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Liquid chromatography-tandem mass spectrometry and its applications: A review

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Abstract
Liquid chromatography-tandem Mass Spectrometry (LC-MS/MS) is the most advanced hyphenated technique for the analysis of samples. It is the Liquid Chro"? Matographic techniques like HPLC or UPLC coupled with Tandem Mass Spectrometer. In Liquid Chromatography the mixtures of components of the sample gets separated according to their affinity towards both stationary phase and mobile phase and enter the Tandem Mass Spectrometer (MS/MS or MS^n) through an interface. Tandem mass spectrometry is the Mass Spectrometer coupled with another Mass Spectrometer. In MS/MS, the separated components from Liquid Chromatography are ionized and ions of selected molecular weight are allowed to enter collision cell where the ions get fragmented and are analyzed. This hyphenated technique offers increased selectivity where the lowest concentration of the component of the sample can be detected. It has a wide range of applications like proteomics, pharmacokinetic studies, forensic science, agrochemistry, etc.

Keywords: LC-MS/MS, hyphenated technique, tandem mass spectrometry, proteomics, etc.

Introduction
The coupling of LC and MS is not a new technique. MS is particularly useful in structural determination and mass information of the molecules. With the use of Tandem Mass Spectrometry (MS^n) (Uday Rakibe, *et al*., 2018) [6] both molecular weight and structural information about a particular molecular species can be determined in a single analysis. MS fails, or atleast, becomes more difficult when samples are not pure or there are matrix interferences that reduce ionization efficiencies of intended sample analyte (Marilyn A. Huestis *et al.,* 2003) [9]. Chromatography, on the other hand, is a separation process and provides information about the chemical composition and constituent interaction with adsorption surfaces and solvents. Therefore, the unification of these two methods of analysis, where the chromatography separates the components of the mixture, while MS provides structural information with regards to each of the eluting molecular species, was an obvious development in analytical sciences.
Long before GC and MS were unified and the technique finds widespread applications in pharmaceutical industry, environmental sciences, forensics, etc. The ease by which MS and GC could be coupled the types of carrier gas and flow rates used in GC were compatible with entry into a Mass Spectrometer ion source and Mass analyzer. However, GC-MS was suitable only for analysis of substances that were relatively non-polar, low molecular weight (Kerstin Greulich *et al.,* 2006) [12], and of course, thermally volatile. LC on the other hand, does not have such limitations and its usefulness extended for analysis of large variety of compounds with high molecular weights and various other molecular attributes.

Basic principles of liquid chromatography and tandem mass spectrometry
Generally, in chromatography there are four principles which are often used, they are: adsorption, partition, ion exchange and size exclusion. Adsorption chromatography arises from interactions between solute and the surface of solid stationary phase. Partition chromatography involves a liquid stationary phase that is immiscible with the eluents and coated on an inert support. Ion exchange chromatography involves a solid stationary phase with anionic or cationic groups on the support to which solute molecules of opposite charge are attracted. Size exclusion chromatography involves a solid stationary phase with controlled pore size between molecules of stationary phase.
The simplest form of Tandem Mass Spectrometry combines two Mass Spectrometers. The first Mass Spectrometer is used to select a single (precursor) mass that is characteristic of analyte of interest in given mixture.

Thus selected mass ions are passed through a region (collision cell) where they are activated in some way that causes them to fall apart to produce fragment (product) ions. Activation of precursor ions is generally done by colliding the ions with neutral gas in collision cell. This type of ion activation is generally called as Collisional Activation (CA) or Collision Induced Dissociation (CID). The second Mass Spectrometer is used to separate the fragment ions according to their mass. The resulting MS/MS spectrum consist only the product ions from the selected precursor.

There are many different ways to increase the internal energy of precursor ion so that chemical bonds will break and fragments are formed. Other than Collision Induced Dissociation (CID), there are other kinds of ion activation methods like Surface Induced Dissociation (SID), Photo dissociation, and Electron Induced Dissociation. If an ion collides with a neutral atom or molecule, some of the ion's kinetic energy is converted to internal energy. This is called Collisional activation. It there is enough excess internal energy to break chemical bonds, the ion will decompose. This is called Collision Induced Dissociation (CID) or Collisionaly Activated Dissociation (CAD). The noble gases such as helium, argon, and xenon are used as target gases as they have high ionization potentials. The precursor ions are made to collide with target gas to yield fragment ions. If target gas is substituted by a target surface in collision chamber, the ions can scatter ("bounce") off the surface. Ions can gain internal energy from the ion-surface collisions and will subsequently decompose. This process is referred as Surface Induced Dissociation (SID). If ions are irradiated with photons (light), they can absorb light and increase their internal energy. This is called Photo dissociation and requires that the ions must have a chromophore that allows them to absorb light and the photons should have higher energies to break the chemical bond. Electron Induced Dissociation is when the ions encounter a high-current electron beam, the ion-electron collisions can result in ion activation.

Instrumentation of LC-MS/MS

LC-MS/MS is a Liquid Chromatography coupled to a Tandem Mass Spectrometer through an interface, given in figure 1. General components are:

- Liquid Chromatography (LC)
- LC-MS Interface
- Tandem Mass spectrometry (MS/MS or MS^n)

Liquid chromatography (LC)

Liquid Chromatography is one of the chromatographic techniques where a solvent or mixture of solvents is used as mobile phase and either solid or liquid is used as stationary phase. Depending on polarity of stationary phase and mobile phase, LC can be operated in two types. Firstly, the REVERSE PHASE where a non-polar stationary phase and a polar mobile phase is used. Next is the NORMAL PHASE where the stationary phase is polar and mobile phase is non-polar. In LC, there are two elution techniques. If the mobile phase composition and concentration of solvents is constant throughout the run time, then it is isocratic elution. If the concentration of solvents in a mobile phase changes with respect to time in a single run time, then it is gradient elution.

Examples of Liquid Chromatography (LC) are Column Chromatography, Thin Layer Chromatography (TLC), Paper Chromatography, High Pressure Liquid Chromatography (HPLC), Ultra High Pressure Liquid Chromatography (UHPLC/UPLC). HPLC and UPLC are most sensitive and advanced chromatographic techniques, often coupled with other analytical tools like Mass Spectrometer, Tandem Mass Spectrometry, etc. The instrumentation of both chromatographic techniques is almost the same with minimum modifications. The difference between HPLC and UPLC is given in the table 1. The various components present in a typical HPLC instrument are:

- Solvent reservoir
- Mixing unit and pumping system
- Sample injection port
- Column
- Detector
- Readout device

Table 1: difference between HPLC and UPLC

Characteristics	HPLC	UPLC
Particle size	3 to 5µm	Less than 2µm
Maximum pressure	3000-8000psi	Up to 15000psi
Analytical column	C8, C18	UPLC BEH C8, C18
Column dimensions	20-50cms length, 1-5mm internal diameter	30-150mm length, 1-5mm internal diameter
Injection volume	5-25µL	1-5µL
Column temperature	30 °C	65 °C
Total run time	15-20 minutes	3-10 minutes
USP resolution	3.2	3.4
Plate count	2000	7500
Flow rate	1-3 ml/min	Less than 1 ml/min
pH	2-8	1-12

Columns in HPLC are generally made of stainless steel which can withstand high pressure maintained in the system with 20-50cms of length and 1-4mm of internal diameter of the column. A stationary phase of reduced particle size is packed tightly in the column. Due to this, the back pressure in column is very high. So pumps are used for optimum flow of mobile phase. The column packing procedure is also a pressurized technique where slurry of stationary phase is prepared and is filled into column under high pressure, after drying columns are checked for any cracks if present.

Solvent reservoir is the vessel or bottle that holds the mobile phase. It is made of material that will not contaminate the mobile phase such as glass, stainless steel etc. Mobile phase from reservoir is delivered to pump by means of Teflon tubing called "inlet line" to the pump. The mobile phase is selected based on PKA value of the sample. Before filling the mobile phase into the reservoir, it should be subjected for degassing and microfiltration process to reduce the

interferences. Degassing can be achieved either by ultrasonication of mobile phase or by passing mobile phase through vacuum pump.

Pumping system is used to develop the pressure in the system. Generally there are two types of pumping systems used in HPLC. First is the low pressure gradient system (LPG) in which only one pump is present with four channels. The pressure maintained is comparatively less. The mixing unit is present before the pumping system i.e., initially the solvents are mixed and then they are pumped into the column. Secondly, the high pressure gradient system (HPG) which is operated at very high pressure up to 8000 psi. It consists of two pumps, each pump with one channel. The mixing unit is present after the pump i.e., mixing is also done at high pressure. The different types of pumps used are constant pressure pumps, constant displacement pumps, reciprocating pumps.

The sample is introduced into the system through sample injection port. There are different types of techniques for sample injection – In stop-injection-flow technique, initially the pump is switched off to attain zero pressure, then sample is injected and again pump is switched on. In loop technique, reodyne injector system is used where loops are attached to which can hold definite volume of sample (10µL, 20µL, etc.). Sample is injected with micro syringe into the loop where sample holds for definite period of time. Most advanced sample injection is auto sampler. The sample to be analyzed is filled into the vials and placed into trays. It is operated through software. The sample along with mobile phase enters the column and separation of components takes place.

After elution, separated component enter into the detector. There are two types are detectors used in HPLC. The non-selective or universal detectors are used for detection of any kind of sample. Refractive index detector (RID) and evaporated light scattering detector (ELSD) are two most commonly used universal detectors. Based on sample, selective detectors are used. They are: Ultraviolet-visible spectrophotometer, Flourimeter, photo diode array (PDA) detector, mass spectrometer, etc. The signal from detector is sent to readout device which displays the results in the form of a chromatogram.

Tandem Mass Spectrometry (MS/MS or MSⁿ)

When MASS spectrometer is coupled with another one or more MASS spectrometer, it is said to be Tandem MASS spectrometer. Multiple MASS spectrometers are serially coupled to obtain the back bone/ in detailed structure of analyte of interest. One Mass Spectrometer is connected to another one through a collision cell. The ions from MS^1 are carried to collision cell where the internal energy of these ions are increased by breaking chemical bonds and form fragments which are analyzed in MS^2. Instrumentation of MS/MS includes:

- Ionization source
- Mass analyzer
- Detector

All the ionization sources which are used in Mass spectrometer are not compatable with liquid chromatography. The ionization sources which are compatable are, Electrospray ionization technique (ESI), Atmospheric pressure chemical ionization technique (APCI), Atmospheric pressure photo ionization (APPI), Matrix assisted laser desorption ionization (MALDI).

The different Tandem Mass Analyzers used are:
Triple Quadrupole analyzer
Time of flight – time of flight (TOF-TOF)
Quadrupole Ion trap analyzer (Quad-ion trap)
Quadrupole – Time of Flight (Quad – TOF)
Linear trap Quadrupole – Fourier Transform Ion Cyclotron resonance (LTQ - FTICR)
Linear Trap Quadrupole – Orbitrap (LTQ – ORBITRAP)

Essentially the triple quadrupole mass analyser operates under the same principle as the single quadrupole mass analyzer. Each of the two mass filters (Q1 and Q3) contains four parallel, cylindrical metal rods. Both Q1 and Q3 are controlled by direct current (dc) and radio-frequency (rf) potentials, while the collision cell, q, is only subjected to RF potential. The RF potential associated with the collision cell (q) allows all ions that were selected for to pass through it. In some instruments, the normal quadrupole collision cell has been replaced by hexapole or octopole collision cells which improve efficiency.

TOF-TOF mass analyzer simply contains a true collision cell separated by two TOF tubes. Ions generated in the source region are accelerated through the first drift tube and can be dissociated through collisions with an inert gas in the collision cell. The resulting fragment ions are subsequently accelerated through second TOF tube and detected.

A quadrupole ion trap is a type of ion trap that uses dynamic electric fields to trap charged particles (figure 2). It consists of two hyperbolic metal electrodes and a hyperbolic ring electrode. This enables the 3D trap of atomic or molecular ions. The ions are trapped in the space between these three electrodes by AC (oscillating) and DC (static) electric fields. The AC radio frequency voltage oscillates between the two hyperbolic metal end cap electrodes if ion excitation is desired; the driving AC voltage is applied to the ring electrode. The ions are first pulled up and down axially while being pushed in radially. The ions are then pulled out radially and pushed in axially (from the top and bottom). In this way the ions move in a complex motion that generally involves the cloud of ions being long and narrow and then short and wide, back and forth, oscillating between the two states.

The Q-TOF tandem mass spectrometer can be described in the simplest way as a triple Quadrupole with the last quadrupole section replaced by a TOF analyzer. In the usual Q-TOF configuration, an additional r.f. Quadrupole Q0 is added to provide collisional damping, so the instrument consists of three Quadrupoles, Q0, Q1 and Q2, followed by a reflecting TOF mass analyzer.

The final element of the mass spectrometer is the detector. The detector records either the charge induced or the current produced when an ion passes by or hits a surface. In a scanning instrument, the signal produced in the detector during the course of the scan versus where the instrument is in the scan (at what *m/Q*) will produce a mass spectrum, a record of ions as a function of *m/Q*. Typically, some type of electron multiplier is used, though other detectors including Faraday cups and ion-to-photon detectors are also used. Because the number of ions leaving the mass analyzer at a particular instant is typically quite small, considerable amplification is often necessary to get a signal. Microchannel plate detectors are commonly used in modern commercial instruments. In FTMS and Orbitraps, the detector consists of a pair of metal surfaces within the mass analyzer/ion trap region

which the ions only pass near as they oscillate. No direct current is produced, only a weak AC image current is produced in a circuit between the electrodes. Other inductive detectors have also been used.

LC-MS interfaces

The First LC–MS Interfaces

The first experiments to couple LC to MS date back to the late 1960s. Though fascinating at the time of their development, the earliest LC–MS interfaces are now almost obsolete. The introduction of techniques that allow delivery of thermolabile biomolecules into the MS show an exponential increase in the number of publications employing LC–MS.

Direct Liquid Introduction

The first attempts to introduce a liquid into an MS using the classic electron impact ionization (EI)/chemical ionization (CI) source were based on the simple principle that by minimizing the amount of liquid, the vacuum system would remove the solvent leaving the analyte in the gas phase for ionization. By using larger pump systems and differential pumping, maintenance of the vacuum was ensured. The flow from the LC column was reduced by using smaller i.d. columns and/or splitting the liquid flow. In order to assist the evaporation a heated desolvation chamber could be introduced.

Moving Belt/Wire

The moving-belt interface separates the condensed liquid-phase side of the LC from the high vacuum of the MS and uses a belt to transport the analytes from one to the other. The mobile phase of the LC is deposited on a band and evaporated. The analytes remain on the continuously cycling belt and are transported from atmospheric pressure into the vacuum of the ion source through two differentially pumped vacuum locks. A heater in the ion source evaporates the sample from the belt allowing MS analysis. Most moving-belt analyses deal with volatile analytes using CI/EI; however, less volatile molecules such as nucleosides and nucleotides are analyzed using this system.

Thermospray

The TSP interface was developed by M. Vestal and co–workers. A major advantage of TSP over other LC–MS interfaces is its ability to handle the high flow-rates delivered by LC (up to 2 mL/min). As the name thermospray implies, heating the liquid flow leaving an LC system creates a spray of superheated mist containing small liquid droplets. Several techniques are developed to heat and vaporize the effluent; however, the most successful method involves directing the liquid flow through an electrically heated capillary, which can be directly introduced into the MS ion source. The droplets are further vaporized as they collide against the walls of the heated ion source. This ion source is equipped with a mechanical pump line opposite to the spray in order to evacuate the excess solvent vapor. The rapid heating and protective effects of the solvent allow the analysis of non-volatile samples without pyrolysis. The analyte ions are sampled into the MS through a sampling cone, if necessary aided by an applied electric field (repeller or accelerating electrode).

Atmospheric Pressure Ionization

The overwhelming increase in LC–MS applications is mainly the result of the sensitivity and ruggedness of atmospheric pressure ionization (API) LC–MS techniques. API is a general name for all ionization techniques in which the ions are formed at atmospheric pressure. Though very popular today, ionization processes at atmospheric pressure (flames, discharges etc.) have been studied using mass spectrometers for many years. In modern LC–MS applications we find two major techniques: ES and APCI. Electrospray can be subdivided into techniques such as pneumatic-assisted ES, ES, multiple sprayer ES etc., that differ mainly in the formation of a spray from the LC flow. However, all ES variants rely on the same mechanism to form ions from the droplets at atmospheric pressure. The ions formed at atmospheric pressure are transported from the source to the vacuum of the analyser through one or more differentially pumped stages separated by skimmers. The ions are focused and guided through the skimmer openings into the MS by applying appropriate electric fields. Various source designs, ion optics configurations, pumping systems and other experimental parameters are used, but the basic features can be found in all instruments. Where ES has its optimal performance at low flow-rates (nL/min range) APCI operates happily using mL/min flow-rates. ES and APCI perform differently under different chromatographic mode.

Applications of LC-MS/MS

Forced degradation studies

A forced degradation studies on acebutolol was performed to determine its labile behaviour under respective stress condition. The drug was labile to acidic, alkaline and photolytic stress, while it was stable in other neutral, oxidative and thermal conditions. The LC separation studies revealed the formation of four degradation products from the drug. DP-I was formed in acidic and basic condition and DP-IV was generated only in basic condition. The remaining two minor DPs such as DP-II and DP-III were the products of photolytic degradation. The structures of all these degradants were resolved with the help of MS, MS^n, and LCMS/MS analysis. The complete degradation pathway of the drug was established.

Bioanalytical applications

The developed LC–MS/MS method is useful in therapeutic drug monitoring of albendazole and diethyl carbamazine, which is the widely preferred Lymphatic filariasis therapy. The method is suitable for high throughput bioanalysis of diethyl Carbamazine, Albendazole, and Albendazole sulfoxide and Albendazole sulfone owing to a run time of 8min per sample. Assay performance, including linearity, precision and sensitivity were satisfactory for routine pharmacokinetic application. The method is readily applicable for therapeutic drug monitoring of DEC and ABZ in routine clinical use. The improved level of quantitation allowed us to monitor drug concentrations for 48 h for all studied compounds. This method may also enable the identification of patients at increased risk of ABZ toxicities by characterizing the concentration of the metabolites present in plasma. Therefore, the current LC–MS/MS method provides a valuable tool to 10 improve the efficacy and safety of DEC and ABZ therapy.

Food analysis

Semicarbazide belongs to the hydrazine family of chemicals, some members of which are known to possess carcinogenic

potential. Low levels of semicarbazide in a variety of raw and processed foods can be identified and measured with high analytical reliability, using LC–MS/MS.

Acrylamide is a chemical substance formed by reaction between amino acids and sugars. It typically occurs when high starch containing foods like potatoes, root vegetables and bread, are cooked at high temperatures in a process of frying, roasting or baking. It is not added to food but it is the byproduct formed during cooking. LCMS/MS is used for the identification and quantification of Acrylamide in various food matrices.

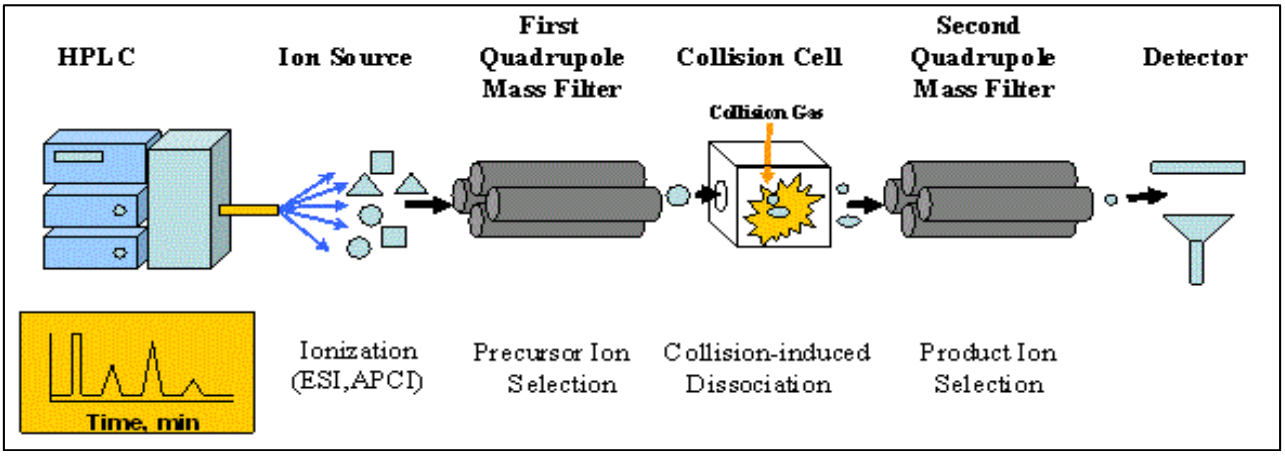

Fig 1: Schematic representation of LC-MS/MS

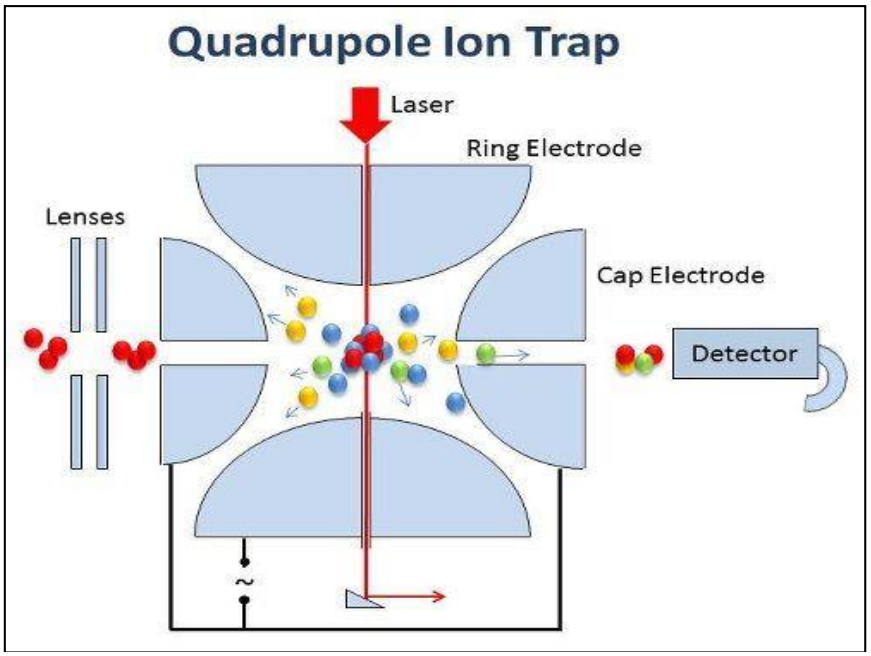

Fig 2: diagram of Quadrupole iontrap analyser.

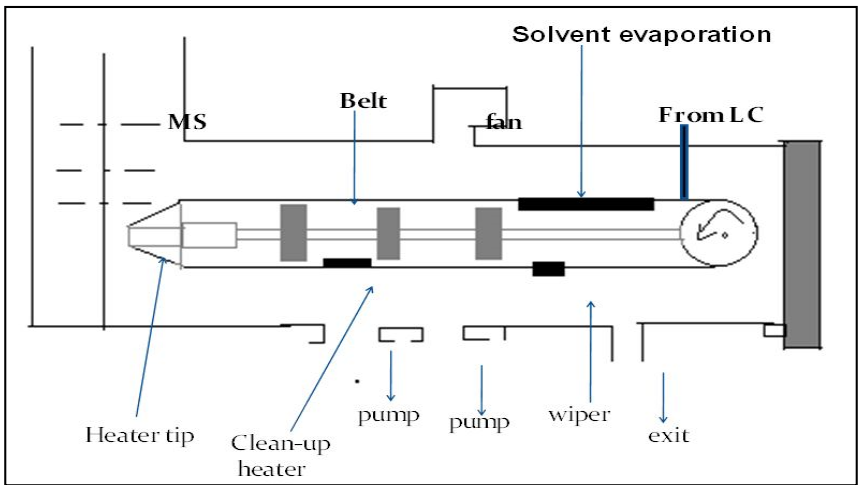

Fig 3: Moving belt LC-MS interface

Proteomics
A multidimensional chromatography peptide separation approach for the large-scale analysis of expressed yeast proteins was evaluated. It was identified 7537 peptides (1504 proteins) during a completely automated analysis.

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