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"AN ANALYTICAL OVERVIEW OF LIQUID CHROMATOGRAPHY-MASS SPECTROSCOPY (LC-MS) INSTRUMENTATION AND APPLICATIONS"

[1] Velpula Aditya Prasanthi*, Dr. P. V Madhavi Latha [2], Dr. P. Umadevi [3], P. Sivalalitha [4]

1.M. Pharmacy, M. Pharmacy Ph.D.,[2] M. Pharmacy, Ph.D.,[3] M. Pharmacy[4]

Viswanadha Institute of Pharmaceutical Sciences, Visakhapatnam, Andhra Pradesh, India

Abstract: LC-MS is a technique that combines physical separation capabilities of liquid chromatography with mass analysis compatibilities of mass spectrometry. It is a method that combines separation power of HPLC with detection power of mass spectroscopy. In LC-MS we remove detector from the column of LC and fit the column to interface of MS In the most of the cases the interfaces used in LC-MS are ionization source. The basic theory of HPLC is for separating a complex mixture into its components. High sensitivity of mass spectroscopy provides the information for identification compounds. As the metabolites appears the end of the column, they enter mass detector where the solvent is removed and metabolites are ionized. The use of LC-MS in toxicology laboratory is for drug conformation testing of an immune assay screen and for broad spectrum drug screening. Multiple different LC-MS platform including LC-TOF and LC-orbitrap have been used for toxicology testing. In addition, multiple different data acquisition modes have been described including target, Acquisition methods with & without data dependent acquisition of product ions spectra. This chapter highlights two LC-MS applications including opioid conformation testing, broad-spectrum drug screening laboratories experience with method development, validation and implementation.

Key words: chromatography, spectrometry, mass analyzer, bioavailability.

INTRODUCTION

When LC and MS are combined into LC-MS, LC separates the mixture into its components, and then MS identifies and quantifies these components based on their mass spectra. This hyphenated technique is powerful because LC separates complex mixtures and MS identifies the separated components accurately. LC-MS is indeed a powerful analytical technique that seamlessly integrates the capabilities of liquid chromatography (LC) and mass spectrometry (MS) to provide comprehensive analysis of complex mixtures. [1]

1. **Purpose of Mass Spectrometry**: Mass spectrometry is crucial for determining the structures of both known and unknown compounds. It provides detailed information about the molecular weight and structure of molecules based on their mass-to-charge ratio.
2. **Challenges with Mixtures**: When dealing with mixtures, mass spectrometry alone may not be sufficient because the mass spectrum of a mixture is complex, often comprising overlapping spectra from individual components. [2]

3. **Integration of LC and MS**: Liquid chromatography (LC) is often coupled with mass spectrometry (MS) to address the challenges posed by mixtures. An interface is used to transfer liquid eluents from LC to MS, allowing for separation of components before MS analysis.
4. **Identification:** MS provides structural information through fragmentation patterns. When ions are fragmented inside the mass spectrometer, the resulting spectra (MS/MS or MS^n spectra) reveal characteristic fragments that can identify the compound. [3]

PRINCIPLE

Liquid chromatography (LC) [4] is indeed a powerful analytical method used extensively for separating compounds from complex mixtures. It operates based on the principle of separating components as they move through a stationary phase under the influence of a mobile phase. High-Performance Liquid Chromatography (HPLC) is a type of LC that is particularly efficient and widely used in various fields. One of the significant advantages of HPLC is its ability to analyze compounds with lower volatility and higher polarity across a wide range of masses without the need for derivatization. This makes it suitable for a broad spectrum of chemical compounds, enhancing its applicability in many scientific and industrial contexts. However, a challenge in liquid chromatography is reliably identifying and quantifying compounds, especially when dealing with complex samples where multiple compounds may co-elute (appear together in a chromatogram). This difficulty is addressed by coupling liquid chromatography with mass spectrometry (LC-MS). Mass spectrometry provides additional information by measuring the molecular weights of compounds, which helps in identifying and distinguishing between different chemicals even when they co-elute in the chromatogram. In summary, while liquid chromatography is effective for separating compounds, the addition of mass spectrometry in LC-MS enhances the method's capabilities by providing molecular weight data that aids in the accurate identification and characterization of compounds in complex mixtures. This combination, Liquid chromatography-mass spectroscopy (LC-MS), is widely used in analytical chemistry for its sensitivity, specificity, and versatility in compound analysis.

INSTRUMENTATION

Liquid chromatography - Mass spectrometry (LC-MS)

LC-MS (Liquid Chromatography-Mass Spectrometry) is indeed a powerful analytical technique that combines the separation capabilities of liquid chromatography with the detection and identification capabilities of mass spectrometry.LC-MS, combines high-resolution chromatographic separation with sensitive mass spectrum detection. This integration represents a significant milestone in chromatographic history, offering precise structural elucidation and identification of sample constituents. Known for its sensitivity and selectivity, LC-MS finds extensive application in pharmaceutical pharmacokinetics and bioanalysis. It is particularly valuable in pharmacognosy, including molecular pharmacognosy, where it aids in identifying components from various phenotypic cloning procedures.

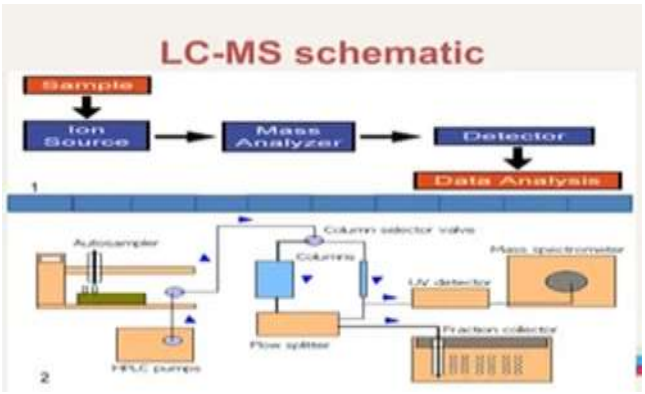

Figure 1: liquid chromatography-mass spectroscopy

LIQUID CHROMATOGRAPHY

High Performance Liquid Chromatography (HPLC) is a versatile technique for separating components within a mixture, employing a solid stationary phase and a liquid mobile phase. This method encompasses various specialized categories tailored for different types of chemical analysis, such as affinity liquid chromatography for biomolecules, reverse phase chromatography for organic compounds, ion-exchange liquid chromatography for charged molecules, chiral separation for enantiomers, and normal phase liquid chromatography for non-polar substances.[5]

a. **Pump** [6] "It consists of materials that are resistant to a wide range of solvents, including organic solvents and aqueous buffers. The system can deliver mobile phase volumes of up to 10 mL/min, facilitated by three main types of pumps: syringe pumps, constant-pressure pumps (isocratic pumps), and reciprocating pumps."

b. **Sample Injector:** "It is used to introduce sample volumes into the chromatographic system, typically ranging from 1µL to 100µL. An injector loop can be employed to increase the injection volume up to 2µL. There are two main types of injectors: automatic and manual. Automatic injectors offer greater precision and reproducibility compared to manual injectors."

c. **Columns:** "The stationary phase consists of carbon chains bonded to silica material. Columns typically range in length from 50 to 300 mm. Common types of columns used in HPLC include Octadecyl (C18), Octyl (C8), Cyano, Amino, and Phenyl packings. The choice of column depends on the specific compound to be separated."

MASS SPECTROMETRY [7]

Mass spectroscopy (MS) is a potent analytical technique utilized for quantifying known materials, identifying unknown compounds with in a sample, and elucidating the structure and chemical properties of diverse molecules. The process involves converting the sample into gaseous ions, with or without fragmentation, followed by characterisation based on their mass-to-charge ratios (m/z) and relative abundances. A triple quadrupole mass spectrometer, commonly used in tandem mass spectrometry (TDM), consists of three quadrupole mass analyzers. The first and third quadrupoles function as filters, while the second quadrupole acts as a collision cell. Here, ions of a predetermined mass are selected in the first quadrupole (Q1) and then fragmented in the collision cell. The third quadrupole (Q3) subsequently filters detects these compound-specific fragment ions. This configuration ensures high analytical specificity, as only fragment ions originating from the selected precursor ions are monitored. The instrument operates with a resolution of approximately one Dalton (Da), enabling it to distinguish ions with slight mass differences when set to near-maximum resolution. Calibration and tuning are performed using pure analyte solutions, and daughter ions are chosen based on their abundance to optimize assay sensitivity.

- Ionization Sources and Interfaces
- Mass Analysers
- Detectors

a) Ionization/Ion Source and Interfaces:

Electrospray Ionization (ESI):

- **Principle**: Electrospray ionization generates ions from a liquid sample by applying a high voltage to a flow of liquid exiting a narrow capillary.
- **Usage**: Widely used for LC-MS due to its ability to ionize a wide range of analytes, especially polar and biomolecules.

Atmospheric Pressure Chemical Ionization (APCI):

- **Principle**: APCI ionizes analytes in a similar fashion to ESI but at higher temperatures and through chemical reactions in the ion source.
- **Usage**: Suitable for less polar compounds that do not ionize well with ESI.

Atmospheric Pressure Photoionization (APPI):

- **Principle**: APPI uses UV light to ionize compounds in the gas phase after they are volatilized from the LC effluent.
- **Usage**: Effective for ionizing compounds with lower proton affinities or those less suited to ESI or APCI.

Thermospray Ionization:

- **Principle**: Similar to APCI, thermospray ionization operates at higher temperatures to volatilize and ionize analytes.
- **Usage**: Historically used in LC-MS but less common today due to advancements in other ionization techniques.

Direct Analysis in Real Time (DART):

- **Principle**: DART ionizes compounds directly from the sample surface using a stream of ionized gas (usually helium or nitrogen).
- **Usage**: Rapid analysis of solid or liquid samples without the need for prior chromatography.

Flow Fast Atom Bombardment (FAB):

- **Principle**: In FAB, a liquid sample containing analyte molecules is dissolved in a matrix, deposited onto a metal surface or probe tip. This matrix-sample mixture is then exposed to a beam of high-energy neutral atoms. These fast atoms collide with the matrix and sample molecules, transferring energy and causing ionization.
- **Usage:** FAB has extensively used for the analysis of proteins and peptides and also used in the analysis of alkaloids & steroids.

b) MASS ANALYZER [8]

High-resolution LC-TOF (Time of flight) mass spectrometers are highly valued in analytical chemistry for their ability to identify unknown compounds in samples lacking standard references. This capability stems from their high-resolution mass spectra, which differentiate closely spaced ions with precision, enabling accurate determination of compound elemental composition and distinction among similar compounds.

Working:

LC-MS (Liquid Chromatography coupled with Mass Spectrometry) facilitates definitive compound identification and enhances the quantitative analysis of samples.

This instrument consists of four vacuum stages and allows filtering from the initial stage, starting from the spray chamber, preventing most solvents from entering the capillary. Only gas ions and a small fraction of solvents can pass through the capillary.

- At the capillary exit, the skimmer filters the ions.
- Ions with higher mass and momentum can easily pass through the skimmer aperture.
- The ions that pass through the skimmer proceed to the second stage of the vacuum system.
- In the second stage, an octopole focuses the ions to efficiently pass through the subsequent two vacuum stages.
- Ions gain momentum due to atmospheric pressure during capillary sampling, enabling them to pass through the octopole.
- Exiting this stage, ions pass through two focusing lenses towards the fourth vacuum stage.
- In the fourth stage of the vacuum system, ions are separated based on their mass-to-charge ratio using a quadrupole mass analyzer.
- The quadrupole mass analyzer utilizes an electromagnetic field to accurately determine ions based on their mass-to-charge ratio, allowing only selected ions to pass through the filter at specific times.
- Finally, ions are focused onto the detector where their presence is recorded and analyzed.

Quadrupole Mass Analyser: [9]

It consists of four hyperbolic rods arranged in a radial array. These rods are parallel to each other and positioned between the ion source and the detector. The quadrupole mass analyzer operates on the principle of ion separation based on their mass-to-charge ratio (m/z) using a combination of direct current (DC) and radio frequency (RF) voltages applied to the rods. When a combination of DC and RF voltages is applied, the ions with a specific m/z ratio follow stable trajectories through the quadrupole while ions with other m/z ratios are destabilized and collide with the rods. Ions of a specific m/z ratio are stable within the electric fields generated by the DC and RF voltages and are transmitted through the quadrupole to the detector. The DC voltage creates a static electric field, while the RF voltage creates an oscillating electric field. As ions pass through the quadrupole, they oscillate in a plane perpendicular to the rod length due to the oscillating RF field. Ions are introduced into the quadrupole with a modest accelerating potential. The combination of DC and RF voltages applied to the rods selectively filters ions based on their m/z ratio.

Time of flight analyser (TFA):[10] In a TOF-MS, ions are generated from a sample in the ion source. It typically operates without a magnetic field, relying instead on electrostatic fields for ion acceleration, focusing, and detection. This simplifies maintenance and calibration compared to instruments that use magnetic fields. After being formed in the ion source, ions are exposed to an accelerating voltage. This voltage accelerates ions of different masses (m) and charges (z) equally, but their velocities differ due to their different masses. The flight time of ions through the TOF-MS tube is directly related to their mass-to-charge ratio (m/z). Lighter ions (lower m/z values) reach the detector sooner than heavier ions (higher m/z values) due to their higher velocities under the same accelerating voltage. Once ions reach the detector, their flight times are recorded. The mass of each ion can be determined from its flight time using the equation $m=(z\cdot(t_{flight})^2)/2.k)$, where m is the mass of the ion, z is its charge, t_{flight} is the flight time, and *K* is a proportionality constant related to the acceleration voltage. In TOF-MS, all ions generated in a single ionization event are typically detected simultaneously as they arrive at the detector over a short time period. This allows for rapid scanning of mass ranges. Also perform fast mass range scanning, which is advantageous for analyzing ions with very large m/z values.

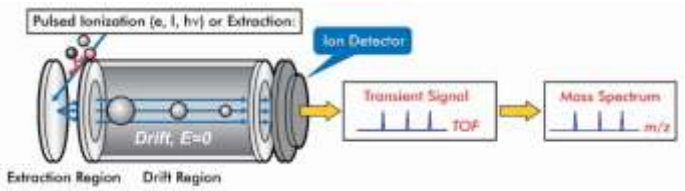

Figure 2: time of flight analyser

c) Detector: After the ions are created and exit the analyzer in a mass spectrometer, they enter the detector stage where they are detected and converted into a measurable signal. The detector in a mass spectrometer plays a crucial role in this process by generating a current or voltage signal that is directly proportional to the number of ions striking it. Different types of detectors are used depending on the specific requirements of the mass spectrometry technique and the characteristics of the ions being detected.

1. Photomultiplier tube: A photomultiplier tube (PMT) is a highly sensitive detector used in various scientific instruments, including mass spectrometers, where it is employed to detect photons generated by interactions with ions or other particles.

i. **Photocathode**: The photomultiplier tube begins with a photocathode, a photosensitive surface typically made of a material such as bialkali materials. When photons strike the photocathode, they cause the emission of electrons through the photoelectric effect.

ii. **Electron Multiplier (Dynode Chain)**: Once emitted from the photocathode, these electrons are accelerated towards a series of electrodes called dynodes.

APPLICATIONS

LC-MS is widely used in various fields such as pharmacodynamics (effects of drugs on the body), bioavailability (rate and extent of drug absorption), bioequivalence (comparison of generic drugs to brand-name drugs), and dissolution (release of drug from its dosage form). This combination is effective in providing detailed molecular information in complex biological samples and pharmaceutical formulations. In summary, LC-MS is a powerful analytical technique used to overcome the limitations of analyzing mixtures with mass spectrometry alone, making it invaluable in pharmaceutical and biological research.

Preparative LC-MS systems offer significant advantages in the purification of chemicals from complex mixtures. Here are some key benefits across various industries

1. **Speed and Efficiency**: LC-MS systems can rapidly identify and purify target compounds from mixtures, saving time compared to traditional purification methods like column chromatography.
2. **High Selectivity**: Mass-directed purification allows for precise isolation of target compounds based on their mass-to-charge ratios (m/z), ensuring high purity of the final product.
3. **Improved Yield**: The ability to optimize conditions and scale up purification processes in preparative LC-MS systems often results in higher yields of purified compounds compared to conventional techniques.
4. **Flexibility**: These systems are adaptable to various types of samples and can handle a wide range of compound polarities and concentrations, making them versatile in different industrial settings.

Overall, preparative LC-MS systems play a crucial role in advancing research capabilities and improving production processes in industries requiring high-purity compounds. Their ability to selectively purify target chemicals from complex mixtures benefits both scientific progress and industrial applications alike.

5. **Blood Withdrawal**: LC-MS methods often require smaller sample volumes compared to traditional blood tests, reducing the need for repeated blood withdrawals.
6. **Plasma Partition**: With LC-MS, there's no need for separating plasma from whole blood, which simplifies the testing process.
7. **Refrigerated Shipment**: LC-MS analysis can sometimes be performed on samples that do not require strict refrigeration during transportation, depending on the stability of the analytes.
8. **Improved Data on Drug Exposure**: LC-MS can provide more accurate and detailed information about drug concentrations specifically in target cells or tissues, offering insights into drug exposure levels that plasma testing may not achieve**.** [11]
9. **Identification of Acylcarnitines and Amino Acids**: By analyzing acylcarnitine's and amino acids, LC-MS/MS can detect abnormalities that indicate the presence of inherited metabolic disorders. These disorders can affect metabolism pathways, leading to characteristic profiles of metabolites in body fluids.
10. **Range of Disorders Detected**: Depending on the specific panel and methodology used, LC-MS/MS can identify up to 45 different inherited disorders. These disorders typically involve defects in enzymes or transport proteins involved in amino acid, organic acid, or fatty acid metabolism.[12]
11. **Clinical Utility**: LC-MS/MS is employed for screening newborns for metabolic disorders, diagnosing patients with unexplained symptoms, monitoring treatment efficacy, and conducting research into metabolic pathways [13]
12. Mass spectrometric techniques are very useful in the identification of hormones such as estrogens and progestogens. [14]

CONCLUSION:

LC-MS and LC-MS/MS is highly effective for analyzing higher polarity and lower volatility chemical compounds across a wide mass range without requiring derivatization. These techniques offer superior specificity and sensitivity, making them particularly valuable for identifying both semipolar and nonpolar compounds. LC-MS/MS, in particular, surpasses immunoassay in reliability for compounds lacking natural chromophores, addressing various limitations associated with immunoassay techniques. LC-MS/MS combines the separation power of HPLC, making it a powerful tool for analytical chemistry especially in the pharmaceutical, environmental, and biomedical fields.

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