CHAPTER 1

Preformulation Studies

- Introduction to Preformulation
- Goals and Objectives
- Study of Physicochemical Characteristics of Drug Substances
- Physical Properties (Physical Form: Crystal &Amorphous)
- Crystalline
- Particle size, shape
- Flow properties
- Solubility Profile (pKa, pH, Partition Coefficient)
- Polymorphism (Polymorphism and Crystal Properties)
- Chemical Properties (Hydrolysis, Oxidation, Reduction, Racemisation and Polymerization)
- BCS Classification of Drugs, its Significant
- Application of PreformulationConsiderations in the Development of Solid, Liquid Oral and Parenteral Dosage Forms and its Impact on Stability of Dosage Forms.

Introduction to Preformulation

The main objective of preformulation studies is *to generate useful information for developing a stable, bioavailable and therapeutically effective formulation that can be manufactured in large scale.*

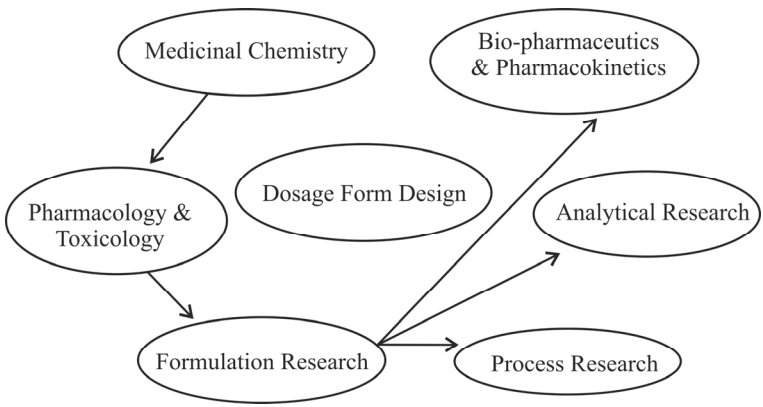


Fig. 1.1 Disciplines involved in dosage form design of a new drug

Development of a suitable dosage form of a new drug substance involves investigation by several departments or disciplines. The ultimate outcome of the entire program can only bring about a suitable dosage form. Figure 1.1 presents the names of disciplines involved in this activity. The development of a suitable dosage form of a new drug depends on certain information about the drug molecule. For example, once a new molecule of therapeutic interest is synthesized, it is first subjected to biological screening. When it passes the test, it is sent for clinical trials. To begin this test some basic information about the physicochemical properties of the drug molecule are necessary like chemical structure, molecular formula, molecular weight, solubility, salt forms, approximate dose, interaction with common excipients, etc. These information are generated during preformulation studies.

Hence, preformulation studies may be defined as *testing of physical and chemical properties of a drug substance alone and in combination with excipients proposed to be used in formulation.*

Goals and Objectives

In science the discovery of a new drug entity is a huge work and it becomes really more important when it passes the toxicity screening tests. It becomes a beneficial work. Finally, the effect of the new chemical entity

depends on its bioavailability after administration through an appropriate route in appropriate form. For this reason, transformation a new chemical entity to a potential new drug is a challenge for the pharmaceutical scientist. In fact, conversion of a new drug to active pharmaceutical formulation itself is a challenge. Thus, the chemical, analytical, pharmacokinetic, and pharmacodynamic properties of a drug are to be thoroughly examined to design a safe, stable and therapeutically effective dosage form.

Preformulation study is a multidisciplinary approach and involves several aspects of pharmacology, toxicology, clinical pharmacy, biochemistry, medicinal chemistry, and analytical chemistry. Therefore, the primary objective of preformulation study is to lay down foundation for transforming a new drug entity into a pharmaceutical formulation so that the drug can be administered in a right way, in right amount, and at right target (site). The secondary objective of preformulation study is to provide longer stability to the developed formulation through proper designing and protecting the drug from environmental condition and to evaluate performance of the prepared formulation. To improve bioavailability and stability efforts are to be made to optimize a molecule inform of salts, solvates, polymorphs, and importantly prodrug.

Salts

About 50% of the drug molecules being marketed as drug products are available in salt form. Conversion of a molecule into its salt form is widely used approach and the performance of the molecule is also enhanced. This improvement can be achieved in the area such as:

- Performance due to enhanced solubility and bioavailability.
- Increased stability due to improved hydrolytic and thermal stability.
- Improved organoleptic properties due to masking of taste.
- Increased patient compliance due to reduced side effects.
- Modified release dosage form due to change in solubility.

Prodrug

On required chemical modification of the molecule, an inactive derivative of the active drug molecule called prodrug is formed. The prodrug has optimized properties and better in vitro performance. About 10% of the drug molecules available in the market are available as respective prodrug. The major purpose of preparing a prodrug is to improve the bioavailability by avoiding first-pass metabolism, to increase the rate of drug absorption,

and organ selective transport. Therefore, prodrug can be defined as inactive form that undergoes biotransformation and is converted to active form to elicit its pharmacological effect. Development of a prodrug depends on specific property of the drug molecule that requires improvement and commonly with respect to stability and improved stability.

Recently the science has classified the drugs into following three categories;

- Cod drugs,
- Hard drugs, and
- Soft drugs

The *cod drug* consists of two pharmacologically active components complexed to form a single molecule, such as sulphasalazine, Levodopa-Entacapone.

Soft drugs are the modified derivatives with predetermined metabolism, so that after exerting therapeutic action for suitable time, its metabolite can be eliminated from the body. The primary intension to develop soft drug is to avoid toxicity associated with formed metabolites.

Hard drugs have the properties just opposite to the soft drugs. The modifications are made in such a way that its original properties are retained but not prone to chemical or biological transformation to avoid generation of metabolites or to increase the biological activity.

The preformulation studies are time bound program. The pharmaceutical industries carry out the preformulation studies on a new drug substance with the goals and objectives that can be divided into following categories –

(a) To find out the necessary physicochemical parameters of the new drug substance,
(b) To determine its kinetic rate profile,
(c) To determine its physical characteristics, and
(d) To assess its compatibility with common excipients.

In the correct viewpoint the following actions take place in an industry after discovery of a new drug entity till it is formulated as a stable product and finally marketed. In most cases, the investigational drug substance can never reach to the market place for one or more reasons as mentioned below:

1. The drug is synthesized and tested in a pharmacological screening test.

2. The drug shows satisfactory performance (result) for further study.
3. The quantity of the drug substance synthesized is so sufficient that
   - Initial toxicity studies could be performed,
   - Initial analytical tests could be performed,
   - Other initial preformulation studies could be performed.
4. Once toxicity study is successfully over, the phase I (Clinical Pharmacological trial) begins and a need for actual formulations is found; although at this point the practical dose level for human beings is not yet determined.
5. Once the phase I clinical trial is found successful, the phase II and phase III clinical trials begin. During this period (preferably phase II) an order of magnitude formula is finalized.
6. After completion of the above, an NDA (New Drug Application) is submitted.
7. Once the NDA gets approval, the production begins for launching of the product.

Fundamental Properties of the molecule

When a molecule is synthesized or isolated from natural sources, the chemical name, chemical structure, molecular formula and molecular weight are known. These are fundamental properties and do not change. Similarly, the solvent used for crystallization and recrystallization become known.

If the technique or method of manufacture of the drug is changed, the solvent used for crystallization and recrystallization may also change. Hence, a molecule is taken or sent for its pharmacological and toxicological evaluation with the knowledge of these.

Study of Physicochemical Characteristics of Drug Substances

The Table 1 presents a list of tests to be carried out during preformulation studies on a new chemical entity (NCE) having therapeutic utility. Most of the new drugs are formulated as solid dosage forms, - tablets and capsules. In fact more than 60% of the marketed formulations are tablet dosage forms. Other dosage forms occupy only 40% or less. More than 45% are tablets and about 15% are capsules.

Table 1.1 List of preformulation tests carried out during preformulation studies

Sl. No.	Tests
1.	Fundamental properties of the Molecule; like, Chemical name, Chemical structure, Molecular formula Molecular weight, Solvent used for crystallization and recrystallization,
2.	Organoleptic properties and Skin sensitivity
3.	Microscopic structure
4.	Physical characteristics Particle size and size distribution, Particle shape and surface area, Density, Flow properties, Compressibility, Hygroscopicity, Polymorphism.
5.	Solution characteristics Solubility, pH of 1% solution, Dissociation constant and pka, Effect of solubilizing agents Partition coefficient, Dissolution rate: intrinsic and particulate,
6.	Chemical properties Chemical identity UV-visible spectroscopy HPLC Analysis TLC Analysis Purity
7.	Therapeutic information Approximate Human dose Lethal dose Bioavailability
8.	Stability data Solid-state stability, Solution-state stability, Physical stability, Chemical stability, Microbiological stability, Therapeutic stability, Toxicological stability,
9.	Recommendation

Physical Properties

Physical form (Crystal & Amorphous)

The microscopic structure of a molecule is studied in the chemistry department after evaluation of fundamental properties. The microscopic structure depends on the method used to manufacture of the substance. Once the method of manufacturing is established the microscopic properties are examined. In fact, this is a property that plays important role in formulation development; because the internal structure and crystal habit (external or outer structure of a crystal shown in Fig. 1.2) can influence the physicochemical properties as well as stability of the compound. In some case the bioavailability of a drug changes with its solvate form; for example ampicillin. Physicochemical properties include flow property. Same compound may have different crystal habits. Depending on the method of crystallization, solvent used and temperature control, etc. different types of crystals are formed; for example prismatic, acicular (needle), bladed, tabular, sphere, spheroid, columnar, pyramidal, cubical, etc. Some of the crystal habits are shown here.

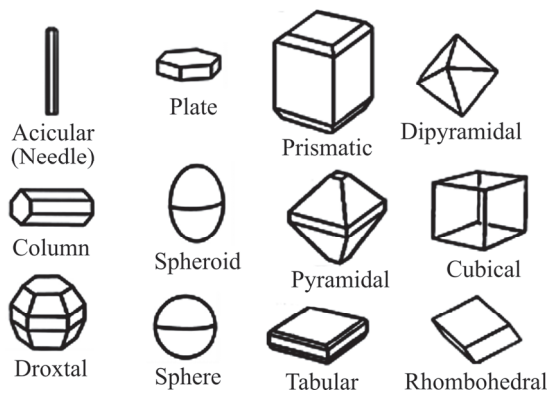


Fig. 1.2 Schematic diagram of some common crystalline structure

The features of crystalline and amorphous forms are given below:

1. Crystalline forms have fixed internal structure; while amorphous forms do not have fixed internal structure.
2. Stability of crystalline form is greater than that of its amorphous forms.
3. Solubility of crystalline solids is less than that of its amorphous solids.
4. On storage crystalline form has lesser tendency to change its form.
5. Thermodynamic energy of amorphous form is higher than its crystalline form.

Amorphous

The amorphous word has been derived from the Greek language; without shape). Non-crystalline solid is a solid that does not contain any long range order that is characteristic of a crystal. In some older books, the term has been used synonymously with glass. Currently, "glassy solid" or "amorphous solid" is being considered to be the concept, and glass is the more special case: Glass is an amorphous solid stabilized below its glass transition temperature. Polymers are frequently amorphous. Other types of amorphous solids are gels, thin films.

The internal structure of amorphous materials is made of interconnected structural blocks. These blocks can be similar to the fundamental structural units found in the equivalent crystalline phase of the same compound. Whether a material is a liquid or solid that depends primarily on the connectivity between its elementary building blocks and the solids can be characterized by a high degree of connectivity whereas structural blocks in liquids or fluids have lower connectivity.

In the pharmaceutical industry, amorphous drugs were shown to have higher bio-availability than their crystalline counterparts. This is due to the high solubility of amorphous powders. However, inside the body certain compounds can undergo precipitation in their amorphous form. They can decrease each other's bio-availability if administered together.

Amorphous materials are used for making thin films. The films contain layers of solid matters may be deposited on a solid surface; the thickness of the film varies from nanometers to micrometer. In general, the amorphous solids have two characteristic properties: When cleaved or broken, they produce fragments with irregular, often curved surfaces; when exposed to x-rays their structural patterns are poorly defined because their constituents are not arranged in a regular range. A glass is translucent, amorphous solid. Most of the substances can solidify in amorphous form if the liquid phase (solvent) is cooled rapidly enough. However, some solids are intrinsically amorphous, because either their components fail to fit together well enough to form a stable crystalline lattice or the impurities present disrupt the lattice. For example, the basic structural units of a quartz crystal and quartz glass have same chemical composition; the former is crystalline while latter is amorphous. Both contain SiO_2 and both consist of linked SiO_4 tetrahedral – the arrangements of the atoms in space are different. Crystalline quartz contains a highly ordered arrangement of silicon and oxygen atoms, but in quartz glass the atoms are randomly arranged. When molten SiO_2 is melted and cooled relatively rapidly, amorphous glass is obtained while

the same is cooled very slowly crystalline arrangement takes place. Similarly, aluminium may be crystalline or amorphous depending on the cooling rate of molten aluminium.

The amorphous phases are produced for particular purposes; for example, studying of growth of thin film. The growth of polycrystalline films is sometimes used and led to an initial amorphous layer; the thickness of the film may be few nm. Wedge-shaped polycrystals are identified by transmission electron microscopy. This can grow out of the amorphous phase only after growth of certain thickness; the exact value depends on deposition temperature, background pressure and various other process parameters.

Crystalline

The word crystal has been derived from the ancient Greek word *krustallos* meaning both ice and rock crystal. For example, snowflakes, diamonds, and table salt are large crystals. Most of the inorganic solids are not crystals but polycrystals. A crystal or crystalline solid can be defined as a solid material whose constituents such as atoms, molecules, or ions are arranged are arranged in a highly ordered microscopic structure, forming a crystal lattice that extends in all directions. In addition, macroscopic single crystals are usually identifiable by their geometrical shape consisting of flat faces with specific, characteristic orientations. The scientific study of crystals and crystal formation is known as crystallography. The process of crystal formation via mechanisms of crystal growth is called crystallization or solidification. Many microscopic crystals when fuse together form a large crystal; for example, metals, rocks, ceramics, and ice. All the solids are not crystals. The crystals have periodic order even when examined microscopically. The crystals and amorphous solids are distinctly different. For example, the process of formation of a glass does not release the latent heat of fusion, but form a crystal. A crystal structure is characterized by its unit cell, a small imaginary box containing one or more atoms in a specific spatial arrangement. The unit cells are stacked in three dimensional spaces to form the crystal. The symmetry of a crystal is constrained by the requirement that the unit cells stack perfectly with no gaps. There are about 219 possible crystal symmetries, called crystallographic space groups.

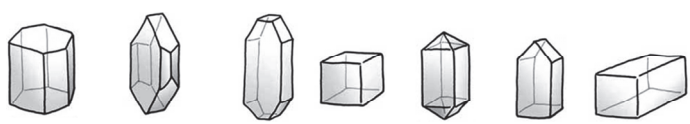


Fig. 1.3 Morphological structure of some crystals

Particle Size, Shape

Particle size distribution and shape can influence various physical as well as biopharmaceutical properties of a drug substance. For example,

- The difference among sizes of powder particles of a drug and other excipients in a mixture can cause mutual sieving (demixing) affecting the homogeneity of the powder mix (*uniformity of content*).
- If the powders are too fine, the particles become sticky and their flowability may decrease (*physical property*).
- Size distribution and the shape of the powders can influence the flow and mixing efficiency. Bioavailability of certain drugs, e.g., griseofulvin, phenacetin, etc. depends directly on the particle size distribution.

Usually the absorption of poorly soluble drug is dissolution rate limited. Thus, the absorption of such drugs can be improved by decreasing their particle size (*Biopharmaceutical property*). The stability of a powder drug depends on the particle size. Due to large surface area fine powders are more susceptible to atmospheric oxygen, moisture, light, heat and other interacting materials than coarse powders.

In general, for most formulations the optimum particle size range is 10-30 μm. If the particles are more than 100μm in size, grinding is required. Otherwise grinding should be avoided; because grinding develops a static electricity, which subsequently develops a tendency to aggregation; as a result apparent hydrophobicity is produced. Grinding damages the solvates and causes polymeric transformation of the substance. Moreover, handling of fines is also difficult. When a drug is administered orally as its solid dosage form, various processes are involved to complete the absorption of the drug. Fig. 1.4 shows these sequential processes of absorption.

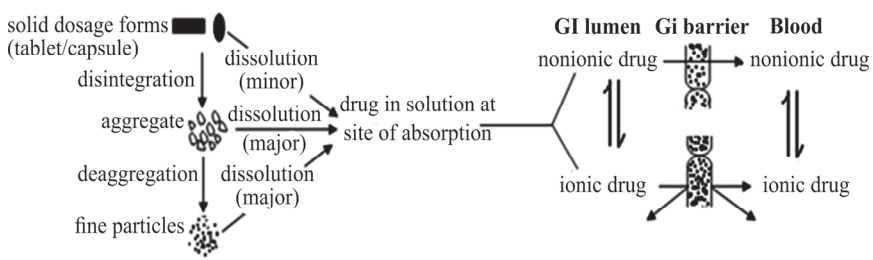


Fig. 1.4 Sequemce of steps involed in absorption of drug from orally administered solid dossage form

There are various methods for determination of particle size and each method has certain advantages as well as limitations with respect to minimum size of the particle, accuracy and ease of operation of the instrument. The Table 1.2 provides some common methods and measurable particle size range.

Table 1.2 Methods of particle size measurement

Method	Particle size range (µm)
Light scattering	0.5 – 50
Permeability	More than 1
Sedimentation	More than 1
Centrifugal	Less than 50
Elutriation	1 – 50
Sieving	More than 50
Microscopic	1 - 100

Microscopy, although tedious, is useful for determining both size and shape of the particles. The flow property, surface area, packing and compaction characteristics of powders depend on the shape of the particles. A sphere has minimum surface area per unit volume.

Density

Density is the weight of a material of unit volume (i.e., weight/volume). It is expressed as g/cc^3 or lb/ft^3. The powders may be porous, nonporous, hydrophilic, and hydrophobic. Density of powders may be determined as true, bulk, tapped density. True or absolute density of powders may be determined after degassing it under vacuum) of a weighed amount. The powders are filled in a container (pycnometer/specific gravity bottle) of known volume. The container is then filled with a liquid that can wet but does not dissolve the powders. By doing so the void spaces around the porous powders are removed and the volume of the packed particles is noted. Thus, the true density of powders, ρ_t can be calculated as;

$$\rho_t = \frac{W}{V_c - V_p}$$

Where W is the weight of the powder (g),

V_c is the volume of the container = volume of powder + volume of liquid

V_p is the volume of liquid in the container,

Hence, the volume of powder in the container = $V_c - V_p$ (ml or cc^3).

This is a traditional method. The density of powders is accurately determined by gas (helium) replacement method.

The information about true density and bulk density of a drug sometimes become very useful for developing a formulation. For example, while developing a suspension formulation, the true density of the dispersed solids helps to design the suspending medium to control the sedimentation. Similarly, to select the capsule size the knowledge about the bulk density of the powders is necessary. The bulk density of the granules helps to select punch size for compression as the dose of the drug is fixed and particularly when the dose size is high.

Bulk density of powders depends on the method of crystallization, milling or method of manufacture of the drug. As the bulk density can be changed either by compaction or by milling, this problem can be solved satisfactorily. Many active pharmaceutical ingredients are porous and hydrophobic. Determination of their density is not easy. It requires special instrument for accurate results. Alternatively, it is determined by suspending the powders in an immiscible solvent of known densities. Each solvent should contain small amount of suitable surfactant, so that the powders are properly wetted. The suspension is shaken vigorously, then mildly centrifuged and kept undisturbed until the powders either float or settle. The medium in which the powders float or suspend freely will have the density equal to that of powders. The density of the medium should be determined using a standard pycnometer. It is better to determine the density of the medium after removal of dispersed phase by filtration, as the medium may contain some dissolved solid which can contribute to the density of the medium.

Bulk density, alternatively called as tapped density, can be measured by tapping a known amount of powder taken in a cylinder using a tap density apparatus. The number of tapping may be done up to about 1000. The volume of the powder after tapping is recorded. The ratio of weight to volume will give the density.

Flow Properties

For manufacture of solid dosage forms, like tablets, capsules, powders, the drug powder need to flow smoothly. Hence, flowability is an important property of the drug powder and should be evaluated during preformulation studies. Flow property of the powders depends on particle size, shape, density, electrostatic charge and adsorbed moisture. During

processing or formulation these properties may change leading to change in flowability. If preformulation study indicates poor flowability of the drug, necessary excipients can be mixed with the drug to improve its flow property. Flow property of powders can be improved by granulating and/or by mixing with a suitable lubricant. The powders or granules should have good flowability to ensure efficient powder mixing and to compress the tablets with acceptable uniformity of weight.

There are various methods to study powder flow[4]; for example angle of repose, flow through an orifice, shear cell, compressibility index, etc.; but none can measure all the parameters of powder flow. For example, angle of repose, very widely used, does not have good sensitivity. The angle of repose can be measured by various methods and a static angle of a heap of powders is calculated. The value of the angle depends on the method used; the value varies from 25^{o} to 45^{o}.

Carr's compressibility index is a simple test to evaluate flowability of a powder. I this method the tapped density or bulk density, ρ_t and fluff (poured or initial bulk density) density, ρ_o are compared and expressed in percent. ρ_o is obtained by simple pouring a known amount of powder into a cylinder and the volume is noted. The tapped density or bulk density is determined by tapping a cylinder filled with the powder. The tapped volume and mass are noted and used to calculate the tapped density, ρ_t. With these two values the Carr's index (%) is calculated as below,

$$\text{Carr's index (\%)} = \frac{\rho_t - \rho_o}{\rho_t} \times 100$$

The Table 1.3 shows the relationship between Carr's index and flowability of powders with few examples.

Table 1.3 Carr's index (%) and flow property of pharmaceutical powders

Carr's index (%)	Flowability	Example
5 - 15	Excellent	Emcompress
12 - 16	Good	
18 - 21	Fair	Lactose monohydrate
23 - 35	Poor	Maize starch,
33 - 38	Very poor	Dicalcium phosphate, dihydrate
More than 40	Very, very poor	

Table 1.3 *Contd...*

Carr's index	Hausner's ratio	Flowability
5–15	1.05–1.18	Excellent
12–16	1.14–1.20	Good
18–21	1.22–1.26	Fair
23–35	1.30–1.54	Poor
33–38	1.50–1.61	Very poor
Greater than 40	Greater than 1.67	Extremely poor

When powders are poured over a flat, circular surface, the powders deposit in the form of a heap under the gravitational force as shown in the Fig 1.5. The angle between the free surface of the heap and horizontal surface is called as static angle of repose. This angle of repose is most commonly used to express flow characteristic of a powder. Most of the pharmaceutical powders or granules show angle of repose between 25° and 45°C. Lower is the angle of repose greater is the flowability.

Sometimes the angle of repose does not indicate flow property correctly. For example, sodium chloride, spray dried lactose having lower angle of repose fail to flow suitably through 6 mm orifice. Hence, the flow property of powders should be evaluated using more than one method.

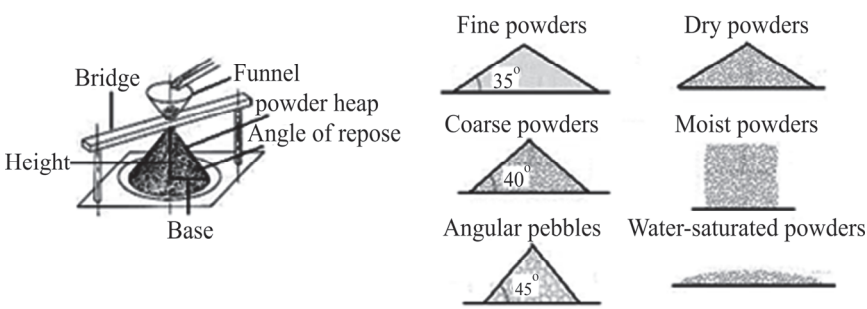


Fig. 1.5 Schematic diagram shwing how the angle of repose varies with the nature of powder

Solubility Profile

Whatever may be the route of administration, a drug must have some aqueous solubility for systemic absorption and pharmacological response. Drugs having aqueous solubility of less than 10 mg/ml show incomplete, erratic and/or slow absorption.

Usually a drug is either a weak acid or weak base. The solubility of a weakly acidic drug is more in alkaline medium and that of a weakly basic

drug is more in acidic medium. In both cases the drug will dissociate with a measurable dissociation constant, pK_a. Thus the solubility will change with change of pH. A drug which is either amorphous or zwitterions, will be soluble in both acidic and alkaline medium. A non-ionizable neutral drug will not show any change in its solubility due to change in pH. The fundamental solubility is the *intrinsic solubility*, C_o. *The solubility of a weakly acidic drug in acidic medium and that of a weakly basic drug in alkaline medium are called as intrinsic solubility*, because this indicates the solubility of unionized drug only.

The solubility has a great influence on the therapeutic efficacy of a drug and its product. This must be considered during formulation development. When a solid drug is administered orally, the drug is to be dissolved in the gastrointestinal fluid first for its absorption. The rate and extent of absorption of the drug depends on its rate of dissolution in gastrointestinal fluid. The absorption of poorly soluble drugs is dissolution rate-limited. If 1g of a drug dissolves in 100ml of water, its absorption will not be affected by its dissolution. At the same time the drug in solution in gastrointestinal fluid must remain stable until it is absorbed. If a drug is very soluble in gastrointestinal fluid but not stable, the absorption of the drug will decrease. In any of the two situations, efforts should be made to modify the solubility of the drug and/or its stability in solution state at pH ranging from 1 to 8.

Determination of solubility

The solubility can be determined quantitatively as follows: At a particular temperature a definite volume of solvent is taken. To this the solute is added gradually in small amounts and the mixture is shaken vigorously after each addition. At each time sample (solution of drug) is withdrawn and analyzed to determine the concentration. The process is continued until the consecutive samples show same concentration and a small amount of solute remains undissolved.

In case of poorly soluble drugs, problem may occur during determination of solubility. While investigating the solubility of poorly soluble drugs it has been found that due to the presence of soluble impurities in the drug, the solubility may appear more than the actual. This can be overcome by using facilitated dissolution method developed by Higuchi et al. According to this method the drug is first dissolved in a water-immiscible solvent and then partitioned into water. The aqueous phase is then analyzed to determine the solubility.

The drugs which degrade in their solution state present difficulty in solubility determinations. A kinetic method has been proposed by Ohnishi and Tanabe. According to their proposal, the rate constants and orders of the reactions for degradation of the drugs (solutes) in the solutions or suspensions are determined (Fig. 1.6). The rate of overall degradation of the drug in the suspension can be expressed as:

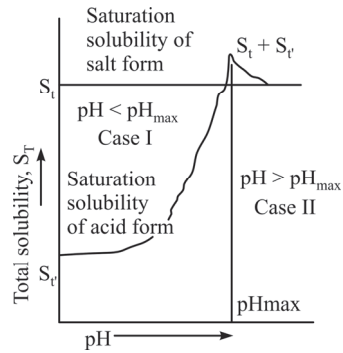


Fig. 1.6 pH – Solubility profile

$$V_s = \Sigma k_n [S]^n$$

Where, V_s is the overall rate of degradation of the drug, n is the order of reaction, k_n is the rate constant of the nth order reaction, and [S] is the concentration of the saturated solution of the drug. Among these V_s, k_n and n are measurable quantities, and hence, [S] can be calculated.

Further, there are certain drugs whose metastable forms transform into more stable forms when come in contact with solvent. For determination of solubility of these drugs, determination of intrinsic dissolution rates should be used. According to Noyes-Nernst equation, the initial dissolution rates for both metastable and stable forms are proportional to the respective solubilities of the polymorphic forms and the proportionality constants for both the forms of a drug will be same. Hence, by determining the intrinsic dissolution rates of metastable and stable forms, and solubility of the stable form, the solubility of the metastable form of the drug can be calculated.

For biopharmaceutical classification equilibrium solubility of a substance is determined under the conditions of physiological pH and temperature. As per FDA, the test is to be conducted at 37 $\pm 1^{o}$C in aqueous media within pH range of 1 – 7.5.

The bioequivalence test protocol prescribes one glass of water for oral administration of a dosage form to a human being. For this reason a drug is considered as the *highest soluble*, when its highest dose strength is completely soluble in 250 ml or less amount of aqueous media.

pKa

It has been mentioned earlier that most of the drugs are either weak acids or weak bases. Their dissolutions are very much dependent on pH. Hence,

Henderson-Hasselbach equation may be considered here:

For weak acid, HA: $pH = pKa + \log_{10} \frac{[A^-]}{HA}$

For weak base, B: $pH = pKa + \log_{10} \frac{[B]}{[BH^+]}$

The above two equations can be utilized;

- To find out the solubility at a particular pH, if the intrinsic solubility and pka are known,
- To select a salt-forming compound with desired solubility – pH profile.

pH

If the drug substances are classified on the basis of their ionic character, less than 5% of the drugs are nonionic or amphoteric. While one fifth (20%) are weak acids and three-forth (75%) are weak bases. Hence, ionization and dissolution of about 95% of the drugs take place simultaneously. The degree of ionization as well as solubility of these drugs depends on the pH of the medium. At a particular pH the saturation solubility will be the sum of the maximum solubility of ionized and unionized form of the drug. *This pH is called as* pH_{max}, *the pH of maximum solubility*. The effect of pH on the solubility and stability of drugs is very much important for liquid dosage forms, which may be intended for oral, topical, parenteral or ophthalmic administration.

Thus, the total amount of a monoprotic weak acidic drug (HA) in solution at a particular pH will be the sum of HA (unionized form) and salt form (A^-).

$$HA + H_2O \overset{k_n}{\longleftrightarrow} [H_3O]^+ + A^-$$

If the drug (HA) is present in excess amount, the amount of the unionized drug will be the maximum and constant. With increase of pH the amount of HA will increase, because the extent of salt formation will be less as shown in the Fig. 1.3. The total amount of drug, ionized and unionized, present in solution at a particular pH can be determined by use of one of the two equations given below depending on whether the pH is less than pH_{max} or more than pH_{max}.

In case of an acidic drug the total solubility, S_T, at a pH less than pH_{max}, $= (HA)_s \left(1 + \frac{k_a}{[H_3O^+]}\right)$ and

At a pH more than pH_{max}, $S_T = (A^-)_s\left(1+\frac{H_3O^+}{k_a}\right)$

Where

S_T = total saturation solubility with respect to the drug (HA) and its salt form (A^-),

$(HA)_s$= saturation solubility of the unionized form of the drug,

$(A^-)_s$= saturation solubility of the ionized form, and

k_a = apparent dissociation constant.

Similarly, for a basic drug substance, BH that ionizes as;

$$BH + H_2O \xleftrightarrow{k_a} [H_3O]^+ + B^-$$

The total solubility at a particular pH can be calculated as follows:

When pH is less than pH_{max}, $S_T = (BH^+)_s\left(1+\frac{H_3O^+}{k_a}\right)$ and

When pH is more than pH_{max}, $S_T = (B)_s\left(1+\frac{H_3O^+}{k_a}\right)$

Where $(BH^+)_s$ represents the solubility of ionized (protonated) form of the drug and $(B)_s$ represents the solubility of unionized form.

Partition Coefficient

As such absorption of drug through biological membrane is a complex process. A solid drug when administered orally the drug must dissolve in gastrointestinal fluid first, then cross the biological membrane for its absorption. In case of relatively insoluble drugs the overall absorption is dissolution rate-limited. While for soluble drugs the overall absorption depends on the permeation through gastrointestinal membrane. It is therefore necessary to study both dissolution and permeation characteristics of the drug during preformulation. The rate of permeation of a drug or any substance mainly depends on; molecular size, relative solubilities in water and lipid, and ionic charge of the molecule. Thus, assessment of permeation behavior of a new chemical entity (NCE) should be done even before studying dissolution characteristics.

The biological membranes are made of protein and lipid. To cross the membrane a drug must have some lipophilicity, that is, the solubility in lipids. Lipids present in biological membrane are complex and it is very

difficult to obtain them in pure form. However, relative lipid solubility can be assessed by measuring partition coefficient of a drug which is the measure of its distribution between a polar solvent (water) and a nonpolar solvent. In other words, *partition coefficient is an index of the hydrophilicity and lipophilicity of the drugs.* Many organic solvents, like chloroform, ether, carbon tetrachloride, isopropyl myristate, amyl acetate, benzene, n-hexane, n-octanol, etc. have been tried for determining partition coefficient of drugs. Out of which n-octanol has been found to produce satisfactory results.

If a definite amount of the drug is added to a mixture of two immiscible liquids, usually n-octanol and water, the drug will distribute itself between the two solvents until equilibrium is reached at a particular temperature. The ratio of concentrations is termed as the partition or distribution coefficient of the drug. The ratio is independent of the concentrations of the dilute solutions. Actually, lipids show some solubility in aqueous phase and vice versa. Hence, before determining the partition coefficient the solvents are to be saturated with respect to each other.

The n-octanol-water (oil-water) partition coefficient is commonly used as a measure of lipophilic character of the drug. The distribution/partition coefficient, K_w^o is expressed as

$$K_w^o = \frac{C_o}{C_w} = P$$

Where C_o and C_w are the concentration of the drug in oil (n-octanol) and water phase respectively. The value of P depends on whether the drug molecules associate or dissociate in the solution. The above equation stands true when the drug molecules associate in solution. When the drug molecules dissociate in solution, the equation may be modified as,

$$P = \frac{C_o}{(1-\alpha)C_w}$$

where α is the degree of ionization

The test is performed during preformulation studies for the selection of a solvent for,

- Extraction of drug,
- Crystallization of a drug,
- Extraction of a drug from its crude extract,
- Extraction and estimation of a drug from its formulation.

The relative polarities of the solvents can be expressed in terms of dielectric constant (ξ), solubility parameters (δ), interfacial tension (γ), or hydrophilic-lipophilic balance (HLB). The best solvent for any solute is one whose polarity matches with that of the solute; i.e. $\delta_{solute} = \delta_{solvent}$.

The most useful and easy method to know the polarity of drug is partition coefficient. The solvent solubility can be related to partition coefficient for majority of the drugs. Octanol is a partially polar solvent and it exhibits following properties similar to biological systems.

- Hydrogen bonding acceptor and donor, similar to many biological macromolecules.
- Inclusion of water, similar to biological lipid membranes.

For these reasons octanol has been widely used solvent for partition coefficient determination and the partition coefficient data has been used to correlate structure activity of the drug molecules also. There are various methods available to determine the partition coefficient of a drug substance, such as

1. Shake-flask method
2. Chromatographic method.
3. Counter current and filter probe method.
4. Tomlinson's filter probe method.
5. Micro-electrometrictitration method
6. Automated instrument is now available.

Polymorphism

Polymorphism and crystal properties

Polymorphism is a property of solid materials to exist in two or more crystalline forms. These forms have different arrangements or conformations of the constituents in the crystal lattice. When a drug substance is prepared either by precipitation or crystallization, the precipitated molecules may either be arranged in regular pattern or in irregular way. The orderly arranged molecules are called *crystals* having specific crystal lattice structure and irregularly or randomly arranged molecules are called as *amorphous.* The crystalline solids may exist in more than one crystalline form with different spacial arrangement in the crystal lattice, these crystal forms are called as *polymorphs* of the drug. The property of having polymorphs is called as *polymorphism*. Sometimes, during crystallization, solvent molecules are entrapped into

the crystals with specific lattice position and in a specific stoichiometry. Such crystals are called as *solvates or pseudo polymorphs*. By controlling the parameters of crystallization, like solvent, temperature, rate of cooling, etc. the number of polymorphs can be controlled. In many cases crystals of single lattice structure can also be obtained. Generally sudden change of cooling temperature or sudden change in the composition of solvent of crystallization or freeze drying (lyophillization) can produce amorphous solids.

The polymorphs of a particular drug mainly differ from each other in physical properties and therapeutic effect also. The polymorphic forms of a drug have different physicochemical properties such as dissolution and solubility, chemical and physical stability, flowability and hygroscopicity; also in various important biological properties such as bioavailability, therapeutic efficacy, and even toxicity. Polymorphic studies are important, because a particular polymorph can have a particular property which might not be exhibited by any other form. Hence, it is necessary to screen the polymorphs and to find out the suitable form for the formulation.

Chemical Properties

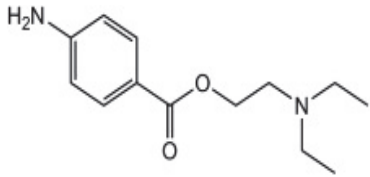
HYDROLYSIS

Hydrolysis is most commonly observed type of drug degradation. This happens due to the presence of water in isolation procedures, formulation strategies, and large number of functional groups that can undergo hydrolysis. Usually, hydrolysis takes place through acid- or base-catalyzed mechanism but it can occur in neutral pH condition where water acts as a base. The carbonyl functional group of esters, lactones, amides, lactams, carbamates, and imides are susceptible to hydrolysis.

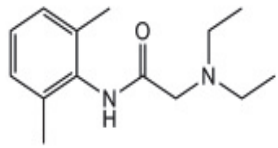
The chemical methods to prevent hydrolysis that may occur in vivo include the steric shielding. For example, the use of a bulky alkyl group close to the functional group hinders the approach of a nucleophile or enzyme, and thus reduces the likelihood of hydrolysis.

Electronic shielding is another method used to replace a functional group; for example, if a labile ester function is replaced with a urethane or amide, the nitrogen can feed electrons into the carbonyl group, making it less reactive. As a result, the chemical and metabolic stability increase. However, sterioelectronic modification can also increase the stability without compromising the activity. By changing the ester group in readily hydrolyzed procaine to an amide and introducing aromatic methyl groups to hinder the attack of the carbonyl group significantly reduce the rate of

hydrolysis. The resulting lidocaine is a longer-acting local anesthetic than procaine.



Procaine



Lidocaine

Hydrolysis can be retarded by modifying the chemical structure of the compound. The compound may stabilized by reducing its solubility.

OXIDATION

Oxidation is an important reason for which pharmaceutical product becomes unstable. Sometimes, not always, the addition of oxygen or removal of hydrogen is involved. When molecular oxygen is consumed, the reaction is said to be auto-oxidation. It takes place simultaneously at room temperature, although at slower rate.

Oxidation or the loss of electrons from an atom, sometimes involves free radicals, and subsequent chain reactions. Only a very small amount of oxygen is required to initiate a chain reaction. In practice, it is easy to remove most of the oxygen from a container, but it is difficult to remove all. Generally, nitrogen and carbon dioxide are used to displace the air in the headspace of the container to reduce or minimize deterioration by oxidation.

Since oxidation reaction is complicated, it is difficult to perform a kinetic study on oxidation processes within a general stability program. The redox potential which is constant and relatively easy to determine, can provide valuable and predictive information. In many oxidative reactions, the rate is proportional to the concentration of the oxidizing species but may be independent of concentration of the oxygen present. The rate is affected by temperature, radiation, and presence of a catalyst. If the temperature is increased, the rate of oxidation will increase. On the other hand, if the temperature of the product is reduced to 0 to 5^{o}C, the rate of oxidation would be reduced to half.

The molecular structure mostly susceptible to oxidation contain hydroxyl group directly bonded to an aromatic ring such as phenol or its derivative, etc. Oxidation products generally are not therapeutically potent. The visibility of oxidation process depends on the dilution of the reactants and eye-sight; for example, colorless epinephrine is oxidized to amber colored product.

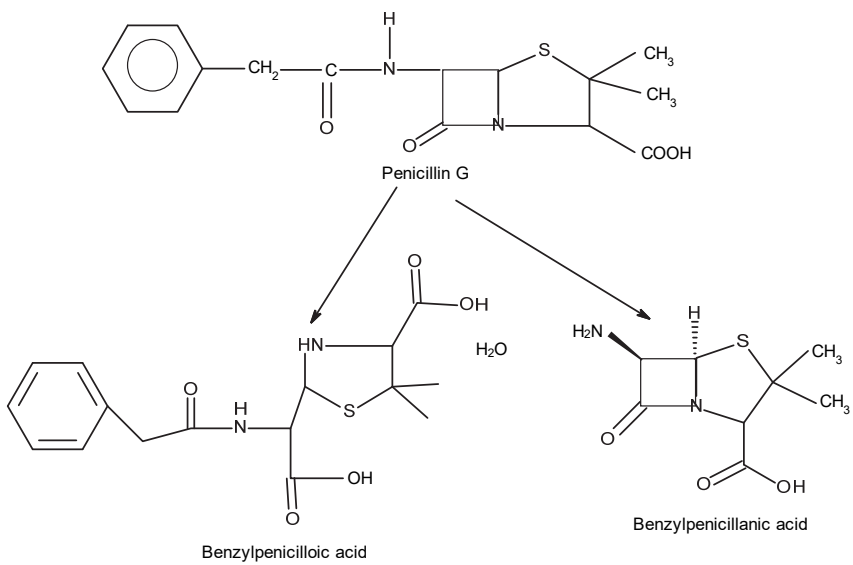
Oxidation is catalyzed by pH values that are higher than optimum, polyvalent heavy metals ions, and exposure to oxygen and UV illumination. Trace amounts of heavy metals such as cupric, chromic, ferrous, ferric ions. As little as 0.2 mg of copper ion per litre can considerably reduce the stability of drugs such as penicillin, epinephrine, phenylephrine, lincomycin, isoprenaline, and procaine HCl, etc.

Hydronium and hydroxyl ions can catalyze oxidative degradation. There is a pH range for maximum stability for any antibiotic and vitamin preparation. The stability of the pharmaceutical preparations can be improved by use of suitable antioxidant, by maintaining a suitable pH, and by using chelating agent.

REDUCTION

It is relatively more common pathway of drug metabolic process. Hepatic microsomes can catalyze various reductive chemical reactions and NADPHs are requiredfor this purpose. Azo and nitro reduction is catalyzed by cytochrome P450.Chloral hydrate is reduced to its active metabolite trichloroethanol by alcohol dehydrogenase. Reduction of prednisolone and cortisone results in the formation of their active metabolites hydrocortisone. Azo dyes used as coloring agents in pharmaceutical products or foods are reduced to form amines in the liver and by the intestinal flora.

RACEMISATION



Many drugs are chiral and racemization is a common mechanism of degradation. This can result in the loss of biological activity. For example, in proteins, a mixture of the D and L enantiomers is formed by base-catalyzed reaction of the natural L configuration. Acid-catalyzed racemization of epinephrine or base-catalyzed racemization of pilocarpine results in loss of pharmacological activity.

In pharmaceutical stability, racemization, or the action or process of changing from an optically active compound into a racemic compound or an optically inactive mixture of corresponding R (rectus) and S (sinister) forms, is a major consideration. Optical activity of a compound may be monitored by polarimetry and reported in terms of specific rotation. Chiral HPLC has beenused in addition to polarimetry to confirm the enantiomeric purity of a sample.

In general, racemization follows first-order kinetics and depends on temperature, solvent, catalyst, and the presence or absence of light. Racemization appears to depend on the functional group bound to the asymmetric carbon atom, with aromatic groups tending to accelerate the process.

POLYMERIZATION

Polymerization is a process is a process of reacting monomer molecules together in a chemical reaction to form polymer chains or three-dimensional networks. There are many forms of polymerization and different systems exist to categorize them.

The chemical compounds undergo polymerization reaction through a variety of reaction mechanisms with varying complexity due to the different functional groups present in the reactants and their inherent steric effects. For example, alkenes form polymers through relatively simple radical reactions; while reactions involving substitution at a carbonyl group require more complex synthesis due to the way in which reactants polymerize. Alkanes can also be polymerized, but only with the help of strong acids.

Since alkenes can polymerize in radical reactions, they form useful compounds such as polyethylene and polyvinyl chloride (PVC), which are produced in high tonnages each year due to their usefulness in manufacturing processes of commercial products, such as piping, insulation and packaging. In general, polymers such as PVC are referred to as homopolymers, as they consist of repeated long chains or structures of the same monomer as copolymers.

Other monomer units, such as formaldehyde hydrates or simple aldehydes, are able to polymerize themselves at quite low temperature such as –80°C to form trimers molecules consisting of 3 monomer units, which can cyclise to form ring structures or undergo further reactions to form tetramers, or 4 monomer-unit compounds. Such small polymers are referred to as oligomers. Formaldehyde is a strong reactive electrophile. It allows nucleophilic addition of hemiacetal intermediates, which are generally short-lived and relatively unstable "mid-stage" compounds that react with other molecules present to form more stable polymeric compounds.

Polymerization is not sufficiently moderated and proceeds at a fast rate can be very hazardous. This phenomenon is known as hazardous polymerization and can cause fires and explosions.

BCS CLASSIFICATION OF DRUGS & ITS SIGNIFICANT

Amidon, et al in 1995 proposed this method that has effectively replaced the repeated in vitro dissolution tests and reduce the time and cost. Based on three parameters – solubility, permeability, and dissolution the drugs are classified into four categories.

Class	Solubility	Permeability	Example
I	High	High	Metoprolol, Diltiazem
II	Low	High	Glibenclimide, Phenytoin
III	High	Low	Cimetidine, Neomycin
IV	Low	Low	Taxol, Hydrochlorothiazide

The objective of BCS classification is *to predict in vivo performance of a formulation from in vitro measurements of permeability and solubility.*

The **significance of BCS classification**:

- For identification of clinical bioequivalence tests, the efficiency of drug development, and review of the process.
- For classification according to dosage form dissolution along with the solubility, permeability characteristics of the drug product.
- To provide regulatory tool for replacing certain bioequivalence studies through accurate in-vitro dissolution tests.

- For reduction of the cost in drug development processes; also reduction of unnecessary drug exposure in healthy objects.
- Guidance to the pharmaceutical industries.

Class I drugs have high solubility and high permeability characteristics; thus these show **high dissolution number** and **high absorption number**. It is known that the rate limiting step is drug dissolution and if the dissolution is very rapid then gastric emptying rate becomes the rate determining step. High permeability indicates that the rate of absorption is higher than rate of excretion. For example, Metoprolol, Diltiazem, Verapamil, Propranolol, etc. belong to this class.

Class II drugs can penetrate the cell membrane easily but these have poor solubility in water. Thus, such drugs have a **high absorption number but a low dissolution number**. In this case, the in-vivo drug dissolution is the rate-limiting step for absorption at a very high dose number. The absorption of Class II drugs is usually slower than that for Class I drugs because of poor solubility. The absorption occurs over a longer period of time. In vitro–in vivo correlation (IVIVC) is usually acceptable for Class I and Class II drugs. The bioavailability of these drugs is influenced by their solvation rates. Hence, a correlation between the in-vivo bioavailability and the in-vitro solvation can be determined. Glibenclimide, phenytoin, mefenamic acid, nifedipine, ketoprofen, naproxen, carbamazepine, and ketoconazole, etc. belong to this class.

Class III drugs are cimetidine, acyclovir, neomycin B, captopril, etc. are highly soluble but can poorly permeate. Thus, their permeability is the rate limiting step for absorption. These drugs exhibit a high variation in the rate and extent of drug absorption. Since their dissolution is rapid, alteration in physiology and membrane permeability is responsible for the variation in their absorption but not the dosage form factors.

Class IV drugs show a lot of problems for effective oral administration; because these drugs have poor solubility and poor permeability. Thus, the drugs of this class show problems in effective oral administration. These compounds have poor bioavailability. These are usually not well absorbed through the intestinal mucosa, and there is high variability is observed. In fact, drugs of this class are rarely developed and marketed. Examples include hydrochlorothiazide, taxol, and furosemide.

Application of Preformulation Considerations in the Development of Solid, Liquid Oral and parenteral Dosage Forms and its Impact on Stability of Dosage Forms

Preformulation testing is considered as the first step of rational development of dosage form of a new drug molecule. The biopharmaceutical principles are used to select the suitable excipients, appropriate composition, correct processing steps, and suitable packaging materials. The ultimate aim is to design an optimum, cost effective, safe, stable, therapeutically effective drug product which is patient friendly. Thus, preformulation testing is the fundamental aspect of developing robust formulations to generate the required data to manufacture the drug product at large scale. It reduces the time of drug development process.

The preformulation testing is conducted in the preformulation stage only. In fact, after the successful synthesis or extraction of a new chemical entity found to have therapeutic effect the required data such as chemical structure, molecular weight, salts available, pharmacological class, expected dose, amount of drug available, expected time of dosage form development, and type of dosage form to be developed are collected and after it passes the toxicity study the formal preformulation testing begins.

Therefore, preformulation study can be considered as a part of research and development process used to study the mechanical as well as physicochemical properties of new drug to develop stable, therapeutically effective and safe dosage form. The information generated in the preformulation study are considered in the development of solid, liquid oral and parenteral dosage forms and its impact on the stability of dosage forms.

On the basis of data generated during detail preformulation studies, the formulation scientist either recommend or reject the proposed formulation. The recommendation or rejection must be supported with logical explanation. For example,

- If the drug is insoluble in water or its solubility cannot be increased adequately, other parameters are found to be satisfactory, the drug is recommended for suspension and ointment dosage forms.
- If the intrinsic solubility of the drug is not adequate but the drug can be solubilized through modification and other parameters are found satisfactory, the drug can be recommended for oral as well as parenteral solution.

- If the drug has good flow property along with normal solubility, the drug is recommended for tablet dosage form. Different crystalline solid forms such as polymorphs, salts, co-crystals.
- If the drug is bitter and the taste cannot be masked properly, the coated tablet is recommended.

During the preformulation study, the stability of the developed dosage form is also tested and accordingly the expiry date and storage conditions for greater stability of prepared dosage form are produced.

A. Multiple Choice Questions

1. The main objective of preformulation studies is
   a) To generate useful information for developing a stable, bioavailable and therapeutically effective formulation that can be manufactured in large scale.
   b) To examine the useful information for developing a stable, bioavailable and therapeutically effective formulation that can be manufactured in large scale.
   c) To evaluate the developed formulation that can be manufactured in large scale.
   d) None of the above
2. The improvement in bioavailability can be achieved through
   a) Increased stability due to improved hydrolytic stability.
   b) Increased stability due to improved thermal stability.
   c) Increased solubility
   d) Decreased solubility
3. The cod drug can be prepared by
   a) Complexing two pharmacologically inactive components to form a single molecule
   b) Complexing two pharmacologically active components to form a single molecule
   c) Complexing one pharmacologically active component with cod liver oil to form a single molecule
   d) Complexing one pharmacologically inactive component with one pharmacologically active component to form a single molecule

4. Why the pharmaceutical industries carry out thepreformulation studies on a new drug substance?
   a) To determine thedissolution rate profile of the drug
   b) To manufacture the dosage form in bulk quantity
   c) To determine the drug degradation rate.
   d) To assess the drug compatibility with common excipients
5. Which one of the following is considered as fundamental property of a drug?
   a) Molecular structure
   b) Particle shape and surface area,
   c) Density,
   d) Flow properties
6. The microscopic structure of a substance……...
   a) Depends on the method used to examine the substance.
   b) Depends on the method used to manufacture the substance
   c) Depends on the particle size of the substance being examined
   d) None of the above
7. Which of the following statements is correct?
   a) Crystalline forms do not have fixed internal structure
   b) Crystalline forms are not stable
   c) Crystalline forms have fixed internal structure
   d) Crystalline forms on storage have a tendency to change its internal structure
8. Thermodynamic energy of amorphous form…….
   a) Is less than its crystalline form
   b) Is equal to than its crystalline form
   c) All of the above
   d) None of the above
9. Particle size distribution and shape can influence……
   a) Various physical and biopharmaceutical properties of a drug substance
   b) Various chemical as well as biopharmaceutical properties of a drug substance
   c) Various chemical properties of a drug substance
   d) Flow properties of a drug substance

10. Which of the methods can be used to determine the particles in size range of 0.5 to 1μm?
    a) Sedimentation
    b) Light scattering
    c) Sieving
    d) Microscopic
11. The density of powders is accurately determined by……..
    a) Nitrogen gas replacement method.
    b) Hydrogen gas replacement method
    c) Helium gas replacement method
    d) Carbon dioxide gas replacement method
12. Bulk density of powders depends on……..
    a) The method of crystallization of the substance
    b) The method of milling of the substance
    c) The method of manufacture of the substance
    d) All of the above
13. According to Carr's index (%) of 18–21 the flow property of pharmaceutical powders would be
    a) Fair
    b) Very poor
    c) Good
    d) Excellent
14. Which of the following statements is correct/
    a) The solubility does not change with change of pH.
    b) An ionizable neutral drug will not show any change in its solubility due to change in pH.
    c) The solubility of a drug change with change of pH.
    d) A non-ionizable neutral drug will change in its solubility due to change in pH.
15. Which of the following statements is correct?
    a) The solubility of a weakly basic drug is more in alkaline medium
    b) The solubility of a weakly acidic drug is more in alkaline medium
    c) The solubility of a weakly acidic drug is more in acidic medium
    d) All of the above
16. Which of the following statements is correct?
    a) A drug is very soluble in gastrointestinal fluid if not stable; the absorption of the drug will increase.

b) A drug is very soluble in gastrointestinal fluid if not stable; the absorption of the drug will not change.

c) The drug in solution in gastrointestinal fluid must not be stable until it is absorbed.

d) The drug in solution in gastrointestinal fluid must remain stable until it is absorbed.

17. The solubility of a drug can be found out at a particular pH, ……

a) If the intrinsic solubility and pka are known

b) If only the intrinsic solubility is known

c) If only the pka value of the drug is known

d) None of the above

18. The effect of pH on the solubility and stability of drugs is very much important …..

a) For solid dosage form

b) For only parenteral dosage forms

c) For liquid dosage forms

d) For only oral dosage forms

19. For determining partition coefficient of drugs which of the following solvents is most suitable?

a) n-hexane, b) Chloroform

c) n-octanol d) Amyl acetate

20. Drugs having high solubility but low permeability are put in the BCS class of

a) I b) IV

c) II d) III

B. Short Questions

1. Enlist the preformulation tests to be conducted on new drug substance.
2. Explain the terms purity and impurity.
3. Explain the relation between pH and solubility.

4. Discuss the relation between absorption through biological membrane and partition coefficient of a drug.
5. Write note on polymorphism and crystal properties.
6. Explain how chemical stability of a drug is influenced by its crystal characteristics.
7. Explain intrinsic dissolution.
8. Write note on solid-state stability.
9. Discuss in brief the effect of temperature on stability of drug.
10. Explain the effect of humidity on stability of a solid drug substance.

C. Long Questions

1. Discuss how particle size distribution and shape can influence various physical as well as biopharmaceutical properties of a drug substance.
2. Define the term solubility. How can it be determined?
3. Describe how the rate of permeation of drugs in solution through the biological membrane is measured by in-vitro method.
4. Explain the factors that influence dissolution rate of a drug.
5. Discuss biopharmaceutical classification system.
6. Discuss how drug-excipient compatibility test is conducted.
7. Explain how the flow property of a drug substance is measured and mention its importance in formulation development.
8. How can compressibility of a solid drug substance be measured and why?
9. How does bioavailability of a drug depend on its crystal characteristics?
10. Explain the bioavailability characteristics of crystalline and amorphous for of drug.

MCQs Answers

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
a	c	b	d	a	b	c	d	a	b	c	d	a	c	b	d	a	b	c	d