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LC-MS A Hyphenated Technique: Review

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ABSTRACT

Liquid Chromatography/Mass spectrum analysis (LC/MS) is quick developing and it's the popular tool of liquid chromatographers. Liquid chromatography-mass spectrum analysis (LC-MS) could be a technique that uses liquid natural process (or HPLC) with mass spectrometry. It is a chemistry technique that combines the physical separation capabilities of liquid natural process (or HPLC) with the mass analysis capabilities of mass spectrum analysis. (LC-MS/MS) is a technique commonly used in laboratories for the qualitative and quantitative analysis of drug substances, drug products, and biological samples. It has long been used in drug development at a variety of stages, including Metabolic Stability Screening, Metabolite Identification, *in vivo* Drug Screening, Impurity Identification, Amide Mapping, Glycoprotein Mapping, Natural Product Dereplication, and Bio-affinity Screening. It is a chemistry-based analytical technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. (LC-MS/MS) LC-MS is now routinely used for routine analysis in a variety of fields, including therapeutic drug monitoring (TDM), clinical and forensic toxicology, and doping control. The need for more powerful analytical and bioanalytical techniques that can accurately and precisely discriminate target analytes from high complexity mixtures in a sensitive and selective manner drove the advancement of LCMS. The use of liquid chromatography (LC) and mass spectrometry (MS) has become a powerful two-dimensional (2D) hyphenated technology due to recent advancements in instrumentation.

INTRODUCTION:

Modern physical analysis methods are so sensitive that they can provide precise and detailed information from even small samples. These are commonly used and, in general, are amenable to automation. As a result of these factors, these are now used in product development, manufacturing and formulation control, as a stability check during storage, and monitoring the use of drugs and medicines.

There are various methods used in Quantitative Analysis which may be broadly classified as

1. Chemical/classical Method (Titrimetric, Volumetric and Gravimetric method)
2. Instrumental Method (Spectrophotometry, Polarography, HPLC, GC)

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

It is a method of analyzing that combines the physical separation capabilities 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[1, 2].

Basic Principle of LCMS:

A. Liquid chromatography (HPLC):

Present day liquid natural process typically utilizes terribly tiny particles packed and operating at comparatively air mass, and is noted as high performance liquid chromatography (HPLC); fashionable LC-MS ways use HPLC instrumentation, primarily exclusively, for sample [3]. The basic principle in HPLC is surface assimilation. In HPLC, the sample is forced by a liquid at high pressure (the mobile part) through a column that's filled with a stationary phase typically composed of on an irregular basis or spherically formed particles chosen or derivative to accomplish particular styles of separations [4]. HPLC ways area unit traditionally divided HPLC ways area unit traditionally divided into 2 completely different sub-classes supported stationary phases and also the corresponding needed polarity of the

mobile part. Use of octadecylsilyl (C18) and connected organic-modified particles as stationary part with pure or pH-adjusted water organic mixtures like water-acetonitrile and water-methanol area unit utilized in techniques termed as reversed part liquid natural process (RP-LC) (RP-LC). Use of materials like colloid as stationary part with neat or mixed organic mixtures area unit utilized in techniques termed traditional phase liquid natural process (NP-LC) (NP-LC). In LC-MS instrumentation, RP-LC is most commonly used because it suggests that samples be introduced into the MS. [5]

Types of HPLC

The types of HPLC used in analysis are generally determined by the phase system used in the process. Normal phase chromatography: Also known as Normal phase HPLC (NP-HPLC), this method separates analytes based on polarity. A polar stationary phase and a non-polar mobile phase are used in NP-HPLC. The polar stationary phase interacted with and retained the polar analyte. Adsorption strengths increase as analyte polarity increases, and the interaction between the polar analyte and the polar stationary phase prolongs elution time. [3, 4]

1. Reversed phase chromatography:

Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. RPC operates on the principle of hydrophobic interactions, which result from repulsive forces between a polar eluent, the relatively non-polar analyte, and the non-polar stationary phase. The analyte's binding to the stationary phase is proportional to the contact surface area around the analyte's non-polar segment upon association with the ligand in the aqueous eluent.

2. Size exclusion chromatography:

Size exclusion chromatography (SEC), also known as gel permeation chromatography or gel filtration chromatography, is a technique used to separate particles based on their size. It is also useful for determining the tertiary structure and quaternary structure of proteins and amino acids. This technique is widely used to determine the molecular weight of polysaccharides.

3. Ion exchange chromatography:

In Ion-exchange chromatography, retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Ions of the same charge are excluded. This type of chromatography is widely used in water purification, ligand-exchange chromatography, protein ion-exchange chromatography, high-pH anion-exchange chromatography of carbohydrates and oligosaccharides, and so on. [3, 4]

4. Bio-affinity chromatography:

Separation based on reversible interactions of proteins with ligands. Ligands are covalently attached to a solid support on a bio-affinity matrix, which retains proteins that interact with the column-bound ligands. [4]

B. 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 ionising chemical compounds to produce charged molecules or molecule fragments and measuring the mass-to-charge ratios of these molecules. [1] In a typical MS procedure, a sample is loaded onto the MS instrument and undergoes vaporization. The components of the sample are ionised 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) (ions). The ions are separated according to their mass-to-charge ratio in an analyzer by electromagnetic fields. Ions are typically detected using a quantitative method. Mass spectra are generated from the ion signal. Furthermore, MS instruments are made up of three modules. An ion source capable of converting gas phase sample molecules to ions. A mass analyzer uses electromagnetic fields to sort ions based on their masses. A detector that measures the value of an indicator quantity, providing data for calculating the abundances of each ion present. The technique can be used for both qualitative and quantitative purposes. Identifying unknown compounds, determining the isotopic composition of elements in a molecule, and determining the structure of a compound by observing its fragmentation are examples of these. Other applications include determining the concentration of a compound in a sample and studying the fundamentals of gas phase ion chemistry (the chemistry of ions and neutrals in a vacuum). MS is now widely used in

analytical laboratories to investigate the physical, chemical, or biological properties of a wide range of compounds. [7]

Mass analyzer:

There are numerous mass analyzers available for use in LC/MS. Single quadrupole, triple quadrupole, ion trap, time of flight (TOF), and quadrupole-time of flight (QTOF) are a few examples.

Interface:

For a long time, the interface between a liquid section technique that continuously flows liquid and a gas phase technique delivered in a very vacuum was difficult. This was altered by the appearance of electrospray ionization. The interface is most commonly associated with an electrospray particle supply or a variant such as a nanospray source; however, a gas pressure chemical ionization interface is also used. [1] Various deposition and drying techniques, such as the use of moving belts, have also been used; however, the most common of these is off-line MALDI deposition. A replacement method still in development is the Direct-EI LC-MS interface, which combines a nano HPLC system with a spectrometer equipped with lepton ionization.

Combination of HPLC and MS:

HPLC not only separates things, but it also provides some additional information about how a chemical might be. In fact, it is difficult in HPLC to be certain about the purity of a specific peak and whether it contains only a single chemical. Adding a Mass Spectrometry to this will give you the masses of all the chemicals present in the peak, which can be used to identify them and is a great way to check for purity. Even a simple mass spec can be used as a mass-specific detector for the chemical under investigation. More sophisticated mass detectors, such as triple quadrupole and ion-trap instruments, can be used to perform more detailed structure-dependent analysis on what is eluting off the HPLC system [6].

Instrumentation of LCMS:

A. High Performance Liquid Chromatography (HPLC):

A high-pressure flow of a liquid through a column containing the stationary phase is used in analytical separation. Stationary phase: This can be either a solid (LSC) or a liquid (LLC). As the compounds pass through the column, a mixture of compounds injected at one end

separates. Compounds that have been separated are detected electronically as they elute at the other end of the column.

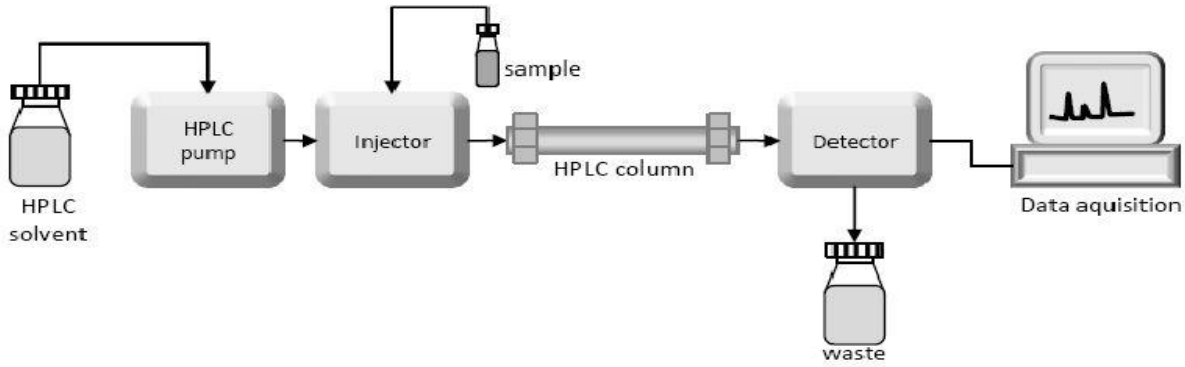

Figure No. 1: Flow Diagram of HPLC Instrumentation

1. Solvent Reservoir

A glass reservoir holds the contents of the mobile phase. In HPLC, the mobile phase, or solvent, is typically a mixture of polar and non-polar liquid components, the concentrations of which vary depending on the sample composition.

2. Pump

A pump draws the mobile phase from the solvent reservoir and forces it through the column and detector of the system. Operating pressures of up to 42000 kPa (about 6000 psi) can be generated depending on a variety of factors such as column dimensions, particle size of the stationary phase, flow rate, and mobile phase composition.[18]

3. Sample Injector

Although a variety of injectors are available, and their selection is often critical to the success of the analysis, a single type of injector is almost exclusively used in HPLC. The loop injector is, as previously stated, merely a convenient way of introducing a liquid sample into a flowing liquid stream, and consists of a nominal volume loop into which sample is introduced with a conventional syringe. While the loop is being filled, mobile phase is pumped through the valve to the column at the desired flow rate to keep the column in equilibrium with the mobile phase and maintain chromatographic performance. When 'injection' is required, a rotating switch is moved, and the flow is diverted through the loop, flushing its contents onto the column's top. From a quantitative standpoint, the way the injector functions is critical to the precision and accuracy that can be obtained, so these two

parameters are critical. Quantitative precision will be determined by factors such as the extent to which the loop can be filled repeatedly. It is usual to fill the loop completely by having a greater volume in the conventional syringe than the loop capacity(excess goes to waste) and it is important to ensure, as much as is possible, that air bubbles are not introduced in place of the sample. An internal standard should be used to obtain the best precision and accuracy during quantitative measurements (this will be discussed further in Section 2.5 below), and if insufficient sample is available to allow complete filling of the loop, i.e. it is only partially filled, an internal standard must be used if meaningful quantitative results are to be obtained. Loops are not precisely calibrated, and a nominally 20 loop is unlikely to have this exact volume. This has no effect on measurement precision or accuracy, as long as the same loop is used for obtaining quantitative calibration and determining the 'unknowns.

The injector is of little concern from the standpoint of mass spectrometry, except that any bubbles introduced into the injector may disrupt the liquid flow, resulting in an unstable response from the mass spectrometer. A single injection injector or an automated injection system can be used. An injector for an HPLC system should allow for the injection of a liquid sample in the volume range of 0.1-100 mL with high reproducibility and under high pressure (up to 4000 psi) [6].

4. Columns

Columns are typically made of polished stainless steel, range in length from 50 to 300 mm, and have an internal diameter of 2 to 5 mm. They are typically filled with a stationary phase with particle sizes ranging from 3–10 m. Microbore columns are defined as columns with internal diameters of less than 2 mm. During an analysis, the temperature of the mobile phase and the column should ideally be kept constant.

HPLC Column Types

For various analytical applications, a wide range of HPLC column types are now available. A few of the most common types, classified by separation mechanism, are described below.

a. Ion exchange HPLC columns:

A cationic or anionic ion exchange column can be used. The charge of polar molecules is used to separate them in this type of HPLC column. An aqueous buffer serves as the mobile phase. Ion exchange HPLC columns can be used to separate a wide range of analytes, but

they are most commonly used to separate carbohydrates, amino acids, and proteins. In a column, ion exchange and ligand exchange chromatography can be combined. Ion exchange in these combined-mode columns is typically accomplished with metal ions, and the ligands are electron-donor molecules such as hydroxyl groups or amines. This type of HPLC column is frequently used for monosaccharide separation [11].

b. Reversed-phase HPLC columns:

Possess nonpolar packing they are used with organic solvent mobile phases that are aqueous or water-miscible. Acetonitrile, methanol, and tetrahydrofuran are the most commonly used solvents (THF). With reversed-phase columns, both isocratic (constant concentration) and gradient (increasing organic solvent concentration) elution are used. Selectivity and retention times are affected by a variety of factors, including the pH of the mobile phase. The reversed-phase HPLC column is the most versatile and widely used column type, capable of handling a wide range of analytes.

c. Normal-phase HPLC columns:

Polar packing is used. Because the mobile phase is nonpolar, it is typically an organic solvent such as hexane or methylene chloride. This type of HPLC column employs hydrophilic interaction liquid chromatography (HILC), a type of partition chromatography in which the mobile phase contains a low concentration of water. The addition of ionic compounds to the mobile phase of an ion-moderated partition HPLC column, such as ammonium acetate, can both change the retention times of analytes and increase their polarity. This type of HPLC column is used to separate small molecules such as organic acids, drugs, and a variety of biomolecules such as glycosylated proteins. Exclusion of size HPLC columns do not rely on analytes interacting with the column packing, but rather on a sieving effect based on molecular weight. Mesopores and micropores are both present in the packing. The size of molecules in the sample that can diffuse into the pores is determined by the size distribution of the pores. The retention time and elution profile are determined by the extent to which molecules can diffuse into the pores. Molecules too large to enter the pores move quickly through the column, eluting as a single peak after the void volume. Exclusion of size HPLC columns are primarily used to separate proteins and carbohydrates. Affinity, ion exclusion, and displacement chromatography columns are other types of HPLC columns; chiral HPLC columns can be used to resolve racemic mixtures. [11]

5. Detector:

The analytes are detected as they elute from the chromatographic column by the HPLC detector, which is located at the end of the column. UV-spectroscopy, fluorescence, mass-spectrometric, and electrochemical detectors are all commonly used.

Detectors can be classified in a variety of ways, including their application as follows:

a. solute- or solvent-property detectors

b. selective or general (universal) detectors

c. mass- or concentration-sensitive detectors

a. Solute- or Solvent-Property Detectors:

This classification is concerned with whether the detector monitors a solute property (analyte), such as the UV detector, or a change in a solvent property (mobile phase) caused by the presence of an analyte, such as the refractive index detector.[12]

b. Selective or General Detectors:

This classification is concerned with whether the detector responds to a specific feature of the analyte of interest or if it responds to a large number of analytes regardless of their structural properties. According to the previous classification, solute detectors are usually selective detectors, whereas solvent detectors are general detectors. UV absorption is arguably the most widely used HPLC detector methodology, and it can be used as a specific or general detector depending on how it is used. If the wavelength of the analyte's maximum absorption (max) is known, it can be monitored, and the detector can be considered selective for that analyte (s). However, because UV absorptions are generally broad, this type of detection is rarely sufficiently selective. If a diode-array instrument is available, multiple wavelengths can be monitored and the absorbance ratio measured. The agreement of the ratio measured from the 'unknown' with that measured in a reference sample increases confidence that the analyte of interest is being measured, but it does not provide absolute certainty. Many organic molecules absorb UV radiation at 254 nm to some extent, and if this wavelength is used, it may be considered a general detection system. However, it should be noted that not all compounds absorb UV radiation. In these cases, indirect UV detection may be used, in which a UV-active Liquid Chromatography 17 compound is added to the mobile phase. When a compound that does not absorb UV radiation elutes from the HPLC column, the background

signal remains constant. If the mass spectrometer is used in conjunction with indirect UV detection, care must be taken to ensure that the UV active compound added to the mobile phase does not produce an unacceptably high background signal that interferes with interpretation of either the TIC trace or the resulting mass spectra. The refractive index detector is a common general detector that monitors changes in the refractive index of the mobile phase as an analyte elutes from the column. When using gradient elution, the refractive index of the mobile phase changes as its composition changes, resulting in a constantly varying detector baseline. The determination of the position and intensity of a low-intensity analytical signal on a varying baseline is less precise and accurate than the same measurement on a constant baseline with no background signal. It is commonly acknowledged that general detectors are less sensitive than specific detectors, have a lower dynamic range, and do not produce the best results when gradient elution is used. The mass spectrometer, like the UV detector, can be used as either a general detector (when full-scan mass spectra are acquired) or as a specific detector. [10]

c. Mass- or Concentration-Sensitive Detectors:

The final classification is based on whether the intensity of the detector response is proportional to the solute concentration or the absolute amount of solute reaching it. This classification is especially critical for quantitative applications. The concentration of analyte reaching the detector remains constant as the mobile phase flow rate increases, but the amount of analyte increases. Under these conditions, the signal intensity from a concentration-sensitive detector will remain constant, despite the fact that the peak width, i.e. the area of the response, will decrease. A change in flow rate will also reduce the width of the response from a mass-sensitive detector, whereas the signal intensity will increase as the absolute amount of analyte reaching the detector increases, in contrast to a concentration-sensitive detector. As the overall response increases, this may be used to improve the signal quality obtained. Under many experimental conditions, the mass spectrometer acts as a mass-sensitive detector, but under others, such as LC-MS with electrospray ionisation, it can act as a concentration-sensitive detector. The reasons for this behaviour are beyond the scope of this book (interested readers should consult Cole's text [8]), but they highlight the importance of incorporating adequate calibration and standardization procedures into any quantitative methodology to ensure the validity of any results obtained.

6. Data Collection Devices:

The detector's signals can be collected on chart recorders or electronic integrators, which vary in complexity and ability to process, store, and reprocess chromatographic data. The computers integrate the detector's response to each component and display it in an easy-to-read and interpret chromatograph**.**

Advantages of HPLC

1. High speed
2. High resolution
3. High sensitivity
4. Reproducibility of ±1%
5. Accuracy
6. Automation

Applications of HPLC:

HPLC can provide information such as compound resolution, identification, and quantification. It also aids in the separation and purification of chemicals. Other HPLC applications include.

❖ **Pharmaceutical Applications**

1. To maintain drug stability.

2. A study of the dissolution of pharmaceutical dosage forms in tablet form.

3. Pharmaceutical quality assurance**.**

❖ **Environmental Applications**

1. Phenolic compound detection in drinking water.

2. Pollutant biomonitoring.

❖ **Applications in Forensics**

1. Drug quantification in biological samples.

2. Detection of steroids in blood, urine, and other bodily fluids.

3. Textile dye forensic analysis.

4. Determination of cocaine and other drugs of abuse in blood, urine, and other bodily fluids.

❖ **Food and Flavour:**

1. Evaluation of the quality of soft drinks and water.

2. Sugar content of fruit juices.

3. Polycyclic compound analysis in vegetables.

4. Preservative evaluation.

❖ **Applications in Clinical Tests**

1. Urine analysis, antibiotics in blood analysis.

2. Bilirubin and biliverdin analysis in hepatic disorders.

3. Detection of endogenous neuropeptides in brain extracellular fluid, etc.

B. Mass spectroscopy:

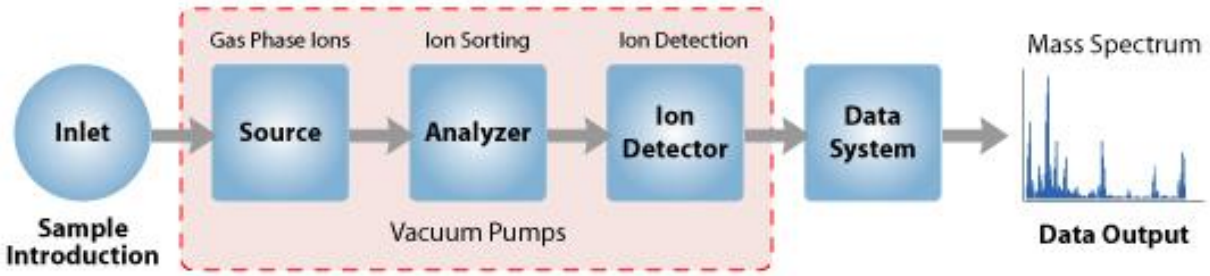

Figure No. 2: Flow Diagram of Mass Spectroscopy Instrumentation

The instrument consists of three major components:

1. Ion Source:

A device used to generate gaseous ions from the substance being studied. With the introduction of atmospheric pressure ionisation (API) techniques, the number of compounds that can be successfully analysed by LC/MS has greatly increased. The analyte molecules are first ionised at atmospheric pressure in atmospheric pressure ionisation. The analyte ions are

then mechanically and electrostatically separated from neutral molecules. The following are examples of common atmospheric pressure ionisation techniques:

1. Electrospray ionization (ESI)
2. Atmospheric pressure chemical ionization (APCI)
3. Atmospheric pressure photoionization (APPI)

a. Electrospray ionization:

Before the analyte reaches the mass spectrometer, electrospray relies on chemistry to generate analyte ions in solution. In the presence of a strong electrostatic field and heated drying gas, the LC eluent is sprayed (nebulized) into an atmospheric pressure chamber.

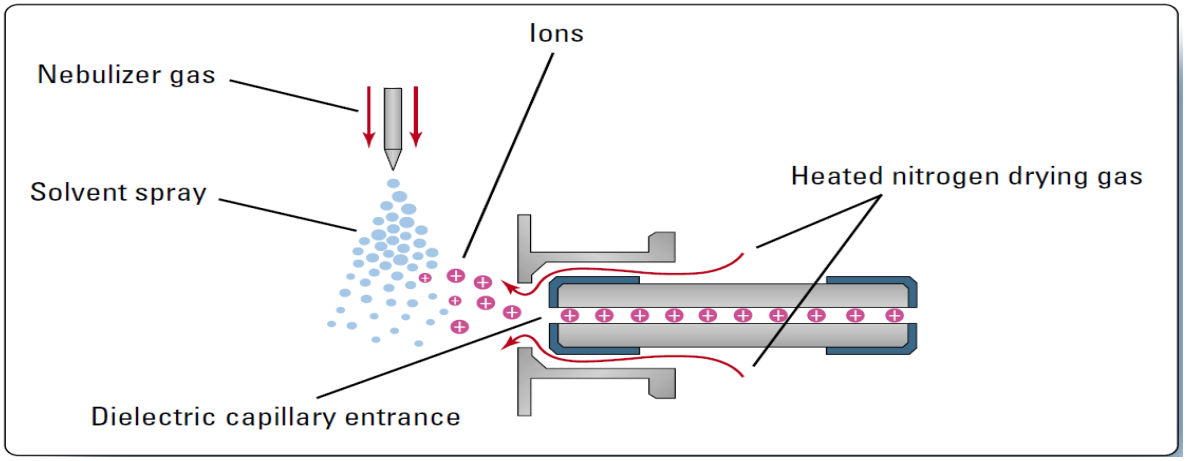

Figure No. 3: Electrospray ion source

The electrostatic field causes further dissociation of the analyte molecules. The heated drying gas causes the solvent in the droplets to evaporate. As the droplets shrink, the charge concentration in the droplets increases. Eventually, the repulsive force between ions with like charges exceeds the cohesive forces and ions are ejected (desorbed) into the gas phase. These ions are attracted to and pass through a capillary sampling orifice into the mass analyzer [27].

b. Atmospheric pressure chemical ionization:

In APCI, the LC eluent is sprayed through a heated (typically 250°C–400°C) vaporizer at atmospheric pressure. The heat vaporizes the liquid. The resulting gas-phase solvent molecules are ionised by electrons discharged from a corona needle. The solvent ions then

transfer charge to the analyte molecules through chemical reactions (chemical ionization). The analyte ions pass through a capillary sampling orifice into the mass analyzer [6].

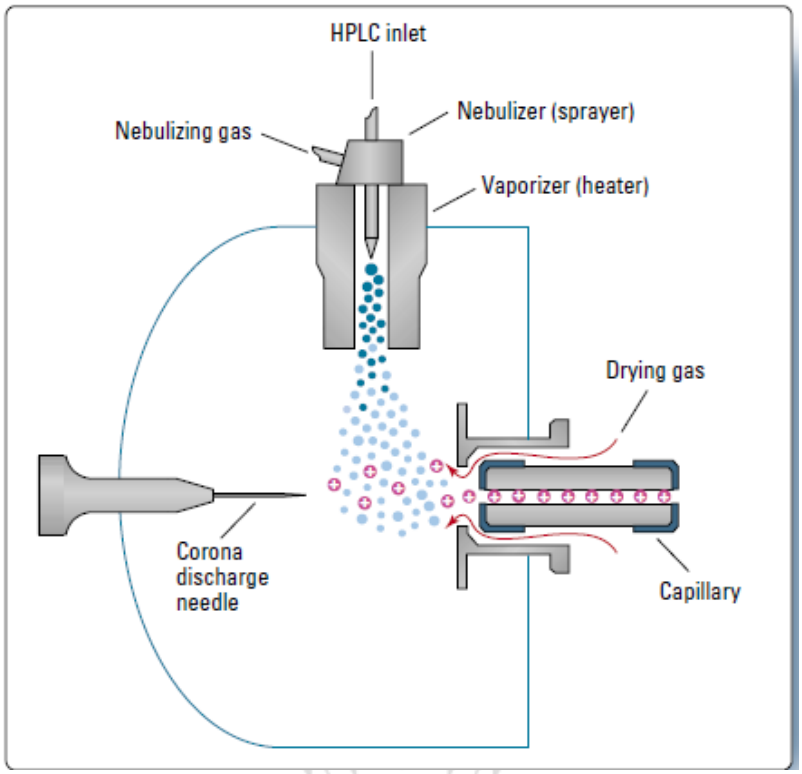

Figure No. 4: APCI ion source

APCI is applicable to a wide range of polar and nonpolar molecules. It rarely results in multiple charging so it is typically used for molecules less than 1,500 u. Due to this, and because it involves high temperatures, APCI is less well-suited than electrospray for analysis of large biomolecules that maybe thermally unstable. APCI is used with normal-phase chromatography more often than electrospray is because the analytes are usually nonpolar.

C. Atmospheric pressure photoionization:

Atmospheric pressure photoionization (APPI) for LC/MS is a relatively new technique. As in APCI, a vaporizer converts the LC eluent to the gas phase. A discharge lamp generates photons in a narrow range of ionisation energies. The range of energies is carefully chosen to ionise as many analyte molecules as possible while minimising the ionisation of solvent molecules. The resulting ions pass through a capillary sampling orifice into the mass analyzer.

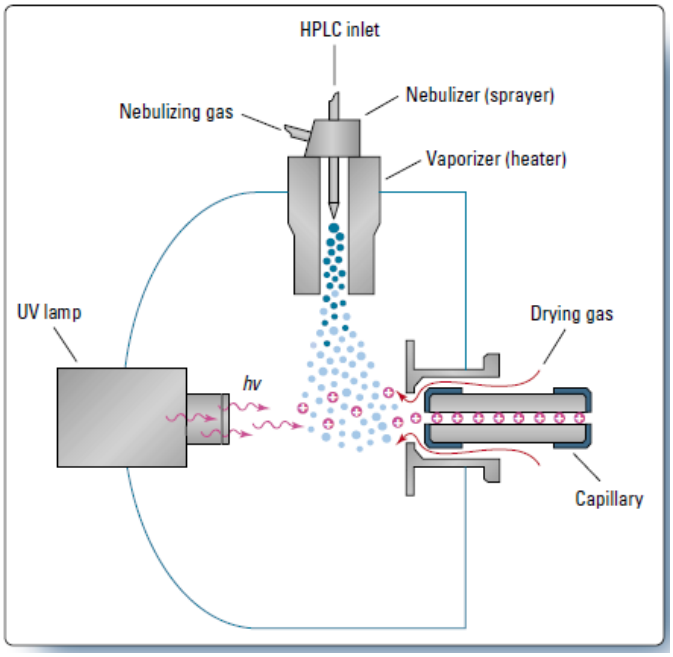

Figure No. 5: APPI ion source

APPI is applicable to many of the same compounds that are typically analysed by APCI. It shows particular promise in two applications, highly nonpolar compounds and low flow rates (<100 µl/min), where APCI sensitivity is sometimes reduced. In all cases, the nature of the analyte(s) and the separation conditions has a strong influence on which ionisation technique: electrospray, APCI, or APPI, will generate the best results. The most effective technique is not always easy to predict. [19]

1. Analyzer:

For resolving the ions into their characteristics mass components according to their mass-to-charge ratio. There are five general types of mass analyzers that can be used for the separation of ions in a mass spectrometry.

Mass Analyzers:

Although in theory any type of mass analyzer could be used for LC/MS, four types:

a. Time-of-flight

b. Ion trap

c. Fourier transform ion cyclotron resonance

d. Quadrupole

a. In a time-of-flight (TOF):

Mass analyzer, a uniform electromagnetic force is applied to all ions at the same time, causing them to accelerate down a flight tube. Lighter ions travel faster and arrive at the detector first, so the mass-to-charge ratios of the ions are determined by their arrival times. Time-of flight mass analyzers have a wide mass range and can be very accurate in their mass measurements.[11]

b. Ion trap:

An ion trap mass analyzer consists of a circular ring electrode plus two end caps that together form a chamber. Ions entering the chamber are "trapped" thereby electromagnetic fields. Another field can be applied to selectively eject ions from the trap. Ion traps have the advantage of being able to perform multiple stages of mass spectrometry without additional mass analyzers**.**

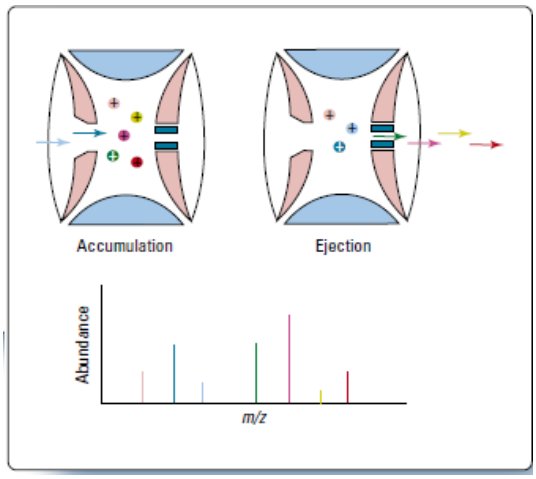

Figure No. 6: Ion trap mass analyzer

c. Fourier transform-ion cyclotron resonance (FT-ICR):

An FT-ICR mass analyzer (also called FT-MS) another type of trapping analyzer. Ions, entering a chamber are trapped in circular orbits by powerful electrical and magnetic fields. When excited by a radio-frequency (RF) electrical field, the ions generate a time dependent current. This current is converted by Fourier transform into orbital frequencies of the ions which correspond to their mass to charge ratios. Like ion traps, FT-ICR mass analyzers can perform multiple stages of mass spectrometry without additional mass analyzers. They also have a wide mass range and excellent mass resolution. They are, however, the most expensive of the mass analyzers. [11]

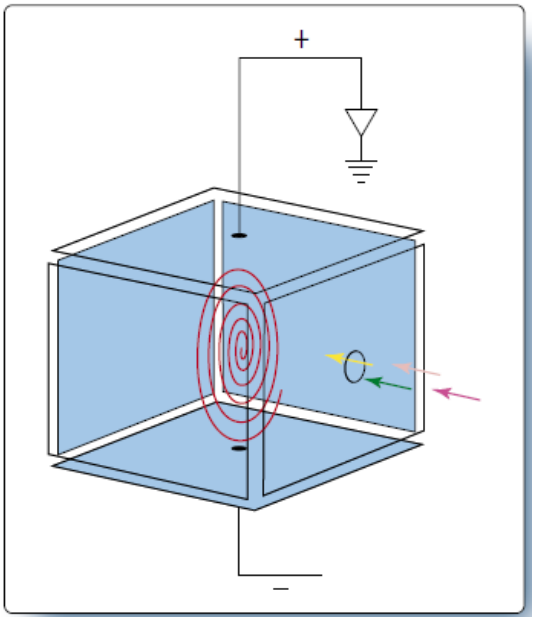

Figure No. 7: FT-ICR mass analyzer

d. Quadrupole Ion Trap Mass Analyzers:

The ions enter the area between the electrodes through one of the end caps. After entry, the electric field in the cavity due to the electrodes causes the ions of certain m/z values to orbit in the space. As the radio frequency voltage increases, heavier mass ion orbits become more stabilized and the light mass ions become less stabilized, causing them to collide with the wall, and eliminating the possibility of travelling to and being detected by the detector.

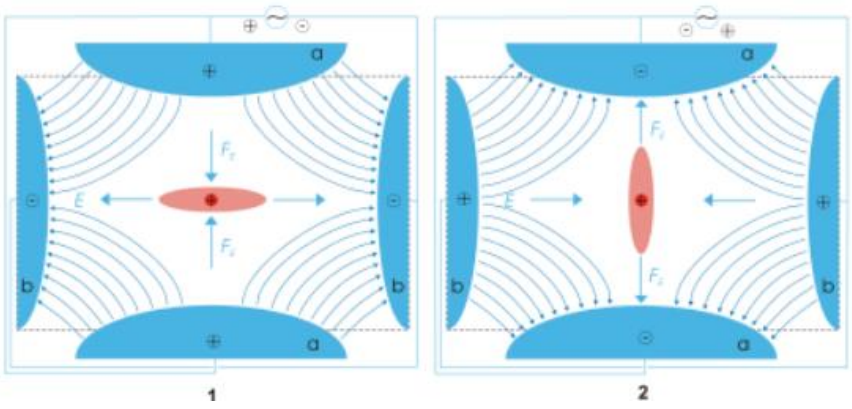

Figure No. 8: Quadrupole Ion Trap Mass Analyzers

The quadrupole ion trap usually runs a mass selective ejection, where selectively it ejects the trapped ions in order of increasing mass by gradually increasing the applied radiofrequency voltage [12].

2. Detector System:

For detecting the ions and recording the relative abundance of each of the resolved ionic species. In addition, a sample introduction system is necessary to admit the samples to be studied to the ion source while maintaining the high vacuum requirements (~10-6 to 10-8 mm of mercury) of the technique; and a computer is required to control the instrument, acquire and manipulate data, and compare spectra to reference libraries.

With all the above components, a mass spectrometer should always perform the following processes:

Produce ions from the sample in the ionization source.

1. Separate these ions according to their mass-to-charge ratio in the mass analyzer.

2. Eventually, fragment the selected ions and analyse the fragments in a second analyzer.

3. Detect the ions emerging from the last analyzer and measure their abundance with the detector that converts the ions into electrical signals.

Process the signals from the detector that are transmitted to the computer and control the instrument using feedback.

❖ **Advantages of mass spectroscopy:**

1. It does away with the need for derivatization.
2. Is capable of separating and identifying similar compounds.
3. Superior analyte sensitivity and selectivity.
4. When used in MRM mode
5. Determination of low-level compound concentrations in biological matrices
6. Broad dynamic range.

For detecting the ions and recording the relative abundance of each of the resolved ionic species. In addition, a sample introduction system is necessary to admit the samples to be studied to the ion source while maintaining the high vacuum requirements (~10-6 to 10-8 mm of mercury) of the technique; and a computer is required to control the instrument, acquire and manipulate data, and compare spectra to reference libraries. For detecting the ions and recording the relative abundance of each of the resolved ionic species. In addition, a sample

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❖ **Application of mass spectroscopy:**

1. Analysis of Biomolecules using Mass Spectrometry:

Mass spectrometry is fast becoming an indispensable field for analysing biomolecules. Till the1970s, the only analytical techniques which provided similar information were electrophoretic, chromatographic or ultracentrifugation methods. The results were not absolute as they were based on characteristics other than molecular weight. Thus the only possibility of knowing the exact molecular weight of a macromolecule remained its calculation based on its chemical structure. The development of desorption ionisation methods based on the emission of pre-existing ions such as plasma desorption (PD), fast atom bombardment (FAB) or laser desorption (LD), allowed the application of mass spectrometry for analysing complex biomolecules [11].

2. Analysis of Glycans:

Oligosaccharides are molecules formed by the association of several monosaccharides linked through glycosidic bonds. The determination of the complete structure of oligosaccharides is more complex than that of proteins or oligonucleotides. It involves the determination of additional components as a consequence of the isomeric nature of monosaccharides and their capacity to form linear or branched oligosaccharides. Knowing the structure of an oligosaccharide requires not only the determination of its monosaccharide sequence and its branching pattern but also the isomer position and the anomeric configuration of each of its glycosidic bonds. Advances in glycobiology involves a comprehensive study of structure, biosynthesis, and biology of sugars and saccharides. Mass spectrometry (MS) is emerging as enabling technology in the field of glycomics and glycobiology [13].

3. Analysis of Lipids:

Lipids are made up of many classes of different molecules which are soluble in organic solvents. Lipidomics, a major part of metabolomics, constitutes the detailed analysis and global characterization, both spatial and temporal, of the structure and function of lipids (the lipidome) within a living system. Many new strategies for mass-spectrometry-based analyses of lipids have been developed. The most popular lipidomics methodologies involve electrospray ionisation (ESI) sources and triple quadrupole analyzers. Using mass spectrometry, it is possible to determine the molecular weight, elemental composition, the position of branching and nature of substituents in the lipid structure.

4. Analysis of Proteins and Peptides:

Proteins and peptides are linear polymers made up of combinations of the 20 amino acids linked by peptide bonds. Proteins undergo several post translational modifications, extending the range of their function via such modifications. The term Proteomics refers to the analysis of complete protein content in a living system, including co- and post-translationally modified proteins and alternatively spliced variants. Mass Spectrometry has now become a crucial technique for almost all proteomics experiments. It allows precise determination of the molecular mass of peptides as well as their sequences. This information can very well be used for protein identification, de novo sequencing, and identification of post-translational modifications [14].

5. Analysis of Oligonucleotides:

Oligonucleotides (DNA or RNA), are linear polymers of nucleotides. These are composed of a nitrogenous base, a ribose sugar and a phosphate group. Oligonucleotides may undergo several natural covalent modifications which are commonly present in tRNA and rRNA, or unnatural ones resulting from reactions with exogenous compounds. Mass spectrometry plays an important role in identifying these modifications and determining their structure as well as their position in the oligonucleotide. It not only allows determination of the molecular weight of oligonucleotides, but also in a direct or indirect manner, the determination of their sequences [8].

❖ **High Performance Liquid chromatography: Mass spectroscopy**

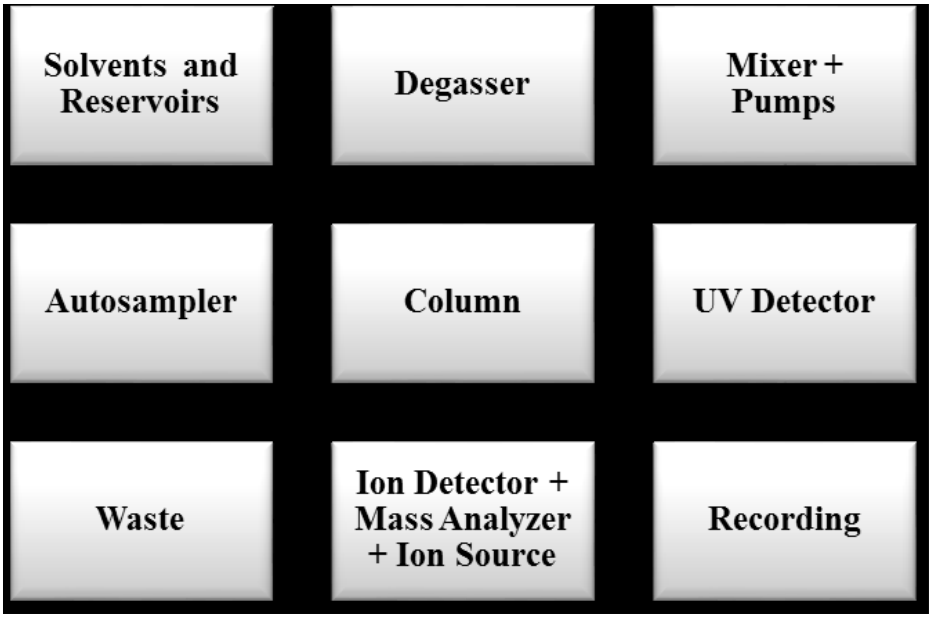

Figure No. 9: Flow Charts of LCMS

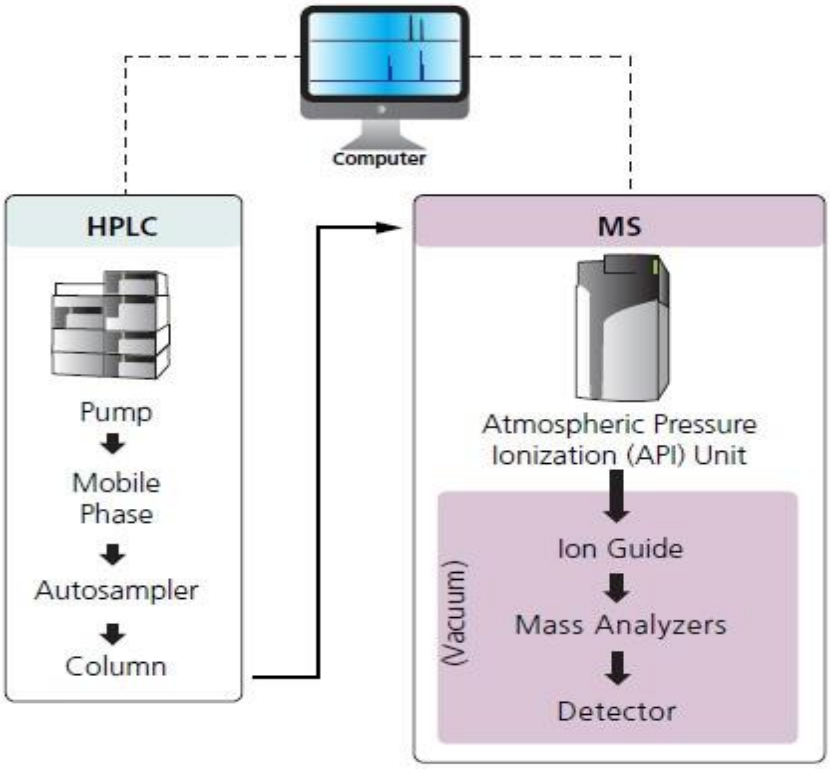

Figure No. 10: Diagram of LCMS

For an LCMS system the instrumentation comprises of:

I. an LC unit,

II. an interface between the LC and MS,

III. an ion source that ionises samples (e.g. API unit),

IV. an ion guide (an electrostatic lens that efficiently introduces the generated ions into the MS,

V. a mass analyzer unit that separates the ions based on their mass-to-charge (m/z),

VI. a detector unit that detects the separated ions [9]

❖ **Applications of High Performance Liquid chromatography: Mass spectroscopy**

LC-MS are most widely used in food industries, pharmaceutical and chemical industries for quantitative and qualitative analysis.

Applications of LS-MS are as follows.

1. Molecular Pharmacognosy:

LCMS determines the contents and categories of different groups of cultured plant cells and select the pair of groups with the biggest different content of ingredient for the study ingredient difference phenotypic cloning [17].

2. Characterization and Identification of Compounds:

Carotenoids:

Because carotenoids are not thermally stable, separation of mixtures and removal of impurities is usually carried out by reversed phase HPLC (particularly HPLC) instead of gas chromatography. The small samples of carotenoids which were isolated from biological matrices such as human serum or tissue prevent structural analysis by Nuclear Magnetic Resonance. Hence, only the most sensitive analytical methods are adequate such as Liquid Chromatography/Mass Spectrometry and HPLC with photodiode-array UV / visible absorbance detection. At the minimum level, carotenoid identification may be confirmed by combining data such as HPLC retention times, photodiode-array absorbance spectroscopy, mass spectrometry and tandem mass spectrometry. Upto date, five LC/MS techniques have

been used for carotenoid analysis including moving belt, particle beam, continuous flow fast atom bombardment, electrospray and Atmospheric Pressure Chemical Ionization (APCI). Among these LC/MS interfaces, electrospray and APCI are probably the easiest to use and are rapidly becoming the most widely available. These techniques provide comparable sensitivity (at the low pmol level) and produce enormous molecular ions [20].

3. Molecular weight determination:

Able to determine the molecule weight of chemical substance, pharmaceutical substances, proteins, etc.

4. Structural determination/elucidation:

Tandem mass spectrometry used to determine structural information using mass spectral fragmentations.

5. Pharmaceutical applications:

It's used to determine the pharmacokinetic profile of the pharmaceuticals like drug, drug metabolites/degradation product, impurities and chiral impurities. The separation and detection of chiral impurities in pharmaceuticals are of great importance because the D-isomer of a drug can have different pharmacological, metabolic and toxicological activity from the L-isomer. [21]

Rapid Chromatography of Benzodiazepines

The information available in a mass spectrum allows some compounds to be separated even though they are chromatographically unresolved. In this example, a series of benzodiazepines was run using both UV and MS detectors. The UV trace could not be used for quantitation, but the extracted ion chromatograms for the MS could be used. The mass spectral information provides additional confirmation of the identity. Chlorine has a characteristic pattern because of the relative abundance of the two most common isotopes.

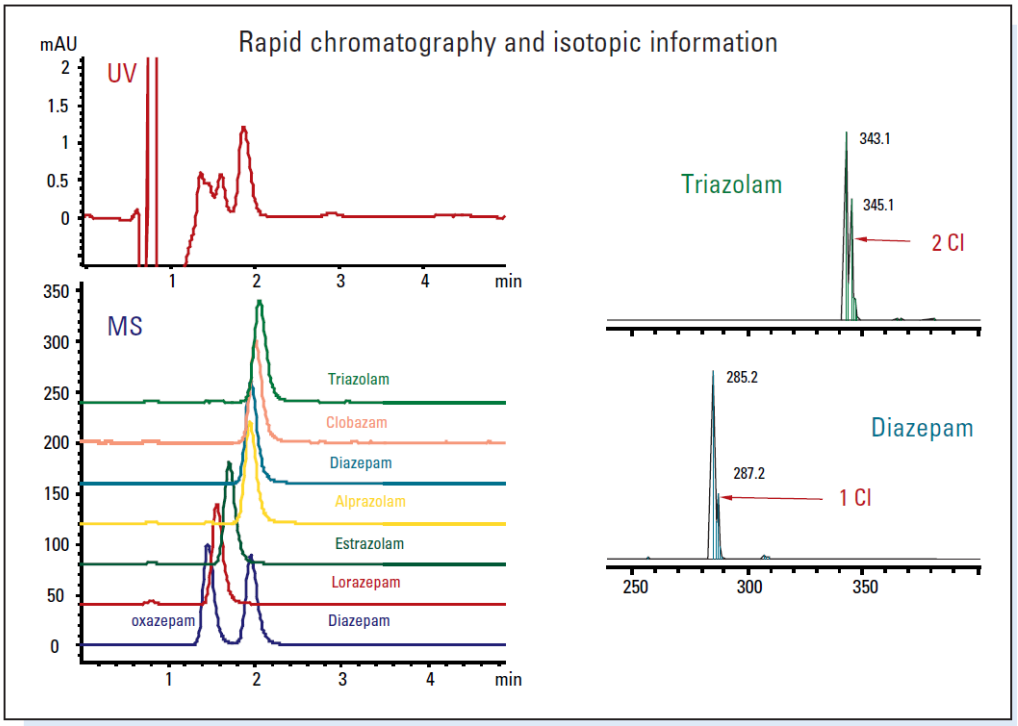

Figure No. 11: Benzodiazepines by API-ES

Detection of Degradation Products for Salbutamol

Detecting degradation products can often be difficult because they can be structurally very similar to the original product. If the chromophoric region is intact, the two compounds cannot be distinguished with a UV detector. The UV spectra for the salbutamol and its degradation products are very similar. The unique mass spectral fragments can be used to identify the compounds.[22]

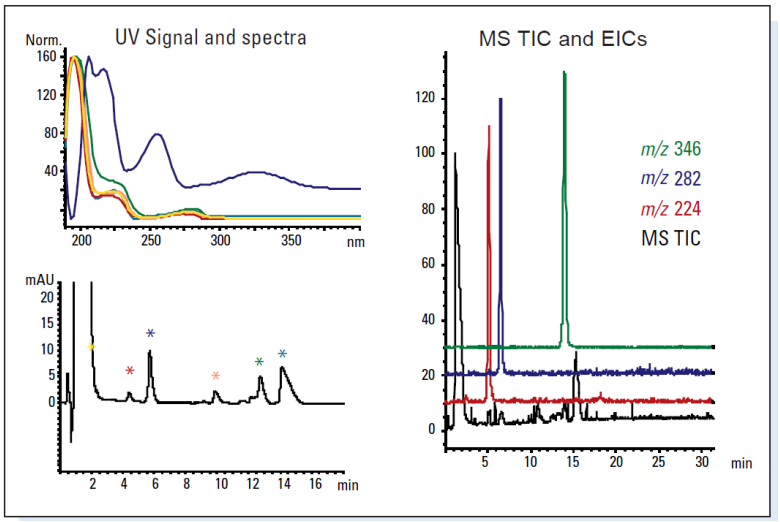

Figure No. 12: Salbutamol degradation products

6. Proteomics:

Liquid Chromatography/Mass Spectrometry (LC/MS) has become a powerful technology in proteomics studies in drug discovery which includes target protein characterization and the discovery of biomarkers. [23]

a. Glycopeptides Characterization:

MS-based glyco-proteomic studies are used to characterise the glycopeptides under examination. This involves pinpointing the glycosylation site, the type of glycan involved and the peptide backbone core. In present, with MS-based strategies, tandem MS fragmentation and data analysis problems provide efficient characterization of intact glycopeptides and then analysis of the peptides is done via Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS).

b. Peptide Mapping

In earlier days protein drugs were made from proteins refined from living organisms. However, they are recently produced using recombinant technology. Insulin, interferon, and erythropoietin are some of the protein drugs made by recombination which are available in the market.

Confirmation of the expression of recombinant proteins is important from the quality control viewpoint. Some of the methods applied for this include analysis of amino acid sequence by peptide sequencer and other simpler methods such as peptide mapping by HPLC or mass mapping by MALDI-TOF MS. For example, Protein analysis and peptide mass mapping of a model sample of horse heart myoglobin is done by LC/MS using a quadrupole mass spectrometer. [24]

C. Products of Degradation

LCMS was used to separate, identify, and characterize degradation products under hydrolytic, oxidative, photolytic, and thermal stress conditions. With the help of LC-MS / MS studies, a complete mass fragmentation pathway of the drug was first established. LC-MS analysis was performed on the stressed samples. It is done to conduct exchange mass studies in order to determine their exact mass, fragment pattern, and number of unstable hydrogens. The MS findings aided in the assignment of provisional structures to degradation products**.** Identification and characterization of Irbesartan degradation products and stressed

Prulifloxacin degradation products are two examples. Hydrolytic degradation, on the other hand, is accomplished by decomposing the drug under hydrolytic conditions, resolving the products on an HPLC column, characterizing the major products using LC-MS/MS studies, and postulating the probable degradation pathways using studies at various time points. Identification and Characterization of Hydrolytic Products of Atorvastatin Phytoconstituents/Plant Metabolomics LC-MS, for example, provides a tool for differentiating this vast plant biodiversity due to its ability to analyse a wide range of metabolites, including secondary metabolites (e.g., alkaloids, glycosides, phenylpropanoids, flavonoids, isoprenes, glucosinolates, terpenoids (oligosaccharides and lipids). LC-MS is one of the most important untargeted analytical techniques for determining global metabolite profiles, as it aids in the identification and relative quantification of all peaks in the chromatogram as ions defined initially by retention time and molecular mass. An improved LC-MS/MS method for continuous determination, qualitative and quantitative analysis of several medicinal plants was developed. Some examples include *Eclipta prostrata* L., a Chinese medicinal tonic, and eleven bioactive constituents of Radix *Angelicae Pubescentis* and its related preparations. The non-targeted LCMS technique was used to analyse active extracts of *Terminalia ferdinandiana* (Kakadu plum) fruit. [24,25]

7. Automated Immunoassay in Therapeutic Drug Monitoring:

TDM of certain drugs with a narrow therapeutic index aids in the improvement of patient outcomes. The requirement for accurate, precise, and standardized drug measurement poses a significant challenge for clinical laboratories and the diagnostics industry. To meet these requirements, various techniques had evolved in the past. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) methods and immunoassays appear to be the most widely used approaches in clinical laboratories today.

Assays based on mass spectrometry can be analytically sensitive, specific, and capable of measuring multiple compounds in a single process. This is a low-cost method of monitoring patients receiving multidrug therapy (e.g., antibacterial therapy for Tuberculosis patients). As demonstrated for immunosuppressant drugs, the selectivity provided by successive mass filtrations is an additional advantage of tandem mass spectrometry over immunoassays. [25]

8. Two Dimensional (2-D) Hyphenated Technology

LCMS has evolved into a powerful two-dimensional (2D) hyphenated technology for use in a wide range of analytical and bioanalytical techniques for the analysis of proteins, amino

acids, nucleic acids, amino acids, carbohydrates, lipids, peptides, and so on, and/or in the main classification in the fields of genomics, lipidomics, metabolomics, proteomics, and so on. LCMS was initially preferred, and this preference may be exacerbated by the need for more powerful analytical and bioanalytical techniques capable of precisely distinguishing target analytes in high complexity mixtures in a sensitive and specific manner. The combination of this hybrid class of HPLC and MS to perform both routine qualitative discovery and quantitative directed analysis of complex mixtures is conceivably one of the most significant combinations in developments and separations, where mass spectrometry has played a significant role in the field of science by detecting various analytical & bioanalytical techniques over the last decade. When combined with an MS system, it improves the robustness and accuracy of their LC systems as well as their detection abilities. [24]

9. Clinical chemistry and toxicology:

For certain clinical chemistry and toxicology analytes, liquid chromatography (LC) combined with tandem mass spectrometry (MS/MS) provides clear advantages over immunoassay testing. Oestradiol, testosterone, thyroid hormones, immunosuppressants, vitamin D, steroids for newborn screening programmes, and clinical and forensic toxicology are among the analytes tested. While immunoassays are widely used in clinical laboratories, the analytical sensitivity and specificity for many of the analytes tested in routine clinical laboratories are subpar. Furthermore, LC-MS/MS can be multiplexed for high testing throughput and detection of multiple analytes. The use of LC-MS/MS in clinical chemistry and toxicology studies will improve as the benefits become more widely known. There are few immunoassays for therapeutic drugs that, if used incorrectly, can cause toxicity. The goal of an untargeted analysis is to identify as many drugs as possible that are of clinical or forensic importance, regardless of the availability of an immunoassay. Urine is usually the preferred sample, but serum and blood are also few important sample types.[26]

10. Clinical and biochemical applications:

SNP genotyping, DNA quantification, gene expression analysis, and DNA and RNA sequencing are all applications for MALDI-TOF MS.

11. Food and Environmental applications:

Use to identify aflatoxins (toxic metabolic product in certain fungi), determine the vitamin D3 in poultry fed supplements, etc. [14-16]

CONCLUSION:

The development of hyphenated techniques, high resolution mass analyzers, and high throughput separation approaches, as well as quantitative and quantitative analysis of pharmaceutical drugs and metabolites, can be accomplished with high sensitivity.

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