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LC-MS Overview: Applications of Small Molecules in Drug Discovery

Selvadurai Muralidharan*, Jaya raja Kumar & Sokkalingam Arumugam Dhanaraj
Faculty of Pharmacy, AIMST University, Malaysia.

Abstract
Mass spectrometry (MS) is one of the most frequently employed techniques in performing quantitative analysis. Its specificity, selectivity and typical limit of detection are more than enough to deal with most analytical problems. This is the result of significant effort, either from the scientists working in the field or from the manufacturing industry, devoted to the development of new ionization methods, expanding the application fields of the technique, and new analysers capable of increasing the specificity mainly by collisional experiments (MS/MS or ''tandem mass spectrometry'') or by high mass accuracy measurements. Thus, the MS panorama is made up of many instrumental configurations, each of which has specific positive and negative aspects and different cost/benefit ratios. These mass spectrometric approaches are usually employed when linked to suitable chromatographic (C) systems. The synergism obtained allows C-MS to be used worldwide and is of considerable interest to researchers involved in basic chemistry, environmental and food controls, biochemistry, biology and medicine. It is to be expected that this diffusion will grow in the future, due to the relevance of the information that quantitative MS can provide, in particular in the field of public health. For this reason, some basic information on the phenomena which form the basis of different instrumental approaches, the general strategy to be employed for the development of a quantitative analysis, the role of the specificity in this context and some theoretical aspects on calibration and data analysis, are of interest and this book aims to cover, as simply as possible, all these aspects [1].

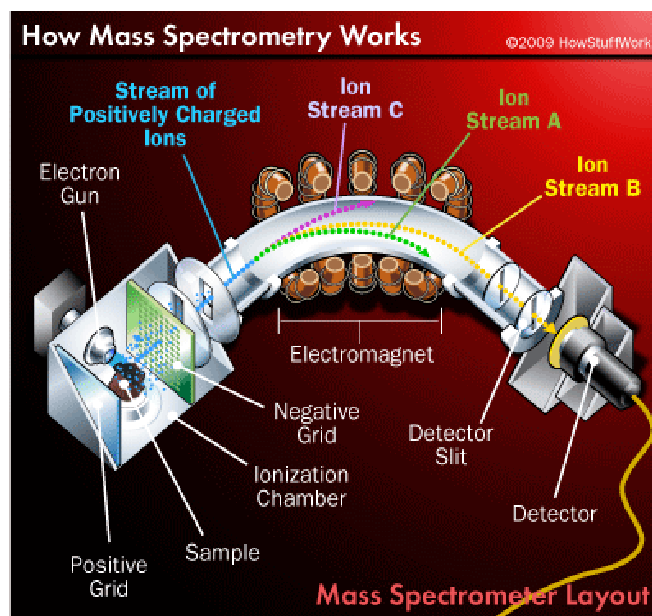
INTRODUCTION

WHAT IS LC-MS?

Chromatography is a separations method that relies on differences in partitioning behavior between a flowing mobile phase and a stationary phase to separate the components in a mixture. A column holds the stationary phase and the mobile phase carries the sample through it. Sample components that partition strongly into the stationary phase spend a greater amount of time in the column and are separated from components that stay predominantly in the mobile phase and pass through the column faster. Mass spectrometers use the difference in mass-to-charge ratio (m/z) of ionized compounds to separate them from each other. Compounds have distinctive fragmentation patterns that provide structural information to specifically detect compounds.

PRINCIPLE OF MASS-SPECTROPHOTOMETER

A mass spectrometer generates multiple ions from the sample under investigation; it then separates them according to their specific mass-to-charge ratio (m/z), and then records the relative abundance of each ion type. The first step in the mass spectrometric analysis of compounds is the production of gas phase ions of the compound, basically by electron ionization. This molecular ion undergoes fragmentation. Each primary product ion derived from the molecular ion, in turn, undergoes fragmentation, and so on. The ions are separated in the mass spectrometer according to their mass-to-charge ratio, and are detected in proportion to their abundance. A mass spectrum of the molecule is thus produced. It displays the result in the form of a plot of ion abundance versus mass-to-charge ratio. Ions provide information concerning the nature and the structure of their precursor molecule. In the spectrum of a pure compound, the molecular ion, if present, appears at the highest value of m/z (followed by ions containing heavier isotopes) and gives the molecular mass of the compound.



Schematic diagram of mass spectrophotometer

TYPES OF MASS SPECTROMETERS

Time-of-flight

The time-of-flight (TOF) analyzer uses an electric field to accelerate the ions through the same potential, and then measures the time they take to reach the detector. If the particles all have the same charge, the kinetic energies will be identical, and their velocities will depend only on their masses. Lighter ions will reach the detector first.[2]

Quadrupole mass filter

Quadrupole mass analyzers use oscillating electrical fields to selectively stabilize or destabilize the paths of ions passing through a radio frequency (RF) quadrupole field created between 4 parallel rods. Only the ions in a certain range of mass/charge ratio are passed through the system at any time, but changes to the potentials on the rods allow a wide range of m/z values to be swept rapidly, either continuously or in a succession of discrete hops. A quadrupole mass analyzer acts as a mass-selective filter and is closely related to the quadrupole ion trap, particularly the linear quadrupole ion trap except that it is designed to pass the untrapped ions rather than collect the trapped ones, and is for that reason referred to as a transmission quadrupole. A common variation of the transmission quadrupole is the triple quadrupole mass spectrometer. The "triple quad" has three consecutive quadrupole stages, the first acting as a mass filter to transmit a particular incoming ion to the second quadrupole, a collision chamber, wherein that ion can be broken into fragments. The third quadrupole also acts as a mass filter, to transmit a particular fragment ion to the detector. If a quadrupole is made to rapidly and repetitively cycle through a range of mass filter settings, full spectra can be reported. Likewise, a triple quad can be made to perform various scan types characteristic of tandem mass spectrometry.

ION TRAPS

Three-dimensional quadrupole ion trap

The quadrupole ion trap works on the same physical principles as the quadrupole mass analyzer, but the ions are trapped and sequentially ejected. Ions are trapped in a mainly quadrupole RF field, in a space defined by a ring electrode (usually connected to the main RF potential) between two endcap electrodes (typically connected to DC or auxiliary AC potentials). The sample is ionized either internally (e.g. with an electron or laser beam), or externally, in which case the ions are often introduced through an aperture in an endcap electrode. There are many mass/charge separation and isolation methods but the most commonly used is the mass instability mode in which the RF potential is ramped so that the orbit of ions with a mass $a > b$ are stable while ions with mass b become unstable and are ejected on the z-axis onto a detector. There are also non-destructive analysis methods. Ions may also be ejected by the resonance excitation method, whereby a supplemental oscillatory excitation voltage is applied to the endcap electrodes, and the trapping voltage amplitude and/or excitation voltage frequency is varied to bring ions into a resonance condition in order of their mass/charge ratio.[3-4]

The cylindrical ion trap mass spectrometer is a derivative of the quadrupole ion trap mass spectrometer.

Linear quadrupole ion trap

A linear quadrupole ion trap is similar to a quadrupole ion trap, but it traps ions in a two dimensional quadrupole field, instead of a three-dimensional quadrupole field as in a 3D quadrupole ion trap. Thermo Fisher's LTQ ("linear trap quadrupole") is an example of the linear ion trap.[5] A toroidal ion trap can be visualized as a linear quadrupole curved around and connected at the ends or as a cross section of a 3D ion trap rotated on edge to form the toroid, donut shaped trap. The trap can store large volumes of ions by distributing them throughout the ring-like trap structure. This toroidal shaped trap is a configuration that allows the increased miniaturization of an ion trap mass analyzer. Additionally all ions are stored in the same trapping field and ejected together simplifying detection that can be complicated with array configurations due to variations in detector alignment and machining of the arrays.[6]

ORBITRAP

These are similar to Fourier transform ion cyclotron resonance mass spectrometers (see text below). Ions are electrostatically trapped in an orbit around a central, spindle shaped electrode. The electrode confines the ions so that they both orbit around the central electrode and oscillate back and forth along the central electrode's long axis. This oscillation generates an image current in the detector plates which is recorded by the instrument. The frequencies of these image currents depend on the mass to charge ratios of the ions. Mass spectra are obtained by Fourier transformation of the recorded image currents. Orbitraps have a high mass accuracy, high sensitivity and a good dynamic range.[7]

FOURIER TRANSFORM ION CYCLOTRON RESONANCE

Fourier transform mass spectrometry (FTMS), or more precisely Fourier transform ion cyclotron resonance MS, measures mass by detecting the image current produced by ions cyclotroning in the presence of a magnetic field. Instead of measuring the deflection of ions with a detector such as an electron multiplier, the ions are injected into a Penning trap (a static electric/magnetic ion trap) where they effectively form part of a circuit. Detectors at fixed positions in space measure the electrical signal of ions which pass near them over time, producing a periodic signal. Since the frequency of an ion's cycling is determined by its mass to charge ratio, this can be deconvoluted by performing a Fourier transform on the signal. FTMS has the advantage of high sensitivity (since each ion is "counted" more than once) and much higher resolution and thus precision.[8-9] Ion cyclotron resonance (ICR) is an older mass analysis technique similar to FTMS except that ions are detected with a traditional detector. Ions trapped in a Penning trap are excited by an RF electric field until they impact the wall of the trap, where the detector is located. Ions of different mass are resolved according to impact time.

STRUCTURE ANALYSIS OF SMALL MOLECULES

The uniqueness of LC/MS is the combined high resolving power of HPLC and superior mass detection capability of MS. The LC/MS and LC/MS/MS techniques provide solutions to a wide range of structural characterization problems in pharmaceutical research. They include identification of trace level impurities and degradants in bulk

drug substances, identification of metabolites in drug metabolism studies as well as unknown identifications in synthetic reaction products. An important aspect of pharmaceutical development is the identification of impurities in bulk drug substances. It not only addresses relevant regulatory issues, but also provides clues to further refine scale-up processes in maximizing yield. The impurity contents of 0.1% or above in the active pharmaceutical ingredient, representing low picomoles of materials in a typical HPLC assay are required to be characterized. The use of LC/MS and LC/MS/MS techniques can rapidly provide structural information of unknown impurities found in production batches. For example, mometasone furoate (MW 520 Da) is a highly potent synthetic dichlorinated corticosteroid. It has been widely used in the treatment of dermatological disorders as topical formulations of ointments, seasonal and perennial allergic rhinitis as an aqueous intranasal spray, and in asthma as a dry powder. Several impurities were found to be present in the course of large-scale production of the drug substance. There are two co-eluting components detected in peak D, corresponding to molecular ions at *m/z* 535 (containing two chlorine atoms) and 581 (containing one chlorine atom) (data not shown). The impurity ion at*m/z* 535 in peak D was established as a 6-keto structure in a previous study,5 although the complete characterization of the ion at *m/z* 581 was not carried out. To fully characterize all the impurities in the sample, we utilized LTQ-Orbitrap hybridmass spectrometer to obtain the structural information. As one of the latest MS instrumentation, LTQ-Orbitrap has the capability of performing high-resolution LC/MS and LC/MS *n* experiments with high resolving power (up to 100 000), excellent mass accuracy (<3 ppm with external calibration) and large dynamic range (over 103). [10–12] Several groups have demonstrated low ppm mass accuracy on mixture analysis, including human urinary proteome studies with identifications of more than 1500 proteins, top-down protein sequencing, and structural identifications of drug metabolites in doping control analysis, as well as in human liver microsomal metabolism.[13–20] In the case of the impurity ion at *m/z* 581, high-resolution LC/MS experiments (at a resolution of 30 000, external calibration) were performed to determine its elemental composition. Based on its isotopic patterns and likely possible element combinations, the best possible elemental composition was determined to be C28H34O9ClS for *m/z* 581 with a mass accuracy of 0.18 ppm. Comparing with the elemental composition of C27H31O6Cl2 for mometasone furoate ([M C H]C), the net addition for the unknown is the moiety of CH3O3S with removal of one chlorine atom. Further high-resolution LC/MS/MS experiments on *m/z* 581 suggest its structure as the addition of sulfur-moiety at the 20-keto position, as supported by accurate mass measurements of product ions. One of the possible mechanisms for formation of the proposed structure for this impurity (*m/z* 581) involves reactions with CH3SO2Cl (reagent).

CONCLUSION

With advancements [21] in ionization methods and instrumentation, liquid chromatography/mass spectrometry (LC/MS) has become a powerful technology for the characterization of small molecules and proteins. This article will illustrate the role of LC/MS analysis in drug discovery process. Examples will be given on high-throughput analysis, structural analysis of trace level impurities in drug substances, identification of metabolites, and characterization of therapeutic protein products for process improvement. Some unique MS techniques will also be discussed to demonstrate their effectiveness in facilitating structural identifications.

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