EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 5
Issue: 5
941-947
Year: 2018

# LIQUID CHROMATOGRAPHY-MASS SPECTROSCOPY(LC-MS)

**Manooj Kumar*, Mohd Shabaz Khan, Mohd Zubair, Shubam Sehgal, Tanvay Jaithliya and Atul Tiwari[1]**

*Faculty of Pharmaceutical Sciences, Mewar University, 312901, Chittorgarh, India.
[1]Assistant Professor, Faculty of Pharmaceutical Sciences, Mewar University, Chittorgarh, 312901, India.

***Corresponding Author: Manooj Kumar**
Faculty of Pharmaceutical Sciences, Mewar University, 312901, Chittorgarh, India.

**Article Received on 19/03/2018** **Article Revised on 09/04/2018** **Article Accepted on 29/04/2018**

## ABSTRACT
Liquid chromatography is a technique of physical parting in which the components of a liquid mixture are distributed between two immiscible phases, i.e., stationary and mobile. Mass spectrometry (MS) is an analytical method that measures the mass-to-charge ratio (*m/z)* of charged particles (ions). Liquid chromatography–mass spectrometry (LC- MS) is an analytical chemistry technique that combines the physical separation abilities of liquid chromatography (or HPLC) with the mass analysis abilities of mass spectrometry (MS). In adding to the liquid chromatography and mass spectrometry devices, an LC-MS system contains an interface that transfers the separated components from the LC column into the MS ion source.

**KEYWORDS:** Liquid chromatography column into the MS ion source.

## Liquid chromatography
Liquid chromatography is a technique of physical parting in which the components of a liquid mixture are distributed between two immiscible phases, i.e., stationary and mobile. The practice of LC can be divided into five categories, i.e., adsorption chromatography, partition chromatography, ion-exchange chromatography, size-exclusion chromatography and affinity chromatography. Among these, the most broadly used different is the reverse-phase (RP) mode of the partition chromatography technique, which makes use of a non-polar (hydrophobic) stationary phase and a polar mobile phase. In common applications, the mobile phase is a mixture of water and other polar solvents (e.g., methanol, isopropanol, and acetonitrile), and the stationary matrix is ready by attaching long-chain alkyl groups (e.g., n-octadecyl or C18) to the surface of irregularly or spherically shaped 5 µm diameter silica particles.[1]

In HPLC, characteristically 20 µl of the sample of attention are vaccinated into the mobile phase stream delivered by a high-pressure pump. The mobile phase containing the analytes infuses through the stationary phase bed in a definite way. The components of the mixture are separated dependent on their chemical affinity with the mobile and stationary phases. The parting occurs after repeated sorption and desorption steps occurring when the liquid interrelates with the stationary bed.[2]

The liquid solvent (mobile phase) is delivered under high pressure (up to 400 bar or 300.000 torr) into a filled column containing the stationary phase. The high pressure is essential to attain a constant flow rate for reproducible chromatography experiments. Dependent on the partitioning between the mobile and stationary phases, the components of the sample will flow out of the column at different times.[3]

The column is the most significant component of the LC system and is designed to withstand the high pressure of the liquid. Conventional LC columns are 100–300 mm long with outer diameter of 6.4 mm (1/4 inch) and internal diameter of 3.0-4.6 mm. For applications involving LC-MS, the length of chromatography columns can be shorter (30–50 mm) with 3-5 µm diameter packing particles. In addition to the conventional model, other LC columns are the narrowbore, microbore, microcapillary and nano-LC models. These columns have smaller internal diameters, allow for a more effective separation, and handle liquid flows under 1 ml/ min (the conventional flow-rate).[2]

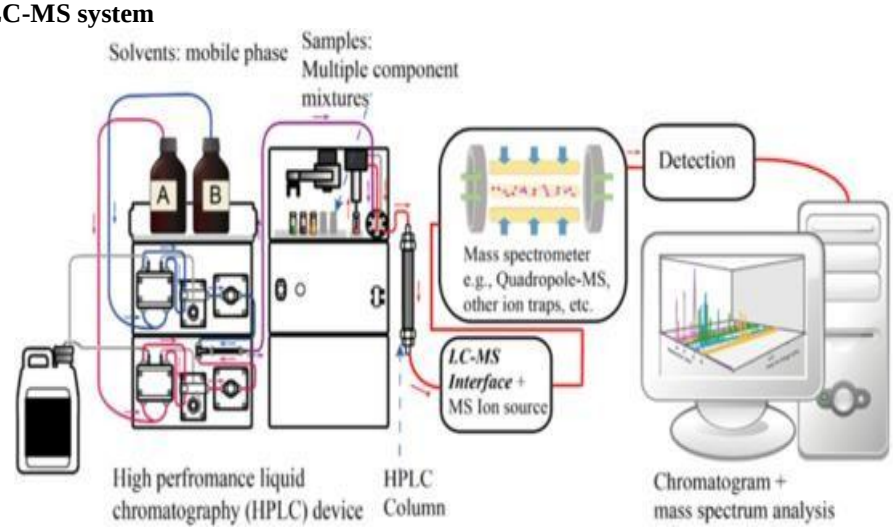
## Mass spectrometry
Mass spectrometry (MS) is an analytical method that measures the mass-to-charge ratio (*m/z)* of charged particles (ions). While there are many different kinds of mass spectrometers, all of them make use of electric or magnetic fields to work the motion of ions produced from an analyte of interest and determine their *m/z.*[4]

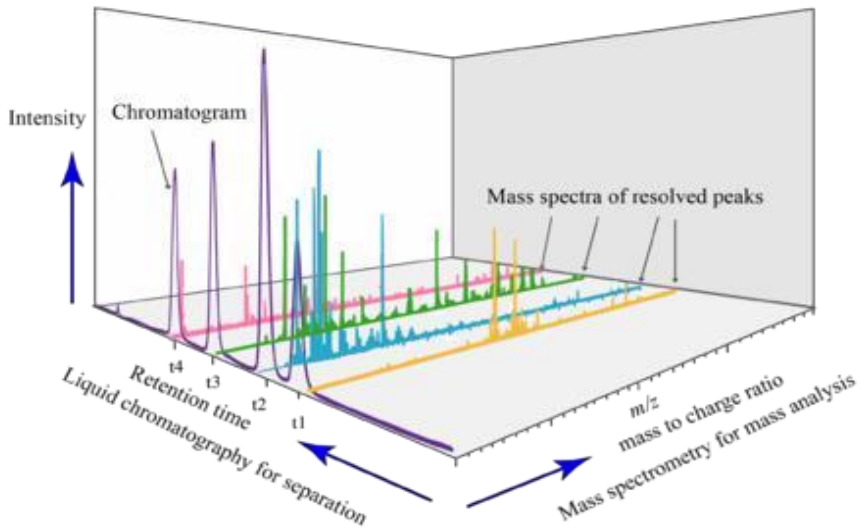
The basic components of a mass spectrometer are the ion source, mass analyzer, detector, and vacuum systems. The ion source is where the components of a sample presented in a MS system are ionized by means of electron beams, photon beams (UV lights), laser beams or corona discharge. In the case of

electrospray ionization, the ion source moves ions that exist in liquid solution into the gas phase. The ion source changes and fragments the neutral sample molecules into gas-phase ions that are sent to the mass analyzer. While the mass analyzer applies the electric and magnetic fields to sort the ions by their masses, the detector measures and amplifies the ion current to calculate the abundances of each mass-resolved ion. In order to generate a mass spectrum that a human eye can easily recognize, the data system records, processes, stores, and displays data in a computer.[1]

**Liquid chromatography–mass spectrometry (LC-MS)** is an analytical chemistry technique that combines the physical separation abilities of liquid chromatography (or HPLC) with the mass analysis abilities of mass spectrometry (MS). Coupled chromatography - MS systems are popular in chemical analysis because the specific abilities of each technique are enhanced synergistically. While liquid chromatography separates mixtures with multiple components, mass spectrometry offers structural individuality of the individual components with high molecular specificity and detection sensitivity. This tandem technique can be used to analyze biochemical, organic, and inorganic compounds usually found in complex samples of environmental and biological origin. Therefore, LC-MS may be useful in a wide range of sectors including biotechnology, environment monitoring, food processing, and pharmaceutical, agrochemical, and cosmetic industries.[5][1]

**Diagram of an LC-MS system**




**LC-MS Spectrum of each resolved peak**

In adding to the liquid chromatography and mass spectrometry devices, an LC-MS system contains an interface that transfers the separated components from the LC column into the MS ion source.[1][6]

The interface is essential because the LC and MS devices are fundamentally mismatched. While the mobile phase in a LC system is a under pressure liquid, the MS analyzers commonly operate under vacuum (around $10^{-6}$ torr). Thus, it is not possible to continuous pump the eluate from the LC column into the MS source. the interface is a

mechanically simple part of the LC-MS system that transfers the maximum amount of analyte, take away an important portion of the mobile phase used in LC and preserves the chemical identity of the chromatography products (chemically inert). As a requirement, the interface should not interfere with the ionizing efficacy and vacuum conditions of the MS system.[1]

Nowadays, most widely applied LC-MS interfaces are based on atmospheric pressure ionization (API) plans like electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo-ionization(APPI). These interfaces became accessible in the 1990s after a two decade long research and development process.[6][2]

**History**

The connection of chromatography with MS is a well developed chemical analysis plan dating back from the 1950s. Gas chromatography (GC) - MS was first presented in 1952, when A.T. James and A.J.P Martin were trying to develop tandem separation - mass analysis techniques.[7]

In GC, the analytes are eluted from the parting column as a gas and the joining with electron ionization (EI) or chemical ionization (CI) ion sources in the MS system was a technically simpler challenge. Because of this, the expansion of GC-MS systems was faster than LC-MS and such systems were first commercialized in the 1970's.[6]

The growth of LC-MS systems took longer than GC-MS and was directly associated to the growth of proper interfaces. V.L. Tal'roze and collaborators started the development of LC-MS in the early 1970s, when they first used capillaries to connect LC columns and MS ion sources.[2][8]

A similar plan was examined by McLafferty and collaborators in 1973. This was the first and most understandable way of coupling LC with MS and was known as the capillary inlet interface. This innovator interface for LC-MS had the same analysis capabilities of GC-MS and was limited to rather volatile analytes and non-polar mixtures with low molecular mass (below 400 Da). In the capillary inlet interface, the disappearance of the mobile phase inside the capillary was one of the main matters. Within the first years of growth of LC-MS, on-line and off-line substitutes were proposed as coupling substitutes. In general, off-line coupling involved fraction collection, disappearance of solvent, and transfer of analytes to the MS using enquiries. Off-line analyte action process was time consuming and there was an inherent risk of sample contamination. Quickly, it was realized that the analysis of complex mixtures would require the growth of a fully automated on-line coupling solution in LC-MS.[2]

**Instrumentation**

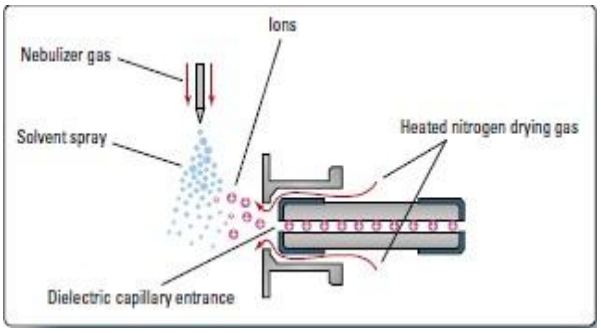
Mass spectrometers work by ionizing molecules and then categorization and identifying the ions according to their mass-to-charge (m/z) ratios. Two components in this process are the ion source, which produces the ions, and the mass analyzer, which sorts the ions. Several different types of ion sources are generally used for LC/MS. Each is suitable for different classes of compounds. Numerous different types of mass analyzers are also used. Each has advantages and disadvantages depending on the type of data needed. Ion Sources Much of the development in LC/MS over the last ten years has been in the development of ion sources and techniques that ionize the analyte molecules and separate the resulting ions from the mobile phase. Past LC/MS systems used interfaces that either did not separate the mobile phase molecules from the analyte molecules (direct liquid inlet, thermospray) or did so before ionization (particle beam). The analyte molecules were then ionized in the mass spectrometer under vacuum, often by customary electron ionization. These approaches were successful only for a very limited number of compounds. The overview of atmospheric pressure ionization (API) techniques greatly prolonged the number of compounds that can be successfully analyzed by LC/MS. In atmospheric pressure ionization, the analyte molecules are ionized first, at atmospheric pressure. The analyte ions are then mechanically and electrostatically separated from neutral molecules.

Common atmospheric pressure ionization techniques are:

- Electrospray ionization (ESI)
- Atmospheric pressure chemical ionization(APCI)
- Atmospheric pressure photoionization(APPI)

**Electrospray ionization**

Electrospray relies in part on chemistry to generate analyte ions in solution before the analyte reaches the mass spectrometer. The LC eluent is sprayed into a compartment at atmospheric pressure in the presence of a strong electrostatic field and heated drying gas. The electrostatic field causes further separation of the analyte molecules.




**Figure**

The heated drying gas effects the solvent in the droplets to evaporate. As the droplets shrink, the charge concentration in the droplets rises. Finally, 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. Some gas-phase reactions, generally proton transfer and charge exchange, can also occur between the time ions are ejected from the droplets and the time they reach the mass analyzer. Electrospray is mainly useful for analyzing large biomolecules such as proteins, peptides, and

oligonucleotides, but can also analyze lesser molecules like benzodiazepines and sulfated conjugates. Large molecules often obtain more than one charge. electrospray can be used to analyze molecules as huge as 150,000 u even though the mass range (or more exactly mass-to-charge range) for a typical LC/MS instruments is around 3000 m/z. For example: 100,000 u / 10 z = 1,000 m/z When a large molecule obtains many charges, a mathematical process called deconvolution is often used to determine the definite molecular weight of the analyte. Evaporation Analyte ion ejected Dielectric capillary entering Nebulizer gas Solvent spray Ions Heated nitrogen drying gas.

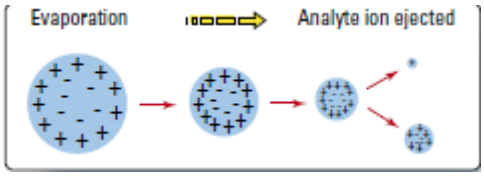
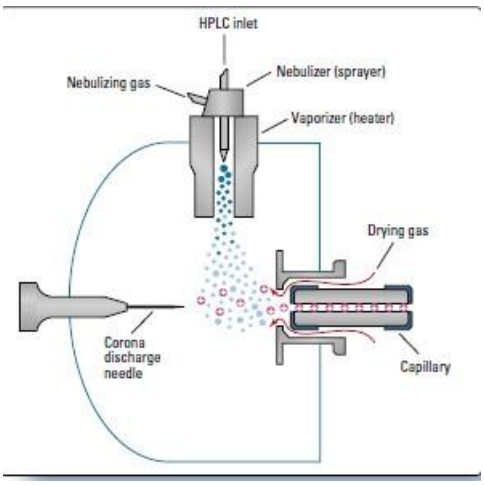
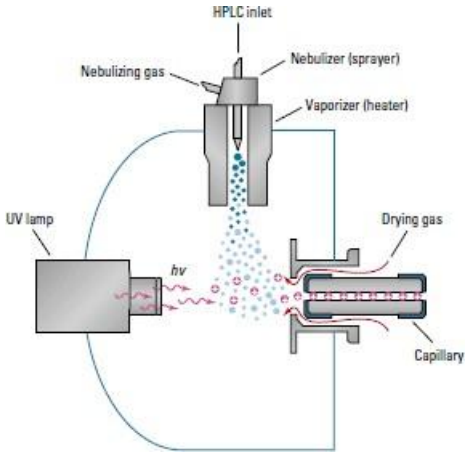



**Figure 2**

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 ionized 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 permit through a capillary sampling orifice into the mass analyzer. APCI is appropriate 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 may be thermally unbalanced. APCI is used with normal-phase chromatography more often than electrospray is because the analytes are usually non-polar.




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 produces photons in a narrow range of ionization energies. The range of energies is carefully chosen to ionize as many analyte molecules as possible while minimizing the ionization of solvent molecules. The resulting ions pass through a capillary sampling orifice into the mass analyzer APPI is appropriate to many of the same compounds that are typically analyzed 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 parting conditions have a strong influence on which ionization technique: electrospray, APCI, or APPI, will generate the best results. The most effective technique is not always easy to predict.



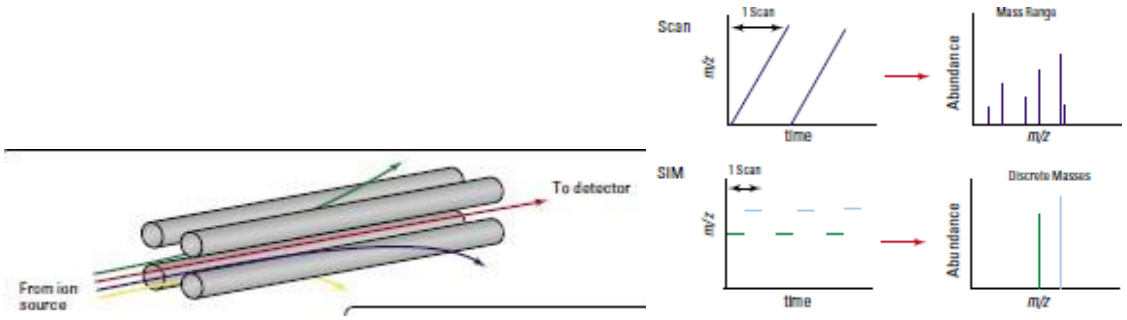

Mass Analyzers Although in theory any type of mass analyzer could be used for LC/MS, four types:

- Quadrupole
- Time-of-flight
- Ion trap
- Fourier transform-ion cyclotron resonance Quadrupole

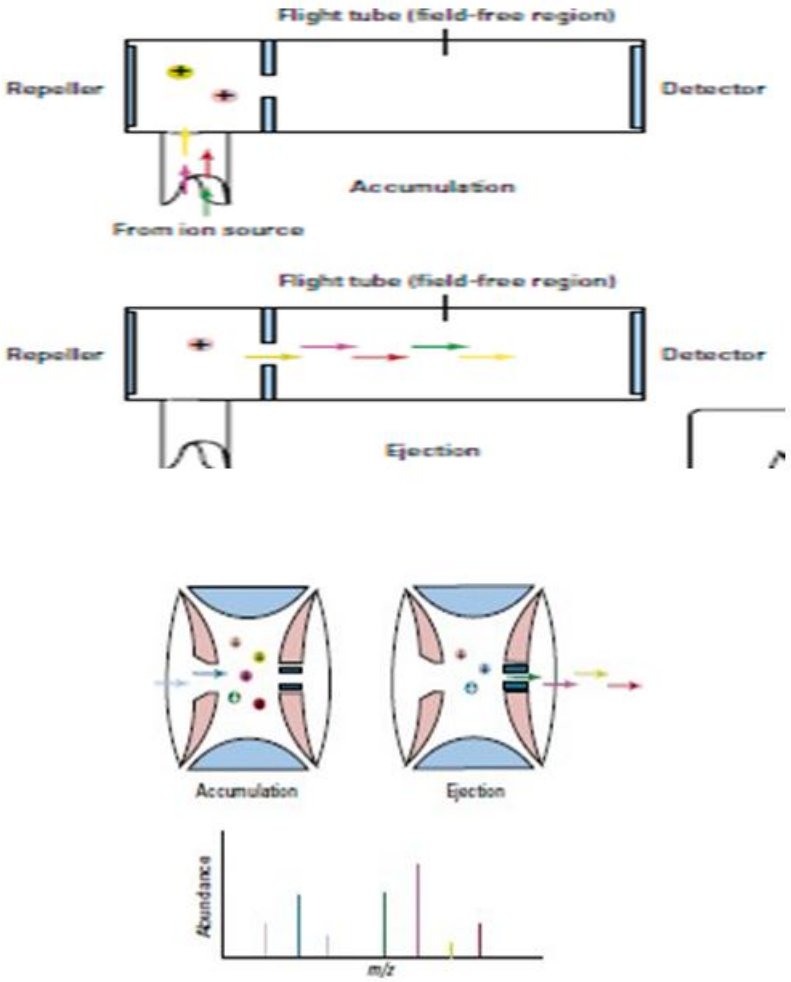
A quadrupole mass analyzer contains of four parallel rods arranged in a square. The analyte ions are directed down the middle of the square. Voltages applied to the rods produce electromagnetic fields. These fields determine which mass-to-charge ratio of ions can pass through the filter at a given time. Quadrupoles tend to be the simplest and least costly mass analyzers. Quadrupole mass analyzers can operate in two modes:

- Scanning (scan) mode
- Selected ion monitoring (SIM) mode

In examination mode, the mass analyzer monitors a range of mass-to-charge ratios. In SIM mode, the mass analyzer monitors only a few mass to charge ratios. SIM mode is significantly more sensitive than examination mode but provides evidence about fewer ions. Scan mode is typically used for qualitative analyses or for quantitation when all analyte masses are not known in advance. SIM mode is used for quantitation and monitoring of target compounds.

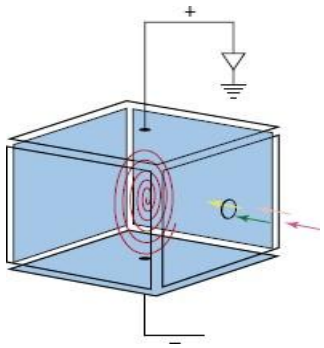



Time-of-flight In a time-of-flight (TOF) mass analyzer, an electromagnetic force is applied to all ions at the same time, causing them to accelerate down a flight tube. Lighter ions travel faster and arrive at the detector first, the mass-to-charge ratios of the ions are determined by their arrival times. Time-off light mass analyzers have a wide mass range and can be very exact in their mass measurements.




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

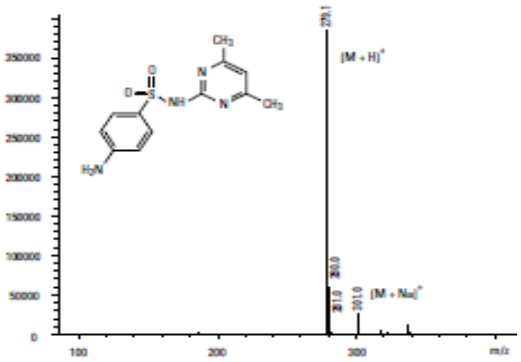
Fourier transform-ion cyclotron resonance (FT-ICR) An FT-ICR mass analyzer is another type of trapping analyzer. Ions incoming a chamber are trapped in rounded orbits by powerful electrical and magnetic fields. When excited by a radio-frequency (RF) electrical field, the ions produce a time dependent current. This current is transformed 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 added mass analyzers. They also have a wide mass range and excellent mass resolution. They are, however, the most costly of the mass analyzers.



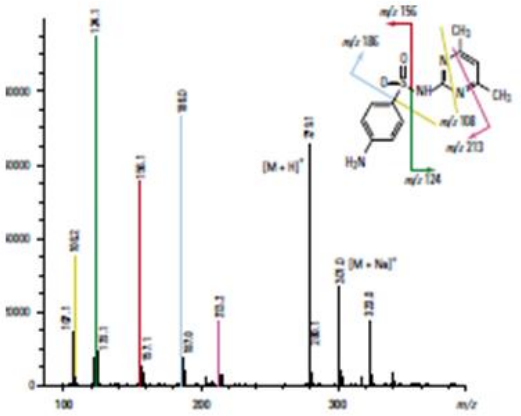
Collision-Induced Dissociation and Multiple-Stage MS.

The atmospheric pressure ionization techniques discussed are all relatively "soft" techniques. They produce primarily:

- Molecular ions M+ or M–
- Protonated molecules [M + H]+
- Simple adduct ions [M + Na]+
- Ions representing simple losses such as the loss of a water [M + H – H2O]+



The resulting molecular weight info is very valuable, but complementary structural information is often needed. To obtain structural info, analyte ions are fragmented by colliding them with neutral molecules in a process known as collision induced dissociation (CID) or collisionally activated dissociation (CAD). Voltages are applied to the analyte ions to add energy to the collisions and create more fragmentation.



**Sample preparation**

Sample preparation usually contains concentrating the analyte and removing compounds that can cause background ions or suppress ionization. Examples of sample preparation include:

- On-column concentration to rise analyte concentration
- Desalting to decrease the sodium and potassium adduct formation that commonly occurs in electrospray
- Filtration to separate a low molecular-weight drug from proteins in plasma, milk, or tissue.[9]

**Advantages**

Liquid chromatography coupled to tandem mass spectrometry (LC–MS has recently become a more and more popular alternative to traditional ligand-binding assays for the quantitative determination of biopharmaceuticals. LC–MS offers several advantages such as improved accuracy and precision, better selectivity, and generic applicability without the need for raising analyte-directed antibodies. Here we discuss the technical requirements for a successful LC–MS method for the quantitation of biopharmaceuticals and evaluate the advantages and disadvantages compared to ligand-binding assays.

**Advancement**

The development of protein‐based pharmaceuticals, or biopharmaceuticals, is by far the fastest growing part of the pharmaceutical industry today. With over 1500 biopharmaceuticals in clinical development and more and more companies shifting their R&D efforts towards this sophisticated and relatively profitable class of drugs, the pharmaceutical landscape has changed beyond recognition compared to 20 or even 10 years ago. As a result, the field of bioanalysis that supports drug development by measuring the concentrations of drugs or relevant endogenous molecules in biological samples has also seen many changes. The quantitative determination of biopharmaceuticals has traditionally been the domain of ligand-binding assays, such as ELISA. However, in the past few years there has been a clear increase in the application of alternative analytical platforms, in particular liquid chromatography coupled to tandem mass spectrometry LC–MS, which has been the workhorse for small-molecule bioanalysis for over 20 years.[10,14]

Over the past decade, there have been many advances in the LC–MS based quantitation of biopharmaceuticals, both from an analytical and a conceptual point of view. In this article, an overview is given of the many aspects of this field of analytical research by reference to a selection of recent applications.

**CONCLUSION**

Liquid chromatography coupled to tandem mass spectrometry (LC–MS has recently become a more and more popular alternative to traditional ligand-binding assays for the quantitative determination of biopharmaceuticals.

Sample preparation usually contains concentrating the analyte and removing compounds that can cause background ions or suppress ionization. Atmospheric pressure photoionization (APPI) for LC/MS is a relatively new technique.

**REFERENCES**


1. *Dass, Chhabil (2007-01-01). Fundamentals of Contemporary Mass Spectrometry. John Wiley & Sons, Inc. pp. 151–194. doi:10.1002/9780470118498.ch5. ISBN 9780470118498.*
2. *Niessen, Wilfried M. A (2006). Liquid Chromatography-Mass Spectrometry, Third Edition. Boca Raton: CRC Taylor & Francis. pp. 50–90. ISBN 9780824740825. OCLC 232370223.*
3. *Ardrey, Robert E. (2003-01-01). Liquid Chromatography – Mass Spectrometry: An Introduction. John Wiley & Sons, Ltd. pp. 1–5. doi:10.1002/0470867299.ch1. ISBN 9780470867297.*
4. *Encyclopedia of Biophysics - Springer. doi:10.1007/978-3-642-16712-6.*
5. *Chaimbault, Patrick (2014-01-01). Jacob, Claus; Kirsch, Gilbert; Slusarenko, Alan; Winyard, Paul G.; Burkholz, Torsten, eds. Recent Advances in Redox Active Plant and Microbial Products. Springer Netherlands. pp. 31–94. doi:10.1007/978-94-017-8953-0_3. ISBN 9789401789523.*
6. *Pitt, James J (2017-03-12). "Principles and Applications of Liquid Chromatography-Mass Spectrometry in Clinical Biochemistry". The Clinical Biochemist Reviews., 30(1): 19–34. ISSN 0159- 8090. PMC 2643089. PMID 19224008.*
7. *James, A. T.; Martin, A. J. P. (1952-03-01). "Gas-liquid partition chromatography: the separation and micro-estimation of volatile fatty acids from formic acid to dodecanoic acid". Biochemical Journal., 50(5): 679–690. doi:10.1042/bj0500679. ISSN 0264-6021. PMC 1197726. PMID 14934673*
8. *Tal'roze, V.L.; Gorodetskii, I.G.; Zolotoy, N.B.; Karpov, G.V.; Skurat, V.E.; Maslennikova, V.Ya. (1978). "Capillary system for continuous introducing of volatile liquids into analytical MS and its application". Adv. Mass Spectrom. 7: 858.*
9. Primer- basics of lc/ms, agilent technologies 1st edition 2001 page no 6-13.
10. R. Bischoff, K.J. Bronsema, and N.C. van de Merbel, Trends Anal. Chem., 2013; 48: 41–51.
11. G. Hopfgartner, A. Lesur, and E. Varesio, Trends Anal. Chem., 2013; 48: 52–61.
12. Ivan den Broek, W.M. Niessen, and W.D. van Dongen, J. Chromatogr.,2013; B929: 161–179.
13. K.J. Bronsema, R. Bischoff, and N.C. van de Merbel, J. Chromatogr., 2012; B893–894: 1–14.
14. D. Wilffert, R. Bischoff, and N.C. van de Merbel, Bioanalysis, 2015; 7: 763–779.