1.0 INTRODUCTION:

In modern era, prescription drug spending is increasing at a rate of over 10% per year and currently represents 11% of all health care expenditures [1]. Major strategy for lowering the health care cost is the reduction of medication costs. So the first effective step regarding this introduces the generics i.e. equivalents of innovators in global market. The national average savings through the use of generic drugs from 1997–2000 was approximately 11% of total prescription costs [2].

In 2007, generic drugs captured the 66% of the prescriptions in US but less than 30% of the cost.

Thus, because of the importance of generic drugs in healthcare, it is imperative that the pharmaceutical quality and in vivo performance of generic drugs should be reliably assessed. As generic drugs are the replacement of innovator products in markets it must be demonstrated that the safety and efficacy of generics are comparable to the safety and efficacy of the corresponding innovator drugs.

Assessment of “inter changeability” between the generic and the innovator product is carried out by a study of “in vivo equivalence” or “Bioequivalence” (BE).

The concept of BE has gained popularity during the last three decades due to its applications to new brand drug and generics. However, the concept of BE and approaches to its assessment were developed in the last 35 years.

In the early 1970s, the “United States Food and Drug Administration” (USFDA) became interested in biological availability of new drugs. During this period, a drug bioequivalence study panel was formed by the Office of Technology Assessment (OTA) to understand the chemical and therapeutic equivalence relationships of drug products. On the basis of the recommendations put forth by this panel, the FDA formulated regulations for the submission of bioavailability data. These regulations are currently incorporated in the 21st volume of Code of Federal Regulation, Part 320 (21CFR320). [3].

In the early 1980s, various statistical methods have been introduced for assessing the BE. The peak methods used are the power approach [4], the confidence interval approach and the Bayesian approach.
A generic drug product is a drug product that is comparable to a brand / reference listed drug product in dosage form, strength, route of administration, quality and performance characteristics, and intended use.

Generic drugs are marketed under a non–proprietary or approved name rather than a proprietary or brand name. Generic drugs are frequently as effective as, but much cheaper than, brand–name drugs. For example, paracetamol is a chemical ingredient found in a number of brand–name painkillers, but is also sold as a generic drug (not under a brand name). Because of their low price, generic drugs are often the only medicines that the poorest can access. The Trade–Related Aspects of Intellectual Property Rights (TRIPS) agreement does not prevent governments from requiring accurate labeling or allowing generic substitution. Indeed, it is argued that competition between drug companies and generic producers has been more effective than negotiations with drug companies in reducing the cost of drugs, in particular those used to treat HIV/AIDS.

A brand name is a name given to a drug by the manufacturer. The use of the name is reserved exclusively for its owner.

<table>
<thead>
<tr>
<th>Brand name Drug</th>
<th>Vs</th>
<th>Generic Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labeling</td>
<td></td>
<td>Labeling</td>
</tr>
<tr>
<td>Pharm/tox</td>
<td></td>
<td>Pharm/tox</td>
</tr>
<tr>
<td>Chemistry</td>
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<td>Chemistry</td>
</tr>
<tr>
<td>Manufacturing</td>
<td></td>
<td>Manufacturing</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td>Controls</td>
</tr>
<tr>
<td>Microbiology</td>
<td></td>
<td>Microbiology</td>
</tr>
<tr>
<td>Inspection</td>
<td></td>
<td>Inspection</td>
</tr>
<tr>
<td>Testing</td>
<td></td>
<td>Testing</td>
</tr>
<tr>
<td>Animal Studies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical Studies</td>
<td></td>
<td>Bioequivalence</td>
</tr>
<tr>
<td>Bioavailability</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Development of Generic Products involves various critical pathways as stipulated in Figure 1.

Figure 1: Pathways for development of generic drug product
1.1 Ultimate endorsement for generics

- Reduce drug cost
- Increase drug use
- Prevent drug shortages
  - Product rationalization
  - Supply distribution

**Global Generics Industry (Global Generics Industry Report, Research and Markets)**

Global generics market was valued at USD63.66 billion during 2005, registering a growth of 20% over the previous year which is 4 times that of patented drugs, which grew at 5% in the same period. North America was the major generics market accounting for about half of the global market in 2005. The US market alone accounted for 44% of the total generics market growing at a rate of 26% in 2005 over the previous year.

1.2 FDA Ratings

The US Food and Drug Administration have rated all generic drugs "A" or "B" (www.fda.gov/cder/ob/default.htm). "A" drugs are considered bioequivalent to the brand name original. They either have been demonstrated to be so by human bioavailability study ("AB"), or are considered inherently unlikely to have bioavailability problems ("AA"); "AA" drugs are usually oral solutions or oral drugs that dissolve readily in water. Other "A" designations (AN, AO, AP, AT) refer to non–oral formulations considered bioequivalent by the FDA. Only "A" rated products are interchangeable with their brand–name equivalents by the FDA. "B" drugs have not been demonstrated to be bioequivalent by an in vivo test. These are generally older drugs that were approved by the FDA on the basis of chemistry, manufacturing controls and in vitro dissolution tests. Less than 3% of marketed generic drugs have a "B" rating.

1.2.1 Regulatory overview of the generic drugs

An Abbreviated New Drug Application (ANDA) is submitted to the Office of Generic Drugs (OGD) and includes supporting data for the review and approval of a generic drug product. For approval, a sponsor of an ANDA must have information to show that the proposed generic product is
pharmaceutically equivalent and bioequivalent and therefore therapeutically equivalent to the RLD. In addition, the sponsor must also demonstrate that the proposed product is appropriately labelled and that it is manufactured in compliance with the current Good Manufacturing Practices (cGMP) guidelines. If approved, an applicant may manufacture and market the generic drug product provided all issues related to patent protection and exclusivity have been resolved.

The primary difference between the requirements of a ‘full’ and an ‘abbreviated’ application is that the preclinical and clinical data in the NDA that establishes the safety and efficacy of the drug product do not need to be repeated for the ANDA. The requirements of an ANDA are based on the premise that an active ingredient is shown to be safe and effective and absorbed into the body to the same rate and extent as that of the innovator product, then it is said to be therapeutically equivalent. Apart from the differing requirements in the submission of clinical data, the remaining requirements including those for chemistry, manufacturing controls, testing and labelling are similar, regardless of whether the application is an ANDA or NDA. The ANDAs are submitted to the OGDs, CDER and are carefully scrutinized to assure that the standards of quality for generic products are the same as those for the brand name products. An ANDA contains data which reveals that the drug product is pharmaceutically equivalent to the RLD.

In vivo and in vitro studies to measure BA and/or establish BE of a product are important elements in support of INDs, NDAs, ANDAs, and their supplements.

As part of INDs and NDAs for orally administered drug products, BA studies focus on determining the process by which a drug is released from the oral dosage form and moves to the site of action. BA data provide an estimate of the fraction of the drug absorbed, as well as its subsequent distribution and elimination. BA can be generally documented by a systemic exposure profile obtained by measuring drug and/or metabolite concentration in the systemic circulation over time. The systemic exposure profile determined during clinical trials in the IND period can serve as a benchmark for subsequent BE studies.
Studies to establish BE between two products are important for certain changes before approval for a pioneer product in NDA and ANDA submissions and in the presence of certain post approval changes in NDAs and ANDAs. In BE studies, an applicant compares the systemic exposure profile of a test drug product to that of a reference drug product (RLD). For two orally administered drug products to be bioequivalent, the active drug ingredient or active moiety in the test product must exhibit the same rate and extent of absorption as the reference drug product [5, 6].

1.2.2 Some terminologies used in BA/BE studies:

➢ Pharmaceutical Equivalents

Pharmaceutical equivalents means drug products in identical dosage forms that contain identical amounts of the identical active drug ingredient, i.e., the same salt or ester of the same therapeutic moiety, or, in the case of modified release dosage forms that require a reservoir or overage or such forms as prefilled syringes where residual volume may vary, that deliver identical amounts of the active drug ingredient over the identical dosing period; do not necessarily contain the same inactive ingredients; and meet the identical compendia or other applicable standard of identity, strength, quality, and purity, including potency and, where applicable, content uniformity, disintegration times, and/or dissolution rates.

➢ Pharmaceutical Alternatives

Pharmaceutical alternatives mean drug products that contain the identical therapeutic moiety, or its precursor, but not necessarily in the same amount or dosage form or as the same salt or ester. Each such drug product individually meets either the identical or its own respective compendial or other applicable standard of identity, strength, quality, and purity, including potency and, where applicable, content uniformity, disintegration times and/or dissolution rates.
➤ **Therapeutic Equivalents**

A medicinal product is therapeutically equivalent with another product if it contains the same active substance or therapeutic moiety and, when administered to the same individual, shows the same efficacy and toxicity as that product, whose efficacy and safety has been established.

c

➤ **Chemical equivalence**

Chemical equivalence indicates that drug products contain the same compound in the same amount and meet current official standards; however, inactive ingredients in drug products may differ.

➤ **Conventional/Immediate Release Dosage Forms**

A conventional dosage form is a formulation or a dosage form from which the active drug is released immediately following administration.

➤ **Modified Release Dosage Forms**

A modified release dosage form is defined as one for which the drug release characteristic of a time course and/or location are chosen to accomplish therapeutic or convenience objectives not offered by conventional dosage form such as solutions, ointments and promptly dissolving forms.

Modified release dosage forms may be recognized as:

(i) Extended Release Dosage Forms and

(ii) Delayed Release Dosage Forms

- **Extended Release Dosage Forms**

  An extended release dosage form is defined as one that allows at least a two-fold reduction in dosing frequency as compared to that drug presented as a conventional dosage form.

  The terms controlled release, prolonged action, sustained release and programmed release etc., are used synonymously with extended release.
- **Delayed Release Dosage Forms**
  A delayed release dosage form is defined as one that releases a drug (or drugs) at a time other than promptly after administration, e.g., enteric coated products.

- **Pharmacokinetic Terms [7]:**
  - $C_{\text{max}}$
    This is the maximum drug concentration achieved in systemic circulation following drug administration.
  - $C_{\text{min}}$
    This is the minimum drug concentration achieved in systemic circulation following multiple dosing at steady state.
  - $C_{\text{pd}}$
    This is the pre dose concentration determined immediately before a dose is given at steady state.
  - $T_{\text{max}}$
    It is the time required to achieve maximum drug concentration in systemic circulation.
  - **Area under the Curve (AUC)**
    Area under the curve is the total area under the biological fluid (serum, blood, etc.) concentration-time curve as determined by the Trapezoidal rule.
  - $\text{AUC}_{0-t}$
    Area under the plasma concentration - time curve to the last quantifiable concentration to be calculated using trapezoidal rule.
  - $\text{AUC}_{0-\infty}$
    Area under the plasma concentration- time curve from 0 h to infinity to be calculated as the sum of $\text{AUC}_{0-t}$ plus the ratio of the last measurable concentration to the elimination rate constant.
  - $K_{\text{el}}$
    Apparent first-order terminal elimination rate constant calculated from a semi-log plot of the plasma concentration versus time curve.
- **T₁/₂**
  
  Elimination half life of a drug is the time necessary to reduce the drug concentration in the blood, plasma, or serum to one half after equilibrium is reached.

- **AUCₜ**
  
  Is the area under the curve from t=0 to t=τ during a dosing interval at steady state.

- **AUC₀⁻ₜ (Single dose)**
  
  \[ \text{AUC}_0^{-\tau} = \text{Area under the plasma concentration-time curve to the last quantifiable concentration to be calculated using trapezoidal rule.} \]

### 1.3 Bioavailability

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

Bioequivalence means 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. Where there is an intentional difference in rate (e.g., in certain extended release dosage forms), certain pharmaceutical equivalents or alternatives may be considered bioequivalent if there is no significant difference in the extent to which the active ingredient or moiety from each product becomes available at the site of drug action. This applies only if the difference in the rate at which the active ingredient or moiety becomes available at the site of drug action is intentional and is reflected in the proposed labeling, is not essential to the attainment of effective body drug concentrations on chronic use, and is considered medically insignificant for the drug.

#### 1.3.1 Assessment of bioavailability

Bioavailability is usually assessed by determining the maximum (peak) plasma drug concentration, peak time and area under the plasma concentration–time curve.
Plasma drug concentration increases with extent of absorption; the peak is reached when drug elimination rate equals absorption rate. Bioavailability determinations based on the peak plasma concentration can be misleading because drug elimination begins as soon as the drug enters the bloodstream. Peak time is the most widely used general index of absorption rate; the slower the absorption, the later the peak time. The most reliable measure of a drug’s bioavailability is the AUC.

The AUC is directly proportional to the total amount of unchanged drug that reaches systemic circulation. Drug products may be considered bioequivalent in extent and rate of absorption if their plasma concentration curves are essentially super imposable.

![Figure 2: Plasma concentration–time relationship after a single oral dose](image)

For drugs excreted primarily unchanged in urine, bioavailability can be estimated by measuring the total amount of drug excreted after a single dose. Ideally, urine is collected over a period of 7 to 10 elimination half-lives for complete urinary recovery of the absorbed drug. After multiple dosing, bioavailability may be estimated by measuring unchanged drug recovered from urine over a 24-h period under steady-state conditions.

1.3.1 Absolute Bioavailability

Absolute bioavailability compares the bioavailability (estimated as the area under the curve, or AUC) of the active drug in systemic circulation following non-intravenous administration (i.e., after oral, rectal, transdermal, subcutaneous, or sublingual administration), with the bioavailability of the
same drug following intravenous administration. It is the fraction of the drug absorbed through non-intravenous administration compared with the corresponding intravenous administration of the same drug. The comparison must be dose normalized if different doses are used; consequently, each AUC is corrected by dividing the corresponding dose administered.

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.

$$ F = \frac{[\text{AUC}]_{po} \times \text{dose}_{IV}}{[\text{AUC}]_{IV} \times \text{dose}_{po}} $$

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.

1.3.2 Relative bioavailability

This measures the bioavailability (estimated as area under the curve, or AUC) 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.

$$ \text{relative bioavailability} = \frac{[\text{AUC}]_{A} \times \text{dose}_{B}}{[\text{AUC}]_{B} \times \text{dose}_{A}} $$

1.4 Bioequivalence

It is defined 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.
Bioequivalence should be conducted for the comparison of two medicinal products containing the same active substances. Several test methods are available to assess equivalence including the following:

- Comparative bioavailability (bioequivalence) studies
- Comparative pharmacodynamic studies in humans
- Comparative clinical trials
- In-vitro dissolution tests

### 1.5 Regulatory requirement for Generic drugs

In order to minimize the variability in reporting the BE results, different regulatory bodies set a standard conditions for conduction of the BE studies. Following are the Regulatory Agency for different countries having there own criteria for bioavailability and bioequivalence studies.

- **USA**: USFDA (United States Foods and Drugs Administration)
- **Europe**: EMA [European Medicines Agency (Formerly European Medicines Evaluation Agency – EMEA)]
- **Brazil**: ANVISA (National Health Surveillance Agency)
- **Japan**: NIHS (National Institute for Health Sciences)
- **Canada**: HPFB/TPD (Health Products and Food Branch/Therapeutic Products Directorate)
- **Australia**: TGA (Therapeutic Goods Administration)
- **New Zealand**: MEDSAFE (New Zealand Medicine and Medical Devices Safety Authority)
- **Saudi Arabia**: SFDA (Saudi Food & Drug Authority)
- **South Africa**: MCC (Medicines Control Council)
- **ACCSQ**: ASEAN (Association of Southeast Asian Nations)
- **South Korea**: KFDA [(South) Korea Food & Drug Administration]
- **Mexico**: Secr. De salud
- **China**: CFDA (China Food & Drug Administration)
Demography:

<table>
<thead>
<tr>
<th>Regulatory body</th>
<th>Age Description</th>
<th>Gender</th>
<th>BMI Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>U.S.A [8]</td>
<td>18 years of age or older</td>
<td>Both Sex</td>
<td>18.5 - 24.9</td>
</tr>
<tr>
<td>Europe [9]</td>
<td>18 years of age or older</td>
<td>Both Sex</td>
<td>18.5 - 30</td>
</tr>
<tr>
<td>Japan [10]</td>
<td>Healthy adult volunteers</td>
<td>----</td>
<td>18.5-25.0</td>
</tr>
<tr>
<td>Australia [9]</td>
<td>Between 18-55</td>
<td>Both Sex</td>
<td>Accepted Normal BMI</td>
</tr>
<tr>
<td>ASEAN [13]</td>
<td>Between 18-50</td>
<td>Both Sex</td>
<td>18.5 and 25 kg/m²</td>
</tr>
<tr>
<td>South Korea [14]</td>
<td>19-55</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mexico [15]</td>
<td>18 and 55</td>
<td>-</td>
<td>Weight 10% from the ideal weight</td>
</tr>
<tr>
<td>China [16]</td>
<td>18 to 40 years of age</td>
<td>Both Sex</td>
<td>Standard weight range.</td>
</tr>
</tbody>
</table>
## Diet and Fluid Restriction

<table>
<thead>
<tr>
<th>Regulatory body</th>
<th>Diet</th>
<th>Fluid</th>
</tr>
</thead>
</table>
| **U.S.A** [8]   | No food should be allowed for at least 4 hours post-dose. 
- Subjects should start the recommended meal 30 minutes prior to administration of the drug product. Study subjects should eat this meal in 30 minutes or less; however, the drug product should be administered 30 minutes after start of the meal. 
- Standardized meals scheduled at the same time in each period of the study. (US FDA BA/BF, 2003) | i) Subjects should be administered the drug product with 240 mL (8 fluid ounces) of water. 
ii) Water is allowed as desired except for one hour before and one hour after drug administration |
| **Europe & Australia** [9] | i) No food is allowed for at least 4 hours post-dose. (fasting study) 
ii) In fed conditions, the timing of administration of the drug product in relation to food intake is recommended to be according to the SmPC of the originator product. If no specific recommendation is given in the originator SmPC, it is recommended that subjects should start the meal 30 minutes prior to administration of the drug product and eat this meal within 30 minutes. (fed study) | i) Test and reference products should be administered with a standardized volume of fluid (at least 150 ml). 
ii) Water is allowed as desired except for one hour before and one hour after drug administration |
| **Japan** [10] | Similar to U.S.A. 
- If bioavailability under fasting conditions is markedly low, or a high incidence of severe adverse effects is indicated, drugs may be given | Similar to Europe |
postprandial. For a postprandial dose, the meal should be eaten within 15 minutes, and the drug administered according to the dosing regimen or 30 minutes. (NIHS Japan, 2000)

<table>
<thead>
<tr>
<th>Country</th>
<th>Requirement</th>
<th>Other Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canada [11]</td>
<td>Similar to Europe&lt;br&gt;- All meals should be standardized and repeated on each study day. (HPB BA/BE, 2009)</td>
<td>Similar to Europe</td>
</tr>
<tr>
<td>Saudi Arabia [12]</td>
<td>Similar to Europe&lt;br&gt;- Standard meals for each study periods can be provided no less than 4 hours after drug administration</td>
<td>The drug product should be administered with 180 ml of water immediately&lt;br&gt;- Water can be allowed ad libitum after 2 hours.</td>
</tr>
<tr>
<td>ASEAN [13]</td>
<td>As per Saudi Arabia</td>
<td>As per Europe&lt;br&gt;- Hot drink or juice may be provided after 3 hours of drug administration</td>
</tr>
<tr>
<td>South Korea [14]</td>
<td>Similar to USA</td>
<td>Similar to USA</td>
</tr>
</tbody>
</table>
## Fasting

<table>
<thead>
<tr>
<th>Regulatory body</th>
<th>Fasting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Europe [9]</td>
<td>At least 8 hours prior to administration of the products and no food is allowed for at least 4 hours post-dose.</td>
</tr>
<tr>
<td>Japan [10]</td>
<td></td>
</tr>
<tr>
<td>Canada [11]</td>
<td></td>
</tr>
<tr>
<td>Saudi Arabia [12]</td>
<td>At least 10 hours of fasting which is continued for at least 4 hours post-dose.</td>
</tr>
<tr>
<td>South Korea [14]</td>
<td></td>
</tr>
<tr>
<td>Saudi Arabia [12]</td>
<td></td>
</tr>
<tr>
<td>USA [8]</td>
<td></td>
</tr>
<tr>
<td>ASEAN [13] and Australia [9]</td>
<td>At least 8 hours prior to administration of the products. If the SmPC contains specific recommendations in relation with food intake related to food interaction effects the study should be designed accordingly.</td>
</tr>
<tr>
<td>Mexico [15]</td>
<td>volunteers must be fasting for at least 10 hours before administering the medication and for at least two hours after administration</td>
</tr>
</tbody>
</table>
## Sample Size

<table>
<thead>
<tr>
<th>Regulatory body</th>
<th>Minimum</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA [8] &amp; South Korea [14]</td>
<td>12</td>
</tr>
<tr>
<td>Europe [9]</td>
<td>12</td>
</tr>
<tr>
<td>WHO [17]</td>
<td>12</td>
</tr>
<tr>
<td>Australia [9]</td>
<td>12</td>
</tr>
<tr>
<td>ASEAN [13]</td>
<td>12</td>
</tr>
<tr>
<td>Malaysia [18]</td>
<td>12</td>
</tr>
<tr>
<td>Argentina [19]</td>
<td>12</td>
</tr>
<tr>
<td>Japan [10]</td>
<td>20</td>
</tr>
<tr>
<td>Brazil [19]</td>
<td>24</td>
</tr>
<tr>
<td>Saudi Arabia [12]</td>
<td>12-24</td>
</tr>
<tr>
<td>New Zealand [20]</td>
<td>12</td>
</tr>
</tbody>
</table>
## Study Design

<table>
<thead>
<tr>
<th>Regulatory body</th>
<th>Diet</th>
<th>Fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>U.S.A [8]</td>
<td>Total of 2 studies: 1 single dose crossover study fasted 1 single</td>
<td>Fasting and fed</td>
</tr>
</tbody>
</table>
|                     | dose crossover study, fed*  
* If food mentioned in the product Monograph if a multiple-dose study design is important, appropriate dosage administration and sampling be carried out to document attainment of steady state. | If a multiple-dose study design is important, appropriate dosage administration and sampling be carried out to document attainment of steady state. |
| Europe & Australia [9] | Total of 1-2 studies: 1 single dose crossover study, Fasted or Fed  | Fasting, fed and steady state                                        |
|                     | condition according to SmPC Recommendations related with food interaction effects. |                                                                  |
| Japan [10]          | Fasting and fed                                                      | Fasting, fed and steady state                                        |
|                     | If Steady-state studies are required, the food and fluid conditions and restrictions noted above should apply on the preceding evening and on the day the plasma profiles are to be obtained. |                                                                  |
| Saudi Arabia [12]   | Fasting and if food effect from document evidence or drug requires to | Fasting and fed                                                      |
|                     | be administered in fed condition in this case fed study required.    |                                                                  |
| South Korea [14]    | Fasting                                                              | Fasting fed and steady state                                        |
## BE Acceptance Criteria for BE

<table>
<thead>
<tr>
<th>Regulatory body</th>
<th>90% CI on log Transformed Data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$C_{\text{max}}$</td>
</tr>
<tr>
<td>Europe &amp; Australia [9]</td>
<td>80-125</td>
</tr>
<tr>
<td>Canada [11]</td>
<td>Ratio must be between 80-125. Need to pass also on potency corrected data. Add-on studies may be allowed if intra-CV greater than expected</td>
</tr>
<tr>
<td>South Africa [21]</td>
<td>75-133</td>
</tr>
<tr>
<td>South Arabia [12]</td>
<td>80-125</td>
</tr>
<tr>
<td>ASEAN [13]</td>
<td>80-125</td>
</tr>
<tr>
<td>South Korea [14]</td>
<td>80-125</td>
</tr>
<tr>
<td>Mexico [15]</td>
<td>80-125</td>
</tr>
</tbody>
</table>
### BE Acceptance Criteria for special classes of drug

<table>
<thead>
<tr>
<th>Regulatory body</th>
<th>Narrow Therapeutics Drugs (90 % CI log transformed data)</th>
<th>Highly Variable Drugs (90 % CI log transformed data)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$C_{\text{max}}$</td>
<td>$AUC_{0-t}$</td>
</tr>
<tr>
<td>USA [8]</td>
<td>80 - 125</td>
<td>80 - 125</td>
</tr>
<tr>
<td>Europe [9]</td>
<td>90 - 111.11</td>
<td>90 - 111.11</td>
</tr>
<tr>
<td>Japan [10]</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Canada [11]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Saudi Arabia [12]</td>
<td>90 - 111</td>
<td>-</td>
</tr>
<tr>
<td>ASEAN [13]</td>
<td>acceptance interval may need to be tightened</td>
<td>acceptance interval may need to be tightened</td>
</tr>
</tbody>
</table>
1.6 Convulsion

Gabapentin (brand name Neurontin) is a medication originally developed for the treatment of convulsion. Presently, Gabapentin is widely used to relieve pain, especially neuropathic pain. Gabapentin is well tolerated in most patients, has a relatively mild side-effect profile, and passes through the body unmetabolized.

Anticonvulsants were discovered by only observations and serendipity rather than rational and target approach. Control over seizures was the main target of the therapy inspite of safety and tolerability. However, experience with thalidomide in the 1960s brought safety to the fore, resulting in an era of much tighter regulatory control that still persists today. A direct consequence of this was an increased emphasis on the importance of evidence from randomized controlled trials on Anticonvulsants.

Convulsion, a disease that has been in existence for ages, continues to affect approximately 50 million individuals worldwide, including about 2.7 million in the United States [22].

Convulsion is the disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiologic, cognitive, psychological, and social consequences of this condition.

An epileptic seizure is a transient occurrence of signs and/or symptom due to abnormal excessive or synchronous neuronal activity in the brain. Signs and symptoms of seizures may include warnings, such as visual or sensory auras, tingling fingers, altered awareness, and abnormal or convulsive movements.

The patho-physiology underlying the Convulsion process includes mechanisms involved in initiation of seizures (ictogenesis), as well as those involved in transforming the normal brain into a seizure-prone brain (epileptogenesis) [23].
1.6.1 Mechanisms of Ictogenesis

Hyper excitation is the key factor underlying ictogenesis.

![Pathophysiology of Epilepsy: Key Players](image)

**Figure 3: Pathophysiology of Convulsion**

Excessive excitation may originate from individual neurons, the neuronal environment, or neuronal networks.

Excitability from individual neurons may arise from structural or functional changes in the postsynaptic membrane; alterations in the type, number, and distribution of voltage- and ligand-gated ion channels; or biochemical modification of receptors that increase permeability to Ca$^{2+}$, producing development of the prolonged depolarization that precedes seizures.

Excitability arising from the neuronal environment may result from both physiologic and structural changes. Physiologic changes include alterations in concentrations of ions, metabolicalterations, and in neurotransmitter levels. Structural changes affect both neurons and glia.

Alterations in the neuronal network may facilitate excitability through sprouting of the axons of the granule cells of the dentate gyrus or mossy fibers; loss of inhibitory neurons; loss of excitatory neurons needed to activate inhibitory neurons; or changes in neuronal firing properties due to channelopathies.
1.6.2 International Classification of seizures [24]

1. **Generalized Seizures**
   - Absence seizures (formerly called petit mal)
   - Myoclonic seizures
   - Clonic seizures
   - Tonic seizures
   - Tonic clonic seizures (formerly called grand mal seizures; also known as convulsions or convulsive seizures).
   - Atonic seizures (drop attacks or astatic or akinetic seizures)

2. **Partial Seizures**
   - Simple partial seizures (consciousness not impaired)
     1. with motor symptoms
     2. with sensory symptoms
     3. with autonomic symptoms
     4. with psychic symptoms
   - Complex partial seizures (with impaired consciousness)
     1. simple partial seizures followed by impairment of consciousness
     2. with impairment of consciousness at seizure onset
   - Partial seizures evolving to secondarily generalized seizures
     1. simple partial secondarily generalized
     2. complex partial secondarily generalized
     3. simple partial evolving to complex partial evolving to generalized

1.6.3 Types of Seizures

The four main sub-categories of seizures are:

- **Generalized Seizures**, which affect the entire brain
- **Partial Seizures**, which affect a part of the brain
- **Non-Epileptic Seizures**, which aren’t related to epilepsy at all, but are caused by other things, like diabetes, a high fever, or something else entirely
- **Status Epilepticus**, which is a continuing seizure and one of the few reasons emergency personnel should be contacted.
Anticonvulsants are the primary option for the management of epilepsy. Although research over the years has led to significant advances in understanding the pathophysiology of epilepsy, the specific causes of several types of epilepsy are unknown and there remains a great need for research on the neural mechanisms that potentially underlie drug resistance.

1.6.4 Characteristics of Gabapentin [25]

<table>
<thead>
<tr>
<th>Name</th>
<th>Gabapentin</th>
</tr>
</thead>
<tbody>
<tr>
<td>IUPAC Name</td>
<td>2-[1-(aminomethyl)cyclohexyl]acetic acid</td>
</tr>
<tr>
<td>Molecular Structure</td>
<td><img src="image" alt="Molecular Structure" /></td>
</tr>
<tr>
<td>Molecular Formula</td>
<td>C9H17NO2</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>171.237 g/mol</td>
</tr>
<tr>
<td>Physical State</td>
<td>Solid</td>
</tr>
<tr>
<td>Melting point</td>
<td>162-167°C (323.6-332-6°F).</td>
</tr>
<tr>
<td>Water Solubility</td>
<td>4490 mg/L</td>
</tr>
<tr>
<td>pH (2% sol’n/water)</td>
<td>6.5-8.0</td>
</tr>
<tr>
<td>Colour</td>
<td>White-almost white</td>
</tr>
<tr>
<td>log P value</td>
<td>1.4</td>
</tr>
<tr>
<td>CAS Registry Number</td>
<td>60142-96-3</td>
</tr>
<tr>
<td>ATC Code</td>
<td>N03AX12</td>
</tr>
<tr>
<td>Trade Name</td>
<td>Fanatrex, Gabarone, Gralise, Neurontin, Nupentin</td>
</tr>
</tbody>
</table>

1.6.5 Taxonomy of Gabapentin

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Organic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classes</td>
<td>Amino Acids</td>
</tr>
<tr>
<td></td>
<td>Carboxylic Acids and Derivatives</td>
</tr>
<tr>
<td>Substructures</td>
<td>Amino Acids</td>
</tr>
<tr>
<td></td>
<td>Hydroxy Compounds</td>
</tr>
<tr>
<td></td>
<td>Acetates</td>
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<tr>
<td></td>
<td>Aliphatic and Aryl Amines</td>
</tr>
<tr>
<td></td>
<td>Carboxylic Acids and Derivatives</td>
</tr>
</tbody>
</table>
1.6.6 Mechanism of action: [26, 27]

The mechanism by which Gabapentin exerts its analgesic action is unknown, but in animal models of analgesia, Gabapentin prevents allodynia (pain-related behavior in response to a normally innocuous stimulus) and hyperalgesia (exaggerated response to painful stimuli). In particular, Gabapentin prevents pain-related responses in several models of neuropathic pain in rats or mice (e.g., spinal nerve ligation models, streptozocin-induced diabetes model, spinal cord injury model, acute herpes zoster infection model). Gabapentin also decreases pain-related responses after peripheral inflammation (carrageenan footpad test, late phase of formalin test). Gabapentin did not alter immediate pain-related behaviors (rat tail flick test, formalin footpad acute phase, acetic acid abdominal constriction test, footpad heat irradiation test). The relevance of these models to human pain is not known.

The mechanism by which Gabapentin exerts its anticonvulsant action is unknown, but in animal test systems designed to detect anticonvulsant activity, Gabapentin prevents seizures as do other marketed anticonvulsants. Gabapentin exhibits antiseizure activity in mice and rats in both the maximal electroshock and pentylene tetrazole seizure models and other preclinical models (e.g., strains with genetic epilepsy, etc.). The relevance of these models to human epilepsy is not known.

Gabapentin is structurally related to the neurotransmitter GABA (gamma-aminobutyric acid) but it does not modify GABA$_A$ or GABA$_B$ radioligand binding, it is not converted metabolically into GABA or a GABA agonist, and it is not an inhibitor of GABA uptake or degradation. Gabapentin was tested in radioligand binding assays at concentrations up to 100 $\mu$M and did not exhibit affinity for a number of other common receptor sites, including benzodiazepine, glutamate, N-methyl-D-aspartate (NMDA), quisqualate, kainate, strychnine-insensitive or strychnine-sensitive glycine, alpha 1, alpha 2, or beta adrenergic, adenosine A1 or A2, cholinergic muscarinic or nicotinic, dopamine D1 or D2, histamine H1, serotonin S1 or S2, opiate mu, delta or kappa, cannabinoid 1, voltage-sensitive calcium channel sites labeled with nitrendipine or diltiazem, or at voltage-sensitive sodium channel sites labeled.
with batrachotoxinin A 20-alpha-benzoate. Furthermore, Gabapentin did not alter the cellular uptake of dopamine, noradrenaline, or serotonin. In vitro studies with radiolabeled Gabapentin have revealed a Gabapentin binding site in areas of rat brain including neocortex and hippocampus. A high-affinity binding protein in animal brain tissue has been identified as an auxiliary subunit of voltage-activated calcium channels. However, functional correlates of Gabapentin binding, if any, remain to be elucidated.

1.6.7 Pharmacokinetics and drug metabolism: [26, 27]

All pharmacological actions following Gabapentin administration are due to the activity of the parent compound; Gabapentin is not appreciably metabolized in humans.

- **Oral Bioavailability**
  Gabapentin bioavailability is not dose proportional; i.e., as dose is increased, bioavailability decreases. Bioavailability of Gabapentin is approximately 60%, 47%, 34%, 33%, and 27% following 900, 1200, 2400, 3600, and 4800 mg/day given in 3 divided doses, respectively. Food has only a slight effect on the rate and extent of absorption of Gabapentin (14% increase in AUC and C\(_{\text{max}}\)).

- **Distribution**
  Less than 3% of Gabapentin circulates bound to plasma protein. The apparent volume of distribution of Gabapentin after 150 mg intravenous administration is 58±6 L (mean ±SD). In patients with epilepsy, steady-state pre dose (C\(_{\text{min}}\)) concentrations of Gabapentin in cerebrospinal fluid were approximately 20% of the corresponding plasma concentrations.

- **Elimination**
  Gabapentin is eliminated from the systemic circulation by renal excretion as unchanged drug. Gabapentin is not appreciably metabolized in humans.
1.6.8 Indication of Gabapentin: [26, 27]

- **Post herpetic Neuralgia**
  Neurontin (Gabapentin) is indicated for the management of post herpetic neuralgia in adults.

- **Epilepsy**
  Neurontin (Gabapentin) is indicated as adjunctive therapy in the treatment of partial seizures with and without secondary generalization in patients over 12 years of age with epilepsy. Neurontin is also indicated as adjunctive therapy in the treatment of partial seizures in pediatric patients age 3 – 12 years.

- **Dosage and administration: [26, 27]**
  Neurontin is given orally with or without food. Patients should be informed that, should they break the scored 600 or 800 mg tablet in order to administer a half-tablet, they should take the unused half-tablet as the next dose. Half-tablets not used within several days of breaking the scored tablet should be discarded.
  If Neurontin dose is reduced, discontinued, or substituted with an alternative medication, this should be done gradually over a minimum of 1 week (a longer period may be needed at the discretion of the prescriber).

- **Post herpetic Neuralgia**
  In adults with post herpetic neuralgia, Neurontin therapy may be initiated as a single 300-mg dose on Day 1, 600 mg/day on Day 2 (divided BID), and 900 mg/day on Day 3 (divided TID). The dose can subsequently be titrated up as needed for pain relief to a daily dose of 1800 mg (divided TID). In clinical studies, efficacy was demonstrated over a range of doses from 1800 mg/day to 3600 mg/day with comparable effects across the dose range. Additional benefit of using doses greater than 1800 mg/day was not demonstrated.
Epilepsy

Neurontin is recommended for add-on therapy in patients 3 years of age and older. Effectiveness in pediatric patients below the age of 3 years has not been established.

Adverse Events: [26, 27]

The most commonly observed adverse events associated with the use of Neurontin in combination with other antiepileptic drugs in patients >12 years of age, not seen at an equivalent frequency among placebo treated patients, were somnolence, dizziness, ataxia (failure of muscular coordination; irregularity of muscular action), fatigue (physical and/or mental exhaustion that can be triggered by stress) and nystagmus (Rhythmic, oscillating motions of the eyes).

Incidence in Controlled Clinical Trials

<table>
<thead>
<tr>
<th>Body as a whole</th>
<th>Fatigue (physical and/or mental exhaustion that can be triggered by stress), headache, weight increase, back pain, peripheral edema</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nervous system</td>
<td>Somnolence (Drowsiness or sleepiness, particularly in excess), dizziness, ataxia (failure of muscular coordination; irregularity of muscular action), nystagmus (Rhythmic, oscillating motions of the eyes), tremor (an involuntary trembling or quivering), dysarthria (a speech disorder caused by disturbances of muscular control because of damage to the central or peripheral nervous system), amnesia (loss of memory), depression, thinking abnormal, twitching (a series of contractions by small muscle units), abnormal co-ordination</td>
</tr>
<tr>
<td>Digestive System</td>
<td>Dyspepsia (painful, difficult, or disturbed digestion), abdominal pain, dry mouth or throat, constipation (an acute or chronic condition in which bowel movements occur less often than usual or consist of hard, dry stools that are painful or difficult to pass), dental abnormalities, increased appetite</td>
</tr>
<tr>
<td>Special Senses</td>
<td>Diplopia (the perception of two images of a single object), amblyopia (Amblyopia is an uncorrectable decrease in vision in one or both eyes with no apparent structural abnormality seen to explain it)</td>
</tr>
<tr>
<td>---------------------</td>
<td>--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Respiratory System</td>
<td>Rhinitis (inflammation of the mucous lining of the nose), pharyngitis (sore throat; inflammation of the pharynx), coughing</td>
</tr>
<tr>
<td>Skin and Appendages</td>
<td>Pruritus (intense chronic itching in the anal region), abrasion (a rubbing or scraping off through unusual or abnormal action)</td>
</tr>
<tr>
<td>Laboratory Deviations</td>
<td>Decrease in White blood cells</td>
</tr>
<tr>
<td>Urogenital System</td>
<td>Impotence (often called erectile dysfunction, is the inability to achieve or maintain an erection long enough to engage in sexual intercourse)</td>
</tr>
<tr>
<td>Musculoskeletal System</td>
<td>Myalgia (muscular pain), fracture</td>
</tr>
<tr>
<td>Cardiovascular System</td>
<td>Vasodilation (Dilation of a blood vessel, as by the action of a nerve or drug)</td>
</tr>
<tr>
<td>Hematologic &amp; Lymphatic Systems</td>
<td>Leucopenia(Any condition in which the number of leukocytes in the circulating blood is lower than normal)</td>
</tr>
</tbody>
</table>

- **Drug interactions:** [26, 27]

  The drug interaction data described in this section were obtained from studies involving healthy adults and adult patients with epilepsy.

- **Phenytoin:** In a single (400 mg) and multiple dose (400 mg TID) study of Neurontin in epileptic patients (N=8) maintained on phenytoin monotherapy for at least 2 months, Gabapentin had no effect on the steady-state trough plasma concentrations of phenytoin and phenytoin had no effect on Gabapentin pharmacokinetics.

- **Carbamazepine:** Steady-state trough plasma carbamazepine and carbamazepine 10, 11 epoxide concentrations were not affected by concomitant Gabapentin (400 mg TID; N=12)
administration. Likewise, Gabapentin pharmacokinetics were unaltered by carbamazepine administration.

- **Valproic Acid**: The mean steady-state trough serum valproic acid concentrations prior to and during concomitant Gabapentin administration (400 mg TID; N=17) were not different and neither were Gabapentin pharmacokinetic parameters affected by valproic acid.

- **Phenobarbital**: Estimates of steady-state pharmacokinetic parameters for phenobarbital or Gabapentin (300 mg TID; N=12) are identical whether the drugs are administered alone or together.

- **Naproxen**: Co-administration (N=18) of naproxen sodium capsules (250 mg) with Neurontin (125 mg) appears to increase the amount of Gabapentin absorbed by 12% to 15%. Gabapentin had no effect on naproxen pharmacokinetic parameters. These doses are lower than the therapeutic doses for both drugs. The magnitude of interaction within the recommended dose ranges of either drug is not known.

- **Hydrocodone**: Co-administration of Neurontin (125 to 500 mg; N=48) decreases hydrocodone (10 mg; N=50) Cmax and AUC values in a dose-dependent manner relative to administration of hydrocodone alone; Cmax and AUC values are 3% to 4% lower, respectively, after administration of 125 mg Neurontin and 21% to 22% lower, respectively, after administration of 500 mg Neurontin. The mechanism for this interaction is unknown. Hydrocodone increases Gabapentin AUC values by 14%. The magnitude of interaction at other doses is not known.

- **Morphine**: A literature article reported that when a 60 mg controlled-release morphine capsule was administered 2 hours prior to a 600 mg Neurontin capsule (N=12), mean Gabapentin AUC increased by 44% compared to gabapentin administered without morphine. Morphine pharmacokinetic parameter values were not affected by administration of Neurontin 2
hours after morphine. The magnitude of interaction at other doses is not known.

- **Cimetidine:** In the presence of cimetidine at 300 mg QID (N=12), the mean apparent oral clearance of Gabapentin fell by 14% and creatinine clearance fell by 10%. Thus, cimetidine appeared to alter the renal excretion of both gabapentin and creatinine, an endogenous marker of renal function. This small decrease in excretion of Gabapentin by cimetidine is not expected to be of clinical importance. The effect of Gabapentin on cimetidine was not evaluated.

- **Oral Contraceptive:** Based on AUC and half-life, multiple-dose pharmacokinetic profiles of norethindrone and ethinyl estradiol following administration of tablets containing 2.5 mg of norethindrone acetate and 50 mcg of ethinyl estradiol were similar with and without coadministration of gabapentin (400 mg TID; N=13). The C_{max} of norethindrone was 13% higher when it was coadministered with Gabapentin; this interaction is not expected to be of clinical importance.

- **Antacid (Maalox®):** Maalox reduced the bioavailability of Gabapentin (N=16) by about 20%. This decrease in bioavailability was about 5% when Gabapentin was administered 2 hours after Maalox. It is recommended that Gabapentin be taken at least 2 hours following Maalox administration.

- **Effect of Probenecid:** Probenecid is a blocker of renal tubular secretion. Gabapentin pharmacokinetic parameters without and with probenecid were comparable. This indicates that Gabapentin does not undergo renal tubular secretion by the pathway that is blocked by probenecid.
1.7 Bioanalysis

Increasing knowledge of the working mechanisms of drugs has led to the development of very potent drugs. Hence, the administered dosages are small, and consequently, the concentration levels in biological fluids are decreasing. Furthermore, biological samples are very complex, because they contain many endogenous substances. Blood fluids, such as serum and plasma, represent an extra problem due to the presence of proteins. Protein binding may affect the extractability of the analytes. Deproteinisation techniques can help to overcome this problem. It may, however, also give rise to even more difficulties, since analytes can be co-precipitated with the proteins. Thus, sample pretreatment techniques are required that retain the analyte(s) of interest, at the same time efficiently removing the endogenous interferences. This has led to the development of “Bioanalysis” [28].

The determination of drugs and related substances in biological samples such as whole blood, plasma, serum, tissues and cells is referred to as bioanalysis. This term was coined in the 1970s in relation to various techniques designed for the study of pharmacokinetics of drugs [29, 30]. Bioanalysis is routinely carried out in forensic medicine and for toxicological studies. However, prior to the analytical determination, the analytes of interest are first subjected to a suitable sample preparation procedure [31]. Currently several techniques are available for extraction of analyte from the biological matrices like; solid phase extraction (SPE), liquid-liquid extraction (LLE) and protein precipitation (PPT) [32, 33].

1.8 Methods of Extraction [34]:

The aim of the sample preparation process is to provide a suitable sample, usually for chromatographic analysis, which will not contaminate the instrumentation and where the concentration in the prepared sample is reflective of that found in the original. The method of sample preparation selected is generally dictated by the analytical technique available and the physical characteristics of the analytes under investigation. The two main sample preparation methods are matrix cleanup or direct injection. In a matrix
cleanup procedure, the aim is to remove as much endogenous material as possible from the drug sample.

Sample preparation is traditionally carried out (a) by liquid-liquid extraction, (b) solid-phase extraction and (c) by precipitation of the plasma proteins, while the final analysis in most cases is accomplished by liquid chromatography interfaced with mass spectrometry or tandem mass spectrometry or capillary gas chromatography.

1.9 Sample Preparation

Sample preparation is necessary for at least two reasons:

a. To remove as many of the endogenous interferences from the analyte as possible.

b. To enrich the sample with respect to the analyte, thus maximizing the sensitivity of the system.

It also serves to ensure that the injection matrix is compatible with the selected column and mobile phase.

Two of the major goals of any sample pretreatment procedure are:

- Quantitative recovery
- A minimum number of steps.

Successful sample preparation has a threefold objective.

- In solution
- Free from interfering matrix elements
- At a concentration appropriate for detection and measurement

The most common approach in analyte separation involves a two phase system where the analyte and interferences are distributed between the two phases. Distribution is an equilibrium process and is reversible. If the sample is distributed between two immiscible liquid phase, the techniques is called liquid-liquid extraction. If the sample is distributed between a solid and a liquid phase, the technique is called liquid-solid adsorption. Often, when analysis involves the measurement of trace amounts of a substance, it is
desirable to increase the concentration of the analyte to a level where it can be measured more easily. Concentration of an analyte can be accomplished by transferring it from a large volume of phase to a smaller volume of phase. Separation can be carried out in a single batch, in multiple batches or by continuous operation.

1.9.1 Liquid-Liquid Extraction Method [35, 36, 37, 38]:

Liquid–liquid extraction was one of the first sample preparation techniques and continues to be widely used for biological sample analysis. Liquid-liquid extraction (LLE), also called solvent extraction technique. Liquid-liquid extraction is useful for separating interferences by partitioning the sample between two immiscible liquids or phases. One phase in LLE often is aqueous and second phase an organic solvent. It is based on the principles of differential solubility and partitioning equilibrium of analyte molecules between aqueous (the original sample) and the organic phases. With proper selection of organic solvent and adjustment of sample pH, very clean extracts can be obtained with good selectivity for the target analytes. Inorganic salts are insoluble in the solvents commonly used for LLE and remain behind in the aqueous phase along with proteins and water-soluble endogenous components. These interferences are excluded from the chromatographic system and a cleaner sample is prepared for analysis.

- **Advantages:**
  - Widely applicable for many drug compounds and is a relatively inexpensive procedure.
  - Clean extracts obtained
  - Greater sample capacity.
  - The technique is simple, rapid and has relatively less cost per sample.

- **Disadvantages:**
  - pH control of the sample is necessary for extraction.
  - During evaporation, since the temperature is increased, the method cannot be used for thermolabile substances.
- Because of its labor-intensive nature with several disjointed vortex mix and centrifugation steps required.
- Organic solvents used are volatile and present hazards to worker safety.
- Emulsions can be formed without warning and can result in loss of sample.
- Unsuitable for hydrophilic compounds.

Liquid-liquid extraction has been demonstrated in collection microplates to provide for high throughput sample preparation. The use of liquid handling workstations reduces the hands-on analyst time required for this technique and offers semi-automation to an otherwise labor-intensive task. Now-a-days traditional LLE has been replaced with advanced and improved techniques like liquid phase microextraction (LPME), single drop-liquid phase microextraction (DLPME) and supported membrane extraction (SME).

1.9.2 Protein Precipitation Technique [33, 35, 36, 37, 38]:

Protein precipitation is a traditional sample preparation technique for the treatment of plasma. Sample matrices for bioanalysis almost always contain some amount of protein, along with other endogenous macromolecules, small molecules, metabolic byproducts, salts and possibly co-administered drugs. These components must be removed from the sample before analysis in order to attain a selective technique for the desired analytes. Therefore it is most important to remove the protein from a biological sample because that protein, when injected into a chromatographic system, will precipitate upon contact with the organic solvents and buffer salts commonly used in mobile phases. The precipitated mass of protein builds up within the column inlet. The result is reduction of column lifetime and an increase in system backpressure. When protein is carried through the analytical system it may reach the mass spectrometer and foul the interface, requiring cleaning. A common approach for removing protein from the injected sample, one that is amenable to high throughput applications in microplates, is precipitation using organic solvents, ionic salts and/or inorganic acids. The precipitated mass can be separated by
either centrifugation or filtration; analysis of the supernatant or filtrate, respectively, is then performed.

**Method of protein precipitation:**

1. **By changing the pH of sample:** At iso-electric pH, i.e., a the net charge goes to zero the proteins which are in their cationic form at low pH form insoluble salts in the acid.

2. **By Addition of organic solvents:** It decreases the dielectric constant of the medium, leads to insolubility thus cause precipitation, or high affinity for the hydrophobic surfaces of the protein leads to denaturing of protein. E.g. Methanol, Acetonitrile.

3. **Salt induced precipitation:** Alts used for precipitation of proteins are citrates, phosphates, acetates etc. Using high concentration of salts solubility of proteins can be decreased thus, they are precipitated.

- **Advantages:**
  - Simple, universal and inexpensive.
  - Can be automated with micro plates.
  - Less time consuming, smaller amounts of organic modifier or other solvents are used.
  - Method can be applied for extraction of both hydrophobic and hydrophilic substances.

- **Disadvantages:**
  - The samples often contain protein residues and it is a non-selective sample cleanup method, there is a risk that endogenous compounds or other drugs may interfere in the LC-system.
  - Endogenous substances may lead to ion suppression.

1.9.3 **Solid phase Extraction Technique [35, 37, 38]:**

Solid-phase extraction (SPE) is a procedure in which an analyte, contained in a liquid phase, comes in contact with a solid phase (sorbent particles in a column or disk) and is selectively adsorbed onto the surface of that solid phase. All other materials not adsorbed by chemical attraction or affinity
remain in the liquid phase and go to waste. SPE is unequivocally the leading sample preparation method used in routine bio-analytical laboratories.

It contains the following steps:

- **Conditioning:** The column is activated with an organic solvent that acts as a wetting agent on the packing material and solvates the functional groups of the sorbent. Water or aqueous buffer is added to activate the column for proper adsorption mechanisms.
- **Sample loading:** After adjustment of pH, the sample is loaded on the column by gravity feed, pumping or aspirating by vacuum.
- **Washing:** Interferences from the matrix are removed while retaining the analyte.
- **Elution:** Disruption of analyte-sorbent interaction by appropriate solvent, removing as little of the remaining interferences as possible.

SPE is chiefly used to prepare liquid samples and extracts of semi-volatile or non-volatile analytes, but may also be used for solids pre-extracted into solvents. The choice of sorbent is the key factor in SPE, because this can control parameters such as selectivity, affinity and capacity. This choice depends strongly on the analytes and their physically chemical properties, which should define the interactions with the chosen sorbent. However, results also depend on the kind of sample matrix and interactions with both the sorbent and the analyte. SPE sorbents range from chemically bonded silica of the C8 and C18 organic group, grafitized carbon and ion-exchange materials up to polymeric materials (PS-DVB, cross-linked styrene-divinylbenzene, PMA, cross-linked methacrylate, MA-DVB and many others), mixed-mode sorbents (containing both non-polar and strong cation or anion), immunosorbents, molecularly imprinted polymers as well as restricted access materials and also recently developed monolith sorbents. Silica sorbents have several disadvantages as compared with polymeric sorbents. Silica sorbents are unstable in a broader pH range and contain silanols, which can cause the irreversible binding of some groups of compounds, e.g., tetracyclines.
Advantages:
- Low concentration of drug can be detected.
- Effective in selective removal of interferences.
- Different types of adsorbents can be used.
- Extending the analytical column life, reduced system maintenance, minimizing ion suppression, high recovery, effective pre-concentration, the need for less organic solvent (compared to LLE), no foaming in the formation of emulsions, ease of operation and greater possibility of automation.

Disadvantages:
- Extraction is difficult for high-density materials.
- In extraction processes a number of steps are to be carried out making it a time consuming process.
- Expensive method.

1.10 Bioanalytical Method Development
Bioanalytical method development can be viewed as a science, an art, a fundamental part of the high-stakes game of pharmaceutical development or an admixture of the above. Specifications can change during development; the latter stages of method development may inconveniently unearth new interferences; clinical samples may show unexpected variation; there may be conflicts with other timelines [39].

Nonclinical and clinical bioanalytical method development activities should be adequately documented to support a reproducible method document for validation. Method development is intended to define the method and provide sound scientific evidence for method design and suitability for its intended purpose [40].

Method development involves evaluation and optimisation of the various stages of sample preparation, chromatographic separation, detection and quantitation [41].
Table 1: An Overview of Method Development Exercise

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyte</td>
<td>Structure</td>
</tr>
<tr>
<td></td>
<td>Properties</td>
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<tr>
<td>Method Optimization</td>
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<tr>
<td>QC Preparation as per Calibration range</td>
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<tr>
<td>Evaluation of Extraction strategies</td>
<td>Phospholipids impact evaluation</td>
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<td>Initial Carryover Evaluation</td>
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<td>Post-column infusion experiment</td>
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<td>Mock Validation Run</td>
<td>Accuracy and Precision</td>
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<td>Carryover</td>
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<td>Freeze / Thaw Stability</td>
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<td>Bench Top (pre-processed) Stability</td>
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<td>Selectivity</td>
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1.10.1 Aspects for Bioanalytical Method development:

- Carryover should be addressed and minimized during method development.” If carryover is inevitable or unavoidable, it should be noted in the method and a non-randomized sequence should be used with extra blanks inserted after the high calibration standard and high quality control (QC) injections.
- Precautions should be taken to eliminate any possibility of contamination of the mobile phases, diluents, wash buffers, stocks, and working stocks.
• Analyte stability (freeze/thaw and use of stabilizer agents) should be assessed during method development to help avoid delays in validation activities due to QC (analyte in matrix) stability issues.

• Selection of the anticoagulant should remain consistent throughout the entire toxicology program (nonclinical and clinical). If the anticoagulant or counter ion is changed for a PK/TK study, at a minimum an equivalency experiment must be performed to show that the existing method can be used for the new anticoagulant. Additional proof of stability may also be necessary.

• Whole blood stability and collection process stability are not often performed as a method development activity; however, assessment may provide valuable information for both nonclinical and clinical studies. The analyte may adsorb to cellular or proteinaceous components during the time period between collection and sample processing.

• If metabolites are known and standards are available, then an evaluation of metabolite impact on quantitation of analyte is required.

• During method development, phospholipids removal should be evaluated and the impact on the assay mitigated by using different sample preparation techniques, different chromatographic gradients, or by employing a diversion valve to shunt the phospholipids to waste before they enter the HPLC column.

• During method development, specific instrument parameters must be clearly defined to ensure a consistent parameter approach can be set and remain fixed during validation: mobile phase composition, gradient profile, ionization mode, HPLC column identity. Minor parameters may be optimized to account for instrument-to-instrument variability and improve response (i.e., voltage and gas settings, precursor and product ion mass adjustment to optimize mass center).

1.10.2 Use of Internal Standards in method development:

To improve precision and accuracy in chromatographic Bioanalytical methods an internal standard is usually added to the samples prior to the sample work-up [42]. An internal standard in analytical chemistry is a chemical substance
that is added in a constant amount to samples, the blank and calibration standards in a chemical analysis. This substance can be used for calibration by plotting the ratio of the analyte signal to the internal standard signal as a function of the analyte standard concentration. The internal standard is a compound that must be show similar behavior to the analyte. This ratio for the samples is then used to obtain their analyte concentrations from a calibration curve. The internal standard used needs to provide a signal that is similar to the analyte signal in most ways but sufficiently different so that the two signals are readily distinguishable by the instrument.

Internal standards are commonly used in LC–MS based quantitative bioanalysis [43]. The main purpose of utilizing internal standards is to correct any variation other than that related to the amount of the analyte present in a sample, such as variability in dilution, evaporation, degradation, recovery, adsorption, derivatization, injection, and detection. Hence, an internal standard (IS) should have the same or very similar physico-chemical properties as its analyte, which means that they usually have similar molecular weights and synthesizing routes for their reference standards. Therefore, cross contributions in mass spectrometry (MS) responses are very common between an analyte and its IS due to chemical impurities and/or isotopic interferences [42, 44].
1.11 Bioanalytical Method Validation

Compliance with good laboratory practices (GLPs) for conducting sample analysis of nonclinical (also known as preclinical) laboratory studies and clinical studies is intended to ensure the quality and integrity of the safety data filed in support of investigational new drug applications (INDs), new drug applications (NDAs), abbreviated new drug applications (ANDAs), supplements in developing bioanalytical method validation information used in human clinical pharmacology, bioavailability (BA), and bioequivalence (BE) studies requiring pharmacokinetic (PK) evaluation [45-51].

Method validation is a process that demonstrates that a method will successfully meet or exceed the minimum standards recommended in the guidelines. 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 parameter for this validation includes [45]:

- Accuracy
- Precision
- Selectivity
- Sensitivity
- Reproducibility
- 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.
1.12 Types of Validation

- **Full 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.

- **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. Typical bioanalytical method changes that fall into this category include, but are not limited to:
  - 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 medication.
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 analyses 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 inter laboratory reliability. Cross-validation should also be considered when data generated using different analytical techniques (e.g., LC-MS-MS vs. ELISA4) in different studies are included in a regulatory submission.

1.13 Process for Bioanalytical Method Validation

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.
3. Application of validated Bioanalytical method to routine drug analysis and acceptance criteria for the analytical run and/or batch.

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 non-commercial establishment. The source and lot number, expiration date, certificates of analyses when available, and/or internally or externally generated evidence of identity and purity should be furnished for each reference standard.

- **Method Development: Chemical Assay**

  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.

- **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.
• **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.

- **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.

1. **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%.
2. Calibration standard/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 standards 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.

- 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.
• **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.

• **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.

• **Long Term Stability:**
  The storage time in a long-term stability evaluation should exceed the 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 from the first day of long-term stability testing.

• **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.

- **Post-Preparative Stability:**
  The stability of processed samples, including the resident time in the auto sampler, 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 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.

- **Application of Validated Method to Routine Drug Analysis:**
  Assays of all samples of an analyte in a biological matrix should be completed within the time period for which stability data are available. In general, biological samples can be analyzed with a single determination without duplicate or replicate analysis if the assay method has acceptable variability as defined by validation data. This is true for procedures where precision and accuracy variabilities routinely fall within acceptable tolerance limits. For a difficult procedure with a labile analyte where high precision and accuracy specifications may be difficult to achieve, duplicate or even triplicate analyses can be performed for a better estimate of analyte.
  A calibration curve should be generated for each analyte to assay samples in each analytical run and should be used to calculate the concentration of the analyte in the unknown samples in the run. The spiked samples can contain more than one analyte. An analytical run can consist of QC samples, calibration standards, and either (1) all the processed samples to be analyzed as one batch or (2) a batch composed of processed unknown samples of one or more volunteers in a study. The calibration (standard) curve should cover the
expected unknown sample concentration range in addition to a calibrator sample at LLOQ. Estimation of concentration in unknown samples by extrapolation of standard curves below LLOQ or above the highest standard is not recommended. Instead, the standard curve should be redefined or samples with higher concentration should be diluted and reassayed. It is preferable to analyze all study samples from a subject in a single run.

Once the analytical method has been validated for routine use, its accuracy and precision should be monitored regularly to ensure that the method continues to perform satisfactorily. To achieve this objective, a number of QC samples prepared separately should be analyzed with processed test samples at intervals based on the total number of samples. The QC samples in duplicate at three concentrations (one near the LLOQ (i.e., 3 x LLOQ), one in midrange, and one close to the high end of the range) should be incorporated in each assay run. The number of QC samples (in multiples of three) will depend on the total number of samples in the run. The results of the QC samples provide the basis of accepting or rejecting the run. At least four of every six QC samples should be within 15% of their respective nominal value. Two of the six QC samples may be outside the 15% of their respective nominal value, but not both at the same concentration.

Acceptance Criteria for the Run

The following acceptance criteria should be considered for accepting the analytical run:

- Standards and QC samples can be prepared from the same spiking stock solution, provided the solution stability and accuracy have been verified. A single source of matrix may also be used, provided selectivity has been verified.
- Standard curve samples, blanks, QCs, and study samples can be arranged as considered appropriate within the run.
- Placement of standards and QC samples within a run should be designed to detect assay drift over the run.
- Matrix-based standard calibration samples: 75%, or a minimum of six standards, when back-calculated (including ULOQ)
should fall within ±15%, except for LLOQ, when it should be ±20% of the nominal value. Values falling outside these limits can be discarded, provided they do not change the established model.

- Quality Control Samples: Quality control samples replicated (at least once) at a minimum of three concentrations (one within 3x of the LLOQ (low QC), one in the midrange (middle QC), and one approaching the high end of the range (high QC)) should be incorporated into each run. The results of the QC samples provide the basis of accepting or rejecting the run.

- At least 67% (four out of six) of the QC samples should be within 15% of their respective nominal (theoretical) values; 33% of the QC samples (not all replicates at the same concentration) can be outside the ±15% of the nominal value. A confidence interval approach yielding comparable accuracy and precision is an appropriate alternative.

- The minimum number of samples (in multiples of three) should be at least 5% of the number of unknown samples or six total QCs, whichever is greater.

- Samples involving multiple analytes should not be rejected based on the data from one analyte failing the acceptance criteria.
1.14 Combined Liquid Chromatography/Mass Spectrometry [52, 53, 54, 55]:

The use of high-performance liquid chromatography combined with mass spectrometry (HPLC–MS) or tandem mass spectrometry (HPLC–MS–MS) has proven to be the analytical technique of choice for most assays used in various stages of new drug discovery [28]. Standard techniques of analyte detection in clinical chemistry rely on indirect characteristics of an analyte, e.g. its absorption of light, chemical reactivity or physical interaction with macromolecules. In mass spectrometric methods, in contrast, analytes are detected directly from molecular characteristics as molecular mass and molecular disintegration patterns. In particular tandem mass spectrometry (MS/MS) instruments hyphenated to liquid chromatography (LC) systems used for sample introduction and pre-fractionation have been implemented in a constantly growing number of clinical laboratories worldwide now. These instruments are mainly used for small molecule analyses in neonatal screening of inborn diseases of metabolism and therapeutic drug monitoring but also in endocrinology and toxicology, while applications for peptide and protein quantification are not yet used in routine laboratories.

LC-MS/MS is attractive for laboratory medicine for three main reasons.

- The development of new methods is in general straightforward and independent from the diagnostic industry, without the need e.g. to develop analytical antibodies
- Highly multiplexed analyses are feasible with very low current costs; the range of potential analytes is practically unlimited; individual “metabolomic analyses” addressing hundreds of analytes from different biochemical pathways and from different chemical classes are possible, as well as a comprehensive and individual description of xenobiotics (“xenobiom”).
- When applying the principle of isotope dilution internal standardization, analyses on a reference method-level of accuracy can be performed in a routine laboratory setting.
Most of the developments in LC-MS/MS automation originated from applications in the pharmaceutical industry. In this field, LC-MS/MS has become a key technology for pharmacokinetic studies and on all levels of drug development. Typically large series of samples have to be analyzed for single drug candidates on a good laboratory practice (GLP) level. Consequently, automation in pharmaceutical industry targets extended batch analyses. In pharmaceutical research laboratories but also in environmental and food testing laboratories typically highly trained technicians are available and the number of different analytical technologies is small. In clinical laboratories, in contrast, the availability of skilled technicians is increasingly a critically limited resource in many countries. Consequently, the need for automation is far more pronounced in medical diagnostics application of LC-MS/MS when compared to “traditional” main areas of application of this technology.

![Diagram of LC-MS System](image)

**Figure 4: Elements of LC-MS System**

**1.14.1 Principle of LC-MS/MS:**

LC/MS is a hyphenated technique, combining the separation power of HPLC, with the detection power of mass spectrometry. Even with a very sophisticated MS instrument, HPLC is still useful to remove the interferences from the sample that would impact the ionization. The mass spectrometer is an instrument designed to separate gas phase ions according to their m/z (mass to charge ratio) value. The "heart" of the mass spectrometer is the analyzer. This element separates the gas phase ions.

The analyzer uses electrical or magnetic fields, or combination of both, to move the ions from the region where they are produced, to a detector, where they produce a signal which is amplified. Since the motion and separation of ions is based on electrical or magnetic fields, it is the mass to charge ratio, and
not only the mass, which is of importance. The analyzer is operated under high vacuum, so that the ions can travel to the detector with a sufficient yield.

1.14.2 LC-MS/MS Instrumentation:
Mass spectrometers work by ionizing molecules and then sorting and identifying the ions according to their mass-to-charge (m/z) ratios. Two key components in this process are the ion source, which generates the ions, and the mass analyzer, which sorts the ions. Several different types of ion sources are commonly used for LC/MS. Each is suitable for different classes of compounds. Several different types of mass analyzers are also used. Each has advantages and disadvantages depending on the type of information needed.

1.14.3 Ion sources:
- Atmospheric Pressure Ionization:
  Atmospheric pressure ionization (API) techniques are soft ionization processes well suited for the analysis of large and small, polar and non-polar, labile compounds. These techniques can be used to rapidly confirm the identity of a wide range of volatile and non-volatile compounds by providing sensitive and accurate molecular weight and fragmentation information. API techniques can be used in metabolite confirmation analysis of most pharmaceutical compounds, and other applications.

Common atmospheric pressure ionization techniques are:
- Electrospray ionization (ESI)
- Atmospheric pressure chemical ionization (APCI)
- Atmospheric pressure photoionization (APPI)
Electro spray ionization (ESI):

Electrospray relies in part on chemistry to generate analyte ions in solution before the analyte reaches the mass spectrometer. The LC eluent is sprayed (nebulized) into a chamber at atmospheric pressure in the presence of a strong electrostatic field and heated drying gas. The electrostatic field causes further dissociation of the analyte molecules. The heated drying gas causes the solvent in the droplets to evaporate. As the droplets shrink, the charge concentration in the droplets increases. Eventually, the repulsive force between ions with like charges exceeds the cohesive forces and ions are ejected (desorbed) into the gas phase. These ions are attracted to and pass through a capillary sampling orifice into the mass analyzer. Some gas-phase reactions, mostly proton transfer and charge exchange, can also occur between the time ions are ejected from the droplets and the time they reach the mass analyzer. Large molecules often acquire more than one charge. Thanks to this multiple charging, electrospray can be used to analyze molecules as large as 150,000 u even though the mass range (or more accurately mass-to-charge range) for a typical LC/MS instruments is around 3000 m/z. For example: 100,000 u / 10 z = 1,000 m/z When a large molecule acquires many charges, a mathematical process.
called deconvolution is often used to determine the actual molecular weight of the analyte.

![Figure 6: Electro spray ion source](image1)

![Figure 7: Desorption of ions from solution](image2)

- **Atmospheric Pressure Chemical Ionization (APCI):**
  In APCI, the LC eluent is sprayed through a heated (typically 250°C – 400°C) vaporizer at atmospheric pressure. The heat vaporizes the liquid. The resulting gas-phase solvent molecules are ionized by electrons discharged from a corona needle. The solvent ions then transfer charge to the analyte molecules through chemical reactions (chemical ionization). The analyte ions pass through a capillary sampling orifice into the mass analyzer. APCI is applicable to a wide range of polar and non-polar molecules. It rarely results in multiple charging so it is typically used for molecules less than 1,500 u. Due to this, and
because it involves high temperatures, APCI is less well-suited than electrospray for analysis of large biomolecules that may be thermally unstable. APCI is used with normal-phase chromatography more often than electrospray is because the analytes are usually non-polar.

![APCI ion source](image)

**Figure 8: APCI ion source**

- **Atmospheric Pressure Photo ionization (APPI):**

  Atmospheric pressure photoionization (APPI) for LC/MS is a relatively new technique. As in APCI, a vaporizer converts the LC eluent to the gas phase. A discharge lamp generates photons in a narrow range of ionization energies. The range of energies is carefully chosen to ionize as many analyte molecules as possible while minimizing the ionization of solvent molecules. The resulting ions pass through a capillary sampling orifice into the mass analyzer. APPI is applicable to many of the same compounds that are typically analyzed by APCI. It shows particular promise in two applications, highly non-polar compounds and low flow rates (<100 l/min), where APCI sensitivity is sometimes reduced. In all cases, the nature of the analyte(s) and the separation conditions has a strong influence on which ionization technique:
electrospray, APCI, or APPI, will generate the best results. The most effective technique is not always easy to predict.

![Diagram of APPI ion source](image)

**Figure 9: APPI ion source**

### 1.14.4 Mass Analyzer:

For LC-MS four types of mass analyzers are there:

1. Quadrupole
2. Time-of-flight
3. Ion trap
4. Fourier transform-ion cyclotron resonance (FT-ICR or FT-MS)

- **Quadrupole:**

  A quadrupole mass analyzer consists of four parallel rods arranged in a square. The analyte ions are directed down the center of the square. Voltages applied to the rods generate electromagnetic fields. These fields determine which mass-to-charge ratio of ions can pass through the filter at a given time. Quadrupoles tend to be the simplest and least expensive mass analyzers. Quadrupole mass analyzers can operate in two modes:
  - Scanning (scan) mode
  - Selected ion monitoring (SIM) mode
Figure 10: Quadrapole Mass Analyzer

Figure 11: Scan and SIM data acquisition
• **Scan Mode:**
  In scan mode, the mass analyzer monitors a range of mass-to-charge ratios. In the scan mode, the instrument detects signals over a mass range (e.g. from 50–2000 m/z) during a short period of time (e.g. 2 sec). During this scan period, the MS electronics sequentially read the signals detected within narrower mass intervals until the full mass range is covered. The spectra that are stored represent the detected signal for the full mass range. Since full mass spectra are recorded, this mode of operation is typically selected for qualitative analysis, or for quantitation when all analyte masses are not known in advance. Samples may be introduced into a mass spectrometer by infusion or through an HPLC. In the latter, it is important to match the peak width and the scan range. The narrower the peaks, the shorter the total scan time must be in order to get proper peak definition. In order to get a short total scan time, it may be necessary to reduce the scan range.

• **SIM Mode:**
  Mass spectrometers can also operate in the selected ion monitoring (SIM) mode. Rather than scanning continuously, they can be set to only monitor a few mass-to-charge ratios (m/z). As a result the quadrupole is able to spend significantly more time sampling each of the m/z values, with a concomitant and large increase in sensitivity. Moreover, because the cycle time between data points is often shorter than it is in scan mode, quantitative precision and accuracy are improved through optimal peak-shape profiling. Since the m/z values to be sampled must be set in advance, SIM is most often used for target compound analysis. For analyses consisting of multiple target compounds, SIM ion sampling choices can be time programmed to match compound elution time windows. No data is collected at m/z values other than those specifically sampled, so SIM is rarely used in qualitative analysis.

• **Time of Flight:**
  In a time-of-flight (TOF) mass analyzer, a uniform electromagnetic force is applied to all ions at the same time, causing them to accelerate down a flight tube. Lighter ions travel faster and arrive at the detector first, so the mass-to-
charge ratios of the ions are determined by their arrival times. Time-off light mass analyzers have a wide mass range and can be very accurate in their mass measurements.

Figure 12: Time of Flight Mass analyzer
Ion Trap:
An ion trap mass analyzer consists of a circular ring electrode plus two end caps that together form a chamber. Ions entering the chamber are “trapped” there by electromagnetic fields. Another field can be applied to selectively eject ions from the trap. Ion traps have the advantage of being able to perform multiple stages of mass spectrometry without additional mass analyzers.

![Ion Trap Mass Analyzer](image)

Figure 13: Ion Trap Mass Analyzer

Fourier Transform-ion Cyclotron Resonance (FT-ICR):
An FT-ICR mass analyzer (also called FT-MS) is another type of trapping analyzer. Ions entering a chamber are trapped in circular orbits by powerful electrical and magnetic fields. When excited by a radio-frequency (RF) electrical field, the ions generate a time dependent current. This current is converted by Fourier transform into orbital frequencies of the ions which correspond to their mass-to-charge ratios. Like ion traps, FT-ICR mass analyzers can perform multiple stages of mass spectrometry without additional mass analyzers. They also have a wide mass range and excellent mass resolution. They are, however, the most expensive of the mass analyzers.
1.15 Statistics [56]

Pharmacokinetic measures assessed by bioequivalence derive directly from drug concentration curve along time, which is characterized by the quantification of a given number of biological samples related to previously established collection times.

The first and most important measure assessed is the area under the curve of the drug plasma concentration versus time, often used to measure absorption length or the total amount of drug absorbed by the body after a single-dose administration of a medication. The determination of bioequivalence between two medications results from the comparison of AUC’s obtained from the experiment. By its mathematical representation:

\[ \text{AUC} = \frac{D}{K_e \cdot V_D} \]

The area under the curve of concentration versus time (AUC) may also be extrapolated and calculated from zero time up to the time related to the complete elimination of the drug. This measure is referred to in literature as the area under the curve from zero time to infinite. The additional portion is expressed by a relationship between the last concentration measured \( C_k \) and the drug elimination speed constant \( K_e \). The elimination constant is calculated for each volunteer as the slope coefficient of regression line adjusted for the 4 to 6 last values converted to \( \log_{10} \), multiplied by \(-2.303\). The area under the curve from zero to infinite is obtained as follows:

\[ \text{AUC}_{\infty} = \text{AUC}_t + \frac{C_k}{K_e} \]

1.15.1 Methods of bioequivalence analysis

Bioequivalence analysis between two reference (R) and test (T) formulations has been carried out based on the pharmacokinetic measures by considering summary measures of longitudinal data i.e. mean, individual, and population bioequivalence. The first one considers analysis by focusing R and T formulation means, whereas the last ones consider the existing variability of pharmacokinetic measures. The more commonly used method is “Average bioequivalence”.

The choice of “Designs and Statistical Methods” for data analysis are two most important aspects in a bioequivalence study. The most used experimental planning in bioavailability / bioequivalence assays is the crossover design.
The concepts of elimination period (washout) and carryover effects (carryover effects) in a crossover design have a great impact on the bioavailability statistical inference between formulations. The crossover experiment shall be used when there is no carryover effect in the treatments.

Crossover is the planning of modified randomized blocks in which each block receives more than a formulation of a same drug in different periods. A block may be an individual or a group of individuals. Individuals in each block receive a different sequence of formulations.

➢ Advantage of cross over design

- Each individual serves as his / her own control, which allows for a comparison of the individual with himself / herself for the different formulations.
- Inter-individual variability is removed from the comparison between formulations, which generally makes the test of treatment difference more powerful.
- With an appropriate individual randomization for the sequence of formulation administrations, planning produces the best non-biased estimations for the difference (or ratio) between formulations.

➢ Types of Designs:

- 2×2 cross over design

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- Replicated cross over design
  - 4 (Sequence) × 2 (Period) cross over design (Balaam’s Design)

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- 2 (Sequence) × 3 (Period) cross over design

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• Cross over design for three products (T1, T2 & T3) (Williams’ design) [57]

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➢ Crossover design for four medications (Williams’ design)

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1.16 Need and Objective

- Pharmaceutical companies face a unique challenge in developing their innovative drugs due to the research and development costs incurred in bringing the innovative drugs to the market. The current estimate of the average cost for a pharmaceutical company to bring an innovative drug to the market is nearly $800 million to $1 billion. Even with this enormous investment, there is no assurance that the innovative drugs will achieve market exclusivity and profitability. Consequently, it is crucial for the innovative drug maker to have in place effective patent protection for the innovative drug. Although a full term for a U.S. patent is twenty years, pharmaceutical companies often file for their patent applications during early stages of drug discovery and development. As a result, the effective patent term at the time of drug launch is significantly reduced. In order to recoup the R & D investments and achieve profitability from selling an innovative drug, pharmaceutical companies often set high drug prices, which results in a lack of affordable medicines for customers in need. In US Congress passed the Drug Price Competition and Patent Term Restoration Act of 1984, commonly called “the Hatch-Waxman Act,” to balance the needs of innovative and generic drug manufacturers (Certain provisions of the Hatch-Waxman Act were amended in 2003 in the Medicare Modernization Act to address concerns arising from applying the Act to the generic drug approval.). On one hand, the innovative drug manufacturers seeking regulatory approval of new drugs were given greater patent protection in the face of expensive and time-consuming regulatory hurdles. On the other hand, the generic drug manufacturers were given an abbreviated, less expensive regulatory approval process for generic versions of innovative drugs, as well as incentives to challenge the patent protection of the innovative drugs. Thus, the generic drug manufacturers got faster entry into the market for the generic version of innovative drugs. This abbreviated drug approval process, known as “Abbreviated New Drug Application (ANDA),” did not require the generic drug manufacturers to conduct independent safety and efficacy studies for the generic drug. Instead, the generic drug manufacturers can rely on the previously submitted safety and efficacy data by the innovative drug makers. Generic companies are only
required to demonstrate bioequivalence, i.e., the generic drug has the same active ingredient, the same basic pharmacokinetics.

- Gabapentin is novel antiepileptic agents and one of the first compounds to emerge from this era was Gabapentin. To understand the safety and efficacy of Gabapentin, bioequivalence study in health volunteer is required. Gabapentin has since gained world-wide recognition, not just for its antiepileptic properties, but also its efficacy in the management of chronic pain syndromes, especially neuropathic pain. Although the anti-spastic effects of Gabapentin proved to be modest, the drug demonstrated considerable efficacy in a range of experimental seizure model. Gabapentin (brand name Neurontin) is a medication originally developed for the treatment of epilepsy. Presently, Gabapentin is widely used to relieve pain, especially neuropathic pain. Gabapentin is well tolerated in most patients, has a relatively mild side-effect profile, and passes through the body un metabolized.

- The Objective of this study was to compare the rate and extent of absorption of Gabapentin capsules USP 400 mg of Alkem Laboratories Limited, with Neurontin® (Gabapentin Capsules USP 400 mg) of Pfizer, USA in healthy adult male human subjects under both fasting and fed conditions. The objective was to monitor the safety of a single dose of Gabapentin capsules USP 400 mg in healthy adult male human subjects. The objective was also to help the human being regarding safe, efficacy and effectiveness of drug and to reduce the cost or price of drugs and easily available to patients.
1.17 Literature Review

The past 15 years has witnessed the unprecedented development of novel antiepileptic agents [58]. One of the first compounds to emerge from this era was Gabapentin which was licensed for the treatment of refractory localization-related epilepsies in the UK and Europe in 1993.

Gabapentin has since gained world-wide recognition, not just for its antiepileptic properties, but also its efficacy in the management of chronic pain syndromes, especially neuropathic pain [59].

Pregabalin is structurally related to Gabapentin and has been marketed for the treatment of seizures and neuropathic pain in the UK since mid-2004. Both drugs are derivatives of the inhibitory neurotransmitter g-aminobutyric acid (GABA; with Gabapentin originally designed as a GABA mimetic agent that could freely cross the blood–brain barrier. Gabapentin was initially evaluated as an anti-spastic compound because of its structural similarity to baclofen and its ability to attenuate the polysynaptic spinal reflex in animal models of spasticity [60].

Although the anti-spastic effects of Gabapentin proved to be modest, the drug demonstrated considerable efficacy in a range of experimental seizure models [61]. These observations led to its initial development as an antiepileptic agent, with its antinociceptive effects emerging, somewhat serendipitously, at a later stage [62]. Pregabalin can be considered as a successor to Gabapentin, at least in terms of its basic chemical structure and therapeutic profile. Clinical experience with Gabapentin encouraged the search for additional GABA derivatives with efficacy in both epilepsy and pain syndromes, and Pregabalin emerged as one of the most promising candidate compounds [63].

Gabapentin (brand name Neurontin) is a medication originally developed for the treatment of epilepsy. Presently, Gabapentin is widely used to relieve pain, especially neuropathic pain. Gabapentin is well tolerated in most patients, has a relatively mild side-effect profile, and passes through the body unmetabolized.
Gabapentin is an antiepileptic drug structurally related to the neurotransmitter Gamma Amino Butyric Acid (GABA). A recent structure–activity study reported that the efficacy of a series of Pregabalin analogues in animal models of seizure, anxiety and pain was directly related to affinity for the L-amino acid transporter [7]. However, contemporary opinion suggests that, although the interaction of Gabapentin with the transporter is essential for, and directly correlates with, the absorption of Gabapentin from the gastrointestinal tract and their distribution across the blood–brain-barrier, it is pharmaco-dynamically neutral and does not contribute to the clinical efficacy of either compound [64, 65].

A series of recent research reports has, however, addressed the possibility that Gabapentin may exert its effects, at least in part, by activation of presynaptic GABA_B receptors. Investigators continued to offer credible experimental evidence to support the original observation.

Gabapentin was shown to reduce potassium evoked calcium influx via voltage-gated calcium channels in a mouse pituitary cell line that constitutively expresses GABA_B receptors comprising the functional gb1a–gb2 subunit heterodimer [66, 67].

Gabapentin interacts with cortical neurons at auxillary subunits of voltage-sensitive calcium channels. Gabapentin increases the synaptic concentration of GABA, enhances GABA responses at non-synaptic sites in neuronal tissues, and reduces the release of mono-amine neurotransmitters.

Prolonged exposure of Gabapentin increased GABA uptake in primary hippocampal neurone cultures, an effect that was ascribed to the redistribution of GAT-1 protein from intracellular locations to the cell membrane [68].

GBP has been reported to augment ATP-sensitive potassium channel conductance in rat hippocampal and human neocortical slices [69]. In addition to effects on potassium channels, GBP has also been reported to modulate the hyperpolarisation-activated cation current (Ih) [70].
One of the mechanisms implicated in this effect of Gabapentin is the reduction of the axon excitability measured as an amplitude change of the presynaptic fibre volley (FV) in the CA1 area of the hippocampus. This is mediated through its binding to presynaptic NMDA receptors. Other studies have shown that the antihyperalgesic and antiallodynic effects of Gabapentin are mediated by the descending noradrenergic system, resulting in the activation of spinal alpha2-adrenergic receptors. Gabapentin has also been shown to bind and activate the adenosine A1 receptor.

Gabapentin is a hydrophilic, zwitterionic, antiepileptic agent with a unique PK profile that is not metabolized or bound to plasma proteins and is excreted unchanged in urine. [71]. Gabapentin has an elimination half life of 7 to 7 hours in healthy subjects and it is well organized that Gabapentin displays dose dependent and saturable absorption pattern.

Absorbed in part by the L-amino acid transport system, which is a carrier-mediated, saturable transport system; as the dose increases, bioavailability decreases. Bioavailability ranges from approximately 60% for a 900 mg dose per day to approximately 27% for a 4800 milligram dose per day. Food has a slight effect on the rate and extent of absorption of Gabapentin (14% increase in AUC).

- **Graeme J Sills et al., 2006** [72]: studied on the mechanisms of action of Gabapentin and pregabalin and concluded that GBP and PGB are structurally related agents with similar spectra of antiepileptic and antinociceptive activity.

- **Kenneth C. Cundy et al., 2008** [73]: Investigated on Clinical Pharmacokinetics of XP13512, a Novel Transported Prodrug of Gabapentin and suggested that XP13512 may provide enhanced absorption, more predictable Gabapentin exposure, reduced interpatient variability, and decreased dosing frequency compared with commercial Gabapentin.

- **Eduardo Abib Junior et al., 2011** [74]: Conducted the bioequivalence study of Gabapentin using Liquid Chromatography Coupled to Mass Spectrometry and concluded that that Gabapentin 400 mg capsule was
bioequivalent to Neurontin® 400 mg capsule according to both the rate and extent of absorption and the test product can be considered interchangeable in medical practice.

- **Ritu Lal et al., 2009** [75]: Investigated the pharmacokinetics and tolerability of Gabapentin enacarbil up to supratherapeutic doses and the effects of Gabapentin enacarbil on cardiac repolarization in healthy volunteers, and to provide a dose reference for a future definitive QT/corrected QT (QTc) study and concluded that Gabapentin enacarbil was associated with dose-proportional Gabapentin exposure at doses up to 6000 mg and was generally well tolerated in these healthy subjects.

- **Toufigh Gordi et al., 2008** [76]: compared the pharmacokinetics of an oral, gastric-retentive, Gabapentin extended-release (G-ER) formulation with a Gabapentin immediate-release (G-IR) formulation after single and multiple daily doses in healthy subjects and found that in these healthy subjects, the daily exposure provided by less frequent G-ER dosing was not significantly different from same daily dose with G-IR, administered more frequently.

- **Ritu Lal et al., 2012** [77]: studied on the description of a population pharmacokinetic analysis of Gabapentin enacarbil in patients with varying degrees of renal function, using data from an open-label study of Gabapentin enacarbil in patients with renal impairment (XenoPort, Inc. protocol XP066), to determine whether dosage adjustments are necessary in patients with renal impairment and suggested that adjustments to Gabapentin enacarbil dosage are necessary in patients with renal impairment.

- **Pablo Kimos et al., 2006** [78]: studied on analgesic action of Gabapentin on chronic pain in the masticatory muscles based on randomized controlled trial and suggested that Gabapentin may be effective in the treatment of other chronic musculoskeletal problems based on results of clinical trial.

- **Dan Chen et al., 2012** [79]: Evaluated the Gabapentin Enacarbil on Cardiac Repolarization thorough QT/QTc Study in Healthy Adults and
concluded that in this population of healthy adults, Gabapentin enacarbil at doses of 1200 and 6000 mg was not associated with QT prolongation and was generally well-tolerated.

- **Torsten E. Gordh et al., 2007** [80]: Conducted to evaluate the efficacy and safety of Gabapentin in the treatment of neuropathic pain caused by traumatic or postsurgical peripheral nerve injury and found that there was no statistically significant difference between the treatments for the primary outcome efficacy variable. However, Gabapentin provided significantly better pain relief (p = 0.015) compared with placebo.

- **Dwayne A. Pierce et al., 2008** [81]: Investigated on probable Case of Gabapentin-Related Reversible Hearing Loss in a Patient with Acute Renal Failure and reported that a patient with acute renal failure who developed hearing loss, myoclonus, and confusion with hallucinations in the presence of elevated Gabapentin concentrations.

- **Manzumeh-Shamsi Meymandi et al., 2006** [82]: Investigated that Gabapentin enhanced the antinociceptive effect of both analgesic and subanalgesic doses of morphine in a dose dependent manner and concluded that co-administration of Gabapentin with low doses of morphine produced therapeutic analgesia which could have important clinical application.

- **M. Segerdahl et al., 2006** [83]: Investigated the effect of Gabapentin on muscle and cutaneous pain in healthy volunteers and concluded that single or repeated dosing of Gabapentin reduced cutaneous but not muscle pain in healthy volunteers.

- **Kok-Yuen Ho et al., 2006** [84]: Reviewed the evaluation the efficacy and tolerability of perioperative Gabapentin administration for the control of acute postoperative pain and concluded that Gabapentin has an analgesic and opioid-sparing effect in acute postoperative pain management when used in conjunction with opioids.

- **M. Baulac et al., 1998** [85]: evaluated the Gabapentin add-on therapy in a large population under conditions close to real practice and to determine the therapeutic doses as reached with adaptable dosages and
found that in 190 patients led to similar efficacy levels, with a tendency for more frequent somnolence and asthenia.

- **Robert H. Dworkin et al., 2008** [86]: Conducted a randomized, placebo-controlled trial of oxycodone and of Gabapentin for acute pain in herpes zoster and found that the results of this clinical trial provide a foundation for evidence-based treatment for acute pain in herpes zoster.

- **Adam Bisaga et al., 2006** [87]: Conducted that a randomized placebo-controlled trial of Gabapentin for cocaine dependence and concluded that when combined with weekly individual relapse-prevention therapy, Gabapentin 1600 mg bid was no more effective than placebo in the treatment of cocaine dependence.

- **David j. Berry et al., 2003** [88]: Studied on the absorption of Gabapentin following high dose escalation and concluded that larger than recommended doses of GBP can be efficiently absorbed by some patients and also that GBP plasma levels do not fluctuate greatly between dosage intervals, therefore, twice daily dosage is a possibility

- **Bernd Huber et al., 2003** [89]: Assessed the antiepileptic efficacy and tolerability of GBP in routine therapy and concluded that the efficacy of GBP in learning disabled patients with highly therapy-resistant partial seizures is limited

- **Grigoris Zoidis et al., 2005** [90]: Investigated on the novel GABA adamantane derivative (AdGABA) w.r.t. design, synthesis, and activity relationship with Gabapentin and concluded that AdGABA was found to antagonize the pentylenetetrazole (PTZ) and semicarbazide (SCZ) induced tonic convulsions and exhibits analgesic activity in the hot plate test on mice.

- **N.V.S. Ramakrishna et al., 2006** [91]: Developed liquid chromatography/tandem mass spectrometry (LC–MS/MS) method for the quantification of Gabapentin, a newantiepileptic drug, in human plasma using its structural analogue, 1,1-cyclohexane diacetic acid monoamide (CAM) as internal standard and finally validated the method for estimation of Gabapentin in human plasma.
• **Carmen E. Burgos-Lepley et al., 2006** [92]: Studied on Carboxylate bioisosteres of Gabapentin and observed that when the carboxylate was replaced by a tetrazole, this group was recognized by the a2-d protein.

• **Olcay Sagirli et al., 2006** [93]: Developed the Determination of Gabapentin in human plasma and urine by high-performance liquid chromatography with UV–vis detection and finally validated for the determination of Gabapentin (GBP) in human plasma and urine and found that the method is precise (relative standard deviation, R.S.D. <4.05%) and accurate (relative mean error, RME <0.15%); mean absolute recoveries were 72.21% for plasma and 72.73% for urine.

• **Anthony B. Ciavarella et al., 2007** [94]: Developed and applied of a validated HPLC method for the determination of Gabapentin and its major degradation impurity in drug products and found that method was used successfully for the quality assessment of four Gabapentin drug products.

• **Laura Mercolini et al., 2010** [95]: Analysed using original high-performance liquid chromatographic method with fluorescence detection for the simultaneous determination of the three antiepileptic drugs Gabapentin, vigabatrin and topiramate in human plasma and found that the method seems to be suitable for the therapeutic drug monitoring (TDM) of patients treated with Gabapentin, vigabatrin and topiramate.

• **Farhan Ahmed Siddiqui et al., 2010** [96]: Developed the Spectrophotometric determination of Gabapentin in pharmaceutical formulations and using ninhydrin and p-acceptors and found that the proposed methods are simple, rapid, accurate, precise and economical for the routine analysis of Gabapentin in pharmaceutical quality control laboratories.

• **Abhay Gupta et al., 2008** [97]: Developed and applied of a validated HPLC method for the analysis of dissolution samples of Gabapentin drug products and a simple isocratic reversed-phase HPLC method was developed and validated for the analysis of dissolution samples of
Gabapentin tablets and capsules and this method was used successfully for the quality assessment of five Gabapentin drug products.

- **Ahmed N. Allam et al., 2011** [98]: Reviewed the bioavailability of Pharmaceutical drugs and found that the bioavailability of drugs was based on biopharmaceutical classification system and stated that this approaches to improves drug solubility as well as drug permeability are the two main strategies in order to enhance the bioavailability of drugs.

- **Raimar LoÈbenberg et al., 2000** [99]: Reviewed the Modern bioavailability, bioequivalence and biopharmaceutics classification system and new scientific approaches to international regulatory standards and reported a brief overview of the BCS and its implications.

- **Laszlo Endrenyi et al., 1998** [100]: Studied on individual bioequivalence for forthcoming draft Guidance of the Food and Drug Administration and suggested that their resolution should be carefully and widely discussed, and that more research and experience is needed before the possible implementation of the new approach.

- **Mei-Ling Chen et al., 2011** [101]: provided a summary of the workshop entitled ‘‘Harmonization of Regulatory Approaches for Evaluating Therapeutic Equivalence and Interchangeability of Multisource and Complex Drug Products’’ and stated that this workshop provided an opportunity for pharmaceutical scientists from academia, industry and regulatory agencies to have open discussions on current regulatory issues and industry practices, facilitating harmonization of regulatory approaches for establishing therapeutic equivalence and interchangeability of multisource drug products.

- **Thomas Mathew et al., 2008** [102]: Assessed on Pilot–pivotal trials for average bioequivalence and provided that how to deals with the design of a pivotal trial, based on the evidence from the pilot trial.

- **L.Z. Benet et al., 1999** [103]: Studied on understanding the bioequivalence study and stated that how bioequivalence studies are carried out at present, the limits of differences allowed for acceptable
products, and some history concerning actual differences between
generic and innovator products as provided by the FDA

- **Herman P. Wijnand et al., 1994**[^104]: Updated of bioequivalence
  programs (including statistical power approximated by Student's t) and
demonstrated that good approximations of power can be obtained by
Student's t-statistic

- **Vangelis Karalis et al., 2009**[^105]: Compare the performance of the
  reference scaled average bioequivalence (scABER) method proposed
  with other approaches focusing on the human exposure expressed as
  the product sample size×periods of drug administration and stated that
  the classic 0.80–1.25 limits were favoured at low intrasubject
  variability and high exposure, whereas the levelling-off limits
demonstrated a preferred overall performance when variability was
  high and exposure was limited.

- **Peter Meredith et al., 2003**[^106]: commented on Bioequivalence and
  Other Unresolved Issues in Generic Drug Substitution and stated that
  measures of individual and population bioequivalence are proposed to
  be more accurate than measures of average bioequivalence.

- **Marc Lindenberg et al., 2004**[^107]: Reviewed on classification of
  orally administered drugs on the World Health Organization Model list
  of Essential Medicines according to the biopharmaceutics
  classification system and reported that orally administered drugs on the
  Model list of Essential Medicines of the World Health Organization
  (WHO) are assigned BCS classifications on the basis of data available
  in the public domain.

- **Gudrun Freitag et al., 2007**[^108]: studied on a nonparametric test for
  similarity of marginals with applications to the assessment of
  population bioequivalence and suggested a completely nonparametric
  test for the assessment of similar marginals of a multivariate
  distribution function.

- **Vangelis Karalis et al., 2003**[^109]: Studied on Pharmacodynamic
  considerations in bioequivalence assessment: comparison of novel and
  existing metrics and stated that all BE indices of either purely PK or
PD nature were classified in a semiquantitative manner according to their strictness in declaring BE.

- **Venkata Ramana S. Uppoor et al., 2001** [110]: Studied on Regulatory perspectives on in vitro (dissolution) / in vivo q (bioavailability) correlations and concluded that IVIVC can impart in vivo meaning to the in vitro dissolution test and can be useful as surrogate for bioequivalence.

- **K.K. Midha et al., 1994** [111]: Studied on the application of partial areas in assessment of rate and extent of absorption in bioequivalence studies of conventional release products and stated that the partial area method is applicable to the evaluation of both relative rate and extent of absorption from conventional release products.