Chapter 2

ANALYTICAL TECHNIQUES
2.1 CHROMATOGRAPHY

Chromatography is a technique used to separate and analyze mixtures. Classification of liquid chromatography is shown in the fig. 2.1 and 2.2. The components to be separated are distributed between two phases: stationary phase and mobile phase, which percolate through the stationary bed. The phases are chosen such that the components of the sample have differing solubilities in each phase. A component which is quite soluble in the stationary phase, will take longer time to travel through it than a component which is not highly soluble in stationary phase but in mobile phase. As a result of their difference in mobilities sample component get separated from each other as they travel through the stationary phase. The separated molecules leave the column and get detected by one or more on-line electrical devices with signals proportional to the concentration of the analytes [1-3]. All chromatographic methods require one static part (the stationary phase) and one moving part (the mobile phase). The techniques rely on one of the following phenomena: adsorption; partition; ion exchange; or molecular exclusion.

2.1.1. Adsorption

Adsorption chromatography was developed first. It has a solid stationary phase and a liquid or gaseous mobile phase. (Plant pigments were separated at the turn of the 20th century by using a calcium carbonate stationary phase and a liquid hydrocarbon mobile phase. The different solutes travelled different distances through the solid, carried along by the solvent. Each solute has its own equilibrium between adsorption onto the surface of the solid and solubility in the solvent, the least soluble or best adsorbed ones travel more slowly. The result is a separation into bands containing different solutes. Liquid chromatography using a column containing silica gel or alumina is an example of adsorption chromatography. The solvent that is put into a column is called the eluent, and the liquid that flows out of the end of the column is called the eluate.

2.1.2. Partition

In partition chromatography the stationary phase is a non-volatile liquid which is held as a thin layer (or film) on the surface of an inert solid. The mixture to be
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separated is carried by a gas or a liquid as the mobile phase. The solutes distribute
themselves between the moving and the stationary phases, with the more soluble
component in the mobile phase reaching the end of the chromatography column first.
Paper chromatography is an example of partition chromatography.

2.1.3. Ion exchange

Ion exchange chromatography is similar to partition chromatography in that it
has a coated solid as the stationary phase. The coating is referred to as a resin, and has
ions (either cations or anions, depending on the resin) covalently bonded to it and ions
of the opposite charge are electrostatically bound to the surface. When the mobile
phase (always a liquid) is eluted through the resin the electrostatically bound ions are
released as other ions are bonded preferentially, eg. Domestic water softeners.

2.1.4. Molecular exclusion

Molecular exclusion differs from other types of chromatography in that no
equilibrium state is established between the solute and the stationary phase. Instead, the
mixture passes as a gas or a liquid through a porous gel. The pore size is designed to
allow the large solute particles to pass through uninhibited. The small particles, however,
permeate the gel and are slowed down so the smaller the particles, the longer it takes for
them to get through the column. Thus separation is according to particle size.

![Liquid chromatography diagram]

Fig. 2.1. Classification of liquid chromatographic techniques
Fig. 2.2 Different types of liquid chromatography

2.2.1 High performance liquid chromatography (HPLC)

Among all separation tools, high performance liquid chromatography (HPLC) takes a central role in pharmaceutical analysis. Liquid chromatography (LC) is a separation method of great importance to the chemical, pharmaceutical and biotechnological industry. The principle is that a sample of a solution of the substances is injected into a column of a porous material (stationary phase) and a liquid (mobile phase) is pumped through the column. The separation of substances is based on differences in rates of migration through the column arising from difference in partition of the substances between the stationary and the mobile phase. Depending on the partition behavior of the different types of substances, these will elute at different times from the column outlet. The technique was originally developed by the Russian Botanist M.S. Tswett in 1903 [4, 5] and since then there has been an enormous development of this technique. The definite break through for liquid
chromatography of low molecular weight compounds was the introduction of chemically modified small diameter particles (3 to 10 μm), e.g. Octadecyl groups bound to silica in the late 1960s [6]. The details of new technique, which became rapidly a powerful separation technique, were summarized by Giddings [7]. For biological samples where the analytes often are non-volatile and/or occur in an aqueous matrix, the reversed-phase mode, using a hydrophobic stationary phase and an aqueous mobile phase is extremely useful. The usefulness and popularity of HPLC was further increased by the possibility to automate and computerize the systems providing unattended operations and high sample capacities. Many Nobel Prize awards have been based upon the work in which chromatography played an important role [8]. Recently, the 2002 Nobel Prize in Chemistry was awarded to “the development of methods for identification, structure analyses of biological macromolecules and bioanalytical applications” in which HPLC and Mass spectrometry (MS) were used [9].

In analytical chromatography, the aim of the separation is to obtain quantitative and qualitative information about the compounds of interest (analytes) in a sample. Analytical chemists have to analyze a variety of complex samples often originating in difficult matrices to answer questions about the quality and quantity of different analytes. A complex sample often contains a wide range of components with varying solubilities. Therefore the sample preparation and the separation methods must be highly selective and sensitive. These requirements are satisfied with HPLC especially if combined with an advanced detection technique such as diode – array detection (DAD) or mass spectrometry (MS). A very large number of reports have been published in the analytical and bioanalytical (analysis of drugs in biological fluids) areas using these techniques.

2.2.2 Normal phase HPLC

Also termed as adsorption chromatography, this method separates analytes based on adsorption. Normal phase-HPLC uses a polar stationary phase and a non-polar mobile phase. It works effectively for separating analytes readily soluble in non-polar solvents. The analyte associates with and is retained by the polar stationary phase.
2.2.3 Reverse phase HPLC

In this case silica is modified to make it non-polar with either 8 or 18 carbons. A polar solvent is used - example, a mixture of water and an alcohol such as methanol. In this case, there will be a strong attraction between the polar solvent and polar molecules in the mixture being passed through the column. There will not be as much attraction between the stationary phase and the polar molecules in the solution. Polar molecules in the mixture will travel faster with the solvent and elute first. Non-polar compounds in the mixture will tend to attract with the hydrocarbon groups because of Van der Waals dispersion forces. They therefore spend less time in solution in the solvent and this will slow the movement through the column and elute later. Reversed phase HPLC is the most commonly used form of HPLC in pharmaceutical analysis.

2.2.4. THEORETICAL & PRACTICAL ASPECTS OF LC-MS/MS

The combination of high performance liquid chromatography and mass spectrometry (LC/MS) has a significant impact on drug development over the past decade. Continual improvements in LC/MS interface technologies combined with powerful features for structure analysis, qualitative and quantitative resulted in the widened scope of applications. These improvements coincide with breakthroughs in combinatorial chemistry, molecular biology and overall industry trend of accelerated drug development. The integration of new technologies in the pharmaceutical industry created a situation where the rate of sample generation far exceeds the rate of sample analysis. As a result, new paradigms have been developed for the analysis of drugs and related substances and both pharmaceutical and instrument manufacturing industries have mutually benefited. The growth of LC/MS applications has been extensive with retention time and molecular weight, emerging as an essential analytical feature from drug target to product. LC/MS based methodologies that involve automation, predictive or surrogate models and open access systems have become a permanent fixture in the drug development landscape. At preset LC/MS has become widely accepted analytical tool for the drug development process.
The combination of chromatography and mass spectrometry (MS) has attracted much interest over the last forty years. The combination of gas chromatography (GC) with mass spectrometry (GC/MS) was first reported in 1958 and made available commercially in 1967. Since then, it has become increasingly utilized and is probably the most widely used 'hyphenated' or 'tandem' technique, as such combinations are often known. The GC/MS technique is applicable to those compounds which are volatile in nature and thermally stable. So this technique is not allowed for the analysis of a wide range of compounds for those which are non-volatile in nature and thermally unstable compound. To overcome this problem, LC/MS technique has been introduced.

2.2.5. ADVANTAGES OF LC-MS/MS

a. LC/MS interfaces are used for studying a wide range of analytes, from low molecular weight drugs and metabolites (<1000 Da) to high-molecular weight biopolymers (>100 000 Da).

b. The mass spectrometer provides the most definitive identification of all the HPLC detectors. It allows the molecular weight of the analyte to be determined.

c. This is the single most discriminating piece of information that may be obtained which together with the structural information that may be generated often allows an unequivocal identification to be made.

d. The high selectivity of the mass spectrometer often provides this identification capability on chromatographically unresolved or partially resolved components.

e. This selectivity allows the use of isotopically labelled analytes as internal standards and this, coupled with high sensitivity, allows very accurate and precise quantitative determinations to be carried out.

2.3 INTRODUCTION TO LIQUID CHROMATOGRAPHY

A block diagram of an HPLC system, illustrating its major components, is shown in Figure 2.1 These components are discussed below in detail.
2.3.1 Pump

The pump must provide stable flow rates between 10 µL min⁻¹ and 2 mL min⁻¹ with the LC/MS requirement, dependent upon the interface being used and the diameter of the HPLC column. For example, the electrospray interface used at lower flow rates ranges from 0.01 mL min⁻¹ to 1.5 mL min⁻¹ and where as APCI interface uses up to 2.0 mL min⁻¹.

A number of different types of pumps are available [1,3], but the most popular pump used today is the reciprocating pump. From a mass spectrometry perspective, the pump must be pulse free, i.e. it must deliver the mobile phase at a constant flow rate. Pulsing of the flow causes the total ion current (TIC) trace, the primary piece of information used for spectral analysis i.e to show increases in signal intensity when analytes are not being eluted and this makes interpretation more difficult.

2.3.2 Sample Introduction (Injector)

The loop injector (sometimes known as the valve injector) is a convenient way of introducing a liquid sample into a flowing liquid stream and consists of a loop of a nominal volume into which the sample is introduced by using a conventional syringe. While the loop is being filled, mobile phase is pumped, at the desired flow rate, through the valve to the column to keep the column in equilibrium with the mobile phase and maintain the chromatographic performance. When ‘injection’ is required, a rotating switch is moved and the flow is diverted through the loop, thus flushing its contents onto the top of the column.

2.3.3 Mobile Phase

In HPLC the relative interaction of an analyte with both the mobile and stationary phases determines the retention characteristics. Hence, it is the varying degrees of interaction of different analytes with the mobile and stationary phases which determines whether or not they will be separated by a particular HPLC system [4-6]. A separation involving a mobile phase of constant composition (irrespective of the number of components it contains) is termed isocratic elution, while that in which the composition of the mobile phase changed is termed as gradient elution. In the latter, a
mobile phase is chosen which provides adequate separation of the early eluting analytes and a solvent which is known to elute the longer retained compounds is added over a period of time. The rate at which the composition is changed may be determined by 'trial and error' or more formal optimization techniques may be used [7-9].

Buffers are used in HPLC to control the degree of ionization of the analyte and thus the tailing of responses and the reproducibility of retention occurs. A range of buffers is available but those most widely used are inorganic, and thus in volatile, materials, such as potassium or sodium phosphate. One of the functions of an LC/MS interface is to remove the mobile phase and this results in buffer molecules being deposited in the interface and/or the source of the mass spectrometer with a consequent reduction in detector performance. Methods involving the use of volatile buffers, such as ammonium acetate are therefore preferred.

2.3.4 Stationary Phase

The majority of HPLC analyses employ reversed-phase systems. The most widely used columns contain a chemically modified silica stationary phase, with the chemical modification determining the polarity of the column. Very popular stationary phase is one in which a C18 alkyl group is bonded to the silica surface.

2.3.5 Detectors

Detectors are classified in a number of ways, including their use as the following:

(a) Solute or solvent-property detectors
(b) Selective or general (universal) detectors
(c) Mass or concentration-sensitive detectors

(a) Solute or Solvent Property Detectors

This classification is concerned with whether the detector monitors the property of the solute (analyte), e.g. the UV detector or a change in some property of the solvent (mobile phase) caused by the presence of an analyte, e.g. the refractive index detector.
(b) Selective or General Detectors

This classification is concerned with whether the detector responds to a specific feature of the analyte of interest or whether it will respond to a large number of analytes, irrespective of their structural properties. In terms of the previous classification, it may be considered that the solute detectors are also usually selective detectors, while solvent detectors are general detectors.

(c) Mass or Concentration-Sensitive Detectors

This classification is particularly important for quantitative applications. If the mobile phase flow rate is increased, the concentration of analyte reaching the detector remains the same, but the amount of analyte increases. Under these circumstances, the signal intensity from a concentration-sensitive detector will remain constant, although the peak width decreases, i.e. the area of the response decreases. A change in flow rate will also reduce the width of the response from a mass-sensitive detector, while, in contrast to a concentration-sensitive detector, the signal intensity increases as the absolute amount of the analyte reaching the detector increases. Since the overall response increases, this may be used to improve the quality of the signal obtained.

Under many experimental conditions, the mass spectrometer functions as a mass-sensitive detector, while in others, with LC-MS using electrospray ionization being a good example, it can behave as a concentration-sensitive detector [10]. An advantage of the mass spectrometer as a detector is that it may allow differentiation of the compounds with similar retention characteristics or may allow the identification and/or quantitative determination of components that are only partially resolved chromatographically or even those that are totally unresolved.

2.4 INTRODUCTION TO MASS SPECTROMETRY

A mass spectrometer consist of three major components

(i) Source - Produces the ions
(ii) Analyzer - separates the ions
(iii) Detector - Detects the ions
2.4.1 Source

Various ionization sources utilized in LC/MS includes electron ionization (EI), chemical ionization (CI), electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), fast-atom bombardment (FAB) and Liquid Secondary Ion Mass Spectrometry (LSIMS).

2.4.2 Electron Ionization (EI)

In electron ionization firstly the analyte must be vaporized. This is usually achieved by heating the probe tip containing a droplet of the analyte in solution. If the sample is thermally unstable, this will often be the first cause of sample fragmentation. Once in the gas phase, the analyte is passed into an EI chamber where it interacts with a homogeneous beam of electrons typically at 70 electron volts energy. The electron beam is produced by a filament (rhenium or tungsten wire) and steered across the source chamber to the electron trap. A fixed magnet is placed, with opposite poles slightly off-axis, across the chamber to create a coil in the electron beam. This is to increase the chance of interactions between the beam and the analyte gas. There are no actual collisions between the analyte molecules and electrons, where the ionization is caused by electron ejection from the analyte or by the analyte decomposition.

Figure 2.2: Schematic side view of an EI source.
2.4.3 Chemical Ionization (CI)

Chemical ionization (CI) is a lower energy alternative to EI for volatile analytes. In CI, there is a reagent gas (user ammonia or methane) in the ion chamber. Scheme 2.1 shows ion formation in Chemical ionization using methane as the reagent gas.

Scheme 2.1 Some of the ion formation reactions that can occur during methane Chemical ionization.

2.4.4 Electrospray Ionization (ESI)

The significance of electrospray ionization technique was introduced in 20th century [11]. In ESI the analyte is introduced in to the source in solution either from a syringe pump or as the eluent flow from liquid chromatography. Flow rates are typically of the order of 1µL min⁻¹. The analyte solution flow passes through the electrospray needle that has a high potential difference (with respect to the counter electrode) applied to it (typically in the range from 2.5 to 4 kV). This forces the spraying of charged droplets from the needle with a surface charge of the same polarity to the charge on the needle. The droplets are repelled from the needle towards the source sampling cone on the counter electrode (shown in blue). As the droplets travels through the space between the needle tip and the cone, the solvent evaporation occurs.
As the solvent evaporation occurs, the droplet shrinks until it reaches the point that the surface tension can no longer sustain the charge (the Rayleigh limit) at which point a "Coulombic explosion" occurs and the droplet is ripped apart. This produces smaller droplets that can repeat the process as well as the naked charged analyte molecules. These charged analyte molecules (they are not strictly ions) can be singly or multiply charged. This is a very soft method of ionization as very little residual energy is retained by the analyte upon ionization. The mechanism of ion formation in ESI is shown in Fig.2.4.
2.4.5 Atmospheric pressure chemical ionization (APCI)

Atmospheric pressure chemical ionization (APCI) is an analogous ionization method to chemical ionization (CI). The significant difference is that APCI occurs at the atmospheric pressure and has its primary applications in the areas of ionization of low mass pharmaceutical compounds (APCI is not suitable for the analysis of thermally labile compounds). The general source set-up (Fig.2.5) shows a strong resemblance to electro spray ionization (ESI) and as such is most commonly used in conjunction with HPLC or other flow separation techniques. Where APCI differs ESI is, in the way the ionization occurs. In ESI, ionization is bought about through the potential difference between the spray needle and the cone along with rapid and gentle desolvation. In APCI, the analyte solution is introduced into a pneumatic nebulizer and desolvated in a heated quartz tube before interacting with the corona discharge creating ions.

![Schematic diagram of an APCI source.](image)

The corona discharge replaces the electron filament in chemical ionization, the atmospheric pressure would quickly "burn out" any filaments and produces primary N₂⁺ and N₄⁺ by electron ionization. These primary ions collide with the vaporized solvent molecules to form secondary reactant ions e.g. H₃O⁺ and (H₂O)ₙH⁺. These reactant ions then undergo repeated collisions with the analyte resulting in the
formation of analyte ions. The high frequency of collisions results in a high ionization efficiency and thermalisation of the analyte ions. This results in the spectra of predominantly molecular species and adducts ions with very little fragmentation. Once the ions are formed, they enter the pumping and focussing stage in much the same as the other atmospheric pressure ionization sources.

2.4.6 Matrix-assisted Laser Desorption/Ionization (MALDI)

In the early 1960's, it was demonstrated that the irradiation of low-mass organic molecules with a high-intensity laser pulse lead to the formation of ions that could be successfully mass analysed. This was the origin of laser desorption (LD) ionization. After the next few decades, the technique underwent substantial development, culminating in the extension of the technique to the volatilisation of non-volatile biopolymers and organic macromolecules. There was, however, a sharp cut of in mass at about 5-10 kDa, limiting the technique's application. The other main limitation was that the ions were created in bursts which prevented the technique from being coupled to scanning mass analysers. In fact LD was only the really successful one when coupled to Time of flight (TOF) mass analysers in mass analysis.

Figure 2.6: Schematic diagram of the mechanism of MALDI.
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The mechanism of MALDI is believed to consist of three basic steps:

(i) **Formation of a 'Solid Solution':** It is essential for the matrix to be in access thus leading to the analyte molecules being completely isolated from each other. This eases the formation of the homogeneous 'solid solution' required to produce a stable desorption of the analyte.

(ii) **Matrix Excitation:** The laser beam is focused onto the surface of the matrix-analyte solid solution. The chromophore of the matrix couples with the laser frequency causing rapid vibrational excitation, bringing about localized disintegration of the solid solution. The clusters ejected from the surface consist of analyte of the solid solution. The clusters ejected from the surface consist of analyte molecules surrounded by matrix and salt ions. The matrix molecules evaporate away from the clusters to leave the free analyte in the gas-phase.

(iii) **Analyte Ionization:** The photo-excited matrix molecules are stabilized through proton transfer to the analyte. Cation attachment to the analyte is also encouraged during this process. It is in this way that the characteristic \([M+X]^+\) \((X= H, Na, K \text{ etc.})\) analyte ions are formed. These ionization reactions take place in the desorbed matrix-analyte cloud just above the surface. The ions are then extracted into the mass spectrometer for the analysis.

2.4.7. **Fast Atom Bombardment (FAB) and Liquid Secondary Ion Mass Spectrometry (LSIMS):**

The techniques of FAB and LSIMS are very similar in concept and designed as they both involve the bombardment of a solid spot of the analyte/matrix mixture on the end of a sample probe by a fast particle beam. The matrix (small organic species like glycerol or 3-nitro benzyl alcohol) is used to keep a homogenous sample surface. The particle beam is incident onto the surface of the analyte/matrix spot, where it transfers its energy bringing about localised collisions and disruptions. Some species are ejected (sputtered) from the surface as the secondary ions by this process. These ions are then extracted and focussed before passing to the mass analyser. The polarity of ions produced depends on the source potentials and Fig.2.7 shows a positive ion beam being formed.
In FAB (Fig 2.7), the particle beam is a neutral inert gas (Ar or Xe) at 4-10 keV and in LSIMS, the particle beam is ions (usually Cs\(^+\)) at 2-30 keV. Both methods are comparatively 'soft' ionization methods, very little residual energy is possessed by the ions after desorption, making them particularly suited to the analysis of low volatile analytes. The resulting spectra consist largely of intact molecular species (e.g. \([M+H]^+\) and \([M+Na]^+\)) with some minor structural fragmentation. The low mass region of the spectra is however dominated by matrix and matrix/salt cluster ions.

![Schematic diagram of the mechanism of fast particle beam ionization mass spectrometry (FAB and LSIMS)](image)

**Figure 2.7: Schematic diagram of the mechanism of fast particle beam ionization mass spectrometry (FAB and LSIMS)**

### 2.5 ANALYZERS

The most common analyzers used in LC-MS were

1. Quadrupole analyser
2. Ion trap analyser
3. Magnetic sector
4. Time of flight analyser
5. Fourier-Transform Ion Cyclotron Resonance
2.5.1 Quadruple Mass analyzer

The quadrupole mass analyser was developed in parallel with the quadrupole ion trap by the third Nobel Prize winning mass spectrometry pioneer, Wolfgang Paul [12-13]. A quadrupole mass analyser consists of four parallel rods (Fig.2.8) that has fixed DC and alternating RF potentials applied to them. Ions produced in the source of the instrument are then focussed and passed along the middle of the quadrupoles. Their motion will depend on the electric fields so that only ions of a particular \( m/z \) will have a stable trajectory and thus pass through the detector. The RF is varied to bring ions of different \( m/z \) into focus on the detector and thus build up a mass spectrum. The trajectory of the ions through the quadrupole is actually very complex.

![Figure 2.8: Schematic diagram of a quadrupole mass analyser.](image)

The two opposite rods in the quadrupole have a potential of '+ ' \((U+V\cos(\delta t))\) (labelled '+ ' on the Fig.2.8 and the other two '-' \((U+V\cos(\delta t))\) where 'U' is the fixed potential and V\( \cos(\delta t) \) is the applied RF of amplitude 'V' and frequency '\( \delta \)'. The applied potentials on the opposed pairs of rods varies sinusoidally as \( \cos(\delta t) \) cycles with time 't'. This results in ions being able to travel the field through free region along the central axis of the rods but with oscillations amongst the poles themselves. These oscillations result in complex ion trajectories depends on the \( m/z \) of the ions. Specific combinations of the potentials 'U' and 'V' and frequency '\( \delta \) ' will result in specific ions having a stable...
trajectory through the quadrupole to the detector. All other $m/z$ values will have unstable trajectories and will hit the quadrupoles and not be detected.

2.5.2 Ion trap analyzer:

The ions, produced in the source of the instrument, enter into the trap through the inlet and are trapped through the action of the three hyperbolic electrodes: the ring electrode and the entrance and exit end cap electrodes. Various voltages are applied to these electrodes which results in the formation of a cavity in which the ions are trapped. The ring electrode RF potential, an a.c. potential of constant frequency but variable amplitude, produces a 3D quadrupolar potential field within the trap. This traps the ions in a stable oscillating trajectory. The exact motion of the ions is dependent on the voltages applied and their individual mass-to-charge ($m/z$) ratios. For the detection of the ions, the potentials are altered to destabilise the ion motions resulting in the ejection of the ions through the exit end cap. The ions are usually ejected in the order of increasing $m/z$ by a gradual change in the potentials. This 'stream' of ions is focussed onto the detector of the instrument to produce the mass spectrum. The nature of trapping and ejection makes a quadrupolar ion trap especially suited for performing MS$^n$ experiments in the structural elucidation studies.

Figure 2.9: Schematic view of Ion Trap Mass Analyser
2.5.3 Magnetic sector analyzer:

The sector mass spectrometer is one of the most common types of mass analyzer and probably the most familiar to the everyday scientist. In the 1950's, the first commercial mass spectrometers were sector instruments. They consist of some combination of a large electromagnetic ('B' sector) and some kind of electrostatic focusing device ('E' sector) different manufactures use different geometries. Fig 2.10 shows a schematic diagram of a standard 'BE' geometry double focussing instrument that is, a dual sector instrument consisting of a magnetic sector followed by an electrostatic sector.

![Figure 2.10: A Schematic diagram of a sector mass spectrometer](image)

Ions enter the instrument from the source (bottom left) where they are initially focused. They enter the magnetic sector through the source slit where they are deflected according to the left-hand rule. Higher-mass ions are deflected less than lower-mass ions. Scanning the magnet enables the ions of different masses to be focused on the monitor slit. At this stage, the ions have been separated only by their masses. To obtain a spectrum of good resolution i.e. where all ions with the same \( m/z \) appear as coincident as one peak in the spectrum, then ions will be filtered by their kinetic energies. After another stage of focusing the ions enter the electrostatic sector where the ions of same \( m/z \) have their energy distributions corrected for and are focused at the double focusing point on the detector slit.
2.5.4 Time-of-Flight (TOF) analyzer:

Time-of-flight mass spectrometry (TOF-MS) (Fig 2.11) is probably the simplest method of mass measurement to conceptualize, although there are hidden complexities when it comes to higher resolution applications. The first commercial TOF instrument was marketed by the Bendix Corporation in the late 1950's. Their design was modified based on the Wiley & MacLaren instrument that was published in 1955 [14]. TOF-MS has really come into its own in recent years as being an essential instrument for biological analysis applications, this is especially the case with the coupling of TOF-MS to MALDI and ESI ionization methods and the development of high-resolution and hybrid instruments (for example Q-TOF and TOF-TOF configurations). The inherent characteristics of TOF-MS have an extreme sensitivity (all ions are detected), almost unlimited mass range and speed of analysis (modern instruments can obtain full spectra in seconds). This makes TOF-MS one of the most desirable methods of mass analyses.

2.5.5 Fourier-transform Ion Cyclotron Resonance (FT-ICR):

Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS or just FT-MS) (Fig.2.12) is often thought of as being the most complex method of mass analysis and detection. It is one of the most sensitive methods of ion detection in existence and has almost unlimited resolution i.e \(10^7\) is possible, with most of the
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experiments. The frequency of rotation of the ions is dependent on their \( m/z \) ratio. At this stage, no signal is observed because the radius of the motion is very small. Excitation of each individual \( m/z \) is achieved by a swept RF pulse across the excitation plates of the cell. Each individual excitation frequency will couple with the ions of natural motion and excite them to a higher orbit where they induce an alternating current between the detector plates. The frequency of this current is the same as the cyclotron frequency of the ions and the intensity is proportional to the number of ions. When the RF goes off to the resonance for that particular \( m/z \) value, the ions drop back down to their natural orbit (relax) and the next \( m/z \) packet is excited. Although the RF sweep is made up of a series of stepped frequencies, it can be considered as all frequencies simultaneously. This results in the measurement of all the ions in one go producing a complex frequency vs. time spectrum containing all the signals i.e the FID. Deconvolution of this signal by FT methods results in the deconvoluted frequency vs. intensity spectrum which is then converted to the mass vs. intensity spectrum. It is also usual to correct the mass errors at this stage by applying a calibration.

**Figure 2.12:** A Schematic view of FT-ICR-MS showing the ion trapping, detection and signal generation.

Due to the ion trap nature of FT-MS, it is possible to measure the ions without destroying them, this enables further experiments to be performed on the ions. The most common of these would be some kind of fragmentation study (MS/MS or MS\(^3\)) for the structural elucidation experiments, but also other gas-phase reactions and studies can be performed.
2.6 TANDEM MASS SPECTROMETRY (MS–MS)

The great strength of mass spectrometry as a technique is that it can provide both the molecular weight of an analyte (the single most discriminating piece of information in structure elucidation) and information concerning the structure of the molecule involved. The ionization techniques most widely used for LC–MS, however, are termed as ‘soft ionization’ in that they produce primarily molecular species with little fragmentation. It is unlikely that the molecular weight alone will allow a structural assignment to be made and it is therefore desirable to be able to generate structural information from such techniques. There are two ways in which this may be done, one of which, the so-called ‘cone-voltage’ or ‘in-source’ fragmentation, is associated specifically with the ionization techniques of electrospray and APCI. The other, termed as mass spectrometry– mass spectrometry (MS–MS) or tandem mass spectrometry, is applicable to all forms of ionization, provided that appropriate hardware is available.

Tandem mass spectrometry (MS–MS) is a term which covers a number of techniques in which one stage of mass spectrometry, not necessarily the first, is used to isolate an ion of interest and a second stage is then used to probe the relationship of this ion with others from which it may have been generated or which it may generate on decomposition. The two stages of mass spectrometry are related in specific ways in order to provide the desired analytical information. There are a large number of different MS–MS experiments that can be carried out but the four most widely used are (i) the product-ion scan, (ii) the precursor-ion scan, (iii) the constant-neutral-loss scan and (iv) selected decomposition monitoring. Before considering these four scan modes in detail, it is worth while considering the types of instrument that have the MS–MS capability because, as two stages of mass spectrometry are involved, as not all systems will provide this facility.

2.7 TRIPLE QUADRAPOLE MASS ANALYSER

This is probably the most widely used MS/MS instrument. The hardware, as the name suggests, consists of three sets of quadrupole rods in series (Fig.2.13). The second set of rods is not used as a mass separation device but as a collision cell, where
fragmentation of ions transmitted by the first set of quadrupole rods is carried out and as a device for focusing any product ions into the third set of quadrupole rods. Both sets of rods may be controlled to allow the transmission of ions of a single $m/z$ ratio or a range of $m/z$ values to give the desired analytical information. When the first quadrupole of a triple quadrupole is replaced by a double-focusing mass spectrometer, the instrument is termed as hybrid (i.e. a hybrid of magnetic sector and quadrupole technologies). Fig.2.13 shows the MS1 unit as a forward-geometry instrument although there is no reason why this could not be of reversed or even tri-sector geometry. The advantage of this configuration is that the MS1 instrument can be used under high-resolution conditions to select the ion of interest. Despite the efficiency of modern chromatography, complete resolution of components, especially if complex mixtures are involved, is not always possible and background signals may be observed. Low-resolution mass spectrometers allow us to differentiate between components with similar retention properties but whose mass spectra contain ions at different $m/z$ ratios, while high-resolution mass spectrometers allow us to do this but also to differentiate between components whose mass spectra contain ions with the same nominal mass but have different atomic compositions. In this way, further specificity is conferred on the analyses.

![Schematic view of a triple quadrupole mass analyzer.](image)

Figure 2.13: Schematic view of a triple quadrupole mass analyzer.

2.8 TANDEM MASS SPECTROMETRY ON THE TOF ANALYSER

Ions are produced in the source of the mass spectrometer and are accelerated into the flight tube for mass analysis. If the ion fragments during its passage through the flight tube, there are a number of mechanisms which may cause this, the product ions are formed, this is analogous to the formation of fragment ions in a collision cell
in other forms of MS–MS, which have the same velocity as their precursor but a reduced kinetic energy. The kinetic energy of the product ions is directly related to the ratio of their mass to that of the precursor, i.e. a product ion with half the mass of the precursor ion will have half of its energy. In instruments without a reflectron, both the precursor and the product ions reach the detector at the same time and are not separated. The reflectron, however, is an energy analyser and the product ions with different energies, after passage through the reflectron, will have different flight times to the detector and may be separated and their m/z ratios are determined. This is known as post-source decay (PSD) [17]

2.8.1 The Quadrupole–Time-of-Flight Instrument

In this instrument, the final stage of the triple quadrupole is replaced by an orthogonal time-of-flight (TOF) mass analyser, [Fig 2.14.] The configuration is the typical of the latest generation of TOF instruments in which a number of reflectrons, in this case two are used to increase the flight path of the ions and thus increase the resolution that may be achieved. In other words, in contrast to other mass analysers which are scanned sequentially through the m/z range of interest and provide MS–MS spectra of user-selected masses, the ToF analyser detects all the ions that enter it at a specific time.

![Figure 2.14: Schematic diagram of a Quadrupole–Time-of-Flight Instrument](image)

It is therefore possible, particularly in view of the high-scan-speed capability of this instrument, to provide, continuously, a full MS–MS product ion spectrum of each ion produced in the source of the mass spectrometer. The disadvantage of this
mode of operation is that it renders the Q–ToF system unable to carry out precursor and constant-neutral-loss scans.

2.8.2 Tandem Mass Spectrometry on the Ion-Trap

More recently, certain MS–MS scans have been made available on the ion-trap instrument. This type of system differs from those described previously in which the MS–MS capability is associated only with the way in which the ion-trap is operated, i.e. it is software controlled and does not require the addition of a collision cell and a further analyzer. This is because, ion selection, decomposition and the subsequent analysis of the product ions are all carried out in the same part of the instrument, with these processes being separated solely in time, rather than time and space as is the case for the instruments described previously.

2.9 IDENTIFICATION BY MASS SPECTROMETRY

For identification purposes, a mass spectrum covering all the m/z ratios that are likely to be generated by the analyte is required. Very rarely, however, a single mass spectrum provide us with complete analytical information for a sample, particularly if mass spectral data from a chromatographic separation taking perhaps up to an hour, is being acquired. The mass spectrometer is therefore set up to scan, repetitively, over a selected m/z range for an appropriate period of time. At the end of each scan, the mass spectrum obtained is stored for the subsequent manipulation before a further spectrum is acquired.

2.10 QUANTITATION BY MASS SPECTROMETRY

Quantitation using mass spectrometry involves the comparison of the intensity of a signal generated by an analyte in a sample to be determined with that obtained from standards containing known amounts/concentrations of that analyte. There are two types of quantification modes available in mass spectrometry.

1. Selective ion monitoring using single quadrupole analyzer and ion trap analyzer

2. Multiple reactions monitoring using triple quadrupole analyzer
2.11 PROCESSING OF MASS SPECTRAL DATA

It is true that in many cases the quality of data acquired during the analysis is directly proportional to the quality of the result that may be obtained, it is also true that in many cases the power of modern computer systems attached to analytical equipment of all sorts can be used to provide 'better' results that might be thought possible from a cursory examination of the raw data. Even when chromatography is used to separate the components of a mixture and simplify the job of the analyst, the computer may still allow information hidden in the vast amount of data generated to be extracted. A full description of a mass spectrometry data system is:

(a) Control of the mass spectrometer (ionization mode and scan mode).

(b) The acquisition of mass spectral data and its subsequent storage.

(c) The processing of the stored data to allow an analytical result to be obtained.

2.11.1 The Total-Ion-Current Trace

During a chromatographic separation, the mass spectrometer is set to scan repetitively, over an appropriate mass range, with the upper mass limit being defined by the highest molecular weight of the analyte (s) thought/likely to be present and the lower limit by the background ions – in the case of LC–MS, usually from the mobile phase. At the end of each scan, a spectrum is stored and the sum of the intensities of the ions present, i.e. the total-ion current, is computed and stored. After an appropriate time, determined by the retention time of the longest retained component, scanning of the mass spectrometer is halted and the data are available for processing. The fundamental piece of information on which the subsequent spectral analysis is based on the total-ion-current (TIC) trace. Such a trace, obtained from the LC–MS analysis of a sample, is shown in Fig. 2.15, together with the UV trace recorded simultaneously.
2.11.2 Detectors

The role of the detector is to convert the energy of incoming particles into a current signal that is registered by the electronic devices and transferred to the computer of the acquisition system of the mass spectrometer. When an incoming particle strikes the detector, the energy from the impact causes emission of secondary particles, for example, electrons or photons. The number of secondary particles created by an impact most often depends on the energy and/or the velocity of the incoming particles. Hence, if all ions are accelerated to the same kinetic energy, as in the TOF mass analyzer, then the detection sensitivity is lower for high mass (slow) ions than for low mass (fast) ions. To increase the detection sensitivity, the ions are often post-accelerated before they strike the detector. A detector preferably should have high efficiency for converting the energy of the incoming ion to electrons or photons, a linear response, low noise, short recovery time and minimal variations in transit time (narrow peak width). In this section some of the detectors currently (or recently) used in mass spectrometers are described. Hybrids of different detectors are also frequently used.
Figure 2.16: Schematic diagram of (a) a photoplate detector, (b) a Faraday cup, (c) a discrete-dynode electron multiplier (EM) of venetian blind type, and (d) a continuous dynode EM.

(a) Photoplate Detector

The photoplate detector is one of the oldest types of MS detectors (Fig.2.16(a)). J.J. Thomson used photoplates to record mass spectra [18]. Photoplates were, one of the most common detectors for multicomponent detection for a long time. In more recent times the photoplate has mainly been used in conjunction with spark source (SS) MS. In SSMS ion-sensitive photoplates are used to simultaneously
integrate ion beams separated by a double focusing sector analyzer. Even though the photoplate has advantages such as simultaneous detection and possibilities of signal integration, compared to modern electrical devices the disadvantages are numerous, including poor sensitivity, short linear range, off-line image processing, off-line calibration and shortage of commercial suppliers.

(b) Faraday Detector

Unlike the photoplate, the Faraday detector (or Faraday cup) is still very much in use today. The main reasons for its lasting popularity are accuracy, reliability and rugged construction. The simplest form of Faraday detector is a metal (conductive) cup that collects the charged particles and is electrically connected to an instrument that measures the produced current (Fig. 2.16 (b)). Faraday cups are not particularly sensitive and the signal produced in most applications must be significantly amplified. An important application for Faraday detectors is precise measurements of ratios of stable isotopes [19].

(c) Electron Multipliers

An electron multiplier (EM) amplifies a weak current of incoming particles by using a series of secondary emission electrodes or dynodes to produce a considerably higher current at the anode [20]. When a particle impinges on the dynode, energy is transferred directly to the electrons in the dynode material and a number of secondary electrons are emitted. The dynode often consists of an alloy of an alkali or alkali earth metal with a more noble metal. Thus, a thin insulating film of oxidized alkaline metal is formed on a conducting support. A good dynode material should emit many secondary electrons per primary incoming particle, have linear gain for high currents, and have low thermionic emission, that is, low noise. Today the EM detectors (Fig 2.16 (c) ) are the most common MS detectors and are available in a variety of designs.

(d) Continuous-Dynode Electron Multiplier.

Very compact electron multipliers that consist of one continuous dynode (Fig. 2.16 (d)) are also often used [21]. The upper limit of the gain is here set by the onset of ion feedback. Ion feedback is caused by positive ions produced in the high charge
density region at the output of the channel that drift back to the input. In the case of large single channels, ion feedback can be suppressed simply by bending or twisting the channels. Another example of a compact electron multiplier configuration with short transit times is one of the most frequently used detectors in mass spectrometers, the microchannel plate (MCP, also sometimes referred to as multichannel plate) [21]. An MCP consists of a parallel array of channel electron multipliers. The inner surfaces of the channels are treated with a semiconductor material acting as a secondary electron emitter. The flat end surfaces of the channels are coated with a metallic alloy to allow a potential difference to be applied over the MCP. When a particle strikes the channel wall, secondary electrons are generated and accelerated down the channel towards the output end. The secondary electrons also hit the channel wall and mass of electrons is generated. Each channel thus acts as a continuous dynode that generates up to 10^4 electrons per impact. Two MCPs are often stacked together to achieve higher gain.

(e) Focal Plane Detector

Focal plane detectors are used primarily to detect ions separated in space by, for example, magnetic sector analyzers. The objective of an ideal focal plane detector is to simultaneously record the location of every ion in the spectrum. In many ways the photoplate is the original focal plane detector, but it has today been more or less replaced with designs that rely on EM detectors. A common arrangement is to allow the spatially dispersed ion beams simultaneously to impinge on an MCP. The secondary electrons generated by the ion impacts, then strike a one or two dimensional array of the metal strips and the current from the individual electrodes is recorded.

(f) Scintillation Detector

The scintillation detector makes use of the fact that certain materials, when struck by a particle, emit a small flash of light, so-called scintillation. Generally the detector consists of a scintillating material that is optically coupled to an amplifying device such as a photomultiplier. When an ion passes through the scintillator, it excites the atoms and molecules, causing light to be emitted. The light is transmitted to the photomultiplier, where it is converted into a weak current of photoelectrons,
which is further amplified by an electron-multiplier system. The light output of a scintillator is directly proportional to the excitation energy of the incoming particle. Since the photomultiplier also is a linear device, the amplitude of the final electrical signal will also be proportional to the energy of the incoming particle. Scintillation detectors have fast recovery time and therefore accept high count rates.

(g) Cryogenic Detector

Cryogenic or energy-sensitive calorimetric detectors measure the heat generated by a particle impacting on a superconducting thin film. One example of such a cryogenic detector is the so-called superconducting tunnel junction. Cryogenic detectors measure low energy solid-state excitations (below 5 millielectronvolts) and must therefore be operated at temperatures typically below 2 K to avoid excessive thermal excitations. Compared to ionization-based detectors, which rely on electronvolt energies needed to produce secondary electrons or electronic excitations, cryogenic detectors are more sensitive to slow-moving (large) ions. An advantage of cryogenic detectors is that they are able to distinguish the charged state of the ion. Another advantage is that, contrary to EM detectors such as the MCP, for example, the detection efficiency of cryogenic detectors is independent of the velocity of the ions. Cryogenic detectors have recently been coupled to TOF mass spectrometers and shown encouragingly good sensitivity for high-mass ions in the mega dalton mass range. The main disadvantages of cryogenic detectors are the extensive cooling necessary for operation and the practical problems when connecting the cooled detector with a spectrometer at room temperature.

(h) Solid-State Detector

Solid-state detectors (SSD) consist of silicon or germanium that emits electrons in response to ionizing radiation. SSDs are mainly used to measure the energy of a particle as a compliment to an m/z determination. The addition of an SSD to determine the residual energy of the ion allows the unambiguous determination of mass, charge and energy independently, instead of obtaining just ratios of energy to charge or mass to charge. The two areas of application are space science and isotope ratio measurements [20].
(i) Image Current Detection

Image current detection is (currently) the only non-destructive detection method in MS. The two mass analyzers that employ image current detection are the FTICR and the orbitrap. In the FTICR, ions are trapped in a magnetic field and move in a circular motion with a frequency that depends on their m/z. correspondingly, in the orbitrap ions move in harmonic oscillations in the z-direction with a frequency that is m/z dependent but independent of the energy and spatial spread of the ions. For detection the ions are made to pass close to a pair of detection plates. When each time an ion passes a plate, a small current (or image charge) will be induced. Thus, the frequency of each generated current corresponds to a certain m/z. An important advantage of non-destructive detection is that it allows for the re measurement of the already detected ions.


The discovery and development of a new drug costs around 1 billion dollars and it may take approximately 10 years for the drug to reach the market. Drug discovery and development is the process of generating compounds and evaluating all of their properties to determine the feasibility of selecting one New Chemical Entity (NCE) to become a safe and efficacious drug. Among many criteria, obtaining experimental Pharmacokinetics (PK) data from laboratory animals in the non-clinical stage is critical to evaluate a drug candidate before it can be qualified to be tested in the clinical trials for safety and efficacy evaluation. A key parameter in PK is the plasma or tissue concentration of the new drug after its administration to laboratory animals. Therefore, developing an accurate and fast analytical method for measuring the concentrations of a compound in plasma or tissue is the first step in order to yield the PK of a compound. Currently, the principal technique used in quantitative bioanalysis is High Performance Liquid Chromatography coupled with Tandem Mass Spectrometry (HPLC-MS/MS) using either electrospray ionization.
2.13. PHARMACOKINETIC PARAMETERS

**Peak Plasma Concentration (C\textsubscript{max})**: The point of maximum concentration of drug in plasma is called as the peak concentration of drug at peak and is known as peak plasma concentration. It is also called as peak height concentration and maximum drug concentration. C\textsubscript{max} is expressed in ng/mL or µg/mL.

**Time of Peak Concentration (T\textsubscript{max})**: The time for drug to reach peak concentration in plasma is called as the time of peak concentration. T\textsubscript{max} is expressed in hrs.

![Pharmacokinetic Parameters Diagram](image)

**Figure 2.17**: A typical figure showing the major pharmacokinetic parameters after oral dosing of the drug.

**Area under the Curve (AUC)**: It represents the total integrated area under the plasma level profile and expresses the total amount of drug that comes into the systemic circulation after its administration. AUC is expressed in µg/mL X hours or ng/mL X hours.

**Elimination Half-Life (T\textsubscript{1/2})**: It is defined as the time taken for the amount of drug in the body as well as plasma concentration to decline by one-half or 50% its initial value. T\textsubscript{1/2} is expressed in hours.
Elimination Rate Constant \((K_{el})\): Elimination rate constant is a value used in pharmacokinetics to calculate the rate at which drugs are removed from the system. \(K_{el}\) is expressed in hours.

**Bioavailability:** Bioavailability is defined as the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and become available at the site of action.

**Bioequivalence:** Bioequivalence is defined as the absence of a significant difference in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalent or pharmaceutical alternative becomes available at the site of drug action when administered at the same molar under similar conditions in an appropriately designed study.

### 2.14. LC-MS APPLICATIONS

Due to wide range of advantages on LC-MS, it is being used for the following applications

#### 2.14.1. Identification of Degradation Products:

The proactive characterization and identification of degradants during the preclinical development phase of drug development offer advantages in the evaluation of drug candidates and their subsequent performance during clinical trials. The early knowledge of degradants provides insight into the stability and toxicity. The value of early knowledge of degradants is to provide insights into critical issues and the development of corrective measures prior to the clinical trials.

During the course of drug development, the bulk drug and drug formulation are studied under a variety of stress conditions such as temperature, humidity, acidity, basicity, oxidation and light. Qin and colleagues described the use of stressing conditions that may cause degradation [22]. The resulting samples may be used to validate analytical monitoring methods and to serve as predictive tools for future formulation and packaging studies. A traditional approach for studying degradants formation involves similar time-consuming scale-up and preparation steps as described for metabolite and impurity analysis. Similarly, this area of pharmaceutical
analysis has experienced the issues associated with faster drug development cycles. Rourick and co-workers described the proactive approaches to obtain degradants information with LC/MS methods during the preclinical development stage [23]. The procedure incorporates qualitative and quantitative process changes that occur during the analyses. The structural information necessary for successful the drug development is emphasized. The corresponding structural information provides insight for decisions, based on which leads are to be developed for clinical testing. The early structural information on the degradants of a drug candidate offers a unique capability for synthetic modification to minimize degradation. Structural information can also facilitate planning of preclinical drug development in process research, formulation development and safety assessment.

Volk and co-workers described the use of LC/MS approaches for the identification of butorphanol degradants present in LTSS samples during the clinical development stage [23]. Butorphanol, the active ingredient in Stadol NS, is formulated as an intranasal analgesic product and is used in the treatment of the pain associated with postsurgical situations, dental intervention and migraine [24-25]. Careful monitoring of degradants formation is an important aspect in determining the stability of the drug. Identification of the degradants is useful for determining potency and providing insight into the improved formulations.

2.14.2 Impurity Identification

Synthetic impurities are of particular concern during process research and safety evaluation activities. Often, impurities are the result of synthetic by-products or starting materials of the scale-up process. Impurities provide a comprehensive indicator of the chemical process and are diagnostic of overall quality. Process chemists use the resulting information to guide process optimization. Knowledge of the identity and relative amount of impurities are needed to diagnose process reactions, so that the changes in the reagents and reaction conditions lead to better yields and higher quality material. Although it is often difficult to assign an exact time period for the completion of chemical process research activities, it is usually the rate-determining step for preclinical development activities. With an increasing number of
novel lead candidates that enter into preclinical development, considerable resources are necessary to identify the impurities. LC/MS-based approaches provide integrated sample cleanup and the structure analysis procedures for the rapid analysis of impurities. This advantage was demonstrated during the preclinical development of TAXOL®. LC/MS played an important role for the identification of impurities contained in extracts and process intermediates from Taxus brevifolia and T. baccata biomass [26-27]. Because drugs derived from natural sources often have a very diverse set of structural analogues, it is important to determine which analogues are carried through the purification process and ultimately appear as impurities. This task presents a unique challenge during the preclinical stage of the drug development due to the highly complex nature of the samples. Similar to the approaches described previously for natural products dereplication during the drug discovery, traditional strategies for impurity identification rely on scale-up, extraction, isolation and detailed structure analysis. Unfortunately, these methods are slow, time-consuming and problematic for accelerated preclinical development activities. Rapid structure identification methods which use LC/MS and LC/MS/MS protocols are ideal for handling large numbers of drug candidates and are applicable to a diverse range of compound classes. Furthermore, the sensitivity of LC/MS-based methods is sufficient to study the impurities and active compounds at extremely low levels (0.2–2 nmol). Kerns and co-workers developed a structure identification strategy that incorporates LC/MS and LC/MS/MS techniques for rapid, sensitive and high-throughput impurity analysis [26].

This approach integrates traditional steps of sample preparation, separation, analysis and the data management into a single instrumental method. The resulting multidimensional data include retention time, molecular weight, UV and substructure information. A structure database is developed for each candidate and is used to rapidly identify the same impurities in new samples. Structures are proposed based on using the drug candidate as a structural template and with the use of a standard method approach, consistency for the comparison of results throughout the preclinical development process is ensured.
2.14.3 Metabolite Identification

Metabolite identification is central to many of the activities in preclinical development. A more complete characterization of pharmacokinetic properties is performed in animals (typically rats and dogs) during this stage. The knowledge of the biotransformation pathways of the lead candidate to its metabolites is used to indicate the magnitude and duration of the activity. Metabolite identification is critical to many of these activities and plays an important role in establishing the dose and toxicity levels. The identification of metabolite structures with LC/MS and LC/MS/MS techniques are an effective approach due to their ability to analyze trace mixtures from complex samples of urine, bile and plasma. The key to structure identification approaches is based on the fact that the metabolites generally retain most of the core structure of the parent drug [28]. Therefore, the parent drug and its corresponding metabolites would be expected to undergo similar fragmentations and to produce the mass spectra that indicate the major substructures.

2.14.4 Drug–Drug Interaction

LC/MS-based screening approaches are used to evaluate the potential of drug–drug interactions either in vitro [29] or in vivo [30-31]. The strategy of these experiments is to obtain early indication of the inhibition or induction of the metabolism of compounds by the cytochrome P450 (CYP) isozymes. Specific CYP isozymes, typically those that are most commonly responsible for the metabolism of drugs, are studied because inhibition of CYP mediated metabolism is often the mechanism for drug–drug interactions.

These studies provide information on whether a drug may inhibit the biotransformation of another when the two are co administered. LC/MS approaches are routinely used to identify/ select drug candidates that have a lower potential for drug–drug interactions.

2.14.5 Combinatorial Mixture Screening

The increased popularity of LC/MS-based methods combined with limited resources resulted in advances that effectively matched the combinatorial chemistry
samples (i.e., complexity) with instrument time. Richmond, Yates and coworkers [32-33] demonstrated the use of flow injection analysis (FIA)-LC/MS systems for rapid purity assessment and combinatorial mixture screening, respectively. These LC/MS based applications addressed two critical bottlenecks.

2.14.6 Structure Confirmation

The open access LC/MS procedure described by Pullen and co-workers, in that the samples were directly introduced from solution for ease of automation and sample preparation. The LC/MS analyses were performed with either thermo spray ionization (TSI) or particle beam (PB) interfaces. These systems successfully analyzed the labile, polar or higher mass compounds, whereas a complementary gas chromatography (GC/MS) system was used for volatile compounds. The LC/MS system is proved to be widely applicable to a range of chemically diverse compounds. The TSI and PB systems were both successful for 80-90% of the compounds analyzed. Automated, open access LC/MS analyses perform well because sample throughput was expected to reach 250,000 in 1995. This throughput corresponds to approximately 1000 samples per day.

Combinatorial Library Screening LC/MS formats which provide accurate mass capabilities have compelling analytical features for the screening of the combinatorial libraries. The unambiguous confirmation/identification of combinatorial library components from small quantities of material have been illustrated using QTOF [34], TOF [35] and Fourier transform ion cyclotron resonance (FTICR) [36-38] mass spectrometers. These formats provide accurate isotope patterns or “isotopic signature,” and unique mass differences that are obtained between isobaric compounds.

2.14.7 Quantitative Bioanalysis—Selected Reaction Monitoring and Selected Ion Monitoring

After the work of Fouda and colleagues, a tremendous growth in the LC/MS application for the quantitative bioanalysis ensured in the clinical development stage of the drug development. Similar LC/MS-based approaches that used SIM methods
for the quantitation to the highly selective SRM methods [40] become popular. SRM methods that use APCI LC/MS/MS for the quantitative analysis of an antipsychotic agent, clozapine, in human plasma were described by Dear and co-workers [40]. In 1991, Fouda demonstrated the use of APCI-LC/MS for the quantitative determination of the renin inhibitor, CP-80,794, in human serum. Because the pharmacological action is below 200 pg/mL, a quantitative assay in the low pg/mL range is required to monitor the drug’s pharmacokinetic and pharmacodynamic properties.
2.15. REFERENCES


Chapter 2


