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Tangirala Aruna
Department of Pharmaceutical Management and Regulatory Affairs, Hindu College of Pharmacy, Amaravathi Road, Guntur, Andhra Pradesh, India

Chavali Vindu Bhargavi Yadav
Department of Pharmaceutical Management and Regulatory Affairs, Hindu College of Pharmacy, Amaravathi Road, Guntur, Andhra Pradesh, India

MV Nagabhushanam
Department of Pharmaceutical Management and Regulatory Affairs, Hindu College of Pharmacy, Amaravathi Road, Guntur, Andhra Pradesh, India

Brahmaiah Bonthagarala
Department of Pharmaceutical Management and Regulatory Affairs, Hindu College of Pharmacy, Amaravathi Road, Guntur, Andhra Pradesh, India

D Nagarjuna Reddy
Department of Pharmaceutical Management and Regulatory Affairs, Hindu College of Pharmacy, Amaravathi Road, Guntur, Andhra Pradesh, India

G Ramakrishna
Department of Pharmaceutical Management and Regulatory Affairs, Hindu College of Pharmacy, Amaravathi Road, Guntur, Andhra Pradesh, India

Correspondence
Tangirala Aruna
Department of Pharmaceutical Management and Regulatory Affairs, Hindu College of Pharmacy, Amaravathi Road, Guntur, Andhra Pradesh, India

Guidelines for bioavailability and bioequivalence studies: A review

Tangirala Aruna, Chavali Vindu Bhargavi Yadav, MV Nagabhushanam, Brahmaiah Bonthagarala, D Nagarjuna Reddy and G Ramakrishna

Abstract
Bioequivalence is a term in pharmacokinetics used to assess the expected *in vivo* biological equivalence of two proprietary preparations of a drug. If two drugs are bioequivalent it means that they would be expected to be, for all intents and purposes, the same. In determining bioequivalence between two drugs such as a reference drug (Brand) and potential to be test drug (marketed generic drug), pharmacokinetic studies are conducted whereby, each of the drugs are administered in a cross over study to volunteers subjects (healthy individuals). Serum/plasma are obtained at regular intervals and assayed for parent drug (metabolites) concentration. Blood concentration levels are neither feasible nor possible to compare the two drugs, then pharmacodynamic endpoints rather than pharmacokinetic end points are used for comparison. For a pharmacokinetic comparison, the plasma concentration data are used to assess key pharmacokinetic parameters such as area under the curve (AUC), peak concentration (Cmax), time to peak concentration (Tmax), and absorption lag time (tlag). Testing should be conducted at several different doses, especially when the drug displays non-linear pharmacokinetics. If 90% Confidence interval for the ratio of the geometric least square means of natural log transformed Cmax, AUC0-t and AUC0-inf of Test and Reference drugs are within 80.00% to 125.00%, then bioequivalence will be establish.

Keywords: Bioavailability and bioequivalence studies

1. Introduction
Ensuring uniformity in standards of quality, efficacy and safety of pharmaceutical products is the fundamental responsibility of CDSCO. Reasonable assurance has to be provided that various products, containing same active ingredients, marketed by different licensees, are clinically equivalent and interchangeable. Bioavailability and bioequivalence data is therefore required to be furnished with applications for new drugs, as required under Schedule Y, depending on the type of application being submitted. Both bioavailability and bioequivalence focus on the release of a drug substance from its dosage form and subsequent absorption into the systemic circulation. Bioavailability 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 early drug development can serve as a benchmark for subsequent BE studies [1,2].

1.1. Bioavailability
Bioavailability refers to the relative amount of drug from an administered dosage form which enters the systemic circulation and the rate at which the drug appears in the systemic circulation.

1.2. Bioequivalence
Bioequivalence of a drug product is achieved if its extent and rate of absorption are not statistically significantly different from those of the reference product when administered at the same molar dose

1.3. Pharmacokinetic Terms [3, 4]
1.3.1. Cmax
This is the maximum drug concentration achieved in systemic circulation following drug administration.

1.3.2. Cmin
This is the minimum drug concentration achieved in systemic circulation following multiple dosing at steady state.

1.3.4. Cpd
This is the pre-dose concentrations determined immediately before a dose is given at steady state.

1.3.5. Tmax
It is the time required to achieve maximum drug concentration in systemic circulation.

1.3.6. AUC_{0-t}
Area under the plasma concentration - time curve from 0 h to the last quantifiable concentration to be calculated using the trapezoidal rule

1.3.7. Kel
Apparent first-order terminal elimination rate constant calculated from a semi-log plot of the plasma concentration versus time curve.

1.3.8. 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.

2. Scope of the guidelines [4, 5]
Bioavailability and Bioequivalence studies are required by regulations to ensure therapeutic equivalence between a pharmaceutically equivalent test product and a reference product. Several *in vivo* and *in vitro* methods are used to measure product quality**.**

3. When bioequivalence studies are necessary and types of studies required
3.1. *In vivo* studies
For certain drugs and dosage forms, *in vivo* documentation of equivalence, through either a bioequivalence study, a comparative clinical pharmacodynamics study, or a comparative clinical trial, is regarded as especially important. These include [4-6].

a. Oral immediate release drug formulations with systemic action.
b. Non-oral and non-parenteral drug formulations designed to act by systemic absorption (such as transdermal patches, suppositories, etc.).
c. Sustained or otherwise modified release drug formulations designed to act by systemic absorption.
d. Fixed-dose combination products with systemic action.
e. Non-solution pharmaceutical products which are for non-systemic use.

3.2. *In vitro* studies [7]
In following circumstances equivalence may be assessed by the use of *in vitro* dissolution testing:
a. Drugs for which the applicant provides data to substantiate all of the following:
1. highest dose strength is soluble in 250 ml of an aqueous media over the pH range of 1-7.5 at 37°C
2. at least 90% of the administered oral dose is absorbed on mass balance determination or in comparison to an intravenous reference dose

b. Different strengths of the drug manufactured by the same manufacturer, where all of the following criteria are fulfilled:
1. the qualitative composition between the strengths is essentially the same;
2. the ratio of active ingredients and excipients between the strengths is essentially the same, or, in the case of small strengths, the ratio between the excipients is the same;

3.2 When bioequivalence studies are not necessary
In following formulations and circumstances, bioequivalence between a new drug and the reference product may be considered self-evident with no further requirement for documentation:
a. When new drugs are to be administered parenterally (e.g., intravenous, intramuscular, subcutaneous, intrathecal administration etc.) as aqueous solutions and contain the same active substance(s) in the same concentration and the same excipients in comparable concentrations;
b. When the new drug is a solution for oral use, and contains the active substance in the same concentration, and does not contain an excipient that is known or suspected to affect gastro-intestinal transit or absorption of the active substance;

4. Design and conduct of studies [8-10]
4.1. Pharmacokinetic Studies
4.1.1. Study Design
The basic design of an in-vivo bioavailability study is determined by the following:
1. What is the scientific question(s) to be answered.
2. The nature of the reference material and the dosage form to be tested.
3. The availability of analytical methods.
4. Benefit-risk ratio considerations in regard to testing in humans.

Single-dose studies generally suffice. However, situations as described below may demand a steady-state study design:
1. Dose or time-dependent pharmacokinetics.
2. Some modified release products (in addition to single dose investigations)
3. If intra-individual variability in the plasma concentration or disposition precludes the possibility of demonstrating bioequivalence in a reasonably sized single-dose study and this variability is reduced at steady state.

4.1.2. Study Population
Selection of the Number of Subjects
The number of subjects required for a study should be statistically significant and is determined by the following considerations:
1. The error variance associated with the primary characteristic to be studied as estimated from a pilot experiment, from previous studies or from published data.
2. The significance level desired: usually 0.05
3. The expected deviation from the reference product compatible with bioequivalence.

Selection Criteria for Subjects
To minimize intra and inter individual variation subjects should be standardized as much as possible and acceptable. The studies should be normally performed on healthy adult volunteers with the aim to minimize variability and permit detection of differences between the study drugs. Subjects

may be males or females; however, the choice of gender should be consistent with usage and safety criteria.

Risks to women of childbearing potential should be considered on an individual basis. Women should be required to give assurance that they are neither pregnant, nor likely to become pregnant until after the study. This should be confirmed by a pregnancy test immediately prior to the first and last dose of the study. Women taking contraceptive drugs should normally not be included in the studies.

If the drug product is to be used predominantly in the elderly attempt should be made to include as many subjects of 60 years of age or older as possible. If the drug product is intended for use in both sexes attempt should be made to include similar proportions of males and females in the studies.

Genetic Phenotyping
Phenotyping and/or genotyping of subjects should be considered for exploratory bioavailability studies and all studies using parallel group design. It may also be considered in crossover studies (e.g. bioequivalence, dose proportionality, food interaction studies etc.) for safety.

4.1.3. Study Conditions [11]
Standardization of the study environment, diet, fluid intake, post-dosing postures, exercise, sampling schedules etc. is important in all studies. Compliance to these standardizations should be stated in the protocol and reported at the end of the study, in order to reassure that all variability factors involved, except that of the products being tested, have been minimized. Unless the study design requires, subjects should abstain from smoking, drinking alcohol, coffee, tea, xanthine containing foods and beverages and fruit juices during the study and at least 48 hours before its commencement.

4.1.4. Selection of Blood Sampling Points/Schedules
The blood-sampling period in single-dose trials of an immediate release product should extend to at least three-elimination half-lives. Sampling should be continued for a sufficient period to ensure that the area extrapolated from the time of the last measured concentration to infinite time is only a small percentage (normally less than 20%) of the total AUC. The use of a truncated AUC is undesirable except in certain circumstances such as in the presence of entero- hepatic recycling where the terminal elimination rate constant cannot be calculated accurately.

There should be at least three sampling points during the absorption phase, three to four at the projected T_{max}, and four points during the elimination phase. The number of points used to calculate the terminal elimination rate constant should be preferably determined by eye from a semi-logarithmic plot. Intervals between successive data/sampling points used to calculate the terminal elimination rate constant should, in general, not be longer than the half-life of the study drug.

Where urinary excretion is measured in a single-dose study it is necessary to collect urine for seven or more half-lives.

Fasting and Fed State Considerations [12]
Generally, a single dose study should be conducted after an overnight fast (at least 10 hours), with subsequent fast of 4 hours following dosing. For multiple dose fasting state studies, when an evening dose must be given, two hours of fasting before and after the dose is considered acceptable. Fed state studies are also required when fasting state studies make assessment of C_{max} and T_{max} difficult. Studies in the fed state require the consumption of a high-fat breakfast before dosing. Such a breakfast must be designed to provide 950 to 1000 KCals. At least 50% of these calories must come from fat, 15 to 20% from proteins and the rest from carbohydrates.

Steady State Studies
In following cases – an additional "steady state study" is considered appropriate:
- Where the drug has a long terminal elimination half-life and blood concentrations after a single dose cannot be followed for a sufficient time.
- Where assay sensitivity is inadequate to follow the terminal elimination phase for an adequate period of time.
- For drugs, which are so toxic that ethically they should only be administered to patients for whom they are a necessary part of therapy, but where multiple dose therapy is required, e.g. many cytotoxic.

5. Characteristics to be investigated during bioavailability / bioequivalence studies [10-13]
In most cases evaluations of bioavailability and bioequivalence will be based upon the measured concentrations of the active drug substance(s) in the biological matrix. In some situations, however, the measurements of an active or inactive metabolite may be necessary.

Race mates should be measured using an achiral assay method. Measurement of individual enantiomers in bioequivalence studies is recommended where all of the following criteria are met:
a) the enantiomers exhibit different pharmacodynamics characteristics
b) the enantiomers exhibit different pharmacokinetic characteristics
c) primary efficacy / safety activity resides with the minor enantiomer
d) non-linear absorption is present for at least one of the enantiomers

5.1. Bioanalytical methodology
The bioanalytical methods used to determine the drug and/or its metabolites in plasma, serum, blood or urine or any other suitable matrix must be well characterized, standardized, fully validated and documented to yield reliable results that can be satisfactorily interpreted.

Although there are various stages in the development and validation of an analytical procedure, the validation of the analytical method can be envisaged to consist of two distinct phases:
1. The pre-study phase which comes before the actual start of the study and involves the validation of the method on biological matrix human plasma samples and spiked plasma samples.
2. The study phase in which the validated bioanalytical method is applied to the actual analysis of samples from bioavailability and bioequivalence studies mainly to confirm the stability, accuracy and precision.

Pre-study Phase
The following characteristics of the bioanalytical method must be evaluated and documented to ensure the acceptability of the performance and reliability of analytical results:

i. Stability of the drug/metabolites in the biological matrix

Stability of the drug and/or active metabolites in the biological matrix under the conditions of the experiment (including any period for which samples are stored before analyses) should be established. The stability data should also include the influence of at least three freezing and thawing cycles representative of actual sample handling.

ii. Specificity/Selectivity

Data should be generated to demonstrate that the assay does not suffer from interference by endogenous compounds, degradation products, other drugs likely to be present in study samples, and metabolites of the drug(s) under study.

iii. Sensitivity

Sensitivity is the capacity of the test procedure to record small variations in concentration. The analytical method chosen should be capable of assaying the drug/metabolites over the expected concentration range. A reliable lowest limit of quantification should be established based on an intra- and inter-day coefficient of variation usually not greater than 20 percent.

iv. Precision and Accuracy

Precision (the degree of reproducibility of individual assays) should be established by replicate assays on standards, preferably at several concentrations. Accuracy is the degree to which the 'true' value of the concentration of drug is estimated by the assay. Precision and accuracy should normally be documented at three concentrations (low, medium, high) where 'low' Accuracy can be assessed in conjunction with precision and is a measure of the extent to which measured concentrations deviate from true or

v. Recovery

Documentation of extraction recovery at high, medium and low concentrations is essential since methods with low recovery are, in general, more prone to inconsistency. If recovery is low, alternative methods should be investigated. Recovery of any internal standard used should also be assessed.

vi. Range and linearity

The quantitative relationship between concentration and response should be adequately characterized over the entire range of expected sample concentrations. For linear relationships, a standard curve should be defined by at least five concentrations. If the concentration response function is non-linear, additional points would be necessary to define the non-linear portions of the curve. Extrapolation beyond the standard curve is not acceptable.

5.2. Study Phase

In general, with acceptable variability as defined by validation data, the analysis of biological sample can be done by single determination without a need for a duplicate or replicate analysis. The need for duplicate analysis should be assessed on a case-by-case basis. A procedure should be developed that documents the reason for re-analysis.

A standard curve should be generated for each analytical run

for each analyte and should be used to calculate the concentration of the analyte in the unknown samples assayed with that run. It is important to use a standard curve that will cover the entire range of concentrations in the unknown unknowns by extrapolations of standard curves below the lowest standard concentration or above the highest standard concentration is not recommended.

Quality Control Samples

Quality control samples are samples with known concentration prepared by spiking drug-free biological fluid with drug. These samples should be prepared in low, medium and high concentration. To avoid possible confusion between quality control samples and standard solutions during the review process, preparation of quality control samples at concentrations different from those used for the calibration is recommended. For stable analytes, quality control samples should be prepared in the fluid of interest at the time of pre-study assay validation or at the time of study sample collection, and stored with the study samples

Repeat Analysis

In most studies some samples will require re-analysis because of aberrant results due to processing errors, equipment failure or poor chromatography. The reasons for re-analysis of such samples should be stated. The criteria for repeat analyses should be determined prior to running the study and recorded in the protocol / laboratory standard operating procedures.

Statistical Evaluation [14, 15]
Data analysis

The primary concern in bio-equivalence assessment is to limit the consumer's risk i.e., erroneously accepting bioequivalence and also at the same time minimizing the manufacture's risk i.e., erroneously rejecting bioequivalence. This is done by using appropriate statistical methods for data analysis and adequate sample size.

Statistical analysis

The statistical procedure should be specified in the protocol itself. In case of bioequivalence studies the procedures should lead to a decision scheme which is symmetrical with respect to the two formulations (i.e. leading to the same decision whether the new formulation is compared to the reference product or the reference product to the new formulation).

The statistical analysis (e.g. ANOVA) should take into account sources of variation that can be reasonably assumed to have an effect on the response.

The 90% confidence interval for the ratio of the population means (Test/reference) or two one sided-t tests with the null hypothesis of non- bioequivalence at the 5% significance level for the parameter under consideration are considered for testing bioequivalence.

To meet the assumption of normality of data underlying the statistical analysis, the logarithmic transformation should be carried out for the pharmacokinetic parameters C_{max} and AUC before performing statistical analysis. However, it is recommended not to verify the assumptions underlying the statistical analysis before making logarithmic transformation.

The analysis of T_{max} is desirable if it is clinically relevant. The parameter T_{max} should be analyzed using non-parametric methods. In addition to above, summary statistics such as minimum, maximum and ratio should be given.

Criteria for bioequivalence

To establish Bioequivalence, the calculated 90% confidence interval for AUC and C_{max} should fall within the bioequivalence range, usually 80-125%. This is equivalent to the rejection of two one sided-t tests with the null hypothesis of non- bioequivalence at 5% level of significance. The non-parametric 90% confidence interval for T_{max} should lie within a clinically acceptable range.

Tighter limits for permissible differences in bioavailability may be required for drugs that have:

i. A narrow therapeutic index.
ii. A serious, dose-related toxicity.
iii. A steep dose/effect curve, or
iv. A non-linear pharmacokinetics within the therapeutic dose range.

A wider acceptance range may be acceptable if it is based on sound clinical justification.

5.3. Pharmacodynamics Studies

Studies in healthy volunteers or patients using pharmacodynamics parameters may be used for establishing equivalence between two pharmaceutical products. These studies may become necessary if quantitative analysis of the drug and/or metabolite(s) in plasma or urine cannot be made with sufficient accuracy and sensitivity.

The following requirements should be recognized when planning, conducting and assessing the results from a pharmacodynamics study:

i. The response measured should be a pharmacological or therapeutic effect which is relevant to the claims of efficacy and/or safety of the drug.
ii. The methodology adopted for carrying out the study should be validated for precision, accuracy, reproducibility and specificity.
iii. Neither the test nor the reference product should produce a maximal response in the course of the study, since it may be impossible to distinguish differences between formulations given in doses that produce such maximal responses. Investigation of dose-response relationship may become necessary.
iv. A crossover or parallel study design should be used, as appropriate.

5.4. Documentation [15]

With respect to the conduct of bioequivalence/bioavailability studies following important documents must be maintained:

i. Clinical Data
ii. Details of the analytical method validation including the following:
a. System suitability test
b. Linearity range
c. Lowest limit of quantitation
d. QC sample analysis
e. Stability sample analysis
f. Recovery experiment result
i. Analytical data of volunteer plasma samples
ii. Raw data
iii. All comments of the chief investigator regarding the data of the study submitted for review.
iv. A copy of the final report

5.6. Study Report

The bioequivalence or bioavailability report should give the complete documentation of its protocol, conduct and evaluation.

The report should include (as a minimum) the following information:

a. Table of contents
b. Title of the study
c. Names and credentials of responsible investigators
d. Signatures of the principal and other responsible investigators authenticating their respective sections of the report
e. Site of the study and facilities used

5.7. Facilities for conducting bioavailability and/or bioequivalence Studies

Legal identity

The organization, conducting the bioequivalence / bioavailability studies, or the parent organization to which it belongs, must be a legally constituted body with appropriate statutory registrations.

Impartiality, confidentiality, independence and integrity:

The organization shall:

a. have managerial staff with the authority and the resources needed to discharge their duties.
b. have arrangements to ensure that its personnel are free from any commercial, financial and other pressures which might adversely affect the quality of their work.
c. be organized in such a way that confidence in its independence of judgment and integrity is maintained at all times.

Organization and management

The study site organization must include the following:

a. An Investigator who has the overall responsibility to provide of the human subjects. The Investigator(s) should possess appropriate medical qualifications and relevant experience for conducting pharmacokinetic studies.
b. The site should have identified adequately qualified and trained personnel to perform the following functions:
- Data handling and interpretation
- Documentation and report preparation
- Quality assurance of all operations in the center

5.8. Documented Standard Operating Procedures

A partial list of procedures for which documented standard operating procedures should be available includes:

a. Maintenance of working standards (pure substances) and respective documentation.
b. withdrawal, storage and handling of biological samples.
c. maintenance, calibration and validation of instruments.
d. managing medical as well as non-medical emergency situations
e. handling of biological fluids
f. managing laboratory hazards
g. disposal procedures for clinical samples and laboratory wastes
h. documentation of clinical pharmacology unit observations, volunteer data and analytical data
i. obtaining informed consent from volunteers

5.9. Clinical Pharmacological Unit

It must have adequate space and facilities to house at least 16 volunteers. Adequate area must be provided for dining and recreation of volunteers, separate from their sleeping area.

5.10. Maintenance of records of ba/be studies

All records of *in vivo* or *in vitro* tests conducted on any marketed batch of a drug product tC assure that the product meets a bioequivalence requirement shall be maintained by the Sponsor for at least 2 years after the expiration date of the batch and submitted to CDSCO on request.

6. Conclusion

Concept of BE has been adopted by the pharmaceutical industry and national regulatory authorities throughout the world over 20 years. There is continuing attempt to understand and develop more efficient and scientifically valid approaches to assess bioequivalence of various dosage forms including some of tough complex special dosage forms.

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