluent to be changed during the analysis. This often indicates that the concentration gradient is to be generated in a linear manner. However, if the eluent composition is changed in a stepwise fashion, this is called a step gradient.

Two mixing methods are available: low-pressure and high-pressure. **Low-pressure mixing method:** The eluent to be absorbed is switched via electromagnetic valves. One pump is used for mixing. System price is lower. Up to four eluents can be mixed. **High-pressure mixing method:** Two pumps are used. The eluents are mixed after pumping. The response of the gradient is superior because of the small volume from the mixing unit to the column. System price is higher due to the increase in the number of pumps.

**Injector for HPLC:**

A sample is injected into the flow path for analysis. This is accomplished via a manual injector or an auto sampler. Each type is equipped with six-port valves, so that a sample can be injected into the flow path at continuous pressure. For the manual injector, the knob is manually operated to deliver the sample to the column, as shown in Figure 3.

**HPLC Detectors:**

The components eluted from the column are detected, and the detection data are converted into an electrical signal. The detector is selected to suit the sample.
Major types of detector

**UV DETECTOR:**
The light source is a D2 lamp. This detector is used mainly to detect components having an absorption wavelength of 400 nm or less in the ultraviolet region.

**UV–VISIBLE DETECTOR:**
A D2 lamp and a W lamp are used as the light source. This detector is effective in the detection of coloring components such as dyes and stains because of coverage of the visible light region.

**DIODE ARRAY DETECTOR (DAD):**
Data on the spectrum from the ultraviolet to visible light range is also collected.

**FLUORESCENCE (FL) DETECTOR:**
Fluorescent substances can be detected specifically with high sensitivity.

**DIFFERENTIAL REFRACTIVE INDEX (RI) DETECTOR:**
Change in the refractive index is detected. Components absorbing no ultraviolet light can also be detected despite low sensitivity.

**CONDUCTIVITY DETECTOR:**
Mainly inorganic ions are detected by monitoring the conductivity.

The electrochemical detector (ECD), evaporative light scattering detector (ELSD), Corona® Charged Aerosol Detector (CAD), and others are also used. In addition, the LC-MS system, in which the components separated by HPLC are further analyzed using a mass spectrometer, is becoming widely used because of its high sensitivity and the possibility of specific detection.