INTERNATIONAL JOURNAL OF PHARMACY AND ANALYTICAL RESEARCH

ISSN:2320-2831

***IJPAR |Vol.6 | Issue 3 | July - Sep -2017***
***Journal Home page: www.ijpar.com***

***Review article*** ***Open Access***

# HPLC and LCMS – A review and a recent update

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## ABSTRACT

Chromatography is defined as a set of techniques which is used for the separation of constituents in a mixture. This technique involves 2 phases stationary and mobile phases. The separation of constituents is based on the difference between partition coefficients of the two phases. The chromatography term is derived from the greek words namely chroma (colour) and graphein (to write). The chromatography is very popular technique and it is mostly used analytically. There are different types of chromatographic techniques namely Paper Chromatography, Gas Chromatography, Liquid Chromatography, Thin Layer Chromatography (TLC), Ion exchange Chromatography and lastly High Performance Liquid Chromatography (HPLC). This review mainly focuses on the HPLC technique its principle, types, instrumentation and applications. Liquid Chromatography/Mass Spectrometry (LC/MS) is fast developing and it's the preferred tool of liquid chromatographers. Liquid chromatography-mass spectrometry (LC-MS/MS) is a technique that uses liquid chromatography (or HPLC) with the mass spectrometry. It is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. (LC-MS/MS) is commonly used in laboratories for the qualitative and quantitative analysis of drug substances, drug products and biological samples. It has been persistently used in drug development at many different stages including Metabolic Stability Screening, Metabolite Identification as well as In Vivo Drug Screening, Impurity Identification, Peptide Mapping, Glycoprotein Mapping, Natural Products De replication, Bio-affinity Screening. LC-MS is now successfully applied to routine analysis in many areas, including therapeutic drug monitoring (TDM), clinical and forensic toxicology as well as doping control. This advancement in LCMS was originally and still is fueled by the need for more powerful analytical and bio-analytical techniques that can accurately and precisely discriminate target analytes from high complexity mixtures in a sensitive and selective way. With recent advancement in instrumentation, the use of liquid chromatography (LC) and mass spectrometry (MS) has become a powerful two-dimensional (2D) hyphenated technology.

**Keywords:** LCMS, HPLC, Peptide Mapping, Glycoprotein Mapping, Therapeutic Drug Monitoring (TDM), Forensic Toxicology, 2D Hyphenated Technology, Chromatography, Mobile phase, Stationary phase.

# HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

## Introduction

High Performance Liquid Chromatography which is also known as High Pressure Liquid Chromatography. It is a popular analytical technique used for the separation, identification and quantification of each constituent of mixture. HPLC is an advanced technique of column liquid chromatography. The solvent usually flows through column with the help of gravity but in HPLC technique the solvent will be forced under high pressures upto 400 atmospheres so that sample can be separated into different constituents with the help of difference in relative affinities [1-7]. In HPLC, pumps will be used to pass pressurized liquid solvent including the sample mixture which is allowed to enter into a column filled with solid adsorbent material. The interaction of each sample component will be varies and this causes difference in flow rates of each component and finally leads to separation of components of column. Chromatography can be depicted as a mass exchange process including adsorption. HPLC depends on pumps to pass a pressurized fluid and an example blend through a section loaded with adsorbent, prompting the partition of the specimen segments. The dynamic segment of the section, the adsorbent, is regularly a granular material made of solid particles (e.g. silica, polymers, etc.) 2 µm to 50 µm in size. The segments of the example mixture/blend are isolated from each other because of their distinctive degrees of connection with the retentive particles. The pressurized fluid is commonly a blend of solvents (e.g. water, acetonitrile and/or methanol) and is known as 'mobile phase'. Its organization and temperature plays an important part in the partition procedure by affecting the connections occurring between sample segments and adsorbent [8-15]. HPLC is recognized from traditional ("low weight") liquid chromatography because operational pressures are fundamentally higher (50 bar to 350 bar), while normal liquid chromatography regularly depends on the power of gravity to pass the portable stage through the segment. Because of the small sample amount isolated in scientific HPLC, column section measurements are 2.1 mm to 4.6 mm distance across, and 30 mm to 250 mm length. Additionally, HPLC segments are made with smaller sorbent particles (2 µm to 50 µm in normal molecule size). This gives HPLC high determining or resolving power (the capacity to recognize components) while isolating mixtures, which makes it a prominent chromatographic method [16-25].

# HISTORY

Preceding HPLC researchers utilized standard liquid chromatographic methods. Liquid chromatographic systems were to an inefficient because of the flow rate of solvents being reliant on gravity. Separations took numerous hours, and some of the time days to finish. Gas chromatography (GC) at the time was more effective than liquid chromatography (LC), in any case, it was trusted that gas stage partition and investigation of extremely polar high atomic weight biopolymers was impossible. GC was ineffectual for some organic chemists due to the thermal instability of the solutes. Accordingly, alternative techniques were hypothesized which would soon bring about the advancement of HPLC. Taking after on the original work of Martin and Synge in 1941, it was anticipated by Cal Giddings, Josef Huber, and others in the 1960s that LC could be worked in the high-proficiency mode by decreasing the pressing molecule measurement generously beneath the run of the mill LC (and GC) level of 150 µm and utilizing pressure to expand the versatile stage velocity. These expectations experienced broad experimentation and refinement all through the 60s into the 70s. Early developmental exploration started to enhance LC particles, and the innovation of Zipax, an externally permeable molecule, was promising for HPLC technology. The 1970s achieved numerous advancements in equipment and instrumentation. Specialists started utilizing pumps and injectors to make a simple configuration of a HPLC system. Gas amplifier pumps were perfect since they worked at consistent pressure and did not require release free seals or check valves for steady flow and great quantitation. While instrumentational advancements were important, the historical backdrop of HPLC is principally about the history and development of molecule technology. After the presentation of permeable layer particles, there has been a steady pattern to reduced molecule size to enhance efficiency. However, by decreasing molecule size new issues arrived. The disadvantage

from the unnecessary pressure drop is expected to drive versatile liquid through the segment and the trouble of setting up a uniform pressing of to a great degree fine materials. Every time molecule size is diminished altogether, another round of instrument advancement normally should occur to handle the pressure.

## OPERATION

The sample blend to be isolated and dissected is presented, in a discrete little volume (commonly microliters), into the stream of mobile phase permeating through the column. The segments of the sample travel through the segment at various speeds, which are a component of particular physical connections with the adsorbent (likewise called stationary stage). The velocity of every component relies on upon its compound nature, composition of mobile phase. The time at which a particular analyte elutes (rises up out of the column) is called its retention time. The retention time measured under specific conditions is a distinguishing normal for a given analyte [26-36]. Various sorts of columns are available, loaded with adsorbents varying in molecule size, and in the nature of their surface ("surface science"). The utilization of small molecule size packing materials requires the utilization of higher operational pressure ("backpressure") and regularly enhances chromatographic resolution (i.e. the degree of division between sequential analytes rising up out of the column). Sorbent particles might be hydrophobic or polar in nature. Basic mobile phases utilized incorporate any miscible mixture of water with different natural solvents (the most widely recognized are acetonitrile and methanol). Some HPLC systems use without water mobile phases. The aqueous segment of the mobile phase may contain acids, (for example, formic, phosphoric or trifluoroacetic corrosive) or salts to help with the seperation of the sample components. The composition of the mobile phase might be kept constant ("isocratic elution mode") or changed ("inclination elution mode") during the chromatographic examination. Isocratic elution is normally successful in the partition of sample components that are not altogether different in their proclivity for the stationary stage. In gradient elution the organization of the mobile phase is fluctuated ordinarily from low to high eluting quality. The eluting quality of the mobile phase is reflected by analyte maintenance times with high eluting quality delivering quick elution. The selected structure of the mobile phase (additionally called eluent) relies on upon the force of connections between different example parts ("analytes") and stationary stage (e.g. hydrophobic connections in turned around stage HPLC). Dependent upon their partiality for the stationary and mobile stages analytes partition between the two. During the detachment procedure occurring in the sample. This procedure is like what happens amid a liquid–liquid extraction however is continuous, not step-wise. In this case, utilizing a water/acetonitrile angle, more hydrophobic parts will elute (fall off the column) late, once the mobile stage gets more packed in acetonitrile (i.e. in a versatile period of higher eluting quality) [37-45].

## INSTRUMENTATION

The HPLC instrumentation involves pump, injector, column, detector, integrator and display system. In the column the separation occurs. The parts include:

- Solvent Reservoir: The contents of mobile phase are present in glass container. In HPLC the mobile phase or solvent is a mixture of polar and non-polar liquid components. Depending on the composition of sample, the polar and non-polar solvents will be varied.
- Pump: The pump suctions the mobile phase from solvent reservoir and forces it to column and then passes to detector. 42000 KPa is the operating pressure of the pump. This operating pressure depends on column dimensions, particle size, flow rate and composition of mobile phase.
- Sample Injector: The injector can be a solitary infusion or a computerized infusion framework. An injector for a HPLC framework should give infusion of the fluid specimen inside the scope of 0.1 mL to 100 mL of volume with high reproducibility and under high pressure (up to 4000 psi).  Columns: Columns are typically made of cleaned stainless steel, are somewhere around 50 mm and 300 mm long and have an inward distance across of somewhere around 2 and 5 mm. They are generally loaded with a stationary phase with a molecule size of 3 µm to 10 µm. Columns with inner diameters of <2 mm are regularly alluded to as microbore segments.

Preferably the temperature of the mobile phase and the column should be kept consistent during investigation.

- Detector: The HPLC detector, situated toward the end of the column distinguishes the analytes as they elute from the chromatographic column. Regularly utilized detectors are UV-spectroscopy, fluorescence, massspectrometric and electrochemical identifiers.
- Data Collection Devices or Integrator: Signals from the detector might be gathered on graph recorders or electronic integrators that fluctuate in many-sided quality and in their capacity to process, store and reprocess chromatographic information. The PC coordinates the reaction of the indicator to every part and places it into a chromatograph that is anything but difficult to interpret.

The schematic representation of a HPLC instrument ordinarily incorporates a sampler, pumps, and a locator. The sampler brings the sample into the mobile phase stream which conveys it into the column. The pumps convey the mobile phase through the column. The detector generates a sign relative to the measure of sample component rising up out of the segment, consequently taking into consideration quantitative investigation of the example parts. A computerized microchip and software control the HPLC instrument and give information data. A few models of mechanical pumps in a HPLC instrument can combine numerous solvents in proportions changing in time, producing a sythesis slope in the portable stage. Most HPLC instruments likewise have a column broiler that considers altering the temperature at which the partition is performed [46-53].

## TYPES OF HPLC

Depending on the substrate used i.e. stationary phase used, the HPLC is divided into following types [54-63]:

- Normal Phase HPLC- In this method the separation is based on polarity. The stationary phase is polar, mostly silica is used and the non-polar phase used is hexane, chloroform and diethyl ether. The polar samples are retained on column [58].
- Reverse Phase HPLC- It is reverse to normal phase HPLC. The mobile phase is polar and the stationary phase is non polar or hydrophobic. The more is the non-polar nature the more it will be retained.
- Size-exclusion HPLC- The column will be incorporating with precisely controlled substrate molecules. Based on the difference in molecular sizes the separation of constituents will occur.
- Ion-exchange HPLC- The stationary phase is having ionically charged surface opposite to the sample charge. The mobile phase used is aqueous buffer which will control pH and ionic strength [56].

## APPLICATIONS OF HPLC

The HPLC has several applications in the fields of pharmacy, forensic, environment and clinical. It also helps in the separation and purification of compound [57-83].

- Pharmaceutical Applications: The pharmaceutical applications include controlling of drug stability, dissolution studies and quality control.
- Environmental Applications: Monitoring of pollutants and detecting components of drinking water.
- Forensic Applications: Analysis of textile dyes, quantification of drugs and steroids in biological samples.
- Food and Flavour Applications: Sugar analysis in fruit juices, detecting polycyclic compounds in vegetables, analysis of preservatives.
- Clinical Applications: Detecting endogeneous neuropeptides, analysis of biological samples like blood and urine.

### Liquid chromatography-mass spectrometry (LC-MS or HPLC-MS)

### Introduction [84-85]

Modern physical methods of analysis are so sensitive that they provide precise and detailed information from even small samples. These are mostly applied and in general are flexible to automation. Due to these reasons, these are now used in product development, in the control of manufacture and formulation, as a stability check during storage, and in monitoring the use of drugs and medicines.

- There are various methods used in Quantitative Analysis which may be broadly classified as(2)-

Chemical/classical Method (Titrimetric, Volumetric and Gravimetric method)

- Instrumental Method (Spectrophotometry, Polarography, HPLC, GC) .

Liquid chromatography-mass spectrometry (LC-MS or HPLC-MS) is an analytical technique that combines the physical separation abilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. LC-MS is a powerful technique used for many applications which has very high sensitivity and selectivity. It is commonly used in pharmacokinetic studies of pharmaceuticals and is the most frequently used technique in the field of bioanalysis.

LC-MS also plays a role in pharmacognosy especially in the field of molecular pharmacognosy when it comes to the ingredients difference in the aspects of phenotypic cloning. The most important factor that has to be considered is how to make the biggest difference of active ingredients in plant cells between the test group of plants and controlled ones.

## BASIC PRINCIPLE OF LCMS [86-88]

Liquid chromatography- High Performance Liquid Chromatography Present day liquid chromatography generally utilizes very small particles packed and operating at relatively high pressure, and is referred to as high performance liquid chromatography (HPLC); modern LC-MS methods use HPLC instrumentation, essentially exclusively, for sample.

The basic principle in HPLC is adsorption. In HPLC, the sample is forced by a liquid at high pressure (the mobile phase) through a column that is packed with a stationary phase generally composed of irregularly or spherically shaped particles chosen or derivatized to accomplish particular types of separations.

HPLC methods are historically divided into two different sub-classes based on stationary phases and the corresponding required polarity of the mobile phase. Use of octadecylsilyl (C18) and related organic-modified particles as stationary phase with pure or pH-adjusted waterorganic mixtures such as water-acetonitrile and water-methanol are used in techniques termed as reversed phase liquid chromatography (RP-LC). Use of materials such as silica gel as stationary phase with neat or mixed organic mixtures are used in techniques termed normal phase liquid chromatography (NP-LC). RP-LC is most often used as the means to introduce samples into the MS, in LC-MS instrumentation.

### Flow splitting

The flow is often split to the ratio of -10:1 when standard bore (4.6 mm) columns are used. The use of other techniques in tandem such as MS and UV detection are helpful. Nevertheless, the sensitivity of spectrophotometric detectors will decrease if the splitting of flow is towards UV. The mass spectrometry will also shows improved sensitivity at flow rates of 200 μL/min or less.

Mass spectrometry Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio of charged particles. It is used for determining masses of particles, for determining the elemental composition of a sample or molecule, and for elucidating the chemical structures of molecules, such as peptides and other chemical compounds. MS works by ionizing chemical compounds to generate charged molecules or molecule fragments and measuring their massto-charge ratios.[84] In a typical MS procedure, a sample is loaded onto the MS instrument and undergoes vaporization. The components of the sample are ionized by one of a variety of methods (e.g., by impacting them with an electron beam), which results in the formation of charged particles (ions). The ions are separated according to their mass-to-charge ratio in an analyzer by electromagnetic fields. The ions are detected, usually by a quantitative method. The ion signal is processed into mass spectra. Additionally, MS instruments consist of three modules. An ion source, which can convert gas phase sample molecules into ions (or, in the case of electrospray ionization, move ions that exist in solution into the gas phase). A mass analyzer, which sorts the ions by their masses by applying electromagnetic fields. A detector, which measures the value of an indicator quantity and thus provides data for calculating the abundances of each ion present. The technique has both qualitative and quantitative uses. These include identifying unknown compounds, determining the isotopic composition of elements in a molecule, and determining the structure of a compound by observing its fragmentation. Other uses include quantifying the amount of a compound in a sample or studying the

fundamentals of gas phase ion chemistry (the chemistry of ions and neutrals in a vacuum). MS is now in very common use in analytical laboratories that study physical, chemical, or biological properties of a great variety of compounds.

## Mass analyzer

There are many different mass analyzers that can be used in LC/MS. Some of them are Single quadrupole, triple quadrupole, ion trap, time of flight (TOF) and quadrupole-time of flight (QTOF).

## Interface

The interface between a liquid phase technique which continuously flows liquid, and a gas phase technique carried out in a vacuum was difficult for a long time. The advent of electrospray ionization changed this. The interface is most often an electrospray ion source or variant such as a nanospray source; however atmospheric pressure chemical ionization interface is also used.[1] Various techniques of deposition and drying have also been used such as using moving belts; however the most common of these is off-line MALDI deposition. A new approach still under development called Direct-EI LC-MS interface which couples a nano HPLC system with a mass spectrometer equipped with electron ionisation.

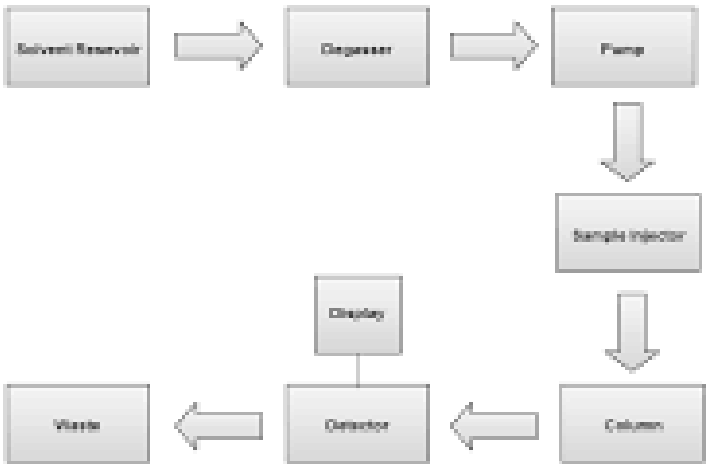
## Combination of HPLC and MS [89]

HPLC not only separates things but also provides little extra information about how a chemical might be. In fact, it is hard in HPLC to be certain about purity of a particular peak, and if it contains only a single chemical. Adding a Mass Spectrometry to this will tell you the masses of all the chemicals present in the peak, which can be used for identifying them, and an excellent method to check for the purity. Even a simple mass spec can be used as a mass specific detector, specific for the chemical under study.

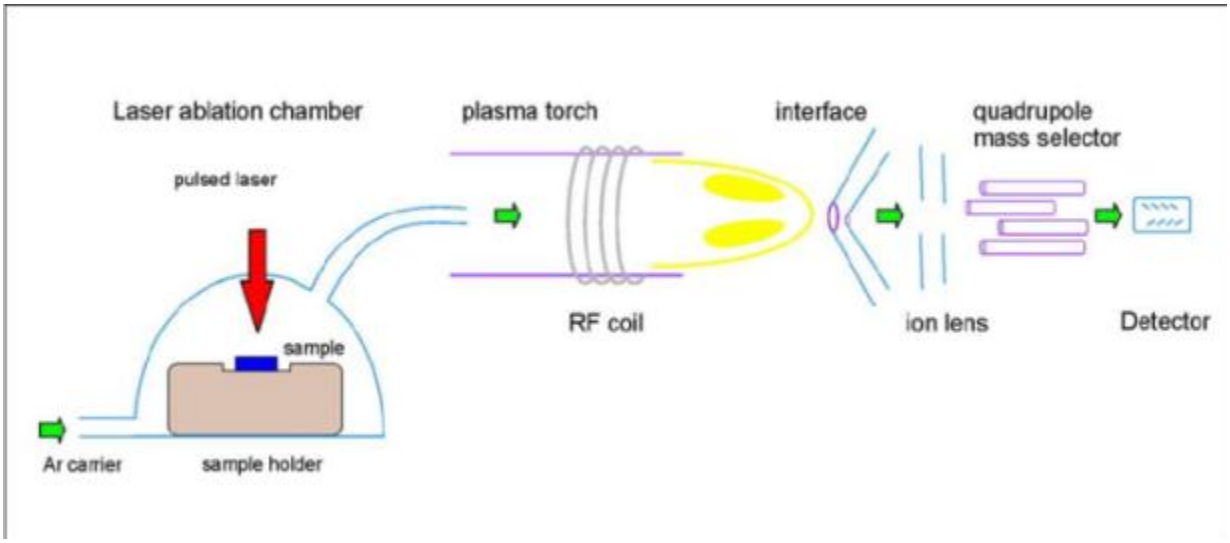
More sophisticated mass detectors such as triple quadrupole and ion-trap instruments can also be used to carry out more detailed structure-dependent analysis on what is eluting off from the HPLC system.

## Instrumentation of LCMS [89]

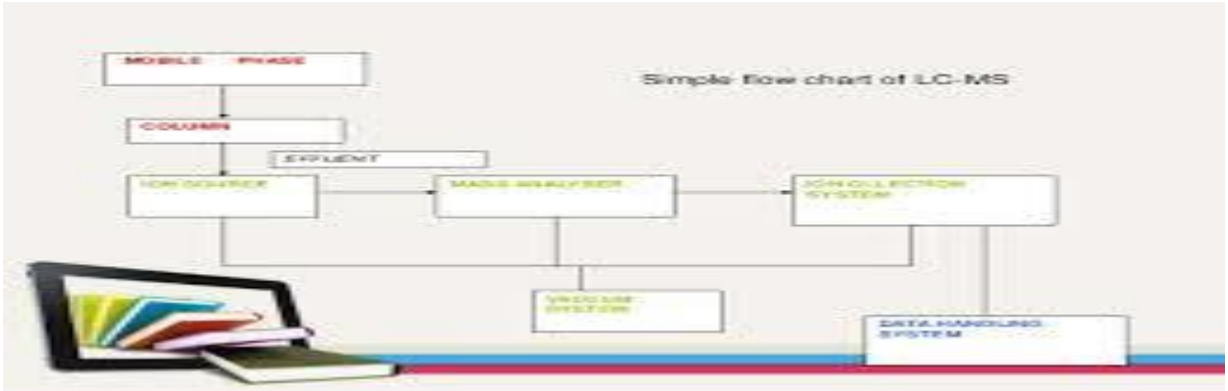
### Instrumentation of HPLC



## Instrumentation of MS




## Instrumentation of LCMS




## Advantages of LCMS [90]

There are various advantages of LCMS over other chromatographic methods of which few are as follows;

- Selectivity: Co-eluting peaks can be isolated by mass selectivity and are not constrained by chromatographic resolution.
- Peak assignment: A molecular fingerprint for the compound under study is generated, ensuring correct peak assignment in the presence of complex matrices.
- Molecular weight information: Confirmation and identification of both known and unknown compounds.
- Structural information: Controlled fragmentation enables structural elucidation of a chemical.
- Rapid method development: Provides easy identification of eluted analytes without retention time validation.
- Sample matrix adaptability: Decreases sample preparation time and hence saves time.
- Quantitation: Quantitative and qualitative data can be obtained easily with limited instrument optimization.

## Various Applications of LCMS [91-110]

1. Molecular Pharmacognosy
2. Characterization and Identification of Compounds
3. Quantitative Bioanalysis of various Biological Samples
4. Qualitative And Quantitative Analysis Of Complex Lipid Mixtures

5. Phytoconstituents / Plant Metabolomics
6. Automated Immunoassay in Therapeutic Drug Monitoring
7. Two Dimensional (2-D) Hyphenated Technology
8. Clinical chemistry and toxicology
9. Proteomics
10. Pharmacovigilance
11. Organic/Inorganic Hybrid Nanoflowers

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