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## Updated Review on LC-MS.

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### ABSTRACT

The current developments in liquid chromatography-mass spectrometry (LC-MS) and its applications to the analysis of pharmaceuticals are reviewed. Various mass spectrometric techniques, including electrospray and nanospray ionization, atmospheric pressure chemical ionization and photoionization and their interface with liquid chromatographic techniques are described. These include high performance liquid chromatography, capillary electrophoresis and capillary electro chromatography and the advantages and disadvantages of each technique are discussed. The applications of LC-MS to the studies of *in vitro* and *in vivo* drug metabolism, identification and characterization of impurities in pharmaceuticals, analysis of chiral impurities in drug substances and high-throughput LC-MS-MS systems for applications in the “accelerated drug discovery” process are described

**Keywords:** Liquid chromatography-mass spectrometry; pharmaceutical analysis; drug metabolism study; drug discovery; impurities identification; high-throughput LC-MS-MS

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## INTRODUCTION

Liquid chromatography–mass spectrometry (LC-MS, or alternatively HPLC-MS) is a chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. LC-MS is a powerful technique used for many applications which has very high sensitivity and selectivity. Generally its application is oriented towards the general detection and potential identification of chemicals in the presence of other chemicals (in a complex mixture). Preparative LC-MS system can be used for fast and mass directed purification of natural-products extracts and new molecular entities important to food, pharmaceutical, agrochemical and other industries. The limitations of LC-MS in urine analysis drug screening are that it often fails to distinguish between specific metabolites, in particular with hydrocodone and its metabolites. LC-MS urine analysis testing is used to detect specific categories of drugs. However, gas chromatography (GC-MS) should be used when detection of a specific drug and its metabolites is required [1].

### Why is LC/MS Important?

- Provides unambiguous compound identity
- Provides sensitive response to most analytes
- Provides compound class information
- Provides compound structure
- Provides sequence information
- Provides molecular weight information
- Provides the five **S**'s
  - **Speed**
  - **Selectivity**
  - **Specificity**
  - **Sensitivity**
  - **\$...Low Cost per Information Content**

### Who uses LC/MS?

- Drug Discovery
- Clinical Analysts
- Proteomics
- Forensic Chemists
- Drug Metabolism
- Environmental Chemists
- Diagnostics.

### ADVANTAGES

Liquid chromatography/mass spectroscopy (LC/MS) is a versatile analytical method commonly employed during all phases of pharmaceutical drug development because of its regarded speed, sensitivity, and selectivity. It is commonly used during final analyses of the

drug approval process. LC/MS identifies proteins, detects metabolites, determines the molecular weight of drugs, verifies molecular structures and detects for impurities and degradants. Purchasing considerations for pharmaceutical LC/MS systems include the desired ionization method, instrument size, ease of use and operation, and the laboratory budget

### **Ultra-High Sensitivity**

By incorporating newly improved ion optics and collision cell technology, the LCMS-8040 provides higher multiple reactions monitoring (MRM) sensitivity. A five-fold increase in sensitivity (Reserpine, S/N ratio) has been achieved by improving ion focusing and minimizing ion losses between multi-pole lenses. These improvements also yield higher sensitivity for scan mode measurements. This higher sensitivity expands the potential range of LC/MS/MS applications.

### **Ultra-High Speed**

The LCMS-8040 was designed to provide significantly higher sensitivity while maintaining the high speed offered by the LCMS-8030. Ultrafast MRM transition speeds, up to 555 MRMs per second, are achieved by Shimadzu's collision cell technology, proprietary high-precision quadrupole machining capabilities, and unique high voltage power supply technology. In addition, the LCMS-8040 features the world's fastest\* polarity switching at 15 msec. With this high-speed performance, the LCMS-8040 can dramatically improve analytical throughput.

### **Ultra-High Reliability**

MRM optimization in Shimadzu's LCMS systems is based on a rapid series of automated flow injection analyses, requiring only minutes to perform. Multiple compounds can be optimized in an unattended sequence, freeing the analyst from tedious work. MRM parameters optimized for the LCMS-8030 can be transferred to the LCMS-8040, making it possible to transfer methods between systems. The LCMS-8040 offers the same ease of maintenance benefits as the LCMS-8030, and all consumables, such as desolvation lines (DL) and ESI capillaries, are interchangeable as well

### **PRINCIPLE [2]**

Generally, Chromatography is an analytical technique used for the separation of complex chemical mixtures into individual components. The sample is carried through a separating column by a mobile phase where the mixture is divided into unique bands based on the amount of interaction between the individual analytes and the stationary phase in the column. As these bands leave the column, their identities and quantities are determined by a detector. When either LC or GC is coupled to MS, then we have LC-MS. In LCMS the mobile phase is below its critical temperature and above its critical pressure, it acts as a liquid, so the technique is liquid chromatography and when the mobile phase is above its critical temperature and below its critical pressure, it acts as a gas so the technique is gas chromatography (GC). GCMS is inapplicable for non volatile or thermally unstable

compounds, unlike LCMS LC is applied mostly in the life sciences and related fields of chemistry. Unlike gas chromatography, which is unsuitable for non volatile and thermally fragile molecules, LC can safely separate a very wide range of organic compounds, from small-molecule drug metabolites to peptides, f-PUFA and its metabolites (PGE, EPA, HETEs, PGDs etc.) and proteins. Mass spectrometers also generate three dimensional data. In addition to signal strength, they generate mass spectral data that can provide valuable information about the molecular weight, structure, identity, quantity, and purity of a sample. Mass spectral data add specificity that increases confidence in the results of both qualitative and quantitative analyses. So when you couple the both technique, LCMS, you'll be sure of high specificity, sensitivity, high through put and devoid of several derivatization and complex sample preparation required by the GCMS. GCMS is better applied in the petrochemical and allied chemical industries (pesticides etc.)

### Liquid Chromatography Mass Spectrometer (LC MS Instrument) [3]

Comprising two components, a liquid-phase separation system and a mass spectrometer, liquid chromatography mass spectrometers (LC MS instruments) couple sample fractionation and analysis. The separation can be accomplished via standard liquid chromatography, HPLC, UPLC, or even nano-LC. Any liquid-phase separation will do, so long as its output can be directed into the mass analyzer (for instance, via an electro spray ionization source). The mass spectrometer is similarly flexible, ranging from the simplest single quadrupole to a Fourier transform ion cyclotron resonance instrument. Just remember, in a liquid chromatography mass spectrometer the column flow is continuous, meaning the mass analyzer must be fast enough to thoroughly analyze one peak before the next one comes off the column. And, unlike with MALDI MS, once a peak is analyzed, it's gone.

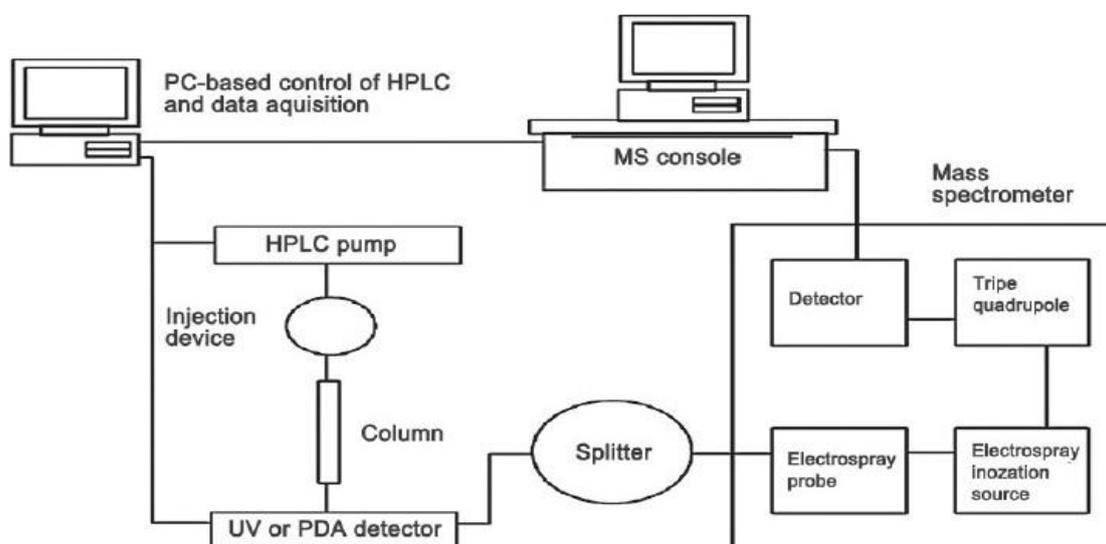


Fig.1 Schematic diagram of LCMS instrument

## **INSTRUMENTATION OF LC-MS [4]**

### **Liquid chromatography**

#### **Flow splitting**

When standard bore (4.6 mm) columns are used the flow is often split ~10:1. This can be beneficial by allowing the use of other techniques in tandem such as MS and UV. However splitting the flow to UV will decrease the sensitivity of spectrophotometric detectors. The mass spectrometry on the other hand will give improved sensitivity at flow rates of 200  $\mu\text{L}/\text{min}$  or less.

#### **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 introductions of techniques that allow delivery of thermo labile bio molecules into the MS show an exponential increase in the number of publications employing LC–MS. However, in order to give somewhat wider overview older LC–MS interfaces will briefly be described

#### **Interface**

Understandably the interface between a liquid phase technique which continuously flows liquid, and a gas phase technique carried out in a vacuum was difficult for a long time. The advent of electro spray ionization changed this. The interface is most often an electro spray ion source or variant such as a nano spray source; however atmospheric pressure chemical ionization interface is also used. Various deposition and drying techniques have also been used such as using moving belts; however the most common of these is off-line MALDI deposition. A new approach still under development called Direct-EI LC-MS interface, couples a nanoHPLC system and an electron ionization equipped mass spectrometer.

#### **Mass spectrophotometry[5]**

Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio of charged particles. It is used for determining masses of particles, for determining the elemental composition of a sample or molecule, and for elucidating the chemical structures of molecules, such as peptides and other chemical compounds. MS works by ionizing chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios. In a typical MS procedure:

- A sample is loaded onto the MS instrument and undergoes vaporization.
- The components of the sample are ionized by one of a variety of methods (e.g., by impacting them with an electron beam), which results in the formation of charged particles (ions).



- The ions are separated according to their mass-to-charge ratio in an analyzer by electromagnetic fields
- The ions are detected, usually by a quantitative method.
- The ion signal is processed into mass spectra.

Additionally, MS instruments consist of three modules

- An ion source, which can convert gas phase sample molecules into ions (or, in the case of electro spray ionization, move ions that exist in solution into the gas phase)
- A mass analyzer, which sorts the ions by their masses by applying electromagnetic fields
- A detector, which measures the value of an indicator quantity and thus provides data for calculating the abundances of each ion present

The technique has both qualitative and quantitative uses. These include identifying unknown compounds, determining the isotopic composition of elements in a molecule, and determining the structure of a compound by observing its fragmentation. Other uses include quantifying the amount of a compound in a sample or studying the fundamentals of gas phase ion chemistry (the chemistry of ions and neutrals in a vacuum). MS is now in very common use in analytical laboratories that study physical, chemical, or biological properties of a great variety of compounds.

### **Ion Sources**

Much of the advancement 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. Earlier LC/MS systems used interfaces that either did not separate the mobile phase molecules from the analyte molecules (direct liquid inlet, thermo spray) or did so before ionization (particle beam). The analyte molecules were then ionized in the mass spectrometer under vacuum, often by traditional electron ionization. These approaches were successful only for a very limited number of compounds. The introduction of atmospheric pressure ionization (API) techniques greatly expanded 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:

- Electro spray ionization (ESI)
- Atmospheric pressure chemical ionization (APCI)
- Atmospheric pressure photo ionization (APPI)

### **Electro spray ionization**

Electro spray relies in part on chemistry to generate analyte ions in solution before the analyte reaches the mass spectrometer. The LC eluent is sprayed (nebulised) into a chamber at atmospheric pressure in the presence of a strong electrostatic field and heated drying gas. The electrostatic field causes further dissociation of the analyte molecules. The

heated drying gas causes the solvent in the droplets to evaporate. As the droplets shrink, the charge concentration in the droplets increases. Eventually, 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, mostly 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.

Electro spray is especially useful for analyzing large biomolecules such as proteins, peptides, and oligo nucleotides, but can also analyze smaller molecules like benzodiazepines and sulphated conjugates. Large molecules often acquire more than one charge. Thanks to this multiple charging, electrospray can be used to analyze molecules as large as 150,000 u even though the mass range (or more accurately mass-to-charge range) for a typical LC/MS instruments is around 3000 m/z. For example:  $100,000 \text{ u} / 10 \text{ z} = 1,000 \text{ m/z}$  When a large molecule acquires many charges, a mathematical process called deconvolution is often used to determine the actual molecular weight of the analyte.

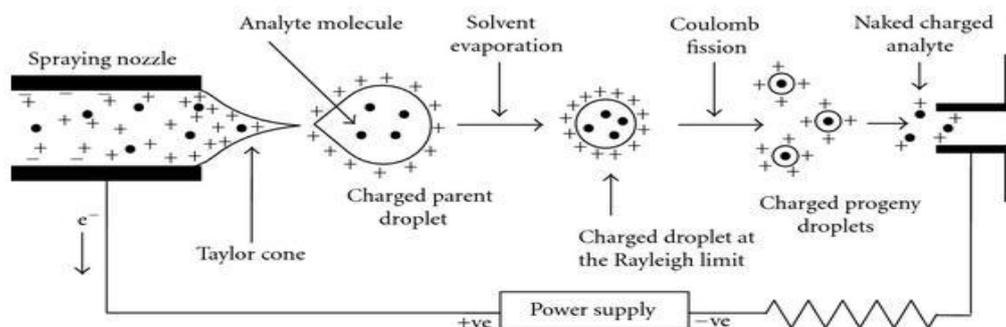


Fig 2 Mechanism of Electro spray ionization

### Atmospheric pressure chemical ionization (APCI)

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 pass through a capillary sampling orifice into the mass analyzer. APCI is applicable 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 electro spray for analysis of large biomolecules that may be thermally unstable. APCI is used with normal-phase chromatography more often than electro spray is because the analytes are usually nonpolar.

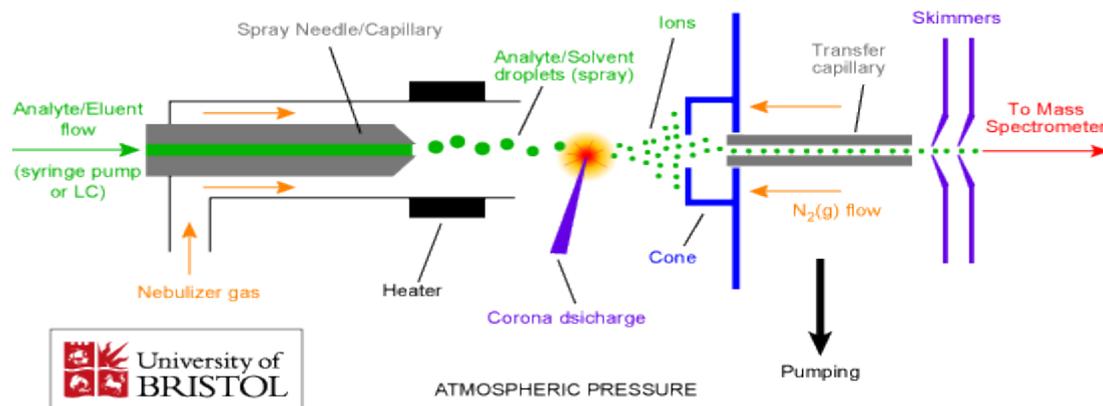


Fig 3 Mechanism of Atmospheric pressure chemical Ionization

### Atmospheric pressure photo ionization

Atmospheric pressure photo ionization (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 generates 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 applicable 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  $\mu\text{l}/\text{min}$ ), where APCI sensitivity is sometimes reduced. In all cases, the nature of the analyte(s) and the separation conditions have a strong influence on which ionization technique: the best results. The most effective technique is not always easy to predict.

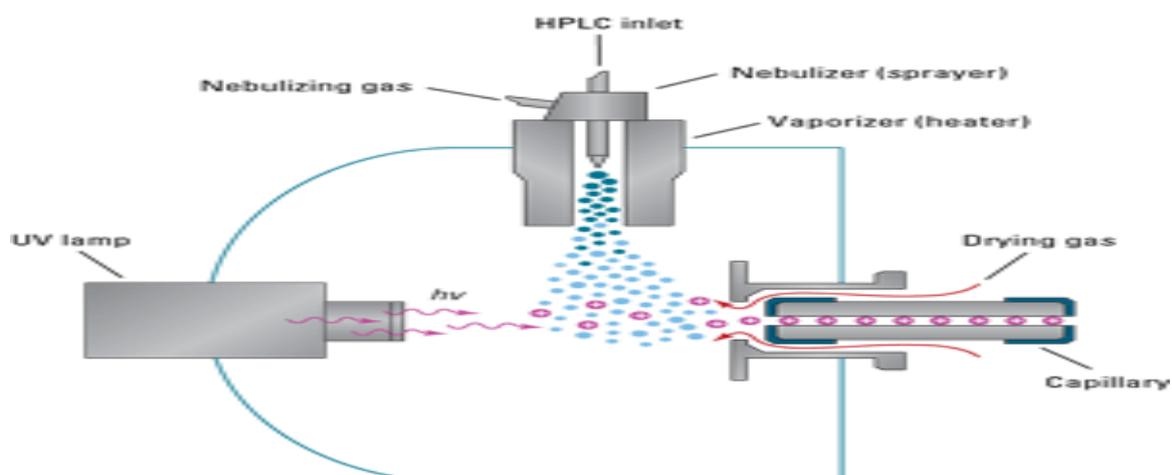


Fig 4 Mechanism of Atmospheric pressure photo Ionization

### Mass analyzer

There are many different mass analyzers that can be used in LC/MS. Single quadrupole, triple quadrupole, ion trap, time of flight (TOF) and quadrupole-time of flight (Q-TOF). 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 (FT-ICR or FT-MS) are used most often. Each has advantages and disadvantages depending on the requirements of a particular analysis.

### Quadrupole

A quadrupole mass analyzer consists of four parallel rods arranged in a square. The analyte ions are directed down the center of the square. Voltages applied to the rods generate 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 expensive mass analyzers. Quadrupole mass analyzers can operate in two modes.

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

In scan 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 scan mode but provides information about fewer ions. Scan mode is typically used for qualitative analyses or for quantization when all analyte masses are not known in advance. SIM mode is used for quantization and monitoring of target compounds.

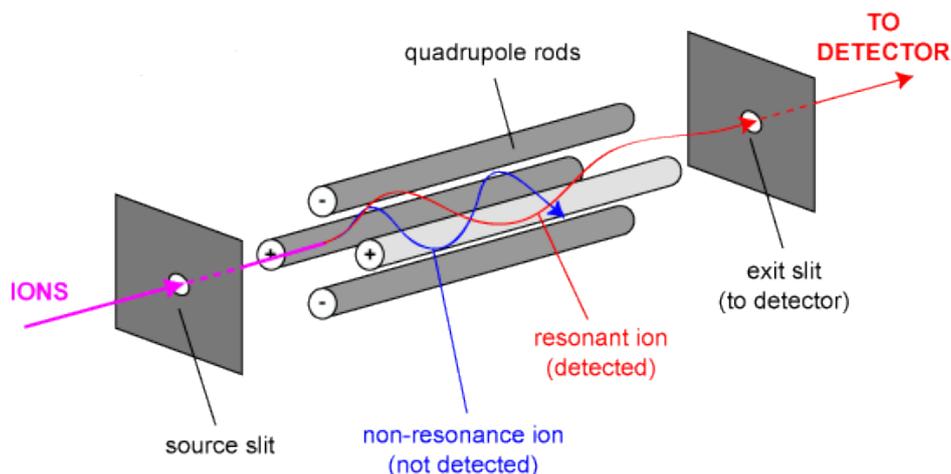


Fig 5 Mechanism of Quadrupole

### Time-of-flight

In a time-of-flight (TOF) mass analyzer, a uniform 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, so the mass-to-charge ratios of the ions are determined by their arrival times. Time-of-flight mass analyzers have a wide mass range and can be very accurate in their mass measurements.

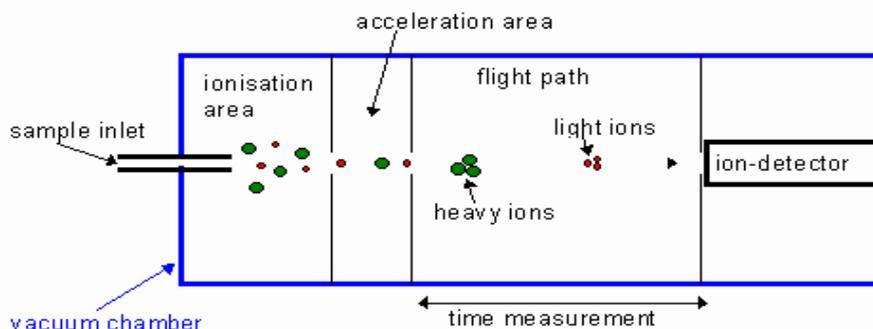


Fig 6 Mechanism of Time-of-Flight

### Ion trap

An ion trap mass analyzer consists of a circular 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 perform multiple stages of mass spectrometry without additional mass analyzers.

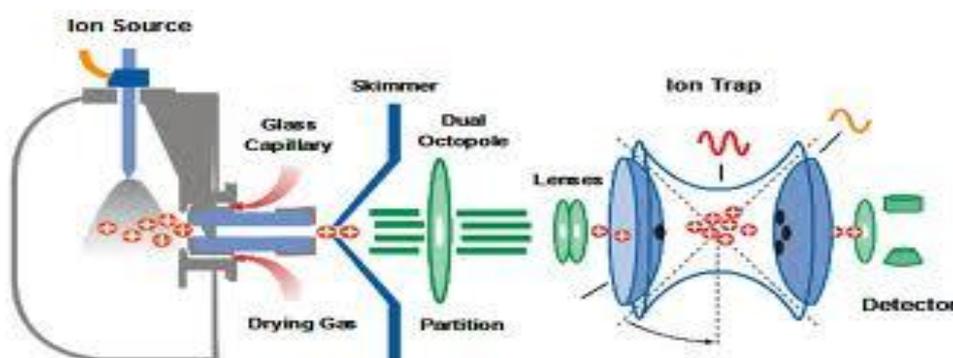


Fig 7 Mechanism of Ion trap

### Fourier transform-ion cyclotron resonance (FT-ICR)

An FT-ICR mass analyzer (also called FT-MS) is another type of trapping analyzer. Ions entering a chamber are trapped in circular orbits by powerful electrical and magnetic fields. When excited by a radio-frequency (RF) electrical field, the ions generate a time dependent current. This current is converted 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 additional mass analyzers. They also have a wide mass range and excellent mass resolution. They are, however, the most expensive of the mass analyzers.

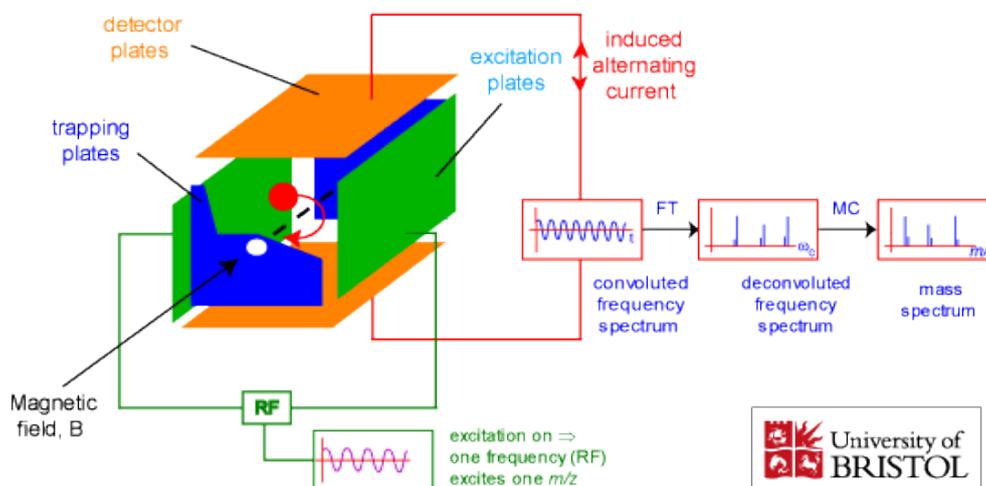


Fig 8 Mechanism of Fourier transform-ion cyclotron resonance

### Sample preparation

Sample preparation generally consists of concentrating the analyte and removing compounds that can cause background ions or suppress ionization. Examples of sample preparation include ,On-column concentration to increase analyte concentration, Desalting to reduce the sodium and potassium adduct formation that commonly occurs in electro spray , Filtration to separate a low molecular-weight drug from proteins in plasma, milk, or tissue.

### Chemistry of Ionization

Because formation of analyte ions in solution is essential to achieving good electro spray results, careful attention must be paid to proper solution chemistry. For electro spray:

- ✓ Select more volatile buffers to reduce the buildup of salts in the ion source
- ✓ Adjust solvent pH according to the polarity of ions desired and the pH of the sample
- ✓ Use solvents that have low heats of vaporization and low surface tensions to enhance ion desorption
- ✓ Make sure that gas-phase reactions do not neutralize ions through proton transfer or ion pair reactions if pH adjustments interfere with proper chromatography, post column modification of the solvent may be a good solution. This can improve MS response without compromising chromatography. Solution chemistry is less critical for APCI operation because ionization occurs in the gas phase, not the liquid phase, but solvent selection can still have a significant effect on APCI analyte signal response.
- ✓ Select more volatile solvents
- ✓ Select solvents with a lower charge affinity than the analyte
- ✓ Protic solvents generally work better than nonprotic solvents for positive ion mode
- ✓ For negative ionization, solvents that readily capture an electron must be used
- ✓ Ammonium salts in the mobile phase can cause ammonium adduct formation Vaporizer temperature also affects APCI ionization results. The temperature must be hot enough to vaporize the solvent but not so hot as to cause thermal degradation of the analyte molecules.



## ROLE OF LC-MS IN PHARMACEUTICAL CHEMISTRY & ANALYSIS

### Rapid chromatography of benzodiazepines

The information available in a mass spectrum allows some compounds to be separated even though they are chromatographically unresolved. In this example, a series of benzodiazepines was analyzed using both UV and MS detectors. The UV trace could not be used for quantization, but the extracted ion chromatograms from the MS could be used. The mass spectral information provides additional confirmation of identity. Chlorine has a characteristic pattern because of the relative abundance of the two most abundant isotopes.

### Identification of bile acid metabolites

The MS capabilities of the ion trap mass spectrometer make it a powerful tool for the structural analysis of complex mixtures. Intelligent, data-dependent acquisition techniques can increase ion trap effectiveness and productivity. They permit the identification of minor metabolites at very low abundances from a single analysis. One application is the identification of metabolic products of drug candidates.

### Rapid protein identification using capillary LC/MS/MS and database searching

Traditional methods of protein identification generally require the isolation of individual proteins by two-dimensional gel electrophoresis. The combination of capillary LC/MS/MS with intelligent, data-dependent acquisition and probability-based database searching makes it possible to rapidly identify as many as 100 proteins in a single analysis.

### Identification of aflatoxins in food

Aflatoxins are toxic metabolites produced in foods by certain fungi. The total ion chromatogram from a mixture of four aflatoxins. Even though they are structurally very similar, each aflatoxin can be uniquely identified by its mass spectrum

### Detection of phenyl urea herbicides

Many of the phenyl urea herbicides are very similar and difficult to distinguish with a UV detector. Monuron and diuron have one benzene ring and differ by a single chlorine. Chloroxuron has two chlorines and a second benzene ring attached to the first by oxygen. The UV-Vis spectra are similar for diuron and monuron, but different for chloroxuron. When analyzed using electro spray ionization on an LC/MS system, each compound has a uniquely identifiable mass spectrum.

### Bio analytical applications

Bio analytical methods are widely used to quantitate drugs and their metabolites in physiological matrices, and the methods could be applied to studies in areas of human clinical pharmacology and nonhuman pharmacology/toxicology. Bio analytical method

employed for the quantitative determination of drugs and their metabolites in biological fluids, plays a significant role in the evaluation and interpretation of bioequivalence, pharmacokinetic (PK), and toxicokinetic studies.<sup>1</sup> The major bioanalytical services are method development, method validation and sample analysis (method application). Whatever way the analysis is done it must be checked to see whether it does what it is purported to do; i.e. it must be validated. Each step in the method must be investigated to determine the extent to which environment, matrix or procedural variables can effect the estimation of analyte in the matrix from the time of collection up to the time of analysis. Both HPLC and LCMS-MS can be used for the bioanalysis of drugs in plasma. Each of the instruments has its own merits and demerits. HPLC coupled with UV, PDA or fluorescence detector can be used for estimation of many compounds but it does not give the high sensitivity as required by some of the potent low dose drugs and lacks selectivity. The main advantages of LCMS-MS include low detection limits, the ability to generate structural information, the requirement of minimal sample treatment and the possibility to cover a wide range of analytes differing in their polarities. Despite their high sensitivity and selectivity LC/MS/MS instruments are limited to some extent due to matrix-induced differences in ionization efficiencies and ion suppression/enhancement effects due to biological matrix. HPLC coupled with UV, PDA or fluorescence detector offers a cost effective bioanalytical method. Depending on the sensitivity, selectivity and cost effectiveness of the method a choice needs to be made between HPLC AND LCMS-MS [6]

### **Miscellaneous applications**

#### **Pharmacokinetics**

LC-MS is very commonly used in pharmacokinetic studies of pharmaceuticals and is thus the most frequently used technique in the field of bioanalysis. These studies give information about how quickly a drug will be cleared from the hepatic blood flow, and organs of the body. MS is used for this due to high sensitivity and exceptional specificity compared to UV (as long as the analyte can be suitably ionised), and short analysis time. The major advantage MS has is the use of tandem MS-MS. The detector may be programmed to select certain ions to fragment. The process is essentially a selection technique, but is in fact more complex. The measured quantity is the sum of molecule fragments chosen by the operator. As long as there are no interferences or ion suppression, the LC separation can be quite quick. It is common now to have analysis times of 1 minute or less by MS-MS detection, compared to over 10 mins with UV detection.

#### **Proteomics/metabolomics**

LC-MS is also used in proteomics where again components of a complex mixture must be detected and identified in some manner. The bottom-up proteomics LC-MS approach to proteomics generally involves protease digestion and denaturation (usually trypsin as a protease, urea to denature tertiary structure and iodoacetamide to cap cysteine residues) followed by LC-MS with peptide mass fingerprinting or LC-MS/MS (tandem MS) to derive sequence of individual peptides. LC-MS/MS is most commonly used for proteomic

analysis of complex samples where peptide masses may overlap even with a high-resolution mass spectrometer. Samples of complex biological fluids like human serum may be run in a modern LC-MS/MS system and result in over 1000 proteins being identified, provided that the sample was first separated on an SDS-PAGE gel or HPLC-SCX. Profiling of secondary metabolites in plants or food like phenolics can be achieved with liquid chromatography–mass spectrometry.

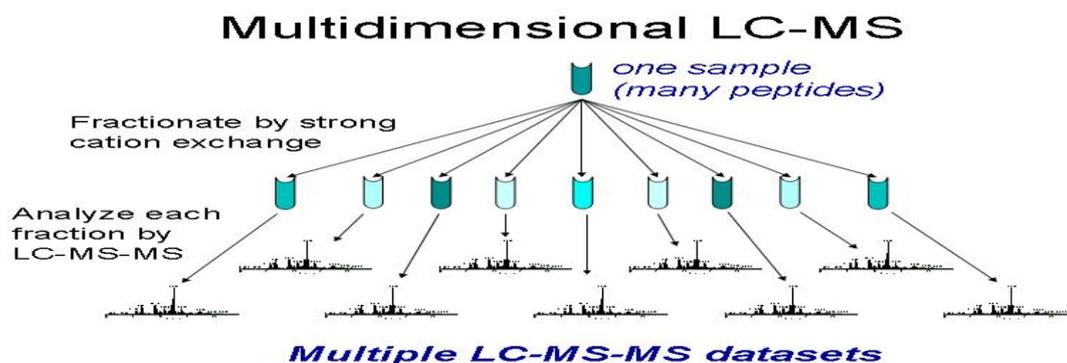


Fig 9 multiple LC/MS/MS database

## Drug development

LC-MS is frequently used in drug development at many different stages including peptide mapping, glycoprotein mapping, natural products dereplication, bio affinity screening, *in vivo* drug screening, metabolic stability screening, metabolite identification, impurity identification, quantitative bio analysis, and quality control.

## Molecular Weight Determination

One fundamental application of LC/MS is the determination of molecular weights. This information is key to determining identity.

## Structural Determination

Another fundamental application of LC/MS is the determination of information about molecular structure. This can be in addition to molecular weight information or instead of molecular weight information if the identity of the analyte is already known.

## LC-MS Method Development expertise includes

- Liquid/Liquid, SPE and Protein Precipitation in 96-Well and Standard Formats
- Hydrophilic Interaction Chromatography (HILIC)
- Ion Exchange SPE and Chromatography
- Chiral Analysis
- Analysis of Drugs in Ocular Tissues
- Analysis of Peptides and Pegylated Drugs and Metabolites<sup>7</sup>

## Future Developments

A very clear goal since the beginnings of LC–MS and still an important trend in newly developed instruments is robustness. Both separation science and mass spectrometry are very specialized research domains and often scientists are focused on only one of them. Thus, when applying the hyphenated LC–MS techniques the other half “just has to work”. The chromatographer wants MS to work as a reliable detector that can be hooked up to a column (no matter what flow-rate or kind of separation), whilst the mass spectrometrists needs a system for introducing these liquid samples (sometimes mixtures) containing polar, thermo labile, in volatile bio molecules, pharmaceuticals, environmental contaminants, pesticides etc. As sample availability and sensitivity are always an issue in analytical (bio)chemistry, miniaturization will be a continuing trend in LC–MS. Miniaturization of the separation techniques and consequent development of the appropriate interfaces will proceed, including chip-based technology for both separation and interfacing to MS. Techniques that are today still considered as off-line techniques, for example, MALDI, 2D gel electrophoresis etc., will be modified and new techniques developed to couple with existing MS and LC–MS systems [8].

## CONCLUSION

Although it is a good starting point, modelling analytical techniques in bio analytical method development should not be restricted to pure and neat analyte solutions. Unknown compounds originating from the biological matrix could interfere with the analyte response and should also be taken into account in the modelling process. Carefully built models provide a good description of the behaviour of the complete analytical system under different conditions. These results could be considered as a summary of the results of the performed experiments. Also, method development could be performed much more structured and the models could be helpful for future use for trouble shooting purposes. Care should be taken to include responses into the models that are not affected by unexpected effects. Besides matrix effects affecting the analytical response, other effects could also blur the results. Solvent evaporation of extracts may lead to incomplete reconstitution due to the loss of analyte or adsorption effects and a decrease in the amount of thermal, oxidation sensitive or pH labile compounds may be interpreted as a low recovery. Also, enzymatic activity in biological matrices may decrease the analyte concentration resulting in misinterpreted recovery values. Moreover, one has to be sure that a state of equilibrium has been reached and that reactions have been complete as in protein precipitation. A bio analytical method which was set up using theoretical models and where matrix effects are taken into account could be considered as a controlled method since the effect of changes in analytical parameters can be predicted quantitatively. Validation of new methods in analytical toxicology is an integral part of quality assurance, accreditation, and publication. Methods intended for routine use or publication must be fully validated to objectively demonstrate their applicability for the intended use. For methods used for analysis of rare analytes or in single cases, the number of validation experiments and parameters to be evaluated may be reduced, but a minimum validation is necessary to ensure sufficient quality of the results.



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