Chapter 1

Introduction

1.1. Pharmacokinetics and metabolism in drug discovery and development

The search for a new drug is a long and complex process that encompasses diverse scientific disciplines with huge amount of money being invested. Drug discovery and drug development are two important stages in the process of finding a novel therapeutic agent. The drug discovery step involves identification and evaluation of biological activity of a new compound, whereas in the drug development stage, the focus is on determining the safety and toxicity of the new drug candidate. The drug development stage is further sub-divided as pre-clinical and clinical phases. The drug candidate that succeeds in passing the preclinical drug development stage (drug metabolism, pharmacokinetics, safety and toxicity testing, etc) [1], is submitted as an investigational new drug application (IND) to regulatory bodies for approval of clinical development: Phase I (safety and tolerability in healthy volunteers), Phase II (efficacy and dose-effect relationships using a small number of patients), and Phase III (efficacy studies using a large number of patients) [2]. If the IND passes all three clinical phases, it is submitted as a new drug application (NDA) and upon approval from regulatory bodies, it eventually enters for the clinical use/marketplace.

In drug discovery process, the failure rate of compounds is very high and it is mainly due to lack of efficacy and safety when used in humans. The efficacy and safety of new chemical entity (NCE) is affected by its pharmacokinetic and metabolic properties. Poor absorption, too long or too short half life ($t_{1/2}$) and extensive first pass metabolism are the undesirable pharmacokinetic properties that affect the bioavailability and hence the efficacy of a drug. Similarly, the metabolic fate of a drug has direct role on the safety of a drug. The drug can undergo metabolism to give inactive, active or toxic metabolite. Thus, accurate pharmacokinetic and metabolic data in the pre-clinical stages help in early evaluation of efficacy and safety of the new drug to reduce the failure rate at a later stage of the drug discovery process [3].

1.1.1. Preclinical pharmacokinetics

Pharmacokinetics (PK) is the study of the time course of absorption, distribution, metabolism and elimination (ADME) of drugs in biological system. It helps to understand the relationship between pharmacological and toxicological effect and concentration of a drug and its metabolite in the body fluid. Preclinical pharmacokinetics is referred to *in vitro* and *in vivo* drug metabolic and

pharmacokinetic (DMPK) properties of NCE in experimental animal species supporting drug development program. It can be broadly divided into three areas: (i) Discovery enabling DMPK studies (ii) Preclinical regulatory pharmacokinetics and metabolism and (iii) Toxicokinetics or PK in toxicological evaluation of NCE in a 14-day or 28-day toxicity study at three dose levels.

1.1.2. In vitro studies

Assessment of ADME properties are now conducted at very early stages of drug discovery for the purpose of accelerating the conversion of 'Hits' and 'Leads' into qualified developmental candidates. To meet this need, high throughput *in vitro* tests have been developed that can profile NCEs in a model for each of the major barriers to good bioavailability post-oral dose, and are often able to screen hundreds of compounds per week with better projections for PK properties of compounds in humans [4-6]. A number of new *in vitro* techniques are available to screen compounds for key ADME characteristics such as absorption and metabolic stability, which, when applied within a rational strategy, can make a major contribution to the design and selection of successful NCEs [7, 8]. One or all of them have a major impact on the exposure of individuals to orally administered drugs and to their efficacy. These assay models have been applied not only to screening and ranking of potential drug candidate but also to the understanding of the mechanisms leading to *in vivo* pharmacokinetic outcomes.

1.1.2.1. Absorption

Good absorption is one of the important criteria for the selection of a compound in the drug development process. Various *in vitro* techniques are available to assess the absorption of NCE. They include the use of everted intestinal sacs, everted intestinal rings, isolated brush border, basolateral membrane and 'Ussing diffusion cells' [9-12]. However, the use of these techniques have limitations associated with them. The use of Caco2 cell monolayer method for drug absorption and permeability studies has increased dramatically in recent years. Caco2 cell line is derived from human colorectal carcinoma. Caco2 cell lines are used to assess the mode of transport of drug molecule and also to know the regulation of drug transport across intestinal epithelium [13]. Drug absorption studies using Caco2 cell lines are easy to perform and require small quantity of compounds, and hence, it can be performed for screening of drug molecules for absorption in early stages of drug discovery.

1.1.2.2. Metabolism

Metabolism is the biotransformation process by which lipophilic drugs are converted into hydrophilic metabolites, which can be eliminated from body in bile or urine. Liver is the major organ of metabolism (hepatic metabolism), which has abundance of metabolic enzymes. Lungs, kidneys and intestine are the other organs with metabolic activity but they account for only 10-20% of total metabolism. The metabolism of most of the drugs takes place in two phases. In phase I metabolism, the drug is converted in to suitable polar substrate for Phase II reactions. The common reactions of phase I metabolism are oxidation, hydroxylation, dealkylation and hydrolysis. Cytochrome P450 (CYP450) is the group of oxidation enzymes, which plays major role in the phase I metabolism of drugs. In phase II metabolism, conjugation reactions like glucuronidation, sulfation of the metabolites formed in phase I metabolism occurs. For some drugs, conjugation reactions take place directly. The metabolism of any xenobiotic is important to eliminate it from the biological system and prevent its accumulation. However, faster and extensive metabolism of drug affects its bioavailability, duration of action and efficacy. The metabolic stability of any drug is desirable property to improve its efficacy.

Early information on the metabolism of NCE is important to select suitable animal species for toxicity studies and to predict the possible clinical drug-drug interactions. However, *in vivo* metabolism studies in humans are not carried out until the later stage of drug development. Therefore, *in vitro* metabolism studies are carried out using liver microsomes [14] or post mitochondrial S9 [15, 16] or hepatocytes fractions [17] and recombinant CYP450 isozymes. These systems retain the physiologic conditions of enzyme systems and simulate the *in vivo* situation better. Microsomes contain the CYP450 oxidizing enzymes and some phase II conjugating enzymes (e.g. UDP-glucuronosyltransferases). The S9 fraction is a cruder preparation than microsomes and contains the microsomal enzymes, as well as additional metabolizing enzymes present in cytosolic fraction. Hepatocytes contain all the liver metabolizing enzymes that are found *in vivo*. Recombinant CYP450 isozymes contain single enzyme that is used to identify the specific enzyme involved in the metabolism of a drug.

The *in vitro* metabolite profile obtained for a drug is generally similar to that of *in vivo* metabolism. *In vitro* metabolism systems are not limited to those derived from the liver. Most pharmaceutical companies have been building liver and tissue banks to permit a cross-comparison of metabolic turnover rates in various tissues from various species. It is common now for liver banks to house tissues from a variety of species, including those from animals treated with enzyme inducers or inhibitors. Therefore, cross-species *in vitro* metabolism comparisons are becoming more feasible and commonplace. The comparison of metabolite profile obtained from animal and human using *in vitro* models helps in the selection of suitable animal species for toxicity studies.

1.1.2.3. Protein binding

The duration and intensity of drug action depends on the unbound drug concentrations at the site of action. As the direct measurement of drug concentrations at the site of action is difficult, plasma concentration is measured which is in equilibrium with the drug concentration at the site of action. Drug molecules circulate in plasma as both protein bound and unbound fractions. The drug can bind to a single blood protein or to multiple proteins (albumin, α1-acid glycoprotein). Albumin is the most abundant protein present in plasma and has number of binding sites, which can bind with wide variety of both endogenous and exogenous compounds. Albumin has higher binding affinity for acidic compounds. α1- acid glycoprotein (AAG) is another plasma protein which binds with basic drugs mediated by one common binding site. The extent and nature of drug-protein complex formation depends on the nature of drug (weak or strong acid or base or neutral compound), the affinity of the drug to protein, the concentration of proteins and drugs [18]. The protein binding is a reversible process and there exists equilibrium between the bound and free form of drug. The concentration of free drug in plasma is dependent on the equilibrium binding and dissociation constant. The free form of drug is available for uptake into tissue and is able to interact with receptors or tissues to exert its pharmacological effect [19].

The protein binding of a drug affects the pharmacokinetic parameters like clearance (Cl) and volume of distribution at steady state (Vss). The extensive protein binding of a drug in plasma results in lower hepatic clearance (Cl_h), as the bound drug is not available to the enzymes, thus protecting the drug from metabolism [20]. The drug-protein complex does not permeate glomerular membranes in nephrons and can affect

the glomerular filtration, active secretion and renal metabolism resulting in decreased renal clearance (Cl_r). The transportation of the bound drug across the tissue membrane is also hindered, leading to variation in the tissue distribution of drugs [21]. Thus the protein binding of a drug may greatly influence the metabolism and pharmacokinetics of the drug depending upon the extent of binding. Therefore, the determination of protein binding of any new drug molecule is important in preclinical pharmacokinetics phase. Plasma protein binding is routinely determined *in vitro* for drugs in discovery and development stage. There are numerous *in vitro* methods for the determination of protein binding, including equilibrium dialysis, dynamic dialysis, ultrafiltration, ultracentrifugation, exclusion chromatography, and circular dichroism. The reliability of these methods has been compared. *In vitro* binding data determined by ultrafiltration and equilibrium dialysis accurately reflect *in vivo* binding situations. However, care still must be exercised in determination of *in vitro* binding when the goal is to represent the *in vivo* situation. For example, in some cases, the metabolite of a drug may also bind to the plasma proteins and thus may be in competition with the parent drug for binding sites. Therefore, an *ex vivo* experiment in which plasma is taken from a species that has already received the drug may better reflect the *in vivo* binding situations.

Although it is easy to determine the plasma protein binding of drugs, the study of tissue binding is hampered by methodological problems. Several methods have been developed for the study of tissue binding. These include perfused intact organs, tissue slices, or tissue homogenates. In principle, these methods allow the direct determination of tissue binding but require removal of tissues from the body, which limits their applicability. Furthermore, the necessary handling of tissues, such as of tissue slices and homegenization, may alter binding properties of the drug *in vitro.*

1.1.3. *In vivo* Studies

Although numerous *in vitro* studies are available for the prediction of *in vivo* pharmacokinetic properties of drug, there is no substitute for actual data obtained from *in vivo* studies. While insight into various aspects of the pharmacokinetic profile (ADME) can be gleaned from *in vitro* techniques, there are, as yet, no methods available for accurately predicting what will happen to a drug when it is put into a

whole animal. The primary information needed about the drug candidate before it can be taken to clinical studies is: whether it can be given orally, its rates of uptake, distribution and elimination. The first such information is generally generated in a rat model.

The development and validation of an assay for measurement of the drug in rat plasma and serum is the first step in the preclinical PK work. While many analytical techniques may be used for this purpose, High Performance Liquid Chromatography-UV visible spectrophotometer (HPLC-UV), Liquid Chromatography-Mass spectrometry (LC-MS) or Liquid Chromatography tandem mass spectrometry (LC-MS/MS) are the commonly used methods. Often the assay developed at this stage is used to measure drug levels in plasma/serum during clinical PK studies. The concentration time data are generally analyzed using compartmental or non-compartmental approach and key PK characteristics such as maximum concentration (C_{max}) in biological fluids, the time to reach C_{max} (T_{max}), half-life ($t_{1/2}$), volume of distribution (V_d), clearance (Cl), area under curve (AUC) [22] are derived. If both oral and intravenous formulations are available, absolute bioavailability ($\%F$) i.e. fraction of an orally administered dose that reaches the blood stream is also calculated.

1.1.4. Key pharmacokinetic parameters

C_{max} and T_{max}

Following intravenous (i.v.) or extravascular drug administration, the maximum observed concentration in serum or plasma (C_{max}) and the time to reach that concentration (T_{max}, which equals 0 for i.v. bolus dosing) are important descriptors of the extent and nature of drug exposure. C_{max}, an indicator of maximum drug exposure, may sometimes relate better to pharmacological or toxicological effects of exposure.

Area under the Curve (AUC)

When blood, plasma or serum drug concentrations are plotted versus time, the AUC is the primary measure of overall exposure following i.v. or extravascular administration of a drug. AUC is most commonly determined using the linear trapezoidal method. AUC is expressed in units of concentration × time (e.g. ng.h/mL).

AUC is used to calculate clearance and bioavailability which, along with volume of distribution, constitute the most important primary PK parameters. In the drug

discovery setting, however, secondary or derived parameters such as $t_{1/2}$ or AUC have increased practical importance.

Clearance (Cl)

Clearance is a proportionality constant that relates the rate of drug elimination (in mass/time) to the concentration of drug in blood or plasma. It is assumed that the amount of drug eventually eliminated from the body equals the amount administered (for an i.v. dose).

Therefore,

$$Dose_{i.v.} = Cl \times AUC_{i.v.} \text{ or } Cl = Dose_{i.v.}/AUC_{i.v.}$$

Clearance is expressed in units of volume/time and can be thought of as the volume of blood or plasma that must be "cleared" of drug per unit time to produce the observed rate of elimination. For drugs that are eliminated only by liver, maximum blood Cl is hepatic blood flow (~1.2 l/h per kg in humans); for drugs cleared by the kidney, maximum blood Cl is the glomerular filtration rate (0.1 l/h per kg); for those excreted passively, maximum blood Cl is renal blood flow (~1.1 l/h per kg) if transport mechanism is involved.

Bioavailability (*F*)

Bioavailability is the fraction of extravascularly administered dose that reaches the systemic circulation. Absolute bioavailability is determined by calculating the ratio of dose normalized AUC_s following i.v. and extravascular administration.

$$\%F_{Absolute} = \frac{AUC_{ext} x Dose_{i.v.}}{AUC_{i.v.} x Dose_{ext}} x100$$

Relative bioavailability between two dose routes, forms or formulations is calculated as

$$\%F_{rel} = \frac{AUC_{test} x Dose_{ref}}{AUC_{ref} x Dose_{test}} x100$$

Volume of Distribution (*V*)

Volume of distribution (V_d) is an indicator of how extensively a molecule is distributed in the body. It is a function of plasma protein and tissue binding.

$$V_d = V_p + V_e \times (f_u/f_{ut})$$

Where V_d is the apparent volume of distribution, V_p is the volume of plasma, V_e is the extra vascular tissue space volume, f_u is the fraction unbound in plasma and f_{ut} is the fraction unbound in tissues. Thus as f_u increases, V_d increases; conversely as f_{ut} increases, V decreases. It is often assumed that unbound plasma concentrations determine *in vivo* efficacy, which is based on the hypotheses that unbound drug is a mediator of pharmacological activity. If V_d approximates plasma volume (~0.04 l/kg in humans), this suggests that the molecule distributes in the vasculature. On the other hand, a V_d~0.6 l/kg indicates distribution into total body water. A volume greater than ~2 l/kg implies extensive distribution in to tissues.

Volume of Distribution at Steady State (V_{ss})

The term volume of distribution at steady state, V_{ss} can be calculated from intravascular PK data using the following equation

$$V_{ss} = Cl \times MRT$$

Where MRT is the mean residence time. Therefore, a steady state parameter can be calculated without steady-state data.

Half-life ($t_{1/2}$)

As implied, the half-life of a drug is the time it takes for its concentration in body fluids or tissues, e.g. blood or plasma to decrease by half. By examining the log drug concentration *versus* time profile, one can determine how many half-life best describe drug loss. Accordingly, when only one phase is observed, there is a single elimination rate constant k_{elim} and half-life is described by,

$$t_{1/2} = \frac{0.693}{k_{e\lim}} = \frac{V}{Cl} x0.693$$

As Cl increases, $t_{1/2}$ decreases; as V increase, $t_{1/2}$ increases. Thus half-life is a secondary parameter, which is a function of clearance and distribution of the drug. For the drugs that display multiple half-lives, an important consideration is determining which half life is more important from pharmacological or PK stand point. This can be assessed by comparing the AUC of each phase relative to total AUC; i.e., determining the half life under which the majority of exposure occurs.

Other important information that is generated at preclinical stage include tissue distribution and *in vitro/in vivo* metabolism of the drug. Tissue distribution studies often require radio-labelled compound, preferably ^{14}C, which reveal information about the drug movement in the body. Radio-labelling technique helps in determining the affinity and accumulation of a drug in a particular tissue, its penetration through the blood brain barrier and its eliminiation in faeces or urine. Plasma, urine and faeces samples also provide an opportunity to search for metabolites.

1.1.5. Predicting human pharmacokinetics from preclinical data

The primary objective of preclinical PK is to generate information describing ADME process in animals that can be used for extrapolation to human ADME process [23]. Approaches used for prediction of PK in relevant population of humans mostly rely on *in vivo* data from animals using allometric scaling or time invariant methods [24]. The growth of *in vitro* and, more recently, *in silico* screens for evaluating pharmaceutical, PK and toxicity properties have been used to predict complex *in vivo* behavior in humans. In most cases, careful and educated application of available approaches provides predictions of PK parameters within 2- or 3- fold of that observed. Attention has now been directed towards integrating information from different sources to increase the precision and accuracy of these PK predictions and to enable a better understanding of the process underlying ADME behavior in humans.

1.2. Validation of bioanalytical method

Selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites (analytes) are critical for the successful conduct of preclinical and clinical pharmacokinetic studies. Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine, is reliable and reproducible for the intended use. Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended analytical applications. The acceptability of analytical data corresponds directly to the criteria used to validate the method.

1.2.1. Validation parameters

There is a general agreement that at least the following validation parameters should be evaluated for quantitative procedures: selectivity, calibration model, accuracy, precision, limit of quantification and stability. Additional parameters which might have to be evaluated include limit of detection, recovery, reproducibility and ruggedness (robustness) [25-33].

Selectivity or Specificity

Selectivity is the ability of the bioanalytical method to measure unequivocally and to differentiate the analyte(s) in the presence of components, which may be expected to be present. Typically, these might include metabolites, impurities, degradants, matrix components, etc. [32]. The requirements established by conference report demands to analyze at least six different sources of blank matrices to establish specificity [30]. In the case of LC-MS/MS based procedures, it is essential that appropriate steps be taken to ensure the lack of matrix effect(s) throughout the application of the method, especially if the nature of the matrix changes from that used during initial method validation.

Calibration Model, Linearity and Range

An important aspect to be considered in assay validation is the appropriate calibration model, to define the concentration of the analytes in mobile phase and biological matrices versus the detector response. The current practice to select the model is by analyzing the replicate samples of calibration standards prior to validation, and treat the data through different transformations and weighting schemes. Previous experiences show that the calibration model should be chosen only after analyzing all validation samples so that one does not over/under-estimate the concentrations of the analytes in unknown samples during pharmacokinetic studies [34]. The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of analyte(s) in the sample. Furthermore, it is stated that for the establishment of linearity, at least 5-8 concentration levels should be studied for linear and, may be more, for nonlinear relationships [35]. The range of analytical procedure is the interval between the upper and lower concentrations of the analyte in the sample for which it has been demonstrated that the analytical procedure

has a suitable level of precision, accuracy, and linearity. The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure.

Accuracy and Precision

Accuracy is defined as the nearness of the calculated concentration to the spiked one while precision deals with the repetitiveness of the calculated concentration. It is established at a minimum of three concentrations, in replicates, of the quality control (QC) samples (low, medium and high). Intra- and inter-batch accuracy is determined by calculating the % bias from the theoretical concentration using the following equation:

$$\% \text{ Bias} = \frac{\text{Observed concentration - Nominal concentration}}{\text{Nominal concentration}} \times 100$$

Inter- and intra-batch precision in terms of coefficient of variation (% C.V.) is obtained by subjecting the data to one way analysis of variance (ANOVA).

Limits

LLOQ based on precision and accuracy (bias) data [25, 26, 29, 31, 32]

This is probably the most practical approach and defines the LLOQ as the lowest concentration of a sample that can still be quantified with acceptable precision and accuracy (bias). In the conference reports, the acceptance criteria for these two parameters at LLOQ are ±20% RSD for precision and ±20% for bias. Only Causon suggested ±15% RSD and ±15% bias, respectively [2]. It should be pointed out, however, that these parameters must be determined using an LLOQ sample independent from the calibration curve. The advantage of this approach is the fact that the estimation of LLOQ is based on the same quantification procedure used for real samples. For LLOQ, S/N is usually required to be equal to or greater than 10.

Upper limit of quantification (ULOQ)

The upper limit of quantification is the maximum analyte concentration of a sample that can be quantified with acceptable precision and accuracy (bias). In general, the ULOQ is identical with the concentration of the highest calibration standard [36].

Limit of detection (LOD)

Quantification below LLOQ is by definition not acceptable [28, 29, 31, 36, 37]. Therefore, below this value, a method can only produce semi-quantitative or qualitative data. However, it can still be important to know the LOD of the method. According to ICH, it is the lowest concentration of analyte in a sample which can be detected but not necessarily quantified as an exact value. According to Conference Report II, it is the lowest concentration of an analyte in a sample that the bioanalytical procedure can reliably differentiate from background noise [32, 37]. The approaches for estimation of the LOD are basically the same as those described for LLOQ. However, for LOD, S/N or k-factor equal to or greater than three is usually chosen [29, 31, 33, 36, 38].

Stability

Stability of the analyte during the whole analytical procedure is a prerequisite for reliable quantification. Therefore, full validation of a method must include stability experiments for the various stages of analysis including storage prior to analysis.

Long-term stability

The stability in the sample matrix should be established under storage conditions, i.e. in the same vessels, at the same temperature and over a period at least as long as the one expected for authentic samples [37-32, 38].

Freeze/thaw stability

As samples are often frozen and thawed, e.g. for reanalyis, the stability of analyte during several freeze/thaw cycles should also be evaluated. The validation programme requires a minimum of three cycles at two concentrations in triplicate. [27, 29, 31-33].

In-process stability

The stability of analyte under the conditions of sample preparation (e.g. ambient temperature over time needed for sample preparation) is evaluated here. There is general agreement, that this type of stability should be evaluated to find out, if preservative have to be added to prevent degradation of analyte during sample preparation [27, 29, 33].

Processed sample stability

Stability of analyte can change in prepared samples also. It is therefore important to test the stability in the prepared samples under conditions of analysis (e.g. autosampler conditions for the expected maximum time of an analytical run). One should also test the stability in prepared samples under storage conditions, e.g. refrigerator, in case prepared samples have to be stored prior to analysis [25-29, 32 and 33]. Stability can be tested by comparing the results of QC samples analyzed before (comparison samples) and after (stability samples) being exposed to the conditions for stability assessment. It has been recommended to perform stability experiments at least at two concentration levels (low and high) [27-29, 33]. For both, comparison and stability samples, analysis of at least six replicates are recommended [29]. Ratios between comparison samples and stability samples of 90-110% with 90% confidence intervals within 80-120% [29] or 85-115% [27] were regarded acceptable. Alternatively, the mean of the reference samples can be tested against a lower acceptance limit corresponding to 90% of the mean of the comparison samples [26, 29].

Recovery

Recovery is calculated by comparison of the analyte response after sample workup with the response of a solution containing the analyte at the theoretical maximum concentration. It has been agreed that the value for recovery is not important, as long as the data for LLOQ, (LOD), precision and accuracy (bias) are acceptable [25, 26, 28, 29, 32, 38]. However, it is preferred that recovery should be consistent.

Ruggedness (Robustness)

Ruggedness is a measure for the susceptibility of a method to small changes, that might occur during routine analysis like small changes of pH values, mobile phase composition, temperature, etc. Full validation must not necessarily include ruggedness testing; it can however be very helpful during the method development/prevalidation phase, as problems that may occur during validation are often detected in advance. Ruggedness should be tested, if a method is supposed to be transferred to another laboratory [29, 36-38].

1.4.2. Principles of bioanalytical method validation

1. The fundamental parameters to ensure the acceptability of the performance of a bioanalytical method validation are accuracy, precision, selectivity, sensitivity, reproducibility, and stability.
2. A specific and detailed description of the bioanalytical method should be written. This can be in the form of a protocol, study plan, report, and/or standard operating procedure (SOP).
3. Each step in the method should be investigated to determine the extent to which environmental, matrix, material, or procedural variables can affect the estimation of analyte in the matrix from the time of collection of the material up to and including the time of analysis.
4. It may be important to consider the variability of the matrix due to the physiological nature of the sample. In the case of LC-MS/MS based procedures, appropriate steps should be taken to ensure the lack of matrix effects throughout the application of the method, especially if the nature of the matrix changes from the matrix used during method validation
5. A bioanalytical method should be validated for the intended use or application. All experiments used to make claims or draw conclusions about the validity of the method should be presented in a report (method validation report).
6. Whenever possible, the same biological matrix as the matrix in the intended samples should be used for validation purposes. (For tissues of limited availability, such as bone marrow, physiologically appropriate proxy matrices can be substituted.)

7. The stability of the analyte (drug and/or metabolite) in the matrix during the collection process and the sample storage period should be assessed, preferably prior to sample analysis.
8. For compounds with potentially labile metabolites, the stability of analyte in matrix from dosed subjects (or species) should be confirmed.
9. The accuracy, precision, reproducibility, response function, and selectivity of the method for endogenous substances, metabolites, and known degradation products should be established for the biological matrix. For selectivity, there should be evidence that the substance being quantified is the intended analyte.
10. The concentration range over which the analyte will be determined should be defined in the bioanalytical method, based on evaluation of actual standard samples over the range, including their statistical variation. This defines the *standard curve*.
11. A sufficient number of standards should be used to adequately define the relationship between concentration and response. The relationship between response and concentration should be demonstrated to be continuous and reproducible. The number of standards used should be a function of the dynamic range and nature of the concentration-response relationship. In many cases, six to eight concentrations (excluding blank values) can define the standard curve. More standard concentrations may be recommended for nonlinear than for linear relationships.
12. The ability to dilute samples originally above the upper limit of the standard curve should be demonstrated by accuracy and precision parameters in the validation.
13. In consideration of high throughput analyses, including but not limited to multiplexing, multicolumn, and parallel systems, sufficient QC samples should be used to ensure control of the assay. The number of QC samples to ensure proper control of the assay should be determined based on the run size. The placement of QC samples should be judiciously considered in the run.
14. For a bioanalytical method to be considered valid, specific acceptance criteria should be set in advance and achieved for accuracy and precision for the validation of QC samples over the range of the standards.

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