13. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY- TANDEM MASS SPECTROMETRY METHOD (LC/MS/MS)

13.1 Introduction to LC-MS

More than 85% of compounds in nature fall into the category of being polar and thermally labile, which are not amenable to GC/MS. In the past, a strong need for a similar combination of HPLC and MS was felt for the analysis of such compounds. Although attempts to combine LC with MS were initiated over 30 years ago, a robust and dependable combination that could be adapted for a wide range of applications has emerged only after the discovery of atmospheric-pressure ionization techniques. Currently, the practice of LC/MS arguably has become the single most widely used analytical technique. A variety of applications has been envisioned for both qualitative and quantitative analysis of complex mixtures of biochemical, inorganic, and organic compounds. HPLC/MS has become the mainstay of proteomics and pharmaceutical laboratories.

HPLC is practiced in the following formats:

*Adsorption chromatography*, in which the stationary phase consists of a high-surface-area solid adsorbent; the solutes physically adsorb on the stationary phase, while the liquid mobile phase tries to dislodge them.
**Partition chromatography** involves distribution of the solutes between a liquid stationary phase and a liquid mobile phase.

**Ion-exchange chromatography**, used for ionic solutes, involves competition for solute ions between immobilized ionic sites on the stationary phase (e.g., ion-exchange resin) and liquid mobile phase.

**Size-exclusion chromatography** separates solutes on the basis of their size. The stationary phase consists of a polymer matrix of various pore sizes. Solutes are separated according to their ability to penetrate the polymer matrix. The small solutes penetrate more and lag behind the larger solutes.

**Affinity chromatography** involves specific interaction between one type of solute molecule and a second, complementary type of molecule that is immobilized on a solid support. Of these, partition chromatography, especially the *reversed-phase (RP) mode*, is the most widely used technique for the separation of several classes of compounds. In RP–HPLC, the stationary phase is a nonpolar matrix and the mobile phase is a polar solvent (e.g., water mixed with a polar organic modifier such as methanol, isopropanol, or acetonitrile). Mobile phase and stationary phase both play prominent roles in the separation mechanism. A sample is applied onto the head of a column filled with an appropriate stationary phase. The
components of a mixture are partitioned differentially between the two phases and are separated when eluted by a stream of a liquid mobile phase. Two common modes of elution are *isocratic*, in which the solvent composition remains constant during elution, and *gradient*, in which the solvent composition is changed either in steps or in a continuous fashion during elution. The basis of *RP–HPLC separation* is the hydrophobic interaction between the analyte and the nonpolar matrix of the stationary phase. A typical stationary phase is prepared by chemically bonding a long-chain alkyl group, such as *n*-octadecyl (C18) to porous silica. The smaller alkyl groups, such as *n*-octyl, *n*-butyl, and *n*-ethyl, can also be used for specific applications. In practice, the nonpolar, hydrophobic solutes interact strongly with the stationary phase. In contrast, relatively hydrophilic compounds spend more time in the polar aqueous mobile phase and thus elute earlier. Increasing the strength of the eluting solvent enables elution of the more strongly retained hydrophobic solutes. The RP–HPLC separation is further fine-tuned by use of appropriate buffers and ion-pairing reagents in the mobile phase. Although a large number of buffer systems has been used in a conventional ultraviolet–visible detection system, volatile ion-pairing reagents work best for LC/MS operation. Partition chromatography is also practiced in the *normal-phase mode*, in which the stationary phase is a polar matrix and the mobile phase
at the start of the separation is a polar solvent. The polar solutes prefer to remain in the stationary phase and elute late.

The challenge of working in the pharmaceutical industry during this time of rapid expansion of our knowledge of the causes and potential cures for many diseases is both exciting and formidable. It is exciting because we are now learning how to make potent drugs that can target specific receptors in order to relieve symptoms or block the progression of a disease. It is formidable because the number of potential targets is large and the size of our chemical libraries that need to be screened against these targets is in the millions and growing even larger. While ultra-high throughput screening effectively reduces these numbers by screening out the inactive compounds, the numbers of compounds that need to be screened through drug metabolism studies can still be overwhelming. The amount of effort in terms of compound screening, lead optimization and attrition is a daunting task. Of the two million compounds that might be screened for activity, perhaps 10,000 are selected and optimized in the drug discovery stage. Next, 20 compounds might be selected for development and five of these may survive the toxicity testing and be suitable for phase I clinical screening. At current rates of success, one of the five compounds would become an approved drug. In a 2003
report by the Tufts Center for the Study of Drug Development, the cost of bringing a new drug to market was estimated to be $897 million.

Over the last 12–15 years, mass spectrometry (MS) has played an increasingly important role in all phases of drug discovery and drug development. In that same time, mass spectrometry has undergone tremendous changes. Mass spectrometers have become more sensitive, easier to use and have been applied to multiple areas of drug metabolism activity. At the same time, new types of mass spectrometers have been introduced. The four of the most widely used types of mass spectrometers; of these four types, the triple quadrupole mass spectrometer (QqQ MS) has become the “gold standard” for quantitative assays in the drug metabolism arena. The focus of this chapter will be on the use of liquid chromatography combined with tandem mass spectrometry (LC–MS/MS) for drug metabolism participation in new drug discovery, specifically in support of in vivo pharmacokinetic (PK) screens and studies.

The fantastic development of mass spectrometry (MS) in the last 30 years has led this technique to be applied practically in all analytical fields. We focus our attention on the application in the organic, biological and medical fields which nowadays represent the environment in which MS finds the widest application. This chapter is devoted to a short description of the different instrumental
approaches currently in use and commercially available. MS is based on the production of ions from the analyte, their analysis with respect to their mass to charge ratio (m/z) values and their detection. Consequently, at instrumental level three components are essential to perform mass spectrometric experiment: (i) ion source; (ii) mass analyser; and (iii) detector. Of course, the performances of these three components reflect on the quality of both quantitative and qualitative data. It must be emphasized that generally these three components are spatially separated and only in two cases [Paul ion trap and Fourier transform mass spectrometer without external source(s)] can they occupy the same physical space and, consequently, the ionization and mass analysis must be separated in time.

The ion production is the phenomenon which highly affects the quality of the mass spectrometric data obtained. The choice of the ionization method to be employed is addressed by the physico-chemical properties of the analyte(s) of interest (volatility, molecular weight, thermolability, complexity of the matrix in which the analyte is contained). Actually the ion sources usually employed can be subdivided into two main classes: those requiring sample in the gas phase prior to ionization; and those able to manage low volatility and high molecular weight samples. The first class includes electron ionization (EI) and chemical ionization (CI) sources which represent
those worldwide most diffused, due to their extensive use in GC/MS systems. The other ones can be further divided into those operating with sample solutions [electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI)] and those based on the contemporary sample desorption and ionization from a solid substrate [matrix - assisted laser desorption/ionization (MALDI) and LDI].

The ever shortening timelines in drug discovery and development have brought about the need for high throughput approaches to methods used to quantitate drugs, metabolites and endogenous biomolecules in biological matrices (blood, plasma, serum, urine, and in-vitro biological samples). The timely advent of the technique of liquid chromatography coupled with tandem mass spectrometry$^{166-169}$ (LC/MS/MS) has greatly enabled bioanalysts to rise to this challenge. It has been possible to drastically reduce the chromatographic run time due to the inherent specificity/sensitivity of this technique compared to bioanalytical LC methods based on traditional detection means, such as UV. The achievement of very short chromatographic run times has in turn brought about the need for high throughput approaches to biological sample preparation that precede the LC/MS/MS analyses.
ION SOURCES

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Electron Ionization

EI is based on the interaction of an energetic (70 eV) electron beam with the sample vapour (at a pressure in the range 10^{-7}–10^{-5} Torr).
This interaction leads to the production of a series of ions related to the chemical properties of the compound(s) under study. The theoretical treatment of EI is beyond the scope of the present book and it is possible to find it in many publications. For the present discussion it is enough to consider that EI generally leads to a molecular ion M⁺, originating by the loss of an electron from the neutral molecule:

\[ M⁻ \rightarrow M⁺ + 2e⁻ \]

and to a series of fragments, generally highly diagnostic from the structural point of view: Some of them originate from simple bond cleavages, while some others are produced through rearrangement processes. What must be emphasized is that EI leads to well reproducible mass spectra. In other words, by different EI sources spectra practically superimposable are obtained and this is the reason for which the only spectrum libraries available are those based on EI data.

The main efforts done in the last decade in the EI field are due to the development of ion sources with the highest possible ion yield. To reach this aim, on the one hand an optimization of ion source geometry has been performed (this has been achieved by the development of suitable ion optics to increase either the ion production or the ion extraction), on the other, to make inert the ion
source walls (originally in stainless steel) so as to avoid the sample loss due to its pyrolysis on the hot metallic surface.

The quantitative data obtained by EI can be strongly affected mainly by two parameters: the first related to sample loss (due to problems related to sample injection lines and to ‘open’ source configuration as well as to thermal decompositions occurring in injection lines and/or source), while the second can be related to a decreased efficiency of ion extraction (nonoptimized extraction field, field modification due to the presence of polluted surfaces). These two aspects reflect not only on the limit of detection (LOD) of the system but also on the linearity of the quantitative response.

The ion most diagnostic from the qualitative point of view is usually considered the molecular one (M⁺). However, wide classes of compounds, easily vaporized, do not lead to the production of M⁺. This is due to the energetics of EI induced decomposition processes. In other words if a decomposition process is energetically favoured (with a particularly low critical energy) it takes place immediately, due to the internal energy content of M⁺. To overcome this problem in the 1960s a new ionization method was developed, based on gas-phase chemical reactions.
Chemical Ionization

To obtain a lower energy deposition in the molecule of interest, reflecting in the privileged formation of charged molecular species, in the 1960s CI methods were proposed. They are based on the production in the gas phase of acidic or basic species, which further react with a neutral molecule of analyte leading to \([M+H]^+\) or \([M-H]^-\) ions, respectively. Generally, protonation reactions of the analyte are those more widely employed; the occurrence of such reactions is related to the proton affinity (PA) of M and the reactant gas, and the internal energy of the obtained species are related to the difference between these proton affinities. Thus, as an example, considering an experiment performed on an organic molecule with PA value of 180 kcal/mol (PAM), it can be protonated by reaction with \(CH^+\)5 (PACH4 ≈ 127 kcal/mol), \(H_3O^+\) (PAH2O ≈ 165 kcal/mol), but not with \(NH^+\)4 (PANH3 ≈ 205 kcal/mol). This example shows an important point about CI: it can be effectively employed to select species of interest in complex matrices. In other words, by a suitable selection of a reacting ion \([AH]^+\) one could produce \([MH]^+\) species of molecules with PA higher than that of A. Furthermore the extension of fragmentation can be modified in terms of the difference of \([PAM - PAA]\). From the operative point of view CI is simply obtained by introducing the neutral reactant species inside an EI ion source in a ‘close’
configuration, by which quite high reactant pressure can be obtained. If the operative conditions are properly set the formation of abundant \([AH]_\text{þ}\) species (or, in the case of negative ions \(B_\text{−}\)) is observed in high yield. Of course, attention must be paid in particular in the case of quantitative analysis to reproduce carefully these experimental conditions, because they reflect substantially on the LOD values. CI, as well as EI, requires the presence of samples in vapour phase and consequently it cannot be applied for nonvolatile analytes. Efforts have been made from the 1960s to develop ionization methods overcoming these aspects and, among them, field desorption (FD) and fast atom bombardment (FAB) resulted in highly effectivemethods and opened new applications for mass spectrometry. More recently new techniques have become available and are currently employed for nonvolatile samples:

APCI, ESI, APPI and MALDI represent nowadays the most used for the analysis of high molecular weight, high polarity samples. For these reasons, we describe these methods.

**Atmospheric Pressure Chemical Ionization**

APCI was developed starting from the consideration that the yield of a gas-phase reaction does not depend only on the partial pressure of the two reactants, but also on the total pressure of the reaction environment. For this reason the passage from the operative pressure
of 0.1–1 Torr, present inside a classical CI source, to atmospheric pressure would, in principle, lead to a relevant increase in ion production and, consequently, to a relevant sensitivity increase. At the beginning of the research devoted to the development of the APCI method, the problem was the choice of the ionizing device. The most suitable and effective one was, and still is, a corona discharge. The important role of this ionization method mainly lies in its possible application to the analysis of compounds of interest dissolved in suitable solvents: the solution is injected in a heated capillary (typical temperatures in the range 350–400 °C), which behaves as a vaporizer. The solution is vaporized and reaches outside from the capillary the atmospheric pressure region where the corona discharge takes place. Usually the vaporization is assisted by a nitrogen flow coaxial to the capillary. The ionization mechanism is typically the same present in CI experiments. The solvent molecules, present in high abundance, are statistically privileged to interact with the electron beam originated from the corona discharge; the ions so formed react with other solvent molecules leading to protonated (in the case of positive ions analysis) or deprotonated (negative ions analysis) species, which are the reactant for the analyte ionization. One problem which, at the beginning of its development, APCI exhibited was the presence of analyte molecules still solvated, i.e. the presence of clusters of analyte
molecules with different numbers of solvent molecules. To obtain a declustering of these species, different approaches have been proposed, among which nonreactive collision with target gases (usually nitrogen) and thermal treatments are those considered most effective and currently employed. Different instrumental configurations, based on a different angle between the vaporizer and entrance capillary (or skimmer) have been proposed; 180° (in line) and 90° (orthogonal) geometries are those most widely employed. In particular, in the case of quantitative analysis, a particular care must be devoted to finding the best operating conditions (vaporizing temperature and solution flow) of the APCI source, which lead to the most stable signals, and carefully maintaining these conditions for all the measurements.

**Electrospray Ionization**

ESI is obtained by injection, through a metal capillary line, of solutions of analyte in the presence of a strong electrical field. The production of ions by ESI can be considered as due to three main steps: (i) production of charged drops in the region close to the metal capillary exit; (ii) fast decreasing of the charged drop dimensions due to solvent evaporation and, through phenomena of coulombic repulsion, formation of charged drop of reduced dimension; (iii)
production of ions in the gas phase originated from small charged droplets.

The experimental device for an ESI experiment is explained below. The analyte solution exits from the metal capillary (external diameter, $r_c$, in the order of $10^{-4}$ m) to which a potential ($V_c$) of 2–5 kV is applied; the counter electrode is placed at a distance ($d$) ranging from 1 to 3 cm. This counter electrode in an ESI source is usually a skimmer with a 10 m orifice or an ‘entrance heated capillary’ (internal diameter 100–500 m; length 5–10 cm), which represents the interface to the mass spectrometric analyser. Considering the thickness of the metal capillary, the electrical field ($E_c$) close to it is particularly high. For example, for $V_c$¼2000 V, $r_c$¼10_4 m and $d$ ¼ 0.02 m, an $E_c$ in the order of $6 \times 10^6$ V/cm has been calculated by Pfeifer and Hendricks. This electrical field interacts with solution and the charged species present inside the solution move in the field direction, leading to the formation of the so called ‘Taylor cone’. If the electrical field is high enough, a spray is formed from the cone apex, consisting of small charged droplets. In the case of positive ion analysis, i.e. when the needle is placed at a positive voltage, the droplets bear positive charge and vice versa in the case of negative ion analysis. A charged drop moves through the atmosphere for the field action in the direction of the counter electrode. The solvent evaporation leads to the reduction
of the drop dimensions and to a consequent increase of the electrical field perpendicular to the droplet surface. For a specific value of droplet radius the ion repulsion becomes stronger than surface tension and in these conditions the droplet explosion takes place. Two mechanisms have been proposed for the formation of gaseous ions from small charged droplets. The first model, called ‘charge residue mechanism’ (CRM) was proposed by Dole in 1968 and describes the process as sequential scissions leading to the production of small droplets bearing one or more charges but only one analyte molecule. When the last, few solvent molecules evaporate the charge(s) remains deposited on the analyte structure, which gives rise to the most stable gaseous ion. More recently, Iribarne and Thomson have proposed a different mechanism, describing the direct emission of gaseous ions from the droplets, after it has reached a certain dimension. This process, called the ‘ion evaporation mechanism’ (IEM) is predominant on the coulombic fission for droplets of radius, r, lower than 10 m. From the above, the reader can consider the factors which can affect the ion production and consequently the sensitivity and reproducibility in ESI measurements. The ion intensity exhibits with respect to analyte concentration a typical trend, analogous to that reported. A linear portion with a slope of about 1 is present for low concentration until 10⁻⁶ M, followed by a slow saturation with a weak
intensity decreasing at the highest concentrations ($10^{-3}$ M). The linear portion, where intensity is proportional to concentration, is the only region suitable for quantitative analysis. The general trend of the plot can be explained considering that in the system there is not just a single analyte: further electrolytes are always present, for example, impurities, co-analytes and buffer. It should be emphasized that for analyte concentrations lower than $10^{-5}$ M, the electrospray phenomenon occur due to the presence of electrolytes as impurities, which lead to the electrical conductivity necessary for the ‘Taylor cone’ production. Also, in the case of ESI sources, ‘in line’ or ‘orthogonal’ geometries have been proposed and employed.

**Atmospheric Pressure Photoionization**

A method recently developed consists in the irradiation, by a normal krypton (Kr) lamp, of the vaporized solution of the sample of interest at atmospheric pressure (APPI). The instrumental set up is very similar to that already described for the APCI system. In this case, the needle for corona discharge is no longer present, while the solution vaporizer is exactly the same as for the APCI source. On a side of the source the Kr lamp is mounted, so that the vapour solution can be irradiated by photons with energies up to 10.6 eV. The photoionization follows a simple general rule: a molecule with ionization energy (IEM) can be ionized by photons with energy $E_n = h\nu$.
only when: IEM  _ En considering that the most of solvents employed in liquid chromatography (LC) methods have an IE higher than 10.6 eV and consequently cannot be ionized by interaction with photon coming from the Kr lamp, the APPI method seems to be, in principle, highly effective for liquid chromatography/mass spectrometry (LC/MS) analysis of compounds exhibiting IE lower than 10.6 eV. In the case of compounds of interest with IE > 10.6 eV, the use of dopants (i.e. substances photoionizable acting as intermediates in the ionization of the molecule of interest) has been proposed. Some investigations have shown that some unexpected reactions can take place in the APPI source, indicating that it can be applied not only for analytical purposes but also for fundamental studies of organic and environmental chemistry.

**Matrix-assisted Laser Desorption/Ionisation:**

MALDI9 consists of the interaction of a laser beam with a solid sample constituted by a suitable matrix in which the analyte is present at very low molar ratio (1:10 000). This interaction leads to the vaporization of a small volume of the solid sample: in the plume of the high density vapour so generated, reactive species originating for the matrix irradiation react with the neutral molecules of analyte, mainly through protonation/deprotonation mechanisms. A detailed
description of the MALDI mechanism is highly complex, due to the presence of many different phenomena:

(i) First of all the choice of the matrix is relevant to obtain effective and well reproducible data.

(ii) The solid sample preparation is usually achieved by the deposition on a metallic surface of the solution of matrix and analyte with concentration suitable to obtain the desired analyte/matrix ratio. The solution is left to dry under different conditions (simply at atmospheric pressure, reduced pressure or under nitrogen stream); in all cases what is observed is the formation of a inhomogeneous solid sample, due to the different crystallization rate of the matrix and analyte. Consequently, the 1:10 000 ratio is only a theoretical datum: in the solid sample different ratios will be found in different positions and the only way to overcome this is to average a high number of spectra corresponding to laser irradiation of different points.

(iii) The photon–phonon transformation, obtained when a photon interacts with a crystal and giving information on the vibrational levels of the crystal lattice, cannot be applied in the laser induced vaporization observed in MALDI experiments, due to the inhomogeneity of the solid sample.

(iv) The laser irradiance is an important parameter: different irradiance values lead to vapour cloud of different density and
consequently different ion–molecule reactions can take place. In other words the MALDI data originate from a series of physical phenomena and chemical interactions originating by the parameterization (matrix nature, analyte nature, matrix/analyte molar ratio, laser irradiation value, averaging of different single spectra), which must be kept under control as much as possible. However, the results obtained by MALDI are of high interest, due to its applicability in fields not covered by other ionization methods. Due to the pulsed nature of ionization phenomena (an N2 laser operating with pulses of 102 ns and with a repetition rate of 5 MHz) the analyser usually employed to obtain the MALDI spectrum is the time-of-flight (TOF) one.

MASS ANALYSERS

The mass analysis of ions in the gas phase is based on their interaction with electrical and magnetic fields. Originally the main component of these devices was a magnetic sector which separates the ions with respect to their m/z ratio. Until the 1960s most of the mass spectrometers devoted to physics, organic and organometallic chemistry were based on this approach, and high resolution conditions were (and still are) generally acquired by the use of an electrostatic sector. The doublefocusing instruments were (and are) of large dimension (at least 2 m2) and required the use of heavy magnet and large pumping systems. In the 1960s, mainly due to the efforts of
the Paul group at Bonn University, the development of devices based on electrodynamic fields for mass analysis led to the production of quadrupole mass filters and ion traps of small dimension, so that the mass spectrometer became a bench-top instrument. The ease of use of these devices, the ease of interfacing them with data systems and, over all, the relatively low cost were the factors that moved mass spectrometry from high level, academic environments to application laboratories, in which the instrument is considered just in terms of its analytical performances.

13.2 Introduction to Bio-analytical method development

For the evaluation and interpretation of bioequivalence, pharmacokinetic (PK), and toxicokinetic studies, bioanalysis is widely used for the quantitative determination of drugs and their metabolites in biological fluids. Efficient, sensitive and selective analytical methods for quantification of drugs and their metabolites are important for such studies\textsuperscript{170-176}.

Bioanalytical method validation is a procedure used to demonstrate that an analytical method employed for quantification of analytes in a biological matrix is reliable and reproducible to achieve its purpose: to quantify the analyte with a degree of accuracy and precision appropriate to the task. Validation data demonstrate that the performance of a method is suitable and reliable for the analytical
applications intended. The quantitative approach used in bioanalytical methods involves the use of a standard curve method with internal standard.

There are various stages in the validation of a bioanalytical procedure. They can be divided into four main steps:

1. Reference standard preparation (Stock solutions, working solutions, spiked calibrators and QCs)
2. Bioanalytical method development where the assay procedure is developed.
3. Bioanalytical method validation and definition of the acceptance criteria for the analytical run
4. Application of validated method to routine sample analysis.

Validation has the primary goal of selecting the best available method for detection and/or quantitation, based on objective performance data. Regulations require that clinical laboratories show documentation and validation data when new tests are introduced. These data are also important for quality control and troubleshooting.

The successful use of an assay depends first on a fundamental understanding of the analytical principles governing the assay. Assay characteristics such as precision, accuracy, and detection limit depend on the properties of the components, the assay format, and the detection system. For example, both manual and automated
assays may have problems associated with timing or reaction rates, but the automated method may be expected to have better precision in reagent delivery. Both may have imprecision as a result of low analyte concentration or a slow approach to equilibrium. Even when the assay has been optimized in theory, practical work requires care and technical skill for optimum performance. Completeness of recovery in each step should be checked independently, for example, by using an independent tracer to follow the progress of a reaction. Equilibration rates may be very sensitive to temperature, the order of addition of reagents and stir rates. Assaying standards in each run exactly the same way as samples counterbalances some minor errors; however, poor timing or temperature control often results in increased coefficients of variation for replicate measurements. Incubation times (when necessary) are often shortened to the minimum, and room temperature incubations are common; if equilibration has not occurred, minor technical variations may have exaggerated effects (e.g., room temperature may vary considerably from summer to winter). Decisions must be made about the method based on the quality of the data produced, and quality criteria must be established. This task is not trivial, since new guidance documents are continually produced to determine whether an analytical method can be used for a quantitative analysis, or whether a new method can replace an
established (standard) one. Guidance documents and regulations have been produced by domestic and international agencies for approval of new methods, in order to protect public health, food quality, and other sensitive areas. Different levels of validation are usually defined. For example, methods proposed to be used worldwide in medically related applications must demonstrate high-quality data related to both precision and accuracy, whereas analytical methods used for research purposes require less stringent validation. High degrees of validation involve several laboratories and the assay of a large number of samples; this tends to be very expensive and impractical for locally or occasionally used methods. International organizations as AOAC International (International Association of Official Analytical Chemists), FAO (Food and Agriculture Organization of the United Nations), IAEA (International Atomic Energy Agency), IUPAC (International Union of Pure and Applied Chemistry), ISO (International Organization for Standardization), have produced international guidelines that can be used in conjunction with the different local policies in effect around the world. In the specific case of biomedical testing, regulations are contained in CLIA (Clinical Laboratory Improvement Amendments), which are the responsibilities of the FDA. Following established validation procedures is a necessary step to obtain regulatory approval to use a new analytical method, or
to use an approved method in a new application. The definitions, methods, and parameters used to validate analytical and bioanalytical methods are not universal; they vary with the type of assay and the regulatory agency. Bioanalytical methods are classified according to the kind of results they provide. Qualitative assays provide information about the presence or absence of the analyte, whereas quantitative assays give the analyte concentration and the uncertainty in this value, in the sample. Semiquantitative methods give an estimate of concentration and are used to determine whether analyte is present at a level that is above or below a reference concentration; therefore the report can state if the concentration in the sample is higher, similar, or lower than the reference value.

The fundamental parameters for bioanalytical method validation include:

- Accuracy
- Precision
- Selectivity
- Sensitivity
- Reproducibility
- Stability
- Range
- Recovery and
• Response function

These parameters were discussed in detail in chapter 8 under method development and validation.