Bioavailability and Bioequivalence

Manas Bhowmik

Introduction

- The therapeutic effectiveness of a drug depends upon the ability of the dosage form to deliver the medicament to its site of action at a rate and amount sufficient to elicit the desired pharmacological response.
- This attribute of the dosage form is referred to as physiological availability, biological availability or simply, bioavailability.
- The term bioavailability is defined as the rate and extent (amount) of absorption of unchanged drug from its dosage form.

Contd.

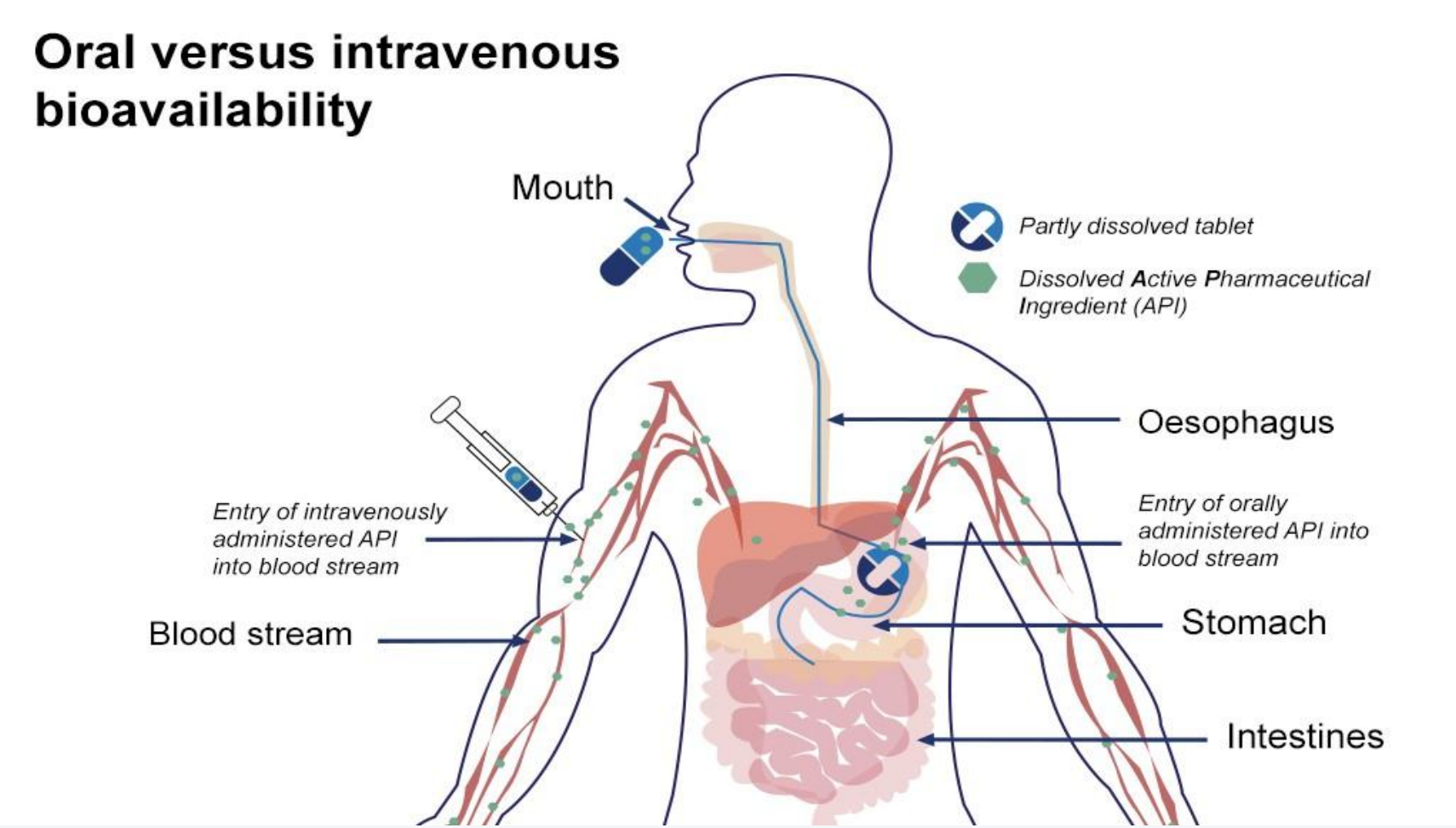
If the size of the dose to be administered is same, then bioavailability of a drug from its dosage form depends upon 3 major factors:

1. Pharmaceutical factors related to physicochemical properties of the drug and characteristics of the dosage form.
2. Patient related factors.
3. Route of administration.

The influence of route of administration on drug's bioavailability is generally in the following order with few exceptions:

Parenteral > Oral > Rectal > Topical

Contd.


Oral versus intravenous bioavailability
Mouth
Partly dissolved tablet
Dissolved Active Pharmaceutical Ingredient (API)
Oesophagus
Entry of intravenously administered API into blood stream
Entry of orally administered API into blood stream
Blood stream
Stomach
Intestines

Contd.

- Within the parenteral route, intravenous injection of a drug results in 100% bioavailability as the absorption process is bypassed. However, for reasons of stability and convenience, most drugs are administered orally.
- In such cases, the dose available to the patient, called as the bioavailable dose, is often less than the administered dose.
- The amount of drug that reaches the systemic circulation (i.e. extent of absorption) is called as **systemic availability** or simply **availability**.
- The term **bioavailable fraction F**, refers to the fraction of administered dose that enters the systemic circulation.

$$F = \frac{\text{Bioavailable dose}}{\text{Administered dose}}$$

Objectives of BA Studies

Bioavailability studies are important in the —

1. Bioavailability data provide an estimate of the fraction of drug absorbed from the formulation, and provide information about the pharmacokinetics of the drug. Relative bioavailability studies compare two drug product formulations.

2. A bioequivalence study is a specialized type of relative bioavailability study. Bioequivalence is defined as the absence of a significant difference in the rate and extent to which the active ingredient or active moiety becomes available at the site of drug action when administered at the same molar dose under similar conditions in an appropriately designed study.

3. Bioavailability and bioequivalence data play pivotal roles in regulatory submissions for marketing approval of new and generic drugs throughout the world.

Cont…

Objectives of BA Studies

4. Primary stages of development of a suitable dosage form for a new drug entity to obtain evidence of its therapeutic utility.

5. Determination of influence of excipients, patient related factors and possible interaction with other drugs on the efficiency of absorption.

6. Development of new formulations of the existing drugs.

7. Control of quality of a drug product during the early stages of marketing in order to determine the influence of processing factors, storage and stability on drug absorption.

8. Comparison of availability of a drug substance from different dosage forms or from the same dosage form produced by different manufacturers.

Contd.

- When the systemic availability of a drug administered orally is determined in comparison to its intravenous administration, it is called as absolute bioavailability. It is denoted by symbol F.

- When the systemic availability of a drug after oral administration is compared with that of an oral standard of the same drug (such as an aqueous or non-aqueous solution or a suspension), it is referred to as relative or comparative bioavailability. It is denoted by symbol Fr.

- In contrast to absolute bioavailability, it is used to characterize absorption of a drug from its formulation. F and Fr are generally expressed in percentages.

Measurement of Bioavailability

Direct and indirect methods may be used to assess drug bioavailability. Bioequivalence of a drug product is demonstrated by the rate and extent of drug absorption, as determined by comparison of measured parameters. The FDA's regulations (US-FDA, CDER, 2014a) list the following approaches to determining bioequivalence.

- In vivo measurement of active moiety or moieties in biological fluid (i.e, a pharmacokinetic study)
- In vivo pharmacodynamics (PD) comparison
- In vivo limited clinical comparison
- In vitro comparison

Measurement of Bioavailability

The methods useful in quantitative evaluation of bioavailability can be broadly divided into two categories — pharmacokinetic methods and pharmacodynamic methods.

I. Pharmacokinetic Methods

These are very widely used and based on the assumption that the pharmacokinetic profile reflects the therapeutic effectiveness of a drug. Thus, these are indirect methods.

The two major pharmacokinetic methods are:

1. *Plasma level-time studies*.
2. *Urinary excretion studies.*

Contd.

II. Pharmacodynamic Methods

These methods are complementary to pharmacokinetic approaches and involve direct measurement of drug effect on a (patho)physiological process as a function of time. The two pharmacodynamic methods involve determination of bioavailability from:

1. *Acute pharmacological response.*
2. *Therapeutic response*.

Contd.

Plasma Level—Time Studies: Unless determination of plasma drug concentration is difficult or impossible, it is the most reliable method and method of choice in comparison to urine data. The method is based on the assumption that two dosage forms that exhibit superimposable plasma level time profiles in a group of subjects should result in identical therapeutic activity.

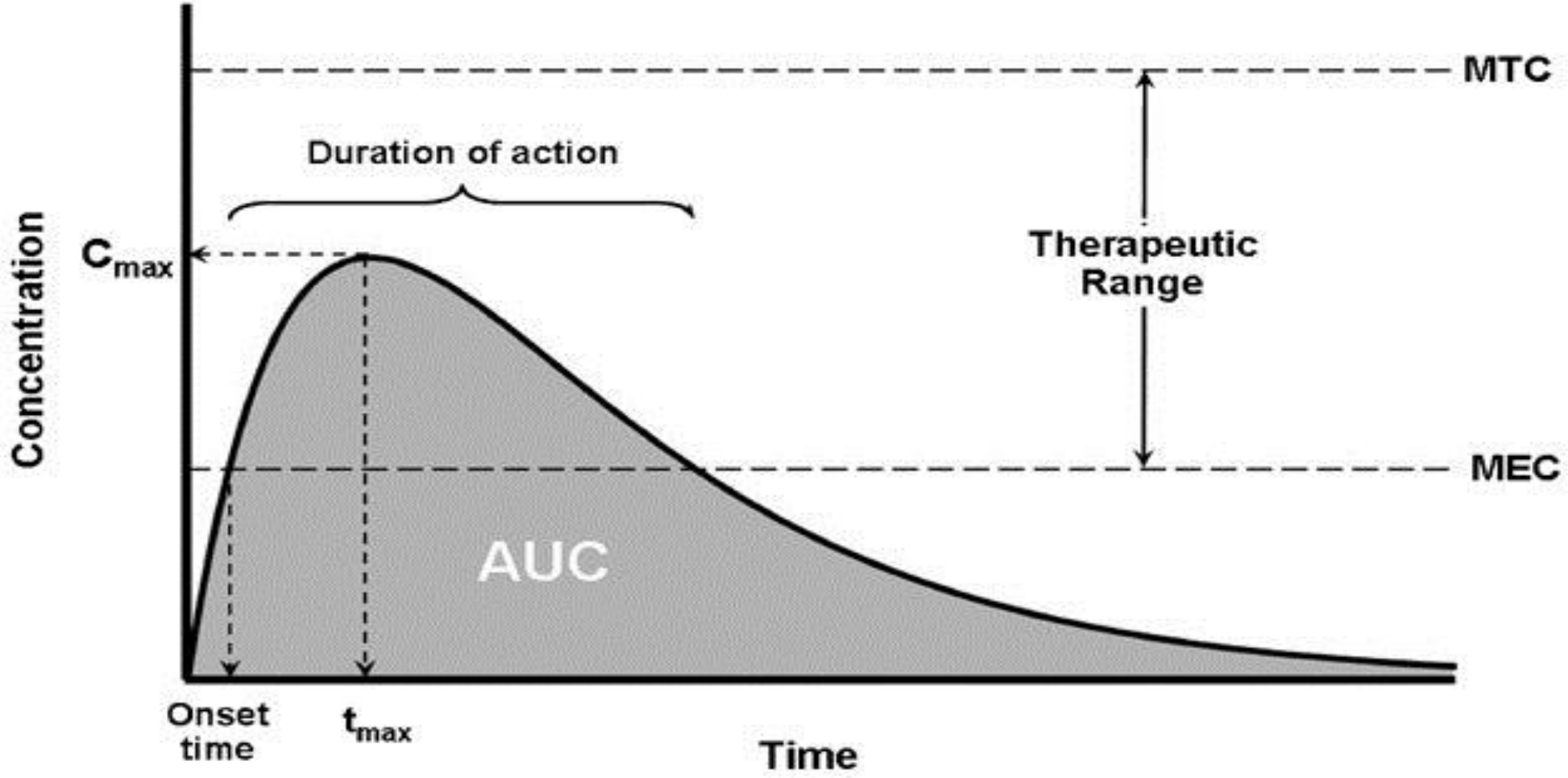
With single dose study, the method requires collection of serial blood samples for a period of 2 to 3 biological half-lives after drug administration, their analysis for drug concentration and making a plot of concentration versus corresponding time of sample collection to obtain the plasma level-time profile.

Contd.

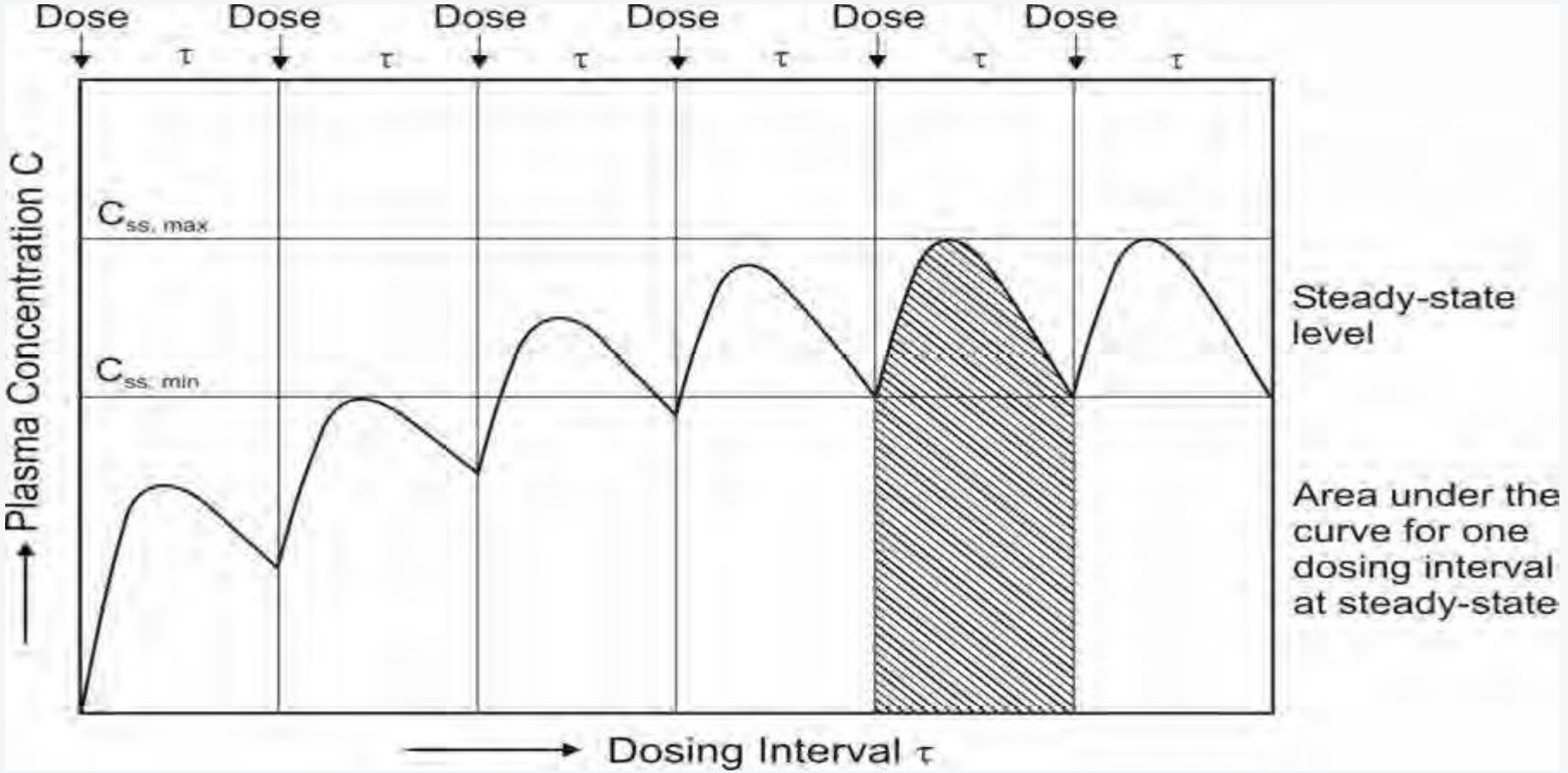
With i.v. dose, sampling should start within 5 minutes of drug administration and subsequent samples taken at 15 minute intervals. To adequately describe the disposition phase, at least 3 sample points should be taken if the drug follows one-compartment kinetics and 5 to 6 points if it fits two compartment model.

For oral dose, at least 3 points should be taken on the ascending part of the curve for accurate determination of Ka. The points for disposition or descending phase of the curve must be taken in a manner similar to that for i.v. dose.

Contd.


MTC
Duration of action
Therapeutic Range
Concentration
C_{max}
MEC
AUC
Onset time
t_{max}
Time

Contd.


Dose
Dose
Dose
Dose
Dose
Dose
τ
τ
τ
τ
τ
τ
Plasma Concentration C
$C_{ss,\,max}$
$C_{ss,\,min}$
Steady-state level
Area under the curve for one dosing interval at steady-state
Dosing Interval τ

Pharmacokinetic parameters

Three pharmacokinetis parameters are C_{max}, t_{max} and AUC

C_{max} : The peak plasma drug concentration obtained after administration of drug.

It is an indication that the drug is sufficiently systemically absorbed to provide a therapeutic response.

it is often used in bioequivalence studies as a measure for the rate of drug bioavailability.

It is a function of **rate and extent of absorption**.

C_{max} will increase with an increase in dose and the rate of absorption and vice versa.

Pharmacokinetic parameters

t_{max}: The time of peak plasma concentration corresponds to the time required to reach maximum drug concentration after drug administration.

At t_{max}, peak drug absorption occurs and the rate of drug absorption exactly equals the rate of drug elimination. **It is an indication of rate of drug absorption**.

Drug absorption still continues after t_{max} is reached, but at a slower rate. The t_{max} will become smaller (indicating less time required to reach peak plasma concentration) as the absorption rate for the drug becomes more rapid.

Pharmacokinetic parameters

AUC: Area under the drug plsma level time curve, is a **measurement of the extent of drug bioavailability**.

The AUC is the area under the drug plasma level–time curve from t = 0 to t = ∞.

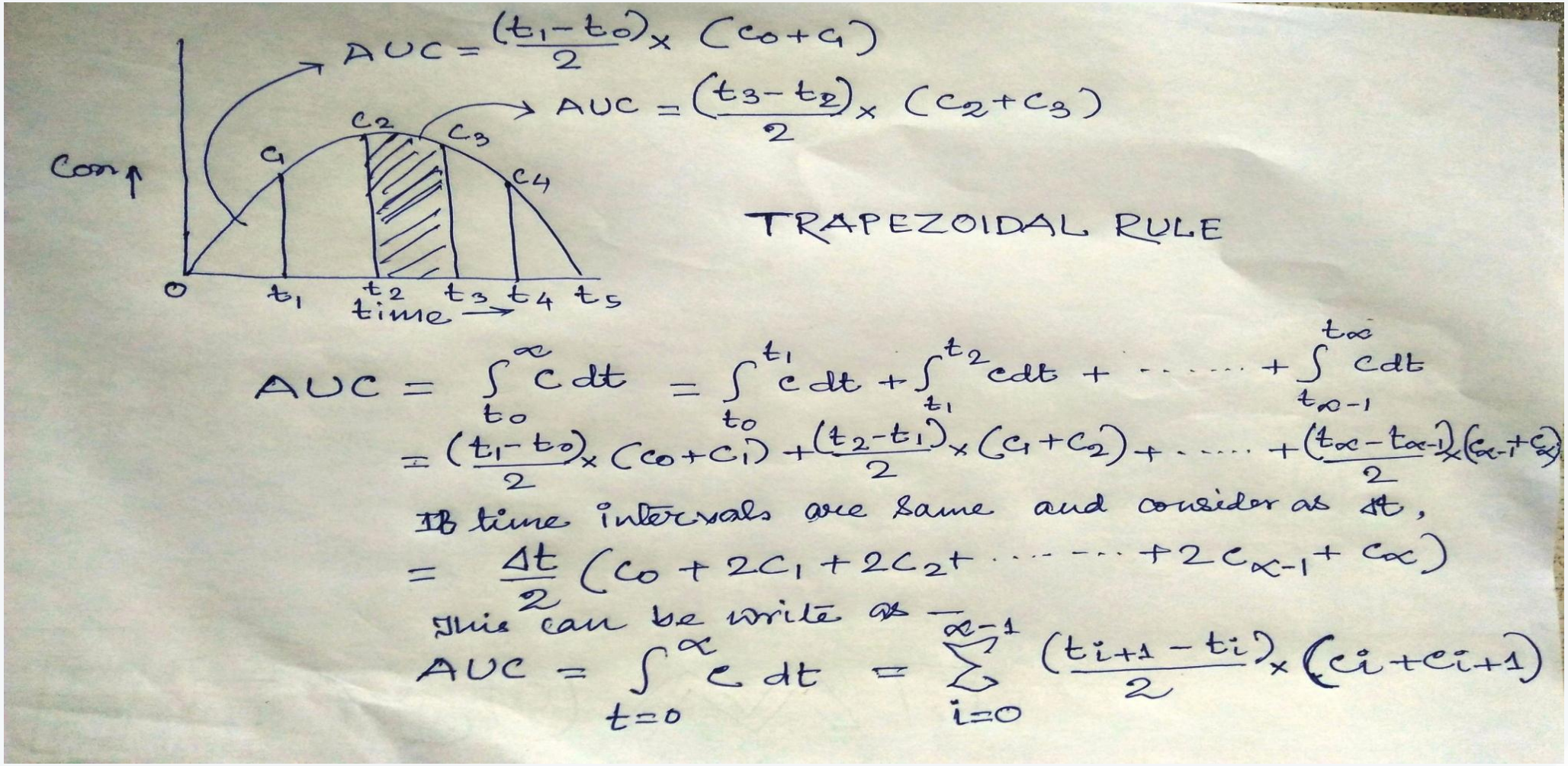
The AUC is dependent on the dose administered and the rate of elimination. In general, AUC is directly proportional to the dose and indirectly proportional to elimination. Sometime AUC is not directly proportional to dose when drug elimination become saturated or capacity limited and show nonlinear relation.

Pharmacokinetic parameters

The AUC curve for intravenous administration generally has two components. The first represents distribution of the drug and the second represents elimination. The AUC curve for orally administered drugs also has two components. The first represents the absorption phase, and the second component represents the elimination phase.

Since elimination is already reflected in the AUC value, there is no need to reintroduce elimination into the calculation when determining absolute bioavailability.

AUC


$AUC = \frac{(t_1 - t_0)}{2} \times (C_0 + C_1)$
$AUC = \frac{(t_3 - t_2)}{2} \times (C_2 + C_3)$
Conc
C_1
C_2
C_3
C_4
O
t_1
t_2
t_3
t_4
t_5
time
TRAPEZOIDAL RULE
$AUC = \int_{t_0}^{\infty} C\,dt = \int_{t_0}^{t_1} C\,dt + \int_{t_1}^{t_2} C\,dt + \cdots\cdots + \int_{t_{\infty-1}}^{t_\infty} C\,dt$
$= \frac{(t_1 - t_0)}{2} \times (C_0 + C_1) + \frac{(t_2 - t_1)}{2} \times (C_1 + C_2) + \cdots\cdots + \frac{(t_\infty - t_{\infty-1})}{2} \times (C_{\infty-1} + C_\infty)$
If time intervals are same and consider as Δt,
$= \frac{\Delta t}{2} (C_0 + 2C_1 + 2C_2 + \cdots\cdots + 2C_{\infty-1} + C_\infty)$
This can be write as —
$AUC = \int_{t=0}^{\infty} C\,dt = \sum_{i=0}^{\infty-1} \frac{(t_{i+1} - t_i)}{2} \times (C_i + C_{i+1})$

AUC

Calculate the area under the plasma curve by the following data:

Time(h)	0	1	2	3	4	5	6	7	8	9	10
Concentration (ng/ml)	0	7.42	5.53	22.85	54.08	46.47	25.25	8.28	26.75	24.55	21.54

Solution:

Time (h)	Concentration (ng/ml)	Individual AUC (t1-t0)*(C0+C1)/2	
0	0		
1	7.42	3.71	
2	5.53	6.475	
3	22.85	14.19	
4	54.08	38.465	
5	46.47	50.275	
6	22.25	34.36	
7	8.28	15.265	
8	26.75	17.515	
9	24.55	25.65	
10	21.54	23.045	
	Total AUC	228.95	ng.hr/ml

Sum of all individual AUC

Contd.

The extent of bioavailability can be determined by following equations:

$$F = \frac{[AUC]_{oral}\ D_{iv}}{[AUC]_{iv}\ D_{oral}}$$

$$F_r = \frac{[AUC]_{test}\ D_{std}}{[AUC]_{std}\ D_{test}}$$

With multiple dose study, the method involves drug administration for at least 5 biological half-lives with a dosing interval equal to or greater than the biological half-life (i.e. administration of at least 5 doses) to reach the steady-state.

$$F_r = \frac{[AUC]_{test}\ D_{std}\ \tau_{test}}{[AUC]_{std}\ D_{test}\ \tau_{std}}$$

Contd.

Bioavailability can also be determined from the peak plasma concentration at steady state Css,max according to following equation:

$$F_r = \frac{[C_{ss,max}]_{test}\ D_{std}\ \tau_{test}}{[C_{ss,max}]_{std}\ D_{test}\ \tau_{std}}$$

Contd.

Urinary Excretion Studies

This method of assessing bioavailability is *based on the principle that the urinary excretion of unchanged drug is directly proportional to the plasma concentration of drug.* Determination of bioavailability using urinary excretion data should be conducted only if at least 20% of administered dose is excreted unchanged in the urine. The study is particularly useful for –

✓ Drugs extensively excreted unchanged in the urine – for example, certain thiazide diuretics and sulphonamides.

✓ Drugs that have urine as the site of action - for example, urinary antiseptics such as nitrofurantoin and hexamine.

Contd.

The method involves –

✓ Collection of urine at regular intervals for a time-span equal to 7 biological half lives.

✓ Analysis of unchanged drug in the collected sample.

✓ Determination of the amount of drug excreted in each interval and cumulative amount excreted.

Contd.

For obtaining valid results, following criteria must be met –

✓ At each sample collection, total emptying of the bladder is necessary to avoid errors resulting from addition of residual amount to the next urine sample.

✓ Frequent sampling of urine is also essential in the beginning in order to compute correctly the rate of absorption.

✓ The fraction excreted unchanged in urine must remain constant.

Contd.

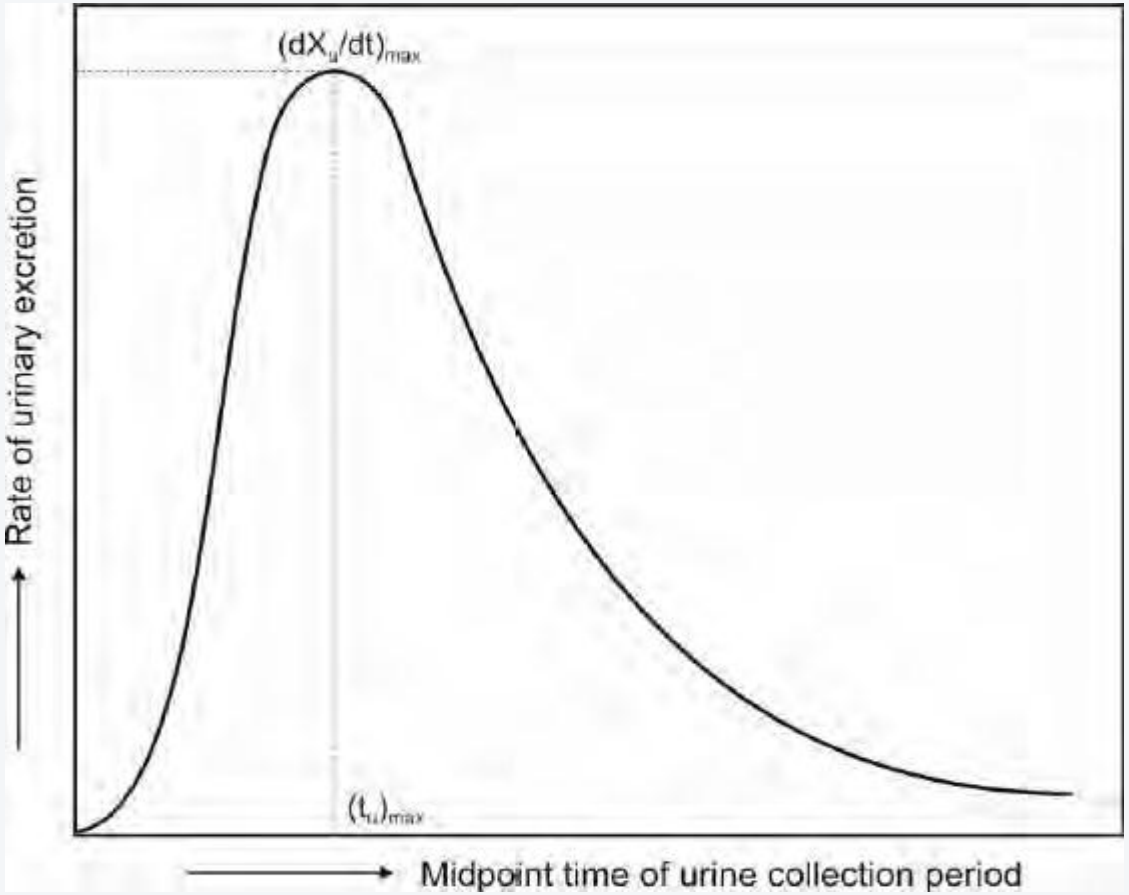
The three major parameters examined in urinary excretion data obtained with a single dose study are:

1. **$(dX_u/dt)_{max}$:** The maximum urinary excretion rate, it is obtained from the peak of plot between rate of excretion versus midpoint time of urine collection period. It is analogous to the C_{max} *derived from plasma level studies since the* rate of appearance of drug in the urine is proportional to its concentration in systemic circulation. Its value increases as the rate of and/or extent of absorption increases.

2. **$(t_u)_{max}$:** The time for maximum excretion rate, it is analogous to the t_{max} *of* plasma level data. Its value decreases as the absorption rate increases.

3. **X_u^{∞}:** The cumulative amount of drug excreted in the urine, it is related to the *AUC of plasma level data and increases as the extent of absorption increases.*

Contd.



Plot of urinary excretion rate versus time.

Contd.

The extent of bioavailability is calculated from equations given below:

$$F = \frac{[Xu\infty]_{oral}\ D_{iv}}{[Xu\infty]_{iv}\ D_{oral}}$$

$$F_r = \frac{[Xu\infty]_{test}\ D_{std}}{[Xu\infty]_{std}\ D_{test}}$$

With multiple dose study to steady-state, the equation for computing bioavailability is:

$$F_r = \frac{[X_{u,ss}]_{test}\ D_{std}\ \tau_{test}}{[X_{u,ss}]_{std}\ D_{test}\ \tau_{std}}$$

Contd.

Acute Pharmacological Response Method

When bioavailability measurement by pharmacokinetic methods is difficult, inaccurate or non-reproducible, an acute pharmacological effect such as a change in ECG or EEG readings, pupil diameter, etc. is related to the time course of a given drug. Bioavailability can then be determined by construction of pharmacological effect-time curve as well as dose-response graphs. The method requires measurement of responses for at least 3 biological half-lives of the drug in order to obtain a good estimate of AUC.

Contd.

Disadvantages of this method include –

1. The pharmacological response tends to be more variable and accurate correlation between measured response and drug available from the formulation is difficult.

2. The observed response may be due to an active metabolite whose concentration is not proportional to the concentration of parent drug responsible for the pharmacological effect.

Contd.

Therapeutic Response Method

Theoretically the most definite, this method is based on observing the clinical response to a drug formulation given to patients suffering from disease for which it is intended to be used. However, the method has several ***drawbacks –***

1. Quantitation of observed response is too improper to allow for reasonable assessment of relative bioavailability between two dosage forms of the same drug.

2. Bioequivalence studies are usually conducted using a crossover design in which each subject receives each of the test dosage forms, and it is assumed that the physiological status of the subject does not change significantly over the duration of the study.

Contd.

3. Unless multiple-dose protocols are employed, a patient who required the drug for a disease would be able to receive only a single dose of the drug every few days or perhaps each week.

4. Many patients receive more than one drug, and the results obtained from a bioavailability study could be compromised because of a drug–drug interaction.

In Vitro Drug Dissolution Rate and Bioavailability

The physicochemical property of most drugs that has greatest influence on their absorption characteristics from the GIT is dissolution rate. The best way of assessing therapeutic efficacy of drugs with a slow dissolution rate is *in vivo determination of* bioavailability which is usually done whenever a new formulation is to be introduced into the market. However, monitoring batch-to-batch consistency through use of such *in vivo* tests is extremely costly, tedious and time consuming besides exposing the healthy subjects to hazards of drugs. It would therefore be always desirable to substitute the *in vivo bioavailability tests with inexpensive in vitro methods. The simple in vitro* disintegration test is unreliable. *The best available tool today which can at least quantitatively assure about the biologic availability of a drug from its formulation is its* ***in vitro dissolution test.***

In Vitro Drug Dissolution Testing Models

For an *in vitro test to be useful, it must predict the in vivo behaviour to such an extent that in vivo bioavailability test need not be performed.*

Despite attempts to standardize the test performance, the *in vitro dissolution technique is still by no means a perfect* approach.

The efforts are mainly aimed at mimicking the environment offered by the biological system.

Contd.

There are several factors that must be considered in the design of a dissolution test.

They are –

- ***Factors relating to the dissolution apparatus*** *such as—the design, the size of the* container (several mL to several litres), the shape of the container (round bottomed or flat), nature of agitation (stirring, rotating or oscillating methods), speed of agitation, performance precision of the apparatus, etc.

Contd.

➢ ***Factors relating to the dissolution fluid such as—*** *composition (water, 0.1N HCl,* phosphate buffer, simulated gastric fluid, simulated intestinal fluid, etc.), viscosity, volume (generally larger than that needed to completely dissolve the drug under test), temperature (generally 37°C) and maintenance of **sink** (drug concentration in solution maintained constant at a low level) or **non-sink** conditions (gradual increase in the drug concentration in the dissolution medium).

➢ ***Process parameters*** *such as method of introduction of dosage form, sampling* techniques, changing the dissolution fluid, etc.

Contd.

The ***ideal features of a dissolution apparatus are:***

1. The fabrication, dimensions, & positioning of all components must be precisely specified & reproducible, run-to-run.

2. The apparatus must have simple design, easy to operate and useable under a variety of conditions.

3. The apparatus must be sensitive enough to reveal process changes and formulation differences but still yield repeatable results under identical conditions.

4. The apparatus should permit controlled variable intensity of uniform, non-turbulent liquid agitation.

5. Nearly perfect sink conditions should be maintained.

6. The apparatus should provide an easy means of introducing the dosage form into the dissolution medium and holding it, once immersed, in a regular reliable fashion.

Contd.

7. The apparatus should provide minimum mechanical abrasion to the dosage form during the test period to avoid disruption of the microenvironment surrounding the dissolving form.

8. Evaporation of the solvent medium must be eliminated, and the medium must be maintained at a fixed temperature within a specified narrow range. Most apparatuses are thermostatically controlled at around 37°C.

9. Samples should be easily withdrawn for automatically or manually without interrupting the flow.

10. The apparatus should be capable of allowing the evaluation of disintegrating, nondisintegrating, dense or floating tablets or capsules, and finely powdered drugs.

11. The apparatus should allow good inter-laboratory agreement.

Contd.

The dissolution apparatus has evolved gradually and considerably from a simple beaker type to a highly versatile and fully automated instrument. The devices can be classified in a number of ways. Based on the absence or presence of sink conditions, there are *two principal types of dissolution apparatus:*

1. **Closed-compartment apparatus:** It is basically a limited-volume apparatus operating under non-sink conditions. The dissolution fluid is restrained to the size of the container, e.g. beaker type apparatuses such as the rotating basket and the rotating paddle apparatus.

Contd.

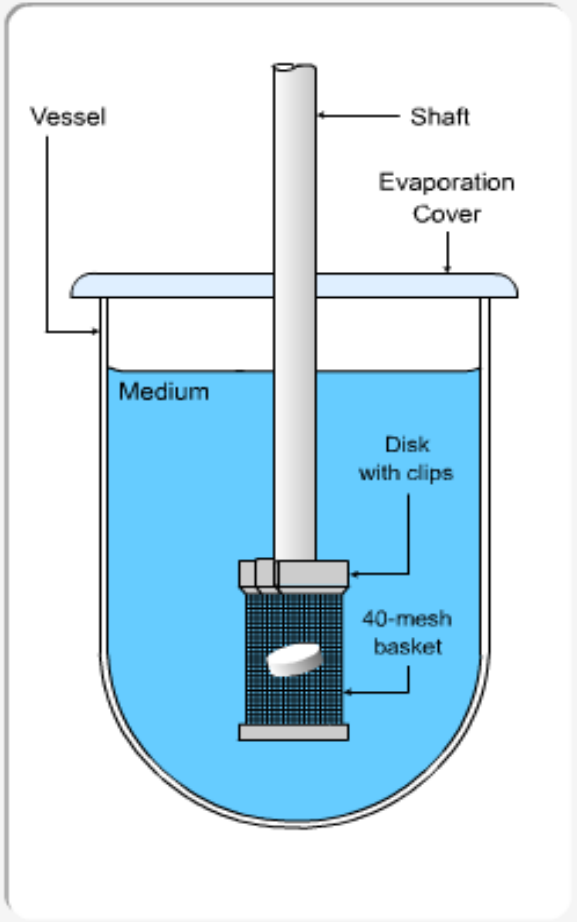
2. **Open-compartment (continuous flow-through) apparatus:** It is the one in which the dosage form is contained in a column which is brought in continuous contact with fresh, flowing dissolution medium (perfect sink condition).

A third type called as **dialysis systems are used for very poorly aqueous soluble drugs** for which maintenance of sink conditions would otherwise require large volume of dissolution fluid. Only the official or compendial methods (USP methods) will be discussed briefly.

Contd.

Rotating Basket Apparatus (Apparatus 1)

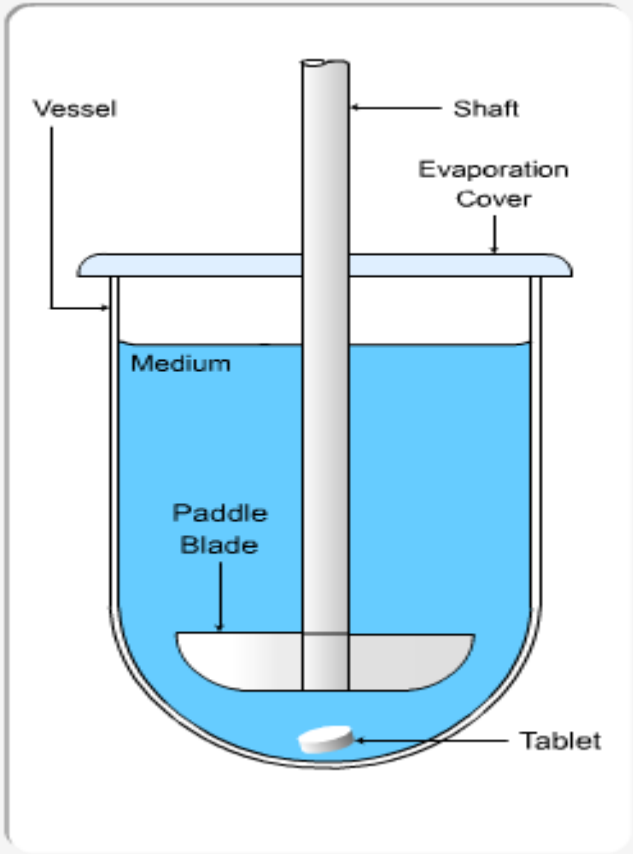
First described by Pernarowski *et al, it is basically a closed-compartment, beaker type* apparatus comprising of a cylindrical glass vessel with hemispherical bottom of one litre capacity partially immersed in a water bath to maintain the temperature at 37°C. A cylindrical basket made of 22 mesh to hold the dosage form is located centrally in the vessel at a distance of 2 cm from the bottom and rotated by a variable speed motor through a shaft. *The basket should remain in motion during drawing of* samples. All metal parts like basket and shaft are made of SS 316.



Contd.

Rotating Paddle Apparatus (Apparatus 2)

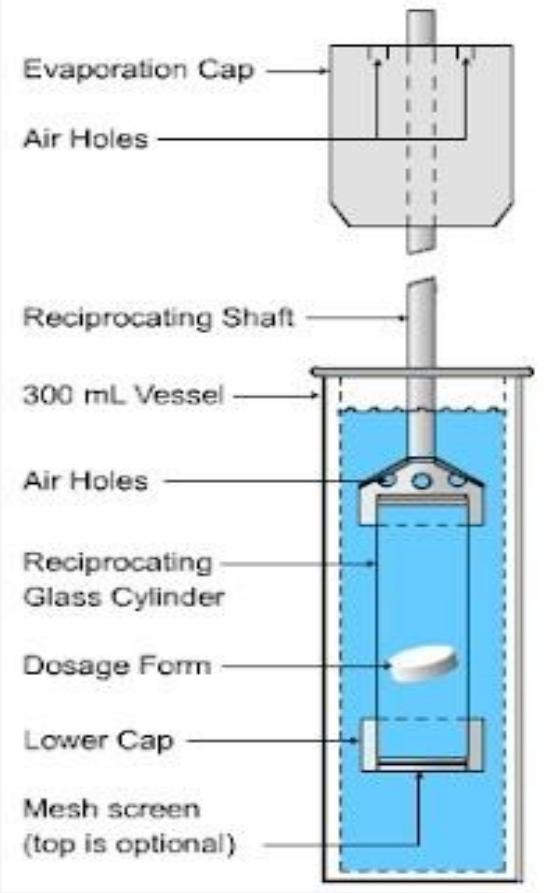
The assembly is same as that for apparatus 1 except that the rotating basket is replaced with a paddle which acts as a stirrer. *The method was first described by* Levy and Hayes. The dosage form is allowed to sink to the bottom of the vessel. Sinkers are recommended to prevent floating of capsules and other floatable forms. A small, loose, wire helix may be attached to such preparations to prevent them from floating.



Contd.

Reciprocating Cylinder Apparatus (Apparatus 3)

This apparatus consists of a set of cylindrical flat-bottomed glass vessels equipped with reciprocating cylinders. *The apparatus is particularly used for* dissolution testing of controlled release bead-type (pellet) formulations.

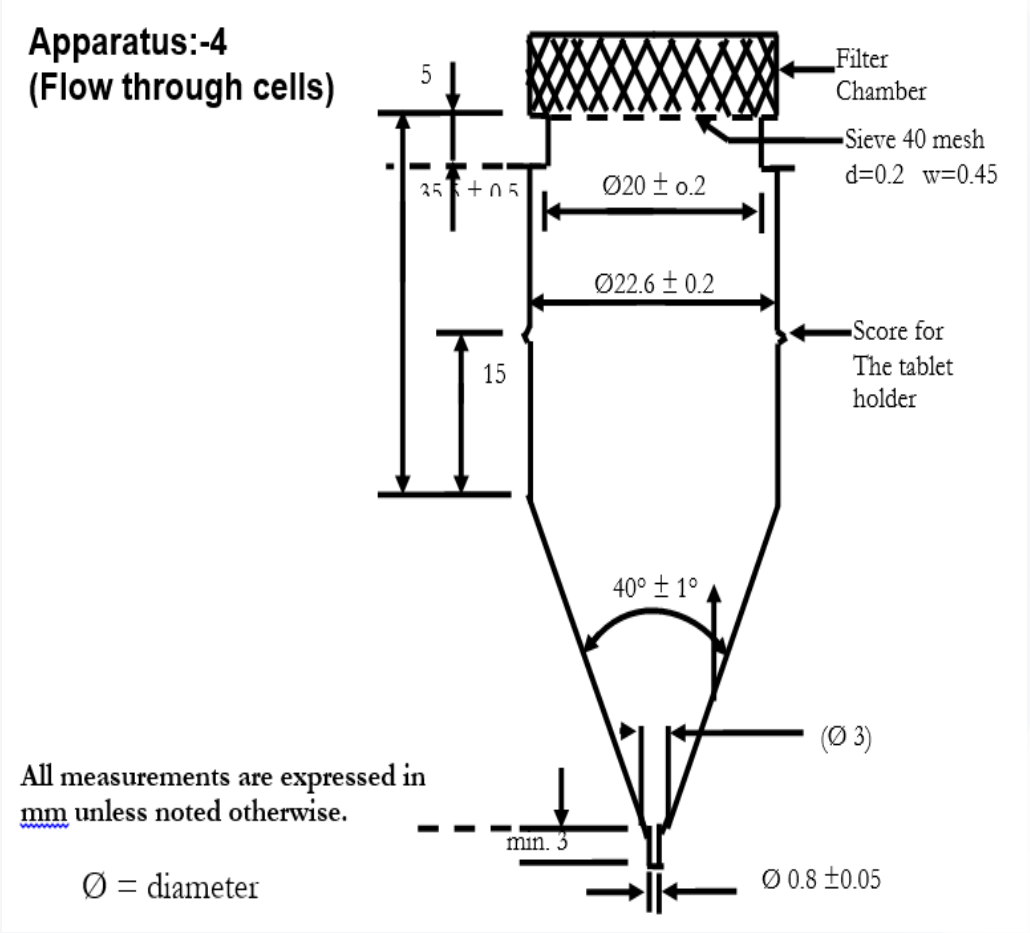


Contd.

Flow-Through Cell Apparatus (Apparatus 4)

The flow-through apparatus consists of a reservoir for the dissolution medium and a pump that forces dissolution medium through the cell holding the test sample. It may be used in either –

- Closed-mode where the fluid is recirculated and, by necessity, is of fixed volume, or
- Open-mode when there is continuous replenishment of the fluids.



Contd.

The material under test (tablet, capsules, or granules) is placed in the vertically mounted dissolution cell, which permits fresh solvent to be pumped (between 240 and 960 mL/h) in from the bottom.

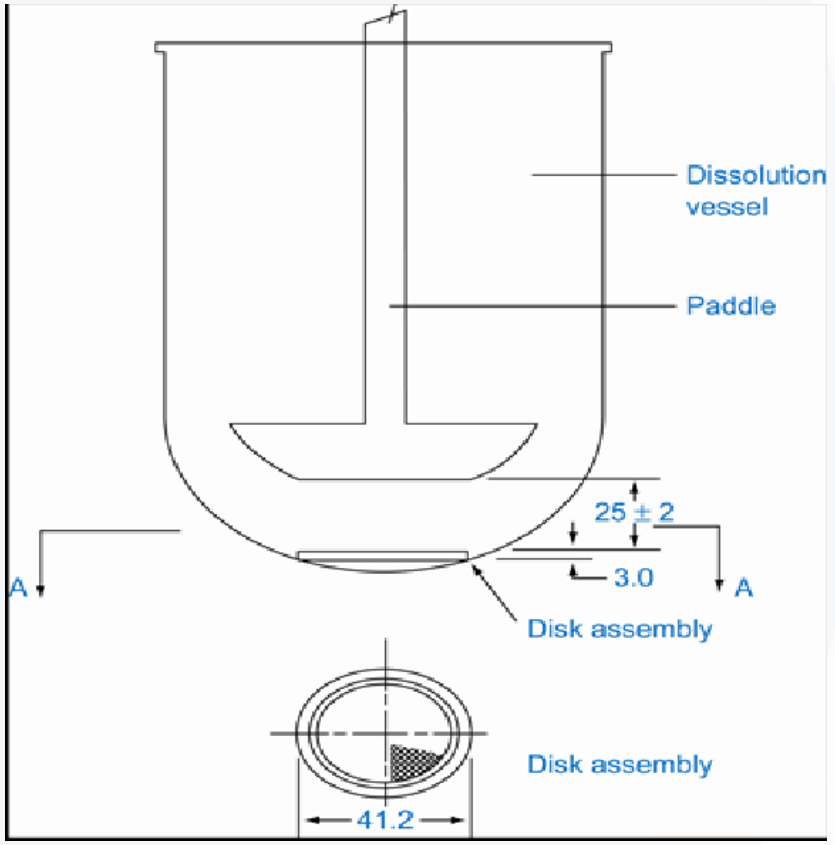
Advantages of this apparatus include –

1. Easy maintenance of sink conditions for dissolution which is often required for drugs having limited aqueous solubility.
2. Feasibility of using large volume of dissolution fluid.
3. Feasibility for automation of apparatus.

Contd.

Paddle Over Disc Apparatus (Apparatus 5)

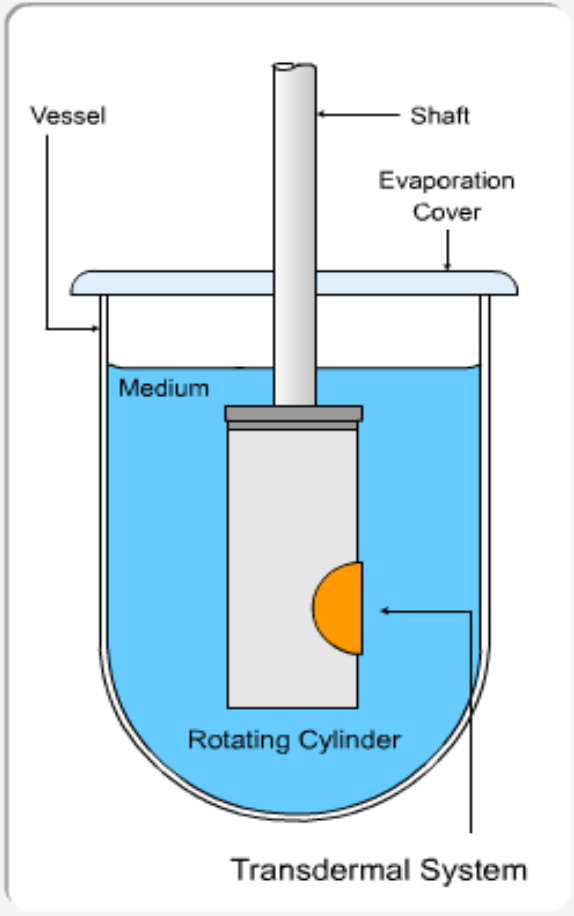
This apparatus is used for evaluation of transdermal products and consists of a sample holder or disc that holds the product. The disc is placed at the bottom of apparatus 2 (rotating paddle apparatus) *and the apparatus operated in the usual way.*



Contd.

Cylinder Apparatus (Apparatus 6)

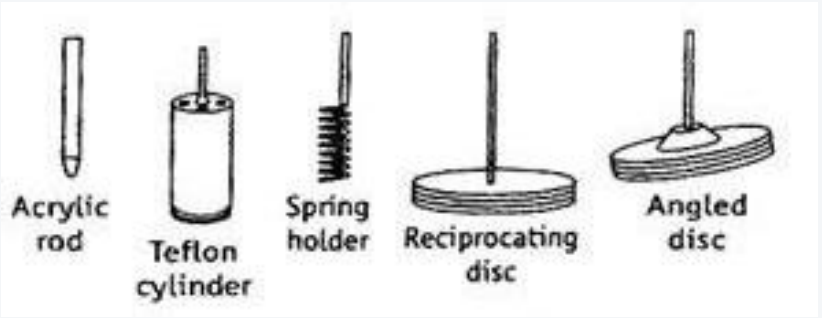
This apparatus is also used for evaluation of transdermal products and is similar to apparatus 1. *Instead of basket, a stainless steel cylinder is used to hold the* sample. The sample is mounted on an inert porous cellulosic material and adhered to the cylinder.



Contd.

Reciprocating Disc Apparatus (Apparatus 7)

This apparatus is used for evaluation of transdermal products as well as nondisintegrating controlled-release oral preparations. The samples are placed on disc-shaped holders *using inert porous cellulosic support which reciprocates vertically* by means of a drive inside a glass container containing dissolution medium. The test is carried out at 32°C and reciprocating frequency of 30 cycles/min.



Dissolution Acceptance Criteria

Stage	Number of dosage units tested	Acceptance criteria
S1	6	No dosage unit is less than Q+5%
S2	6	Average of the 12 dosage units ≥Q% and no dosage unit less than Q-15%
S3	12	Average of the 24 dosage units ≥Q% and not more than 2 dosage units are less than Q-15% and no dosage unit is less than Q-25%

Dissolution Methodology for Immediate Release Products Based on BCS

BCS Class	Dissolution Methodology
I	Single point if NLT 85% Q in 15 minutes Multiple point if Q < 85% in 15 minutes
II	Multiple point
III	Single point if NLT 85% Q in 15 minutes Multiple point if Q < 85% in 15 minutes
IV	Multiple point

Method for Comparison of Dissolution Profile

Model independent methods for comparison of two dissolution profiles:

1. **Determination of difference factor f1**: According to the FDA it is the % difference between 2 curves at each time points and is a measurement of relative errors between two curves.

$f_1 = \frac{\sum_{t=1}^{n}(R_t - T_t)}{\sum_{t=1}^{n} R_t} \times 100$, n is the number of dissolution time point, R_t is the dissolution value of the reference drug product at time t, T_t is the dissolution value of the test drug product at time t,

2. **Determination of similarity factor f2**: According to the FDA it is the logarithmic reciprocal square root transformation of the sum squared error and it is the measurement of the % dissolution between two curves.

$$f_2 = 50 \log \left[\left\{ 1 + \frac{1}{n} \sum_{t=1}^{n} (R_t - T_t)^2 \right\}^{-0.5} \times 100 \right]$$

Method for Comparison of Dissolution Profile

f_1	f_2	interpretation
0	100	Dissolution profiles are identical
≤ 15	≥ 50	Dissolution profiles are similar or equivalent

Similarity evaluation of between dissolution profiles are based on:

1. Minimum 3 dissolution points are measured
2. Minimum numbers of sample tested are 12 for both test and reference
3. Not more than 1 mean value of >85% dissolved for each product
4. SD of mean of any product should not be more than 10% from second to last dissolution time point

Contd.

Objectives of Dissolution Profile Comparison

Comparison of *in vitro dissolution profiles of test drug product and approved drug* product are useful for –

- Development of bioequivalent drug products.
- Demonstrating equivalence after change in formulation of drug product.
- Biowaiver of drug product of lower dose strength in proportion to higher dose strength drug product containing same active ingredient and excipients.

In Vitro—In Vivo Correlation (IVIVC)

A simple *in vitro dissolution test on the drug product will be insufficient to predict its* therapeutic efficacy. Convincing correlation between *in vitro dissolution behaviour of a* drug and its *in vivo bioavailability must be experimentally demonstrated to guarantee* reproducibility of biologic response.

In vitro-in vivo correlation *is defined as the predictive mathematical model that describes the relationship between an in-vitro property (such as the rate and extent of dissolution) of a dosage form and an in-vivo response (such as the plasma drug concentration or amount of drug absorbed).*

Contd.

The main objective of developing and evaluating an IVIVC is to enable the dissolution test to serve as a ***surrogate*** *(alternate) for in vivo bioavailability studies in* human beings.

The ***applications*** *of developing such an IVIVC are —*

1. To ensure batch-to-batch consistency in the physiological performance of a drug product by use of such *in vitro values.*

2. To serve as a tool in the development of a new dosage form with desired *in vivo* performance.

3. To assist in validating or setting dissolution specifications (i.e. the dissolution specifications are based on the performance of product *in vivo).*

Contd.

There are ***two basic approaches*** *by which a correlation between dissolution testing* and bioavailability can be developed:

1. By establishing a relationship, usually linear, between the *in vitro dissolution and* the *in vivo bioavailability parameters.*

2. By using the data from previous bioavailability studies to modify the dissolution methodology in order to arrive at meaningful *in vitro-in vivo correlation.*

Though the former approach is widely used, the latter still holds substance, since to date, there is no single dissolution rate test methodology that can be applied to all drugs.

Contd.

Some of the often used quantitative linear *in vitro-in vivo correlations are* –

1. **Correlations Based on the Plasma Level Data:** Here linear relationships between dissolution parameters such as percent drug dissolved, time of dissolution (t50%), mean dissolution time, rate of dissolution and corresponding parameters obtained from plasma level data such as AUC, Cmax, percent drug absorbed, Cmax, mean absorption time, mean residence time, Ka, etc. are developed; for example, percent drug dissolved versus percent drug absorbed plots.

In-vitro dissolution parameters	In-vivo plasma parameters
t50%	AUC, Cmax
Amount dissolve at specific time point	Fraction of drug absorbed, Ka
Mean dissolution time	Mean dissolution time, Mean residential time, Mean absorption time
Dissolution parameter	Concentration or absorbed amount at time t

Contd.

2. **Correlation Based on the Urinary Excretion Data:** Here, dissolution parameters are correlated to the amount of drug excreted unchanged in the urine, cumulative amount of drug excreted as a function of time, etc.

3. **Correlation Based on the Pharmacological Response:** An acute pharmacological effect such as LD50 in animals is related to any of the dissolution parameters.

Contd.

Statistical moments theory can also be used to determine the relationship such as mean dissolution time (*in vitro) versus mean residence time (in vivo).* Though examples of good correlations are many, there are instances when positive correlation is difficult or impossible; for example, in case of corticosteroids, the systemic availability may not depend upon the dissolution characteristics of the drug. Several factors that limit such a correlation include variables pertaining to the drug such as dissolution methodology, physicochemical properties of the drug such as particle size, physiologic variables like presystemic metabolism, etc.

Statistical moments are parameters that describe the characteristics of the time courses of plasma concentration (area, mean residence time, and variance of residence time) and of the urinary excretion rate that follow administration of a single dose of a drug.

Contd.

In vitro-In vivo Correlation Levels

Three IVIVC levels have been defined and categorised in descending order of usefulness.

Level A – The highest category of correlation, it represents a point-to-point relationship between *in vitro dissolution and the in vivo rate of absorption (or in vivo dissolution) i.e. the in vitro dissolution and in vivo absorption rate curves are* superimposable and the mathematical description for both curves is the same.

Advantages of level A correlation are as follows –

1. A point-to-point correlation is developed. The *in vitro dissolution curve* serves as a surrogate for *in vivo performance. Any change in* manufacturing procedure or modification in formula can be justified without the need for additional human studies.

2. The *in vitro dissolution serves as in vivo indicating quality control* procedure for predicting dosage form‘s performance.

Contd.

Level B – Utilises the principles of statistical moment analysis. The mean *in vitro* dissolution time is compared to either the mean residence time or the mean *in vivo* dissolution time. However, such a correlation is not a point-to-point correlation since there are a number of *in vivo curves that will produce similar mean residence* time values. It is for this reason that one cannot rely upon level B correlation to justify changes in manufacturing or modification in formula. Moreover, the *in vitro* data cannot be used for quality control standards. Alone it can not justify biowaiver.

Contd.

Level C – It is a single point correlation. It relates one dissolution time point (e.g. t50%, etc.) to one pharmacokinetic parameter such as AUC, tmax or Cmax. This level is generally useful only as a guide in formulation development or quality control owing to its obvious limitations. Biowaiver is not possible.

Multiple Level C –parameters to the amount of drug dissolved at various time points. It is correlation involving one or several pharmacokinetic parameters. It may be justify biowaiver provided that the correlation has been established the entire dissolution profile with one or more PK parameters.

Bioequivalence Studies

Need/Objectives of Bioequivalence Studies

If a new product is intended to be a substitute for an approved medicinal product as a pharmaceutical equivalent or alternative, the equivalence with this product should be shown or justified. In order to ensure clinical performance of such drug products, bioequivalence studies should be performed. Bioequivalence studies are conducted if there is:

- A risk of bio-inequivalence and/or
- A risk of pharmacotherapeutic failure or diminished clinical safety.

Contd.

Bioequivalence: It is a relative term which denotes that the drug substance in two or more identical dosage forms, reaches the systemic circulation at the same relative rate and to the same relative extent i.e. their plasma concentration-time profiles will be identical without significant statistical differences.

When statistically significant differences are observed in the bioavailability of two or more drug products, **bio-inequivalence** is indicated.

Types of Bioequivalence Studies

Bioequivalence can be demonstrated either –

- *In vivo, or*
- *In vitro.*

In vivo Bioequivalence Studies:

The following sequence of criteria is useful in assessing the need for *in vivo studies:*

1. Oral immediate release products with systemic action

- Indicated for serious conditions requiring assured response
- Narrow therapeutic margin
- Pharmacokinetics complicated by absorption < 70% or absorption window, nonlinear kinetics, presystemic elimination > 70%
- Unfavourable physiochemical properties, e.g. low solubility, metastable modifications, instability, etc.
- Documented evidence for bioavailability problems
- No relevant data available, unless justification by applicant that *in vivo study is* not necessary.

Contd.

2. Non-oral immediate release products.

3. Modified release products with systemic action.

In vivo bioequivalence studies are conducted in the usual manner as discussed for bioavailability studies, i.e. the pharmacokinetic and the pharmacodynamic methods.

Contd.

In vitro Bioequivalence Studies

If none of the criteria for in-vivo conditions is applicable, comparative *in vitro dissolution studies will* adequate. *In vitro studies, i.e. dissolution studies can be used in lieu of in vivo* bioequivalence under certain circumstances, called as ***biowaivers*** *(exemptions)* –

Contd.

1. The drug product differs only in strength of the active substance it contains, provided all the following conditions hold –

- Pharmacokinetics are linear
- The qualitative composition is the same
- The ratio between active substance and the excipients is the same, or (in the case of small strengths) the ratio between the excipients is the same
- Both products are produced by the same manufacturer at the same production site
- A bioavailability or bioequivalence study has been performed with the original product
- Under the same test conditions, the *in vitro dissolution rate is the same.*

Contd.

2. The drug product has been slightly reformulated or the manufacturing method has been slightly modified by the original manufacturer in ways that can convincingly be argued to be irrelevant for the bioavailability.

3. The drug product meets all of the following requirements –

- The product is in the form of solution or solubilised form (elixir, syrup, tincture, etc.)
- The product contains active ingredient in the same concentration as the approved drug product.
- The product contains no excipients known to significantly affect absorption of the active ingredient.

Contd.

4. An acceptable IVIVC and the *in vitro dissolution rate of the new product is* equivalent with that of the already approved medicinal product.

Moreover,

- The product is intended for topical administration (cream, ointment, gel, etc.) for local effect.
- The product is for oral administration but not intended to be absorbed (antacid or radio-opaque medium).
- The product is administered by inhalation as a gas or vapour. The criteria for drug products listed above indicate that ***bioavailability and bioequivalence are self-evident.***

Contd.

Bioequivalence Experimental Study Design

The various types of test designs that are usually employed in clinical trials, bioavailability and bioequivalence studies are discussed below.

ANOVA (Analysis of Variance)

It is a statistical technique specially designed to test whether the mean of more than two quantitative populations are equal or not. It is classified according to the numbers of criteria considered for test like one way, two way etc.

Assumptions:

1. Variance are distributed normally
2. There should be homogenous relation between variance
3. They are independent of error

ANOVA

To assess the significance of possible variation in dissolution performance of four different batches of tablets of a drug. The results at 15 minutes are given below. Make an analysis of variance data.

Hypothesis that there is no significant difference in the dissolution profiles of the four batches of tablets.

Batch-A	Batch-B	Batch-C	Batch-D
8	12	18	13
10	11	12	9
12	9	16	12
8	14	6	16
7	4	8	15

Calculation process for ANOVA

1. Calculate $\bar{x}$ for each sample
2. Then calculate x^2 for each sample
3. Calculate $\sum x$ and $\sum x^2$ for each sample variable
4. Calculation of correction factor = $\frac{T^2}{N}$, Sum of the variables of all samples T= $\sum X_1 + \sum X_2 + \cdots$,
   N= No. of the all observation
5. Calculation of total sum of square = $\sum {x_1}^2 + \sum {x_2}^2 + \cdots - \frac{T^2}{N}$
6. Calculation of sum of square between (sample) column treatment = $\frac{(\sum x_1)^2}{n_1} + \frac{(\sum x_2)^2}{n_2} + \cdots - \frac{T^2}{N}$
7. Calculation of Mean sum of square between sample = $\frac{Sun\ square\ between\ sample}{DF\ (n-1)}$
8. Calculation of sum of square between (within sample) row treatment = (Total sum of square- sum of square between sample)
9. Calculation of Mean sum of square within sample = $\frac{Sun\ square\ within\ sample}{DF\ (N-n)}$
10. Calculation of F= $\frac{\text{Mean sum of square between sample}}{\text{Mean sum of square within sample}}$
11. Construct an ANOVA table

Calculation process for ANOVA

Batch-A x_1	x_1^2	Batch-B x_2	x_2^2	Batch-C x_3	x_3^2	Batch-D x_4	x_4^2
8	64	12	144	18	324	13	169
10	100	11	121	12	144	9	81
12	144	9	81	16	256	12	144
8	64	14	196	6	36	16	256
7	49	4	16	8	64	15	225
$\sum 45$	$\sum 421$	$\sum 50$	$\sum 558$	$\sum 60$	$\sum 824$	$\sum 65$	$\sum 875$
$\overline{x_1} = 9$		$\overline{x_2} = 10$		$\overline{x_3} = 12$		$\overline{x_4} = 13$	

Calculation process for ANOVA

T= 45+50+60+65= 220

$\frac{T^2}{N} = \frac{(220)^2}{20}$ = 2420

Total sum of square = $\sum {x_1}^2 + \sum {x_2}^2 + \cdots - \frac{T^2}{N}$ = 421+558+824+875 -2420 =258

Sum square between sample = $\frac{(\sum x_1)^2}{n_1} + \frac{(\sum x_2)^2}{n_2} + \frac{(\sum x_1)^2}{n_3} + \frac{(\sum x_1)^2}{n_4} - \frac{T^2}{N}$

= $\frac{45\times45}{5} + \frac{50\times50}{5} + \frac{60\times60}{5} + \frac{65\times65}{5} - \frac{T^2}{N}$ = 2470-2420 = 50

Mean sum of square between sample = $\frac{Sun\ square\ between\ sample}{DF\ (n-1)} = \frac{50}{4-1}$ = 16.7

Sum of square within sample = (Total sum of square- sum of square between sample) = 258-50= 208

Mean sum of square within sample = $\frac{Sun\ square\ within\ sample}{DF\ (N-n)} = \frac{208}{20-4}$ = 13

F= $\frac{16.7}{13} = 1.285$

ANOVA table

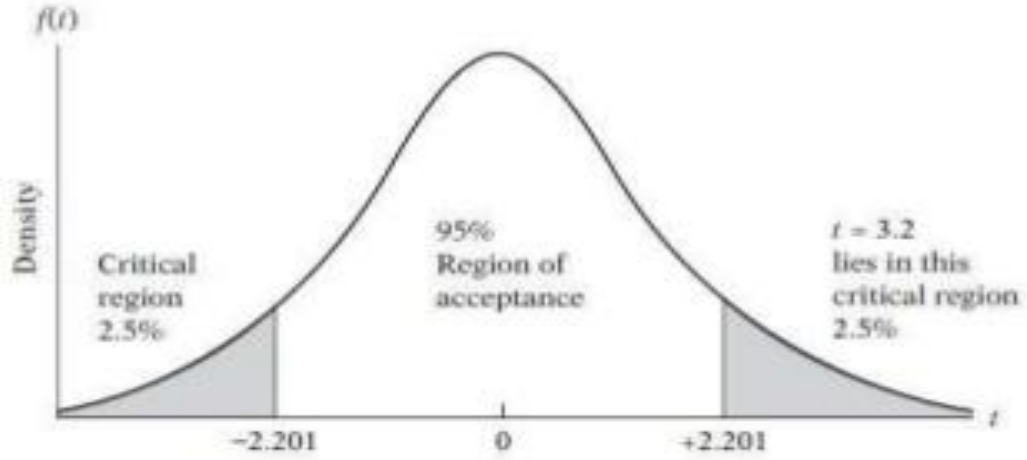
Source of variation	Sum square	Degree of freedom	Mean square	F
Between sample	50	3	16.7	1.285
Within sample	208	16	13	
Total	258	19		

For d.f. 3 and 16 at 5% level of significance F is 3.24 which is less than the calculated value. So, there is no significant difference between mean value of the dissolution profiles of four batches of tablets.

Hypothesis Testing: Confidence Interval VS The Test-of-Significance Approach

The value of t static is , t = (0.7240 − 0.5)/0.0700 = 3.2 which clearly lies in the rejection region.

FIGURE 5.4
The 95% confidence interval for t(11 df).



Student-t-test

When sample size is less or equals to 30 and population standard deviation is unknown , t-test is used. It is applicable for three different types of data. T-distribution has been derived under assumption of a normally distributed population.

1. Test the significance of the mean of random sample $t = \frac{\bar{X}-\mu}{S}\sqrt{n}$, μ is mean of population, $\overline{X}$ is mean of sample, S is standard deviation of sample = $\sqrt{\frac{\sum(X-\bar{X})^2}{n-1}}$, n is sample size
2. Test the difference between means of two samples (independent) $t = \frac{|\overline{X_1}-\overline{X_2}|}{S}\sqrt{\frac{n_1 n_2}{n_1+n_2}}$, S is Standard deviation of sample S= $\sqrt{\frac{\sum(X_1-\overline{X_1})^2+\sum(X_2-\overline{X_2})^2}{n_1+n_2-2}}$
3. Test the difference between means of two samples (dependent) $t = \frac{\bar{d}}{S}\sqrt{n}$, $\bar{d}$ is mean of differences, S= $\sqrt{\frac{\sum d^2 - n(\bar{d})^2}{n-1}}$

Student-t-test

The manufacture of a drug product claims that the mean self life is 25 months with standard deviation of 5 months. A random sample of 6 such product showed self life of 24, 26, 30, 20, 20 and 18 months. Is manufacturer's claim valid at 1% level of significance (at df.5, $t_{0.01} = 3.365$).

Solution: Let us take the hypothesis that there is no significant difference in the mean self life of the product in the sample and that of population. Applying t-test

X	$\bar{X}$	$X - \bar{X}$	$(X - \bar{X})^2$
24	23	1	1
26		3	9
30		7	49
20		-3	9
20		-3	9
18		-5	25
$\sum = 138$			$\sum = 102$

$$S = \sqrt{\frac{\sum(X-\bar{X})^2}{n-1}} = \sqrt{\frac{102}{5}} = 4.51 \qquad \mu = 25$$

$$t = \frac{\bar{X}-\mu}{S}\sqrt{n} = \frac{|23-25|}{4.51}\sqrt{6} = 1.07$$

Degree of freedom = n-1 = 6-1 = 5

The calculated t value is less than the tabulated value at 1% level of significance and degree of freedom 5. So, no significant difference is there and the manufacturer's claim is valid.

Student-t-test

Two types of products of a drug were used on 5 and 7 patients for reducing their weights. Drug A was imported and B was indigenous. The decrease in body weight after 6 months are A: 10, 12, 13, 11, 14; B: 8, 9, 12, 14, 15, 10, 9. Is there any significant difference in the efficiency and which product should buy? ($At\ df.10, t_{0.05} = 2.22$).

Solution: Let us take the hypothesis that there is no significant difference in efficiency between two products. Applying t-test

X_1	$\overline{X_1}$	$X_1 - \overline{X_1}$	$(X_1 - \overline{X_1})^2$
10	12	-2	4
12		0	0
13		1	1
11		-1	1
14		2	4
$\sum = 60$			$\sum = 10$

X_2	$\overline{X_2}$	$X_2 - \overline{X_2}$	$(X_2 - \overline{X_2})^2$
8	11	-3	9
9		-2	4
12		1	1
14		-3	9
15		4	16
10		-1	1
9		-2	4
$\sum = 77$			$\sum = 44$

$$S = \sqrt{\frac{\sum(X_1-\overline{X_1})^2+\sum(X_2-\overline{X_2})^2}{n_1+n_2-2}} = \sqrt{\frac{10+44}{5+7-2}} = 2.32$$

$$t = \frac{|\overline{X_1}-\overline{X_2}|}{S}\sqrt{\frac{n_1 n_2}{n_1+n_2}} = \frac{|12-11|}{2.32}\sqrt{\frac{5\times7}{5+7}} = 0.7$$

Degree of freedom = 5+7-2 = 10

The calculated t value is less than the tabulated value at 5% level of significance and degree of freedom 10. So, no significant difference is there in the efficacy of two drug products and the indigenous product should buy.

Student-t-test

d	d^2
3	9
6	36
-2	4
4	16
-3	9
4	16
6	36
0	0
0	0
2	4
$\sum = 20$	$\sum = 130$

A drug is given to 10 patients and the increment in their BP were recorded to be 3, 6, -2, 4, -3, 4, 6, 0, 0, 2. Is it believe that the drug has no effect on change of blood pressure? ($At\ df.9,\ t_{0.05} = 2.26$).

Solution: Let us take the hypothesis that the drug has no effect in change of BP.

Applying t-test

n= 10, $\bar{d} = \frac{20}{10} = 2$

$$S = \sqrt{\frac{\sum d^2 - n(\bar{d})^2}{n-1}} = \sqrt{\frac{130 - 10 \times 2^2}{10-1}} = 3.162$$

$$t = \frac{2}{3.162}\sqrt{10} = 2$$

Degree of freedom = 10-1= 9

The calculated t value is less than the tabulated value at 5% level of significance and degree of freedom 9. So, no significant difference is there and the drug has no effects on BP.

Contd.

1. Completely randomised designs

A completely randomized design (CRD) is one where the treatments are assigned completely at random so that each experimental unit has the same chance of receiving any one treatment. For the CRD, any difference among experimental units receiving the same treatment is considered as experimental error. Hence, CRD is appropriate only for experiments with homogeneous experimental units, such as laboratory experiments, where environmental effects are relatively easy to control.

Contd.

Advantages

1) The design is extremely easy to construct.

2) It can accommodate any number of treatments and subjects.

3) The design is easy and simple to analyse even though the sample sizes might not be the same for each treatment.

Disadvantages

1) Although the design can be used for any number of treatments, it is best suited for situations in which there are relatively few treatments.

2) All subjects must be as homogeneous as possible. Any extraneous sources of variability will tend to inflate the random error term, making it difficult to detect differences among the treatment (or factor level) mean responses.

Contd.

Method of randomisation

For a microbial culture experiment with four treatments A, B, C and D, each replicated five times taking as example.

Step 1. Determine the total number of experimental plots (n) as the product of the number of treatments (t) and the number of replications (r); that is, n = rt. For our example, n = 5 x 4 = 20. Here, one plot with a single treatment in it may be called a plot. In case the number of replications is not the same for all the treatments, the total number of experimental plots is to be obtained as the sum of the replications for each treatment

Contd.

Step 2. Assign a plot number to each experimental plot in any convenient manner; for example, consecutively from 1 to n.

Step 3. Assign the treatments to the experimental plots randomly using a table of random numbers. Locate a starting point in a table of random numbers by closing your eyes and pointing a finger to any position in a page. For our example, the starting point is taken at the intersection of the sixth row and the twelfth (single) column of two-digit numbers. Using the starting point obtained, read downward vertically to obtain n = 20 distinct two-digit random numbers. For our example, starting at the intersection of the sixth row and the twelfth column, the 20 distinct two-digit random numbers are as shown here together with their corresponding sequence of appearance. Rank the n random numbers obtained in ascending or descending order. For our example, the 20 random numbers are ranked from the smallest to the largest

Random Number	Sequence	Rank	Random Number	Sequence	Rank
37	1	8	86	11	19
80	2	18	30	12	6
76	3	16	67	13	14
02	4	1	05	14	3
65	5	13	50	15	11
27	6	5	31	16	7
54	7	12	04	17	2
77	8	17	18	18	4
48	9	10	41	19	9
73	10	15	89	20	20

Divide the n ranks derived into t groups, each consisting of r numbers, according to the sequence in which the random numbers appeared. For our example, the 20 ranks are divided into four groups, each consisting of five numbers, as follows:

Group Number	Ranks in the Group				
1	8	13	10	14	2
2	18	5	15	3	4
3	16	12	19	11	9
4	1	17	6	7	20

Assign the t treatments to the n experimental plots, by using the group number as the treatment number and the corresponding ranks in each group as the plot number in which the corresponding treatment is to be assigned. For our example, the first group is assigned to treatment A and plots numbered 8, 13, 10, 14 and 2 are assigned to receive this treatment; the second group is assigned to treatment B with plots numbered 18, 5, 15, 3 and 4; the third group is assigned to treatment C with plots numbered 16, 12, 19, 11 and 9; and the fourth group to treatment D with plots numbered 1, 17, 6, 7 and 20.

Plot no.	1	2	3	4
Treatment	D	A	B	B
Plot no.	5	6	7	8
Treatment	B	D	D	A
Plot no.	9	10	11	12
Treatment	C	A	C	C
Plot no.	13	14	15	16
Treatment	A	A	B	C
Plot no.	17	18	19	20
Treatment	D	B	C	D

Analysis of variance (ANOVA)

There are two sources of variation among the *n* observations obtained from a CRD trial. One is the variation due to treatments, the other is experimental error. The relative size of the two is used to indicate whether the observed difference among treatments is real or is due to chance. The treatment difference is said to be real if treatment variation is sufficiently larger than experimental error.

Contd.

2. Randomised block designs

The randomized complete block design (RCBD) is one of the most widely used experimental designs. The design is especially suited for field experiments where the number of treatments is not large and there exists a conspicuous factor based on which homogenous sets of experimental units can be identified. The primary distinguishing feature of the RCBD is the presence of blocks of equal size, each of which contains all the treatments.

First, subjects are sorted into homogeneous groups, called blocks and the treatments are then assigned at random within the blocks.

Contd.

Method of Randomisation

- Different blocks are constructed on a plot (table)
- Several variables or treatments are applied to different blocks
- The plots are arranged in such a way that each contains as many blocks as there are treatments
- It is used to determine the difference in productivity of different treatments
- Subjects having similar background characteristics are formed as blocks. Then treatments are randomised within each block, just like the simple randomisation. Randomisations for different blocks are done independent of each other.

Contd.

Advantages

1) With effective and systematic way of grouping, it can provide substantially more precise results than a completely randomised design of comparable size.

2) It can accommodate any number of treatments or replications.

3) Different treatments need not have equal sample size.

4) The statistical analysis is relatively simple. The design is easy to construct.

5) If an entire treatment or block needs to be dropped from the analysis for some reason, such as spoiled results, the analysis is not thereby complicated.

6) Variability in experimental units can be deliberately introduced to widen the range of validity of the experimental results without sacrificing the precision of results.

Block-I		Block-II		Block-III	
A	D	B	C	C	A
C	B	A	D	D	B
C	D	B	D	B	D
B	A	A	C	A	B

A, B, C and D treatment in three blocks.

Steps:

1. Calculation of correction factor = $\frac{T^2}{N}$

T= $\sum X_1 + \sum X_2 + \cdots$

N= No. of the all observation

2. Calculation of total sum of square = $\sum {x_1}^2 + \sum {x_2}^2 + \cdots - \frac{T^2}{N}$

3. Calculation of sum of square between column treatment = $\frac{(\sum x_1)^2}{n_1} + \frac{(\sum x_2)^2}{n_2} + \cdots - \frac{T^2}{N}$

4. Calculation of sum of square between row treatment = $\frac{(\sum r_1)^2}{n_r} + \frac{(\sum r_2)^2}{n_r} + \cdots - \frac{T^2}{N}$

5. Calculation of sum of square of residual/error = Total sum square – sum square between column and row

6. Calculation of mean sum of square by dividing each sum square by corresponding degree of freedom

7. Construct an ANOVA table

8. Interpretation of the analysis

Contd.

Disadvantages

1) Missing observations within a block require more complex analysis.

2) The degrees of freedom of experimental error are not as large as with a completely randomised design.

Contd.

3. Repeated measures, cross-over and carry-over designs

This is essentially a randomised block design in which the same subject serves as a block. The same subject is utilized for each of the treatments under study. Since we take repeated measures on each subject we get the design name —repeated measures design. The study may involve several treatments or a single treatment evaluated at different points in time. *The administration of two or more treatments one after the other in a specified or random order to the same group of patients is called a* ***crossover design or change-over design.***

Contd.

The drawback of crossover studies is the potential for distortion due to carry-over, that is, residual effects from preceding treatments. To prevent ***carry-over effects,*** *one must always allow for a wash-out period during which most of the drug is* eliminated from the body – generally about 10 elimination half-lives.

Example: *clinical trials to monitor safety and side effects.*

Contd.

Method of Randomisation: Complete randomisation is used to randomise the order of treatments for each subject. Randomisations for different subjects are independent of each other.

Advantages

1) They provide good precision for comparing treatments because all sources of variability between subjects are excluded from the experimental error.

2) It is economic on subjects. This is particularly important when only a few subjects can be utilized for the experiments.

3) When the interest is in the effects of a treatment over time, it is usually desirable to observe the same subject at different points in time rather than observing different subjects at the specified points in time.

Contd.

Disadvantages

1) There may be an order effect, which is connected with the position in the treatment order.

2) There may be a carry-over effect, which is connected with the preceding treatment or treatments.

Contd.

4. Latin square designs

Completely randomised design, randomised block design and repeated measures design are experiments where the person/subject/volunteer remains on the treatment from the start of the experiment until the end and thus are called as ***continuous trial.*** *In a Latin* square, however, each subject receives each treatment during the course of the experiment. In this design the experimental area is divided into blocks that are arranged in a square i.e., no of blocks in row and column are equals and equals to the no of treatments. A treatment can occur only once in a row and column.

Contd.

Latin square design is a two-factor design (subjects and treatments are the two factors) with one observation in each cell.

Such a design is useful compared the earlier ones when three or more treatments are to be compared and carry-over effects are balanced.

In a Latin square design, ***rows represent subjects, and columns represent treatments.***

Contd.

1. Calculation of total sum of square = $\sum {x_1}^2 + \sum {x_2}^2 + \cdots - \frac{T^2}{N}$

3. Calculation of sum of square between column = $\frac{(\sum x_1)^2}{n_1} + \frac{(\sum x_2)^2}{n_2} + \cdots - \frac{T^2}{N}$

4. Calculation of sum of square between row = $\frac{(\sum r_1)^2}{n_r} + \frac{(\sum r_2)^2}{n_r} + \cdots - \frac{T^2}{N}$

5. Calculation of sum of square between treatment = $\frac{(\sum T_1)^2}{n_t} + \frac{(\sum T_2)^2}{n_t} + \cdots - \frac{T^2}{N}$

6. Calculation of Reidual/Error = (total sum of square - sum of square between column- sum of square between row - sum of square between treatment)

7. Calculation of mean sum of square between column, between row and between treatment by dividing each sum square by corresponding degree of freedom

7. Construct an ANOVA table

8. Interpretation of the analysis

Contd.

Analyze the following results using Latin square design. A, B, C and denote the treatments and the figure denote the observations.

Solution: Coding the data (12 as common factor)

A (12)	D (20)	C (16)	B (10)
D (18)	A (14)	B (11)	C (14)
B (12)	C (15)	D (19)	A (13)
C (16)	B (11)	A (15)	D (20)

		x_1^2		x_2^2		x_3^2		x_4^2	Row Total
	A (0)	0	D (8)	64	C (4)	16	B (-2)	4	10
	D (6)	36	A (2)	4	B (-1)	1	C (2)	4	9
	B (0)	0	C (3)	9	D (7)	49	A (1)	1	11
	C (4)	16	B (-1)	1	A (3)	9	D (8)	64	14
Column Total	10	52	12	78	13	75	9	73	44

T= 10+12+13+9 = 44

$\frac{T^2}{N} = \frac{(44)^2}{16} = 121$

Total sum square = 52+78+75+73 - $\frac{T^2}{N}$

= 278-121 =157

Contd.

Treatment:

				Row Total
A (0)	A (2)	A (3)	A (1)	6
B (0)	B (-1)	B (-1)	B (-2)	-4
C (4)	C (3)	C (4)	C (2)	13
D (6)	D (8)	D (7)	D (8)	29
				44

Sum square between columns = $\frac{(\sum x_1)^2}{n_1} + \frac{(\sum x_2)^2}{n_2} + \cdots - \frac{T^2}{N} =$

$\frac{(10)^2}{4} + \frac{(12)^2}{4} + \frac{(13)^2}{4} + \frac{(9)^2}{4} - 121 = 2.5$

Sum square between rows = $\frac{(\sum r_1)^2}{n_r} + \frac{(\sum r_2)^2}{n_r} + \cdots - \frac{T^2}{N}$

$= \frac{(10)^2}{4} + \frac{(9)^2}{4} + \frac{(11)^2}{4} + \frac{(14)^2}{4} - 121 = 3.5$

Sum square between treatment = $\frac{(\sum T_1)^2}{n_t} + \frac{(\sum T_2)^2}{n_t} + \cdots - \frac{T^2}{N} =$

$\frac{(6)^2}{4} + \frac{(-4)^2}{4} + \frac{(13)^2}{4} + \frac{(29)^2}{4} - 121 = 144.5$

Residual/Error sum square = 157-2.5-3.5-144.5 = 6.5

Contd.

ANOVA TABLE

Source of variation	Sum square	Degree of freedom	Mean square	F	$F_{0.05}$
Between column	2.5	3	0.83	0.77	27.91
Between row	3.5	3	1.17	1.08	27.91
Between treatment	144.5	3	48.17	44.6	27.91
Residual/Error	6.5	6	1.08		

The F values for between columns and between rows are less than the tabulated value, so, there is no significant difference between rows and column data. But F value for between treatments is highly significant, so, Latin square design is not advantageous .

Contd.

Advantages

1) It minimizes the inter-subject variability in plasma drug levels.

2) Minimizes the carry-over effects which could occur when a given dosage form influences the bioavailability of a subsequently administered product (intrasubject variability).

3) Minimizes the variations due to time effect.

4) Treatment effects can be studied from a small-scale experiment. This is particularly helpful in preliminary or pilot studies.

5) Makes it possible to focus more on the formulation variables which is the key to success for any bioequivalence study.

Contd.

Disadvantages

1) The use of Latin square design will lead to a very small number of degrees of freedom for experimental error when only a few treatments are studied. On the other hand, when many treatments are studied, the degrees of freedom for experimental error maybe larger than necessary.

2) The randomisation required is somewhat more complex than that for earlier designs considered.

Contd.

3) The study takes a long time since an appropriate washout period between two administrations is essential which may be very long if the drug has a long t½.

4) When the number of formulations to be tested is more, the study becomes more difficult and subject dropout rates are also high. This can be overcome by use of a balanced incomplete block design in which a subject receives no more than 2 formulations.

Methods of determining drug Permeability

A. In vitro method

B. In Vivo method

C. In situ method

In vitro method

In vitro methods are carried out outside of the body and are used to determine the permeability of drug using live animal tissues. In vitro models have been introduced to assess the major factors involved in the absorption process and predict the rate and extent of drug absorption.

Methods of determining in-vitro drug Permeability

Physicochemical methods

1. Partition coefficient
2. Artificial membranes
3. Chromatographic retention indices
4. Brush border membrane vesicles (BBMV)
5. Isolated intestinal cells

6. Tissue techniques:

a). Everted small intestinal sac technique

b) Everted sac modification

c) Circulation techniques

d) Everted intestinal ring or slice techniques

7. Diffusion cell method
8. Cell culture techniques

Artificial membrane in-vitro drug Permeability

Artificial membranes are very useful in studying passive membrane permeability as they are reproducible and are suitable for high throughput screening. In this method, PAMPA model is used. Parallel artificial membrane permeability assay (PAMPA). PAMPA is a method which determines the permeability of substances from a donor compartment, through a lipid infused artificial membrane into an acceptor compartment. The artificial membrane is like a phospholipid membranes supported by filter material. It is prepared by pipetting a solution of lipids in an inert organic solvent on a supporting filter material which is placed on 96- well microtitre plate. A modification of this system is immobilized liposome chromatography(ILC) and on ILC, many compounds with same log P have been shown to demonstrate variable membrane partitioning based on their logs.

Chromatographic retention indices in-vitro drug Permeability

Immobilized artificial membranes (IAM) chromatography along with physicochemical parameters is used for evaluation of passive intestinal absorption. IAM stationary phases consist of monolayers of amphiphilic phospholipids covalently bound to a propylamino-silica skeleton. Micellar liquid chromatography (MLC) is also used for the prediction of passive drug absorption and in this system retention of drug mainly depends on hydrophobic, electronic and steric interactions. In general, chromatographic techniques are easy in operation and have high analytical sensitivity. Retention on IAM is expressed as retention factors corresponding to 100% aqueous mobile phase (logkw). Alternatively, the Chromatographic Hydrophobicity Indices (CHI-IAM), obtained by gradient elution, can be used. CHI-IAM corresponds to the fraction of organic modifier (acetonitrile) which leads to equal concentration between stationary and mobile phase. Usually, there is a good correlation between CHI-IAM and logkw values.

Brush border membrane vesicle (BBMV) in-vitro drug Permeability

A brush border is the name for the microvilli covered surface of simple cuboidal epithelium and simple columnar epithelial cells, found in the small intestine. Both animal and human tissue can be used for this.

Procedure: intestinal tissues are treated with calcium chloride precipitation method using centrifugation. the pellets obtained after centrifugation is resuspended in buffer which results in the formation of vesicles. Vesicles are mixed with drug in buffer solution and filtered after a period of time. The amount of drug taken up by the vesicles gives an account of drug absorption.

Advantage - useful for mechanic studies of drug absorption process.

Isolated intestinal cells in-vitro drug Permeability

Here, the small intestine is perfused with enzyme solutions that release the cells and the cells are treated with chelating agents or enzymes. The freshly isolated cells are suspended in buffer solution. At the time of experiment, the cells are separated, resuspended in buffer containing the drug under O2/CO2 and shaken well. After a specific period of time, the cells are separated by filtration, extracted and drug absorbed is determined.

Tissue technique (Everted small intestinal sac technique) in-vitro drug Permeability

This method involves isolating a small segment of the intestine of a laboratory animal such as rat, inverting the intestine and filling the sac with a small volume of drug free buffer solution.

Both the segments are tied off and the sac is immersed in an ERLENMEYER FLASK containing a large volume of buffer solution that contains the drug. The flask and its contents are then oxygenated and the whole preparation is maintained at 37°C and shaken mildly. At predetermined time intervals, the sac is removed and the concentration of drug in the serosal fluid is determined/ assayed for drug content.

Tissue technique (Everted sac modification) in-vitro drug Permeability

In this method, the test animal is fasted for a period of 20-24 hr and water is allowed. The animal is killed and the entire small intestine is everted. Segments, 5-15 cm in length are cut from a specific region of the intestine. The distal end of the segment is tied and the proximal end is attached to the cannula. The segment is suspended in a mucosal solution which contains the drug. A drug free buffer is then placed in the serosal compartment. For determining the rate of drug transfer, the entire volume of serosal solution is removed from the sac at each time interval with the help of a syringe and replaced with fresh buffer solution.The amount of drug that permeates the intestinal mucosa is plotted against time to describe the absorption profile of drug at any specific pH.

Tissue technique (Circulation technique) in-vitro drug Permeability

In this method, small intestine may or may not be everted. This involves isolating either the entire small intestine of small lab animal or a segment and circulating oxygenated buffer containing the drug through the lumen. Drug free buffer is circulated on the serosal side of the intestinal membrane and oxygenated. Absorption rate from the lumen to the outer solution are determined by sampling both the fluid circulating through the lumen and outside.

Tissue technique (Everted intestinal ring or slice technique) in-vitro drug Permeability

In this technique, the entire small intestine is isolated from the fasted experimental animal and washed with saline solution and dried by blotting with filter paper. The segment is tied at one end and by placing on glass rod it is carefully everted and cut into small rings. The everted intestinal rings are then incubated in drug containing buffer maintained at 37ºC with constant oxygenation. Under optimal conditions, rings remain viable for up to 2 hours and the transport of drug is stopped by rinsing the rings with ice cold buffer and drying them. At selected time interval, the tissue slices are assayed for drug content and expressed as mol/gm/time.

Diffusion cell in-vitro drug Permeability

In this method, small segments of small intestine are mounted between two glass chambers filled with buffer at 37° C. Diffusion cell consist of two compartments :-

I-Donor compartment - which contains the drug solution and the lower end of which contains the synthetic or natural GI membrane that interfaces with the receptor compartment.

II- Receptor compartment - which contain the buffer solution.

Cell culture technique in-vitro drug Permeability

Cell culture is the complex process by which cells are grown under controlled conditions, generally outside their natural environment. In this technique, differentiated cells of the intestine,

originating from CaCo2 cells (cells of carcinoma of colon) are placed on synthetic polycarbonate membrane previously treated with an appropriate material such as collagen which on incubation aids reproduction of cells while not retarding drug permeation characteristics. These models are based on the assumption that passage of drugs across the intestinal epithelium is the main barrier for drugs to reach the circulation.

Cell culture technique in-vitro drug Permeability

Human intestinal cell lines are generally divided into four different groups:

Type I: These cells differentiate spontaneously under normal culture conditions and hence are polarized (i.e. apical and basolateral surface), form domes, have tight junctions and brush border (eg.Caco-2 cells).

Type II: These cells differentiate into enterocytes- type cells only under specific culture conditions e.g. HT29 in presence of glucose, HT29 clone can differentiate into mucus cells.

Type III: These cells form domes but do not express any biochemical or morphological markers of differentiated cells. e.g. T84, SW116 and Col115 cell lines.

Cell culture technique in-vitro drug Permeability

Type IV: These cells do not differentiate. e.g. HCA7 and SE480 cell lines. CaCo-2 is the most widely used cell line and CaCo-2 are a human colon carcinoma cell line. The CaCo-2 (colon cancer cells) cell line is a continuous line of heterogeneous human epithelial colorectal adenocarcinoma cells. Solution of drug is placed on this layer of cultured cells and the system is placed on this layer of cultured cells and the system is placed (R.C) in a bath of buffer solution. The drug that reaches the latter compartment is sampled and analysed periodically. CaCo2 cells express tight junctions, microvilli and a number of enzymes and transporters that are characteristic of enterocytes. CaCo2 monolayer is widely used across the pharmaceutical industry as an in vitro model of the human small intestine mucosa to predict the absorption of orally administered drugs.

Cell culture technique in-vitro drug Permeability

The correlation between the in vitro apparent permeability across CaCo2 cell (human colon adenocarcinoma) monolayers or MDCK (Madin Darby Canine Kidney) cell line and the in vivo fraction absorbed is well established. Cell culture models have been employed in the screening of the intestinal permeability of libraries of new drug entities that have been generated through combinatorial chemistry and high throughput pharmacological screening.

In-vivo drug Permeability

In vitro and in situ techniques gives us an idea about absorption, but in vivo method gives us an idea about some important factor that influence absorption such as gastric emptying, intestinal motility and the effects of drugs on the GIT can be determined.

The in vivo method can be classified into:

1. Direct method
2. Indirect method

Direct method for in-vivo drug Permeability

The drug levels in blood or urine is determined as a function of time. For this, a suitable sensitive reproducible analytical procedure should be developed to determine the drug in the biological fluid. In this method, blank urine or blood sample is taken from the test animal before the experiment. The test dosage form is administered to the animal and at appropriate intervals of time the blood or urine sample are collected and assayed for the drug content. From the data, we can determine the rate and extent of drug absorption.

In this method, the experimental animal chosen should bear some resemble to man. It is reported that pigs most closely resemble to man but are not used due to the handling problems. The other animal that can be used are dogs, rabbits and rats. In this method, the experimental animal chosen should bear some resemble to man. It is reported that pigs most closely resemble to man but are not used due to the handling problems. The other animal that can be used are dogs, rabbits and rats.

Indirect method for in-vivo drug Permeability

When the measurement of drug concentration in blood or urine is difficult or not possible, but a sensitive method is available to test the activity, then absorption studies can be done by this indirect method. In this method, pharmacological response of the drug is related to the amount of drug in the body. The response is determined after the administration of a test dosage form; LD 50 appears to be dependent on the rate of the absorption of drug.

In-situ drug Permeability

It simulates the in vivo conditions for drug absorption and are based on perfusion of a segment of GIT by drug solution and determination of amount of drug diffused through it. In situ refers to those method in which the animal's blood supply remains intact in which the rate of absorption determined from these methods may be more realistic than those determined from in vitro techniques. These models are powerful tools to study the mechanistic aspects of this important process and acts as a bridge between in vivo and in vitro methods.

Thank you