Liquid chromatography - mass spectrometry (LC-MS) is an extremely versatile instrumental technique. Mass spectrometry (MS) combined with the separation power of chromatography has revolutionized the way chemical analysis is done today. LC-MS/MS is a sensitive technique which comprises a high rapid rate of analysis with good separation efficiency. It involves in separation of small fragmented particles and also in study of bioanalysis with less retention time. The method is popular because it is non-destructive, has very broad applicability and may be applied to thermally labile compounds. Mass spectrometry is a powerful analytical technique used to quantify known materials, to identify unknown compounds within a sample, and to elucidate the structure and chemical properties of different molecules. The complete process involves the conversion of the sample into gaseous ions, with or without fragmentation, which are then characterized by their mass to charge ratios (m/z) and relative abundances. This technique basically studies the effect of ionizing energy on molecules. With a selection of LC–MS interfaces now available, a wide range of analytes, from low molecular-weight drugs and metabolites (<1000 Da) to high-molecular-weight biopolymers (>100 000 Da), may be studied. The high selectivity of the mass spectrometer often provides identification capability on chromatographically unresolved or partially resolved components. This article will help in finding the basic information regarding LCMS/Ms instrumentation, working and the parameters involved in functioning.

KEY WORDS: Liquid Chromatography, Tandem Mass Spectroscopy, bio analysis, metabolites.

1. INTRODUCTION

Chromatography comprises different group of methods which are utilized for the separation of closely related components of mixtures. Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (the stationary phase), while the other (the mobile phase) moves in a definite direction over the stationary phase. The phases are chosen such that components of the sample have differing affinities for each phase. The most commonly employed separation technique for Bioanalysis is high performance liquid chromatography (HPLC), also known simply as LC. The method is popular because it is non-destructive, has very broad applicability and may be applied to thermally labile compounds (unlike GC); it is also a very sensitive technique since it can incorporate a wide choice of detection methods. With the use of post-column derivatisation methods to improve selectivity and detection limits, HPLC can easily be extended to trace determination of compounds that do not usually provide adequate detector response. The wide applicability of HPLC as a separation method makes it a valuable separation tool in many scientific and Pharmaceutical fields. HPLC is an important tool for the analysis of Pharmaceutical drugs, for drug monitoring and for quality assurance. The use of LC-MS/MS has grown exponentially in the last decade, due to its un-matched sensitivity, extraordinary selectivity, and rapid rate of analysis. The principle of MS is the production of ions from analyzed compounds that are separated or filtered on the basis of their mass-to-charge ratio (m/z). Most of applications for quantitative bio-analysis use tandem mass spectrometers (MS/MS) that employs two mass analyzers – one for the precursor ion in the first quadrupole and the other for the product ion in the third quadrupole after the collision – activated dissociation of the precursor ion in a collision cell.

Innovative and successful research efforts in the past decades on the design of an effective interface connection between LC (operated under Atmospheric pressure) and MS (operated under a high-vacuum environment) have made LC congenial with MS. Electrospray ionization (ESI) and Atmospheric-pressure chemical ionization (APCI), collectively called Atmospheric pressure ionization (API), have matured into reliable interface necessary for quantitative LC-MS/MS Bioanalysis. More recently, Atmospheric pressure photo-ionization (APPI) also became an interesting alternative ionization source for quantitative LC-MS/MS. (Ardrey, 2003).

Theory of liquid chromatography

Capacity factor: The time taken for an analyte to elute from a chromatographic column with a particular mobile phase is termed its retention time $R_0$. Since this will vary with several factors, including, column length and mobile phase flow rate, it is more useful to use the capacity factor, $k'$. This relates the retention time of an analyte to the time taken by an unretained compound, i.e. one which passes through the column without interacting with the stationary phase, to elute
from the column under identical conditions \( t_0 \) (Michael W Dong, 2006). The capacity factor, \( k' \), (sometimes called the retention factor) is an essential factor in the retention mechanism of analytes. The retention factor for analyte A is defined as a measure of the affinity that an analyte has for the stationary phase (Equation 1.1, Figure 1).

\[
k' = \frac{R_t}{t_0} \quad \text{Equation 1.1}
\]

where \( R_t \): analyte retention time (min) 
\( t_0 \): time taken for the mobile phase to pass through the column (min)

The capacity factor, \( k' \), is an essential factor in the retention mechanism of analytes.\]

Ideally, the capacity factor for an analyte is between two and five. A retention factor less than two indicates the analyte has eluted too quickly and is too close to the void volume so accurate determination of the retention time is difficult. When an analyte's retention factor is more than five, the analyte has eluted too slowly so the run time may be excessively long. The \( k' \) value can be altered by modifying the mobile phase composition or pore size of the column (Hooper, 1992).

**Separation efficiency:** Separation efficiency is a measure of the sharpness of peaks eluting from a specific column. The column efficiency is measured either in terms of the plate height (H), the efficiency of the column per unit length, or the plate number (N), i.e. the number of plates for the column. The plate model supposes that the chromatographic column contains a large number of separate layers, called theoretical plates. Separate equilibrations of the sample between the stationary and mobile phase occur in these "plates". The analyte moves down the column by transfer of equilibrated mobile Phase from one plate to the next. The number of theoretical plates that a real column possesses can be found by examining a chromatographic peak after elution, (Eqn. 1.2)

\[
N = \frac{5.55 \ R_t^2}{w_{1/2}^2} \quad \text{Equation 1.2}
\]

where \( w_{1/2} \): Peak width at half-height

The number of theoretical plates is proportional to the column length. The height equivalent to theoretical plate HETP also describes the efficiency of a given column for unit length of column (Equation 1.3). The higher the number of theoretical plates in a column, \( N \), the better and the lower the plate height the better.

\[
\text{HETP} = \frac{L}{N} \quad \text{Equation 1.3}
\]

where \( L \): Length of the column

The concept of theoretical plates was first introduced by Martin and Synge (Hoffmann, 2007). However, Van Deemter(Ardrey, 2003.) developed a more realistic theory which takes into account the diffusion effects of mass transfer and migration through a packed bed, with the resulting peak shape being affected by the rate of elution (Eqn. 1.4) for the Van Deemter equation for plate height.

\[
\text{HETP} = A + \frac{B}{u} + \frac{C}{u} \quad \text{Equation 1.4}
\]

where \( u \): Average velocity of the mobile phase
- A: Eddy diffusion
- B: Longitudinal diffusion
- C: Resistance to mass transfer

The mobile phase moves through the column which is packed with stationary phase. Eddy diffusion is caused by the movement of molecules from the same analyte, which migrate through the stationary phase at random. This will cause broadening of the solute band, because different paths are of different lengths (Liang Tang, 1993). Longitudinal diffusion occurs due to the concentration of analyte being less at the edges of the band than at the centre. Analyte diffuses out from the centre to the edges causing broadening. If the velocity of the mobile phase is high then the analyte spends less time on the column, which decreases the effects of longitudinal diffusion. The last parameter is due to the amount of time an
The mobile phase will move ahead of the analyte optimized. The optimum parameter with which to control each ion type. The first.

The mass spectrometer provides the most definitive identification of all of the HPLC detectors. The high selectivity of the mass spectrometer provides identification capability on chromatographically unresolved or partially resolved components. This selectivity allows the use of isotopically labeled analytes as internal standards and this, coupled with high sensitivity; the equipment comprises a chromatograph, attached via a suitable interface, to a mass spectrometer. As MS measures the mass to charge (m/z) ratio of ions, most molecules which can be ionized will be suitable for this detector, hence the detector is considered to be a ‘universal’ one. Interfacing the two techniques is not straightforward as the solutes leaving an LC column are dissolved in mobile Phase at Atmospheric pressure, whereas the
MS is set up to detect gas Phase ions in vacuum (May L Chiu, 2010). To ease this transition from the liquid to the gas Phase, a number of ion source interfaces have been developed.

**Sample Ionisation:** The use of Atmospheric Pressure Ionisation (API) techniques allows positive or negative ions to be created and detected. API offers a soft ionisation approach resulting in little or no analyte fragmentation. A typical API spectrum contains only the protonated (positive ion mode) or deprotonated (negative ion mode) molecular ion (Westman-brinkmalm, 2008). The detected ion peaks are generally created through addition or subtraction of a proton adducts of analyte ions of other types such as sodium or potassium can be seen in some circumstances (in positive mode). MS spectra provide valuable molecular weight information of singly and multiply charged ions and identification of the charge state of each peak in the charge-state envelope of a given compound. Software can be used to deconvolute multiply charged mass spectral data of mixtures of proteins and other biopolymers. The vast majority of LC–MS analyses currently in use employ either electrospray ionisation (ESI) or APCI, both which are soft ionization techniques (Ardrey, 2003).

In electrospray ionization (ESI): ESI converts the liquid molecules that elute from the LC into charged gaseous analytes by evaporating the LC solvent and charging the analytes and then pulling the ions into the mass analyser by keeping the capillary at a high voltage of opposite charge and at high vacuum. The only disadvantage of the technique is that very little (usually none) fragmentation is produced directly although this may be overcome through the use of tandem mass spectrometric techniques such as MS/MS. This ionisation technique is very suitable for the analysis of polar, thermally labile molecules such as drugs, DNA, RNA, sugars, peptides and proteins. Nano spray ionisation (NSI) is essentially ESI operating at very low liquid flow rates of 100 nL/min to several μL/min in static or dynamic modes. It is most compatible with capillary LC separations.

Atmospheric pressure chemical ionisation (APCI) vaporises the sample solution at high temperatures up to 600 °C. Application of a high electrical potential produces reagent ion plasma, mainly from the solvent vapour. The sample vapour is formed by ion-molecule reactions with the reagent ions in the plasma. APCI accommodates liquid flows of 100 μL/min to 2 mL/min and is generally employed for larger, poorly ionisable, hydrophobic molecules.

**Mass analysers:** A mass analyser measures gas Phase molecules with respect to their mass-to-charge ratio (m/z), where the charge is produced by the addition or loss of a protons, cations, anions or electrons. The addition of charge allows the molecules to be affected by electric fields thus allowing its mass measurement. There are a number of types of mass analysers available and choice depends on the information required from the ionized analytes (Tang, 2009).

Quadrupole mass analysers have been important in mass analysis for many decades because they are relatively inexpensive, rugged, and have been implemented in a wide variety of instrumental configurations including triple
quadrupole instruments which can do MS/MS experiments. A quadrupole mass analyser consists of four parallel rods that have fixed DC and alternating RF potentials applied to them (Figure no.1.5). Ions produced in the source are focused and passed along the middle of the quadrupoles. Motion of these ions will depend on the electric fields so that only ions of a particular mass to charge ratio (m/z) will have a stable trajectory and thus pass through to the detector (Chen., 2006). Varying the RF brings ions of different m/z into focus on the detector and thus builds up a mass spectrum.

Quadrupoles can also be placed in tandem to enable them to perform fragmentation studies - the most common set-up is the triple quadrupole (Antignac J, 2005) (QQQ) mass spectrometer which enables basic ion fragmentation studies (tandem mass spectrometry MS/MS) to be performed. These instruments are particularly sensitive in selected ion monitoring modes and hence ideal for trace analysis and pharmacokinetic applications.

In a triple quadrupole, the sample enters the ion source and is usually fragmented by either an electron impact or chemical ionisation process. The first quadrupole acts as a filter for the ion of interest; the various charged ions are separated in the usual way and then pass into the second quadrupole section sometimes called the collision cell. This one acts as a collision chamber for the MS/MS to form the product ions of interest.

These product ions are then passed into the third quadrupole which functions as a second analyser to detect the aforementioned ions for quantitation. The second analyser segregates the product ions into their individual masses, which are detected by the sensor, producing the mass spectrum (originally from ions of one mass only). A diagram of a triple Quadrupole Mass Spectrometer is shown in Figure no.6. MS QQQ is an extremely powerful analytical system that can handle exceedingly complex mixtures and very involved molecular structures.

Ion traps (which are normally quadrupole ion traps) are popular because they are relatively inexpensive, rugged, and provide capabilities that enhance selectivity and qualitative analysis. The quadrupole ion-trap consists of a ring electrode with further electrodes, the end-cap electrodes, above and below this. In contrast to the quadrupole, described above, ions, after introduction into the ion-trap, follow a stable (but complex) trajectory, i.e. are trapped, until an RF voltage is applied to the ring electrode. Ions of a particular m/z then become unstable and are directed toward the detector. By varying the RF voltage in a systematic way, a complete mass spectrum may be obtained. Again, this is a low-resolution device, capable of fast scanning and tolerant of relatively high operating pressures.

The Time-of-flight (TOF) analyser is the simplest type of mass analyser. TOF systems require a pulsed ion source, a flight tube and an ion detector. This system relies on the fact that if all of the ions produced in the source of a mass spectrometer, by whatever technique, are given the same kinetic energy then the velocity of each will be inversely proportional to the square root of its mass. As a consequence, the time taken for them to traverse a field-free region (the flight tube of the mass spectrometer) will be related in the same way to the m/z of the ion. A complete mass spectrum is obtained simply by allowing sufficient time for all of the ions of interest to reach the detector. Were ions to be introduced continuously it would be impossible to determine exactly when each began its passage through the flight tube and therefore to calculate its m/z ratio. This is sometimes referred to as a ‘pulsed’ source. The other great advantage of TOF is the virtually unlimited mass range when sampling in the time domain.

Figure no 5: Adapted schematic of a Quadrupole Mass Analyser

Modes of Detection: Typically the mass spectrometer is set to scan a specific mass range. This mass scan can be wide as in the full scan analysis or can be very narrow as in selected ion monitoring. A single mass scan can take anywhere from 10 ms to 1 s depending on the type of scan. Many scans are acquired during an LC-MS analysis. LC-MS data is represented by adding up the ion current in the individual mass scans and plotting the ‘total’ ion current as an intensity against time. (Hoffmann, 2007)

The total ion current (TIC) is a plot of the total number of ions in each MS scan plotted as an intensity point against time. In the TIC plot, ions of every mass over the chosen range are plotted. As many compounds have the same
m/z it can be difficult finding the compound of interest. A specific mass can later be selectively extracted but sensitivity is not as good as the next technique of selected ion monitoring. TIC plots are often overlaid onto UV or other plots and this can give useful information about the compounds being studied.

With selected ion monitoring (SIM) the mass spectrometer is set to scan over a very small mass range, typically one mass unit. As the mass spectrometer can dwell for a longer time over a smaller mass range, this makes SIM more sensitive than TIC. Selected reaction monitoring (SRM) is the most common method used for performing mass spectrometric quantitation. SRM creates a unique fragment (product) ion that can be monitored and quantified in the midst of complicated matrices, thus enabling more confirmatory identification.

Multiple Reactions Monitoring (MRM): In this scan type Q₁ is the 1st Quad which allows the parent ion filtration, Q₂ is the collision cell, in this parent ion get fragmented and focus the fragment ions to the Q₃.

Limitations of MS analysis: Analyzing biological fluids is one on the most difficult tasks faced by an analytical chemist. Most challenging are interferences from matrices and decomposition products combined with low concentrations of target analytes. Matrix components present in biological samples can affect the response of the analyte of interest and can lead to inaccurate quantitation. The primary matrix effect associated with LC-MS/MS methods is ion suppression or enhancement caused by the co-eluting matrix components. Matrix effects occur when molecules co-eluting with the compound/s of interest alter the ionization efficiency of the electrospray interface.

This phenomenon was first described by Tang and Kebarle who showed that electrospray responses of organic bases decreased as the concentrations of other organic bases were increased. The exact mechanism of matrix effects is unknown, but it probably originates from the competition between an analyte and the co-eluting, undetected matrix components. (Liang Tang, 1993)

Matrix effects are also compound dependent. Bonfiglio reported that the chemical nature of a compound has a significant effect on the degree of matrix effects. In a study of four compounds of different polarities under the same mass spectrometric conditions, the most polar was found to have the largest ion suppression and the least polar was affected less by ion suppression. These findings of differential matrix effects have important ramifications particularly when selecting a suitable internal standard for quantification purposes. The possible origins of ion suppression are multiple. The main problem source commonly reported is the presence of endogenous substances, i.e. organic or inorganic molecules present in the sample and that are retrieved in the final extract. Among this first group of ion suppressor agents, can be included ionic species (inorganic electrolytes, salts), highly polar compounds (phenols, pigments), and various organic molecules including carbohydrates, amines, urea, lipids, peptides, analogous compounds or metabolites with a chemical structure close to the target analyte one. Finally, a wide range of molecules can lead to ion suppression especially when they are present in high concentration in the extract and eluted in the same retention window than the analyte of interest.
A second problem source, usually less described, is due to the presence of exogenous substances, i.e. molecules not present in the sample but coming from various external sources during the sample preparation. Among this second group of ion suppressor agents, can be included plastic and polymer residues (Gustavsson, 2001) phthalates, detergent degradation products (alkylphenols), ion pairing (Chaimbault, 1999; Chaimbault, 2000; Keever, 1998), proton-exchanges promoting agents such as organic acids (Roberts, 1998; Chi, 2010) calibration products, buffers, or material released by the solid Phase extraction, LC or GC stationary Phases.

Many components in biological matrices influence the result of an analysis, affecting assay sensitivity and reproducibility. Improved matrix management becomes critical as requirements for higher assay sensitivity and increased process throughput become more demanding (Chiu, 2010) When ion suppression occurs, the sensitivity and lower limit of quantification of a method may be adversely affected.

2. CONCLUSION

LC MS/MS is variably a simple fastest accurate instrument particularly in the bio-analysis. With its rapid rate of elution and short retention times it gained popularity amongst the present techniques. Usage of different ionization techniques and sharp separation methods the compounds of less sensitivity can be known. Most of applications for quantitative bio-analysis use tandem mass spectrometers (MS/MS) as it is highly advantageous which comprises of two mass analyzers – one for the precursor ion in the first quadrupole and the other for the product ion in the third quadrupole after the collision – activated dissociation of the precursor ion in a collision cell. With a high rate of accuracy, sensitivity and selectivity one can complete the analysis of compounds within no time.

REFERENCE

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