CHAPTER 1

GENERAL – INTRODUCTION
INTRODUCTION[1]

1.1 Generic drug
A generic drug is a drug which is bioequivalent to a brand name drug with respect to pharmacokinetic and pharmacodynamic properties. These drugs are usually sold at a lower price than the brand name drug. Generic medicines must contain the same active ingredient at the same strength as the "innovator" brand, be bioequivalent and are required to meet the same pharmacopoeial requirements for the preparation. By extension, therefore, generics are assumed to be identical in dose, strength, route of administration, safety, efficacy and intended use.

1.2 Reasons for cheaper price
The principal reason for the reduced price of generic medicines is that these companies incur less costs in creating the generic drug and are therefore able to offer a lower price and still maintain profitability.

Manufacturers of generic drugs are mainly able to avoid the following three costs that brand name pharmaceutical companies incur 1) costs associated with the research and development of the drug 2) costs associated obtaining regulatory approval (i.e. proving safety and efficacy of a drug) and 3) marketing costs.

First, Generic manufacturers do not incur the cost of drug discovery and instead reverse-engineer existing brand name drugs to allow them to manufacture bioequivalent versions.

Second, generic manufacturers do not bear the burden of proving the safety and efficacy of the drugs through clinical trials - rather, generic manufacturers must prove the bioequivalence to the existing drug.

Third, these companies receive the large benefit of the marketing that goes into pushing the innovator drug. The drugs that generic manufacturers are selling have been on the market for usually a decade or more and do not need additional advertising. For the same reason, generic manufacturers also do not give away
sample doses to promote their products. The significant research & development and marketing costs incurred by the large pharmaceutical companies in bringing a new drug to the market is often cited as the reason for the high cost of new agents. They wish to recover these costs before the patent expires. Generic manufacturers do not incur these costs, with bioequivalence testing and the actual manufacturing process costing relatively little and are able to charge significantly less than the "innovator" brand.

1.3 When can a generic drug be produced?
Generic drugs can be legally produced for drugs where 1) the patent has expired 2) the generic company certifies the brand company's patents are either invalid, unenforceable or will not be infringed 3) for drugs which have never held patents or 4) in countries where a patent(s) is/are not in force. The expiration of a patent removes the monopoly of the patent holder on drug sales licensing. It is also becoming popular for the large pharmaceutical companies to preempt the expiry of their patent by producing their own generic product or license their own product to be branded by generic companies.

Enacted in 1984, the U.S. Drug Price Competition and Patent Term Restoration Act, informally known as the "Hatch-Waxman Act", standardized U.S. procedures for recognition of generic drugs. An applicant files an Abbreviated New Drug Application (or "ANDA") with the Food and Drug Administration (FDA) and seeks to demonstrate therapeutic equivalence to a specified, previously approved "reference listed drug." When an ANDA is approved, the FDA adds the drug to its Approved Drug Products list, also known as the "Orange Book" and annotates the list to show equivalence between the reference listed drug and the approved generic. The FDA also recognizes drugs using the same ingredients with different bioavailability and divides them into therapeutic equivalence groups. For example, as of 2006 diltiazem hydrochloride had four equivalence groups all using the same active ingredient but considered equivalent only within a group. For an explanation of FDA terms and procedures, see "Approved Drug Products with Therapeutic Equivalence Evaluations, Preface."
1.4 Patent lifetime and research cost issues

Pharmaceutical companies may produce a generic drug when the patent expires on the innovator drug. Patent lifetime differs from country to country. The length of time before a patent expires varies for different drugs. Usually, there is no way to renew a patent after it expires. A new version of the drug with significant changes to the compound could be patented but this will require new clinical trials and will not prevent the generic versions of the original drug. Usually, generic drugs are much less expensive than the brand-name product. Some patients and physicians will hesitate to select these medications because of concerns about the quality of generic drugs. When a pharmaceutical company first markets a drug, it is usually under a patent that allows only the pharmaceutical company that developed the drug to sell it. This allows the company to recoup the cost of developing that particular drug. After the patent on a drug expires, any pharmaceutical company can manufacture and sell that drug. Since the drug has already been tested and approved, the cost of simply manufacturing the drug will be a fraction of the original cost of testing and developing that particular drug. The brand-name drug companies have tended to litigate aggressively to extend patent protection on their medicines and keep generic versions off the market, a process referred to by critics as "evergreening."

1.5 Bioequivalence

Bioequivalence is a term in pharmacokinetics used to assess the expected in vivo biological equivalence of two proprietary preparations of a drug. If two products are said to be bioequivalent it means that they would be expected to be, for all intents and purposes, the same.

Birkett (2003) defined bioequivalence by stating that, "two pharmaceutical products are bioequivalent if they are pharmaceutically equivalent and their bioavailabilities (rate and extent of availability) after administration in the same molar dose are similar to such a degree that their effects, with respect to both efficacy and safety, can be expected to be essentially the same. Pharmaceutical equivalence implies the same amount of the same active substance(s), in the same
dosage form, for the same route of administration and meeting the same or comparable standards."

The United States Food and Drug Administration (FDA) has defined bioequivalence as, "the absence of a significant difference in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalents or pharmaceutical alternatives becomes available at the site of drug action when administered at the same molar dose under similar conditions in an appropriately designed study." (FDA, 2003)

1.5.1 Regulatory definition

Australia
In Australia, the Therapeutics Goods Administration (TGA) considers preparations to be bioequivalent if the 90% confidence intervals (90% CI) of the transformed natural log ratios, between the two preparations, of $C_{\text{max}}$ and AUC lie in the range 0.80-1.25. $T_{\text{max}}$ should also be similar between the products (Birkett 2003).

There are tighter requirements for drugs with a narrow therapeutic index and/or saturable metabolism. Thus no generic products exist on the Australian market for digoxin or phenytoin for instance.

Europe
According to European regulations EMEA-CPMP, Note for Guidance on the investigation of Bioavailability and Bioequivalence, London, July 2001 CPMP/EWP/QWP/1401/98 two medicinal products are bioequivalent if they are pharmaceutically equivalent or pharmaceutical alternatives and if their bioavailabilities after administration in the same molar dose are similar to such a degree that their effects, with respect to both efficacy and safety, will be essentially the same.

United States
The FDA considers two products bioequivalent if the 90% CI of the relative mean $C_{\text{max}}$, $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$ of the test (e.g. generic formulation) to reference (e.g.
innovator brand formulation) should be within 80.00% to 125.00% in the fasting state. Although there are a few exceptions, generally a bioequivalent comparison of Test to Reference formulations also requires administration after an appropriate meal at a specified time before taking the drug, a so-called "fed" or "food-effect" study. A food-effect study requires the same statistical evaluation as the fasting study, described above.

1.5.2 Bioequivalence testing

In determining bioequivalence, for example, between two products such as a commercially-available Brand product and a potential to-be-marketed generic product, pharmacokinetic studies are conducted whereby each of the preparations are administered in a cross-over study to volunteer subjects, generally healthy individuals but occasionally in patients. Serum plasma samples are obtained at regular intervals and assayed for parent drug (or occasionally metabolite) concentration. Occasionally, blood concentration levels are neither feasible or possible to compare the two products (e.g. inhaled corticosteroids), then pharmacodynamic endpoints rather than pharmacokinetic endpoints (see below) are used for comparison. For a pharmacokinetic comparison, the plasma concentration data are used to assess key pharmacokinetic parameters such as area under the curve (AUC), peak concentration (C_max), time to peak concentration (T_max) and absorption lag time (T_lag). Testing should be conducted at several different doses, especially when the drug displays non-linear pharmacokinetics.

In addition to data from bioequivalence studies, other data may need to be submitted to meet regulatory requirements for bioequivalence. Such evidence may include analytical method validation in vitro-in vivo and correlation studies.

1.5.3 ADME[^2-4^]

ADME is an acronym in pharmacokinetics and pharmacology for Absorption, Distribution, Metabolism and Excretion; of describes the disposition of a pharmaceutical compound within an animal or human body. These four criteria all...
influence the drug levels and kinetics of drug exposure to the tissues and hence influence the performance and pharmacological activity of the compound as a drug.

Absorption
To exert a pharmacological effect in tissues drug molecules have to pass through biological membranes such as the intestinal mucus to get into the bloodstream. Factors such as poor compound solubility, chemical instability in the stomach and inability to permeate the intestinal wall can all reduce the extent to which a drug is absorbed after oral administration. Oral route is the most clinically acceptable route of administration of drugs.

In pharmacology (and more specifically pharmacokinetics), absorption is the movement of a drug into the bloodstream.

Absorption involves several phases. First, the drug needs to be administered via some route of administration (oral, via the skin, etc.) and in a specific dosage form such as a tablet, capsule and so on.

In other situations, such as intravenous therapy and intramuscular injection, absorption is even more straightforward and there is less variability in absorption and bioavailability is often near 100%.

Absorption is a primary focus in drug development and medicinal chemistry, since the drug must be absorbed before any medicinal effects can take place. Moreover, the drug’s pharmacokinetic profile can be easily and significantly changed by adjusting factors that affect absorption.

The gastrointestinal tract is lined with epithelial cells. Drugs must pass through these cells in order to be absorbed into the circulatory system. One particular cellular barrier that may prevent absorption of a given drug is the cell membrane. Cell membranes are essentially lipid bilayers which form a semipermeable membrane. Pure lipid bilayers are generally permeable only to small, uncharged solutes. Hence, whether or not a molecule is ionized will affect its absorption, since ionic molecules are considered charged molecules by definition.
Distribution

Once the drug enters the bloodstream it needs to be carried to its effector site. The compound will then distribute into various tissues and organs to differing extents based mainly on the lipophilicity of the drug. Biological barriers such as the blood-brain-barrier and transporters play a role in determining the distribution of the drug. The distribution of the drug into target organs is critical in ensuring efficacy.

Metabolism

Drugs are typically chemicals that are not commonly found in the environment and thereby termed "xenobiotics". Several enzyme systems have evolved as man evolved that prevent exposure of humans to xenobiotics. These enzyme systems prevent exposure by chemically modifying the drug molecule mainly by oxidation (Phase I), hydrolysis, and reduction or by conjugation with biologically occurring molecules by enzymes such as UDP-Glucuronyl transferase, sulfotransferase (Phase II). The main purpose of metabolism is to convert molecules to become more polar so that they can be easily excreted. The products of metabolism are called metabolites. Cytochrome P450 or CYP450 which is a heme containing oxidizing enzyme system mainly found in the liver is responsible for the metabolism of a majority of small-molecules. In most cases metabolism inactivates the pharmacological response of the drug; however in some cases metabolites can be pharmacologically active as well. Liver is the main metabolizing organ in the body; however metabolism can occur at many tissues in the body including intestine, blood and organs.

Excretion

The process of removal of the drug and metabolites from the human body is termed "excretion". Intact drug and metabolites can be excreted by the kidneys (urine) or feces (through bile). While urinary and fecal route of elimination are the most important ones, excretion can also occur through breath, sweat and saliva.

1.6 Bioavailability

Bioavailability is a measurement of the rate and extent of a therapeutically active drug that reaches the systemic circulation and is available at the site of action.
In pharmacology, bioavailability is used to describe the fraction of an administered dose of medication that reaches the systemic circulation, one of the principal pharmacokinetic properties of drugs. By definition, when a medication is administered intravenously, its bioavailability is 100%. However, when a medication is administered via other routes (such as by mouth), its bioavailability decreases (due to incomplete absorption and first-pass metabolism). Bioavailability is one of the essential tools in pharmacokinetics, as bioavailability must be considered when calculating dosages for non-intravenous routes of administration. Bioavailability (F), which is the percent of drug that reaches the systemic circulation, is effected by absorption and first-pass metabolism. In first-pass metabolism the drug under go metabolized before reaching the systemic circulation by the intestine and liver. Biopharmaceutics is the study of the release characteristics and permeability of the drug through intestinal membrane barriers.

1.6.1 Absolute bioavailability

Absolute bioavailability measures the availability of the active drug in systemic circulation after non-intravenous administration (i.e., after oral, rectal, transdermal, subcutaneous administration).

In order to determine absolute bioavailability of a drug, a pharmacokinetic study must be done to obtain a plasma drug concentration vs time plot for the drug after both intravenous (IV) and non-intravenous administration. The absolute bioavailability is the dose-corrected area under curve (AUC) non-intravenous divided by AUC intravenous. For example, the formula for calculating F for a drug administered by the oral route (po) is given below.

\[ F = \frac{[AUC]_{po} \times dose_{IV}}{[AUC]_{IV} \times dose_{po}} \]

Therefore, a drug given by the intravenous route will have an absolute bioavailability of 1 (F=1) while drugs given by other routes usually have an absolute bioavailability of less than one.
1.6.2 Relative bioavailability
This measures the bioavailability of a certain drug when compared with another formulation of the same drug, usually an established standard, or through administration via a different route. When the standard consists of intravenously administered drug, this is known as relative bioavailability.

\[
\text{relative bioavailability} = \frac{[\text{AUC}]_A \times \text{dose}_B}{[\text{AUC}]_B \times \text{dose}_A}
\]

1.6.3 Factors influencing bioavailability
The absolute bioavailability of a drug, when administered by an extravascular route, is usually less than one (i.e. F<1). Various physiological factors reduce the availability of drugs prior to their entry into the systemic circulation. Such factors may include, but are not limited to poor absorption from the gastrointestinal tract degradation or metabolism of the drug prior to absorption hepatic first pass effect.

Each of these factors may vary from patient to patient and indeed in the same patient over time. Whether a drug is taken with or without food will affect absorption, other drugs taken concurrently may alter absorption and first-pass metabolism. Intestinal motility alters the dissolution of the drug and may affect the degree of chemical degradation of the drug by intestinal microflora. Disease states affecting liver metabolism or gastrointestinal function will also have an effect.

1.7 Ensuring bioequivalence
In the USA the Food and Drug Administration (FDA) is responsible for making sure that generic drugs are "safe and effective." The approval process for generic drugs began in the late 1960s. Generic drug manufacturers were required to prove that their formulation exhibits bioequivalence to the innovator product. Over the past several years there have been studies that have shown the effectiveness and safety of some generic drugs. Generic drugs are always less expensive and can save patients and insurance companies thousands of dollars supposedly without
compromising the quality of care. Bioequivalence, however, does not mean that
generic drugs are exactly the same as their innovator product counterparts, as
chemical differences do exist. Some doctors and patients emphatically believe that
certain generic drugs are not as effective as the products they are meant to replace
(i.e. Prozac, Oxycontin) and consumers would undoubtedly benefit from more
clinical studies done on drug by drug basis. Generic drugs start out at first being
fairly expensive; however the price of the generic product decreases as the rate of
production increases.

As an interesting case study in the use of generic equivalents of name-brand agents,
Warfarin has been only available under the trade name Coumadin in North America
until recently. Warfarin (either under the trade name or the generic equivalent) has
a narrow therapeutic window and requires frequent blood tests to make sure
patients do not have a subtherapeutic or a toxic level. A study performed in the
Canadian province of Ontario showed that replacing Coumadin with generic
Warfarin was considered safe. In spite of the study, many physicians are not
comfortable in allowing their patients to take the branded generic equivalent
agents\[5-7\].

1.7.1 180 Day Generic Drug Exclusivity

The US FDA offers a 180 days exclusivity period to generic drug manufacturers in
specific cases. During this period only one (or sometimes a few) generic
manufacturers can produce the generic version of a drug. This exclusivity period is
only used when a generic manufacturer argues that a patent is invalid or is not
violated in the generic production of a drug and the period acts as a reward for the
generic manufacturer who is willing to risk liability in court and the cost of patent
court litigation. There is often contention around these 180 days exclusivity periods
because a generic producer does not have to produce the drug during this period
and can file an application first to prevent other generic producers from selling the
drug.
Large pharmaceutical companies often spend thousands of dollars protecting their patents from generic competition. Apart from litigation, companies use other methods such as reformulation or licensing a subsidiary (or another company) to sell generics under the original patent. Generics sold under license from the patent holder are known as authorized generics; they are not affected by the 180 days exclusivity period as they fall under the patent holder's original drug application.

A prime example of how this works is Simvastatin (Zocor), a popular drug created and manufactured by U.S. based pharmaceutical Merck & Co., which lost its US patent protection on June 23, 2006. India-based Ranbaxy Laboratories (at the 80-mg strength) and Israel-based Teva Pharmaceutical Industries (at all other strengths) received 180 days exclusivity periods for Simvastatin; due to Zocor's popularity, both companies began marketing their products immediately after the patent expired. However, Dr. Reddy's Laboratories also markets an authorized generic version of Simvastatin under license from Zocor's manufacturer, Merck & Co.; some packages of Dr. Reddy's Simvastatin even show Merck as the actual manufacturer and have Merck's logo on the bottom.

1.7.2 Research exemption
In patent law, the research exemption or safe Harbour exemption is an exemption to the rights conferred by patents, which is especially relevant to drugs. According to this exemption, despite the patent rights, performing research and tests for preparing regulatory approval, for instance by the FDA in the United States, does not constitute infringement for a limited term before the end of patent term. This exemption allows generic manufacturers to prepare generic drugs in advance of the patent expiration.

1.8 Estimation of Drug from Biological Matrix$^{[9]}$
Selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites (analytes) are critical for the successful conduct of preclinical and/or biopharmaceutics and clinical pharmacology 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. The fundamental parameters for this validation include 1) accuracy 2) precision (3) selectivity 4) sensitivity 5) reproducibility and 6) stability. 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.

Published methods of analysis are often modified to suit the requirements of the laboratory performing the assay. These modifications should be validated to ensure suitable performance of the analytical method. When changes are made to a previously validated method, the analyst should exercise judgment as to how much additional validation is needed. During the course of a typical drug development program, a defined bioanalytical method undergoes many modifications. The evolutionary changes to support specific studies and different levels of validation demonstrate the validity of an assay’s performance. Different types and levels of validation are defined and characterized as follows:

1.8.1 Full Method Validation
- Full validation is important when developing and implementing a bioanalytical method for the first time.
- Full validation is important for a new drug entity.
- A full validation of the revised assay is important if metabolites are added to an existing assay for quantification.

1.8.2 Partial Validation
Partial validations are modifications of already validated bioanalytical methods. Partial validation can range from as little as one intra-assay accuracy and precision determination to a nearly full validation. Some of examples of changes in the method are as below.
Bioanalytical method transfers between laboratories or analysts
- Change in analytical methodology (e.g., change in detection systems)
- Change in anticoagulant in harvesting biological fluid
- Change in matrix within species (e.g., human plasma to human urine)
- Change in sample processing procedures
- Change in species within matrix (e.g., rat plasma to mouse plasma)
- Change in relevant concentration range
- Changes in instruments and/or software platforms
- Limited sample volume (e.g., pediatric study)
- Rare matrices
- Selectivity demonstration of an analyte in the presence of concomitant medications
- Selectivity demonstration of an analyte in the presence of specific metabolites

1.8.3 Cross-Validation

Cross-validation is a comparison of validation parameters when two or more bioanalytical methods are used to generate data within the same study or across different studies. An example of cross-validation would be a situation where an original validated bioanalytical method serves as the reference and the revised bioanalytical method is the comparator. The comparisons should be done both ways.

When sample analysis within a single study are conducted at more than one site or more than one laboratory, cross-validation with spiked matrix standards and subject samples should be conducted at each site or laboratory to establish interlaboratory reliability. Cross-validation should also be considered when data generated using different analytical techniques (e.g., LC-MS-MS vs. ELISA) in different studies are included in a regulatory submission.

All modifications should be assessed to determine the recommended degree of validation. The analytical laboratory conducting pharmacology/toxicology and other preclinical studies for regulatory submissions should adhere to FDA’s Good Laboratory Practices (GLPs) (21 CFR part 58) and to sound principles of quality.
assurance throughout the testing process. The bioanalytical method for human BA, BE, PK, and drug interaction studies must meet the criteria in 21 CFR 320.29. The analytical laboratory should have a written set of standard operating procedures (SOPs) to ensure a complete system of quality control and assurance. The SOPs should cover all aspects of analysis from the time the sample is collected and reaches the laboratory until the results of the analysis are reported. The SOPs should include record keeping, security and chain of sample custody (accountability systems that ensure integrity of test articles), sample preparation and analytical tools such as methods, reagents, equipment, instrumentation and procedures for quality control and verification of results.

The process by which a specific bioanalytical method is developed, validated and used in routine sample analysis can be divided into 1) reference standard preparation 2) bioanalytical method development and establishment of assay procedure and 3) application of validated bioanalytical method to routine drug analysis and acceptance criteria for the analytical run and/or batch.

1.8.4 Reference Standard

Analysis of drugs and their metabolites in a biological matrix is carried out using samples spiked with calibration (reference) standards and using quality control (QC) samples. The purity of the reference standard used to prepare spiked samples can affect study data. For this reason, an authenticated analytical reference standard of known identity and purity should be used to prepare solutions of known concentrations. If possible, the reference standard should be identical to the analyte. When this is not possible, an established chemical form (free base or acid, salt or ester) of known purity can be used.

Three types of reference standards are usually used: 1) certified reference standards (e.g., USP compendial standards); 2) commercially supplied reference standards obtained from a reputable commercial source; and/or 3) other materials of documented purity custom-synthesized by an analytical laboratory or other noncommercial establishment. The source and lot number, expiration date,
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certificates of analyses when available, and/or internally or externally generated evidence of identity and purity should be furnished for each reference standard.

1.8.5 Method Development

The method development and establishment phase defines the chemical assay. The fundamental parameters for a bioanalytical method validation are accuracy, precision, selectivity, sensitivity, reproducibility, and stability. Measurements for each analyte in the biological matrix should be validated. In addition, the stability of the analyte in spiked samples should be determined. Typical method development and establishment for a bioanalytical method include determination of 1) selectivity 2) accuracy precision, recovery 3) calibration curve and 4) stability of analyte in spiked samples.

1.8.5.1 Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. For selectivity, analyses of blank samples of the appropriate biological matrix (plasma, urine, or other matrix) should be obtained from at least six sources. Each blank sample should be tested for interference and selectivity should be ensured at the lower limit of quantification (LLOQ).

Potential interfering substances in a biological matrix include endogenous matrix components, metabolites, decomposition products and in the actual study, concomitant medication and other exogenous xenobiotics. If the method is intended to quantify more than one analyte, each analyte should be tested to ensure that there is no interference.

1.8.5.2 Accuracy, Precision, and Recovery

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected
concentrations is recommended. The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as the measure of accuracy.

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV. Precision is further subdivided into within-run, intra-batch precision or repeatability, which assesses precision during a single analytical run, and between-run, inter-batch precision or repeatability, which measures precision with time and may involve different analysts, equipment, reagents and laboratories.

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium and high) with unextracted standards that represent 100% recovery.

### 1.8.5.3 Calibration/Standard Curve

A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. A calibration curve should be generated for each analyte in the sample. A sufficient number of standards should be used to adequately define the relationship between concentration and response. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte.
The number of standards used in constructing a calibration curve will be a function of the anticipated range of analytical values and the nature of the analyte/response relationship. Concentrations of standards should be chosen on the basis of the concentration range expected in a particular study. A calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard) and six to eight non-zero samples covering the expected range, including LLOQ.

- **Lower Limit of Quantification (LLOQ)**

  The lowest standard on the calibration curve should be accepted as the limit of quantification if the following conditions are met:

  - The analyte response at the LLOQ should be at least 5 times the response compared to blank response.
  - Analyte peak (response) should be identifiable, discrete and reproducible with a precision of 20% and accuracy of 80-120%.

- **Calibration Curve/Standard Curve/Concentration-Response**

  The simplest model that adequately describes the concentration-response relationship should be used. Selection of weighting and use of a complex regression equation should be justified. The following conditions should be met in developing a calibration curve:

  - 20% deviation of the LLOQ from nominal concentration
  - 15% deviation of standards other than LLOQ from nominal concentration
  - At least four out of six non-zero samples should meet the above criteria, including the LLOQ and the calibration standard at the highest concentration. Excluding the standards should not change the model used.

1.8.5.4 **Stability**

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix and the container system. The stability
of an analyte in a particular matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems. Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term (bench top, room temperature) storage and after going through freeze and thaw cycles and the analytical process. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. The procedure should also include an evaluation of analyte stability in stock solution.

All stability determinations should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at known concentrations.

1.8.5.4.1 Freeze and Thaw Stability

Analyte stability should be determined after three freeze and thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples should be refrozen for 12 to 24 hours under the same conditions. The freeze–thaw cycle should be repeated two more times, then analyzed on the third cycle. If an analyte is unstable at the intended storage temperature, the stability sample should be frozen at -70°C during the three freeze and thaw cycles.

1.8.5.4.2 Short Term Temperature Stability

Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed.
1.8.5.4.3 Long Term Stability

The long term stability of drug in plasma should be evaluated at -20°C. If the drug is not stable at -20°C then stability shall be performed at -70°C. The storage time in a Long Term Stability should be the duration of time between the date of first sample collection and the date of last sample analysis. Long Term Stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples. The volume of samples should be sufficient for analysis on three separate occasions. The concentrations of all the stability samples should be compared to the mean of back-calculated values for the standards at the appropriate concentrations.

1.8.5.4.4 Stock Solution Stability

The stability of stock solutions of drug and the internal standard should be evaluated at room temperature for at least 6 hours. If the stock solutions are refrigerated or frozen for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

1.8.5.4.5 Post Preparative Stability

The stability of processed samples, including the resident time in the autosampler, should be determined. The stability of the drug and the internal standard should be assessed over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards.

Although the traditional approach of comparing analytical results for stored samples with those for freshly prepared samples has been referred to in this guidance, other statistical approaches based on confidence limits for evaluation of an analytes stability in a biological matrix can be used. SOPs should clearly describe the statistical method and rules used. Additional validation may include investigation of samples from dosed subjects.
1.9 References

1. http://en.wikipedia.org, the free encyclopedia for bioequivalence


