



## **Physical properties of Drug Molecules**

- Physical properties are properties that can be measured or observed without changing the chemical composition of the substance.
- The study of physical properties involves the specific interaction between the molecules and a well-defined form of energy or external influence.
- Example: The concept of weight uses the force of gravity as an external influence to compare the mass of the objects. Ideally, physical property should be easily measured or calculated.
- Some physical properties of drug molecules and solvents are:
  - Molecular volume
  - Dipole moment
  - Optical rotation
  - Viscosity
  - Surface tension
  - Refractive index
  - Dielectric constant

## **Applications:**

- Identification of drugs
- Determination of percentage composition of drugs
- Drug synthesis
- Elucidation of the structure of molecules
- Formulation of dosage forms
- Evaluation of drug action

### **Group contribution methods:**

- Several physical properties are measured in the laboratory. These are due to the contribution of atoms, groups and their arrangement. Hence it is possible to calculate them theoretically by group contribution method.
- Group contribution method is a method used for calculating the physical properties theoretically based on contribution of atoms, groups and their arrangement.
- Basic principles involved in the group contribution methods are given below.
  - Colligative
  - Additive
  - Constitutive

### **Colligative properties:**

- Colligative properties depend mainly on the number of molecules present in a solution.
- The colligative properties of solutions are osmotic pressure, vapor pressure lowering, freezing point depression, and boiling point elevation.
- The values of the colligative properties are approximately the same for equal concentrations of different nonelectrolytes in solution regardless of the species or chemical nature of the constituents.
- For non volatile solute colligative properties of solid-in-liquid solutions may be vapor pressure.

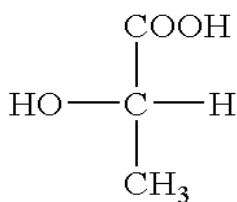
### **Additive properties:**

- Additive properties depend on the total contribution of the atoms in the molecules or on the sum of the properties of the constituents in a solution.

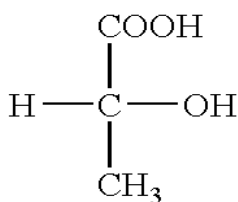
- Example: Molecular weight (because it is the sum of the masses of the constituent atoms).
- The total mass of the solution is the sum of the masses of the individual components.
- Consider the case of acetic acid (CH<sub>3</sub>COOH). Obtaining molecular weight of acetic acid involves addition of molecular weights of individual atoms that makes it. Acetic acid contains; C = 2, H = 4 and O = 2.
- So the molecular weight is calculated as;
 
$$\begin{aligned} \text{Molecular weight of acetic acid} &= \text{C} \times 2 + \text{H} \times 4 + \text{O} \times 2 \\ &= 12 \times 2 + 1 \times 4 + 16 \times 2 \\ &= 60 \text{ g/mol} \end{aligned}$$

### **Constitutive properties:**

- Constitutive properties depend on the arrangement and to a lesser extent on the number and kind of atoms within a molecule.
- These properties give clues to the constitution of individual compounds and groups of molecules in a system.
- The refraction of light, electric properties, surface and interfacial characteristics, and the solubility of drugs are at least in part constitutive and in part additive properties.
- The examples of this type are optical activity, surface tension, viscosity etc.
- Consider the case of lactic acid.
- It exists in two forms namely d-lactic acid and l-lactic acid.
- The specific rotation of d-lactic acid is +3.8° while l-lactic acid shows it as -3.8°.



L(+)-lactic acid



D(-)-lactic acid

### Refractive index:

The ratio between the speed of light in medium to speed in a vacuum is the refractive index. When light travels in medium other than the vacuum, the atoms of that medium continually absorb and re-emit the particles of light, slowing down the speed of light.

Refractive index is defined as “the ratio of the speed of light in a vacuum to its speed in a specific medium”.

Refractive index is also referred to as refraction index or index of refraction. The speed of light in a medium depends on the properties of the medium. In electromagnetic waves, the speed is dependent on the optical density of the medium.

Optical density is the tendency of the atoms in a material to restore the absorbed electromagnetic energy. The more optically dense material is, the slower the speed of light. One such indicator of the optical density of a medium is the refractive index.

### Refractive Index Formula

The refractive index is dimensionless. It is a number that indicates the number of times slower than a light wave would be in the material than it is in a vacuum. The refractive index, represented by symbol  $n$ , is the velocity of light in

vacuum divided by the velocity of light in a medium. The formula of the refractive index is as follows:

$$n = \frac{\sin i}{\sin r}$$

Where,

n is the refractive index

c is the velocity of light in a vacuum (  $3 \times 10^8$  m/s)

v is the velocity of light in a substance

The vacuum has a refractive index of 1. The refractive index of other materials can be calculated from the above equation. **Higher the refractive index, the higher the optical density and slower is the speed of light.** The table below lists the refractive indices of some materials.

**Table: Refractive Indices of Some Materials**

Material	Refractive Index	Material	Refractive Index
Air	1.00029	Crystal	2.00
Water	1.330	Diamond	2.417
Glass, soda-lime	1.510	Ethyl Alcohol	1.36
Vacuum	1.000000 (exactly)	Glass	1.5
Air (STP)	1.00029	Ice	1.309
Acetone	1.36	Iodine Crystal	3.34
Alcohol	1.329	Sodium Chloride	1.544
Crown Glass	1.52	Sugar Solution (30%)	1.38
Sugar Solution (80%)	1.49	Water (20°C)	1.333

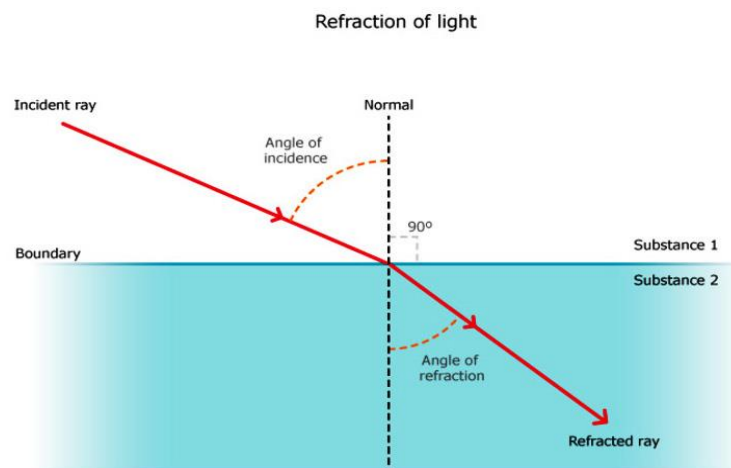
Snell's law states that the refractive index (n) of the liquid is a constant ratio of the angle of incidence to that of the refraction. It is given by:

$$n = \frac{\sin i}{\sin r}$$

Where, i is angle of incidence

r is angle of refraction

When monochromatic light passes through a less dense medium such as air or vacuum and enters a denser medium, the advancing waves at interface are modified and brought closer together. This leads to decrease in speed and shortening of wavelength. When light passes the denser medium, a part of wave slows down more quickly as it passes through interface and makes it bend towards the interface. This phenomenon is called as refraction. If light passes from denser medium to less denser medium then it is refracted away from the interface. This effect observed between mediums is expressed as refractive index ( $n$ ). The refractive index is a constant for a given pair of substances under specified conditions. It can be defined as ratio of speed of light in substance 1 to the speed of light in substance 2. This is usually written  $n_{21}$  and is the refractive index of substance 2 relative to substance 1. The incident light is in substance 1 and the refracted light is in substance 2. When the incident light is in a vacuum this value is called the absolute refractive index of substance.

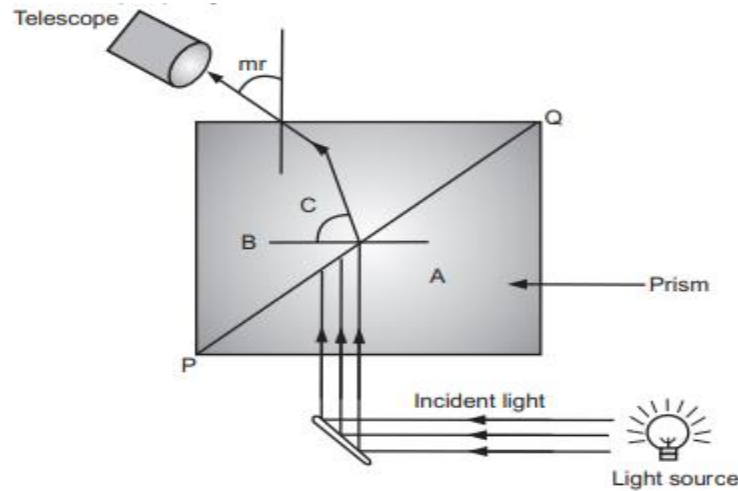


**Figure: Angles of Incidence and Refraction**

Refractive indices of most substance are more than air because the velocity of light in air is greater than in the substance for example, absolute refractive index

of water is 1.330, soda lime glass 1.510. By definition the refractive index of a vacuum is 1. In practice, air makes little difference to the refraction of light with an absolute refractive index of 1.0008.

### Measurement of Refractive Index:



**Figure: Schematic of Abbe's refractometer**

Refractive index is determined by using instrument called **refractometer**. Abbes refractometer, immersion refractometer and Pulfrich refractometer are used for this purpose. **Abbes refractometer** is commonly used at laboratory scale because of its advantages over other refractometers. It is most convenient, reliable and simple instrument with small sample size requirement suitable for range of substances. Ordinary light source, easy maintenance and economy and easy determinations are some of the other advantages of this instrument. The components of Abbes refractometers include light reflection mirror, dispersion compensator, telescope, and index arm and prism box. Abbes refractometer may be calibrated with anyone of the liquid specified in Table at temperatures below 25 °C using D-line of sodium.

**Table: Reference liquids for calibration of Abbes' refractometer**

Reference liquids	Refractive index ( $n_d^{25}$ )	Temperature coefficient
Water	1.3325	-
Carbon tetrachloride	1.4969	0.00057
Toluene	1.4969	- 0.00056
$\alpha$ -methyl-naphthalene	1.6176	- 0.00048

The refractive index varies with temperature and wavelength of light used and hence it is not a constant property.

Recent research has demonstrated the **existence of negative refractive index**. Not thought to occur naturally, this can be achieved with so called **metasubstances** and offers the possibility of perfect lenses and other exotic phenomena such as a **reversal of Snell's law**. The real and imaginary parts of the complex refractive index can be determined as a function of wavelength from an absorption spectrum of the substance. The refractive index of certain media may be different depending on the polarization and direction of propagation of the light through the medium. This is known as **birefringence or anisotropy**. The strong electric field of high intensity light for example, a laser, may cause a medium's refractive index to vary as the light passes through it, giving rise to non-linear optics. If the index varies quadratically with the field it is called the **optical Kerr effect** and causes phenomena such as self-focusing and self phase modulation. If the index varies linearly with the field it is known as the **Pockels effect**. If the refractive index of a medium is not constant, but varies gradually with position, the substance is known as a **gradient index medium**.

### **Why is high refractive index important for optical polymers?**

Optical polymers with high refractive index allow light rays to bend more within the substance, which helps in lowering the profile of the lens. Also, as the



refractive index increases, the thickness of the lens decreases, resulting in less weight.

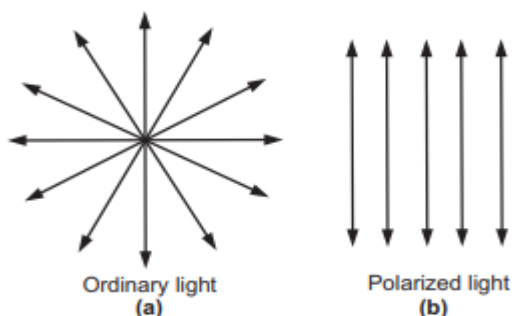
### **Applications:**

- Since refractive index is a fundamental physical property of a substance it is often used to analyze and identify a particular substance, confirm its purity, or measure its concentration.
- Refractive index values are useful in determination of molecular weights and structures of organic compounds from their molar refraction values.
- Refractive index is used to measure refraction characteristics of solids, liquids, and gases.
- Most commonly it is used to measure the concentration of a solute in an aqueous solution.
- For a solution of sugar, the refractive index can be used to determine the sugar content.
- Similarly alcohol content in bioproduction is also determined from the refractometry.
- Dielectric constant and molar polarizability values can be obtained from the refractive index.
- Refractive index of a substance is the most important property of any optical system that uses refraction for example, lenses and prisms.

### **Optical rotation:**

- The optical rotation is the angle through which the plane of polarization is rotated when polarized light passes through a layer of liquid.
- Ordinary light consists of vibrations, which are evenly distributed in all directions in a plane perpendicular to the direction of propagation, called as

**unpolarised light.** When the vibrations of light are restricted to only one plane, the light is said to be **polarized light.**



**Figure: Ordinary and polarized light**

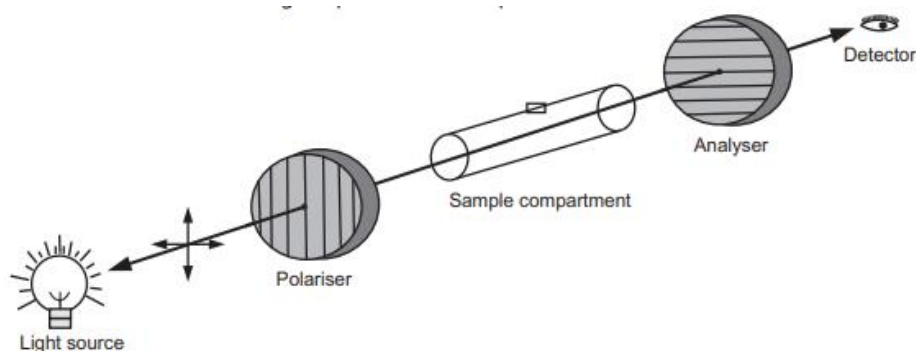
- Some substances rotate the plane of polarized light and are called as **optically active substances.** This property of an optically active substance is measured as **angle of rotation.**
- The property in which rotation of plane polarized light is observed is known as **optical activity** (or)
- Optical activity is the ability of a compound to rotate the plane of polarized light.
- Optically active substances include organic molecules with a central carbon atom to which four different groups are attached, making the molecule very asymmetric (chiral carbon).
- Similarly, laevulose, more commonly known as fructose, causes the plane of polarization to rotate to the left. Fructose is even more strongly levo rotatory than the glucose.
- The substance which rotates the plane of polarized light to the right or clockwise when viewed in the direction of light propagation is called dextro rotatory (d) or (+) substance.

- The use of one name for glucose, dextrose, refers to the fact that it causes linearly polarized light to rotate to the right or dexter side. Those optically active substances that rotate plane polarized light to the left or counter clockwise are known as leavo rotatory (l) or (–) substance. Laevulose, more commonly known as fructose causes the plane of polarization to the left. Fructose is even more strongly leavo rotatory than the glucose.
- Other examples of optically active substances are lactic acid, tartaric acid, 2-methyl -1-butanol etc.
- Optical rotation occurs because of optically active substances have different refractive indices for left and right polarized light. Another way to make this statement is that left and right polarized light travel through an optically active substance at different velocities.
- Optical activity is considered to be due to the interaction of plane polarized radiations with electrons in molecules which shows electronic polarization. This interaction rotates the direction of vibration of radiation by altering electric field.
- Optically active substances can be categorized in to two types:
  1. Those which are optically active only in the crystal state due to their characteristic crystal structure and becomes optically inactive in the fused or dissolved state, for example, sodium chlorate, quartz crystal etc and,
  2. Those which shows optical activity in all states viz. Crystalline (solid), fused (liquid) and gaseous state, due to their structural configurations.

### **Measurement of Optical Activity:**

Measurement of orientation of plane polarized light is called **polarimetry**, and the instrument used is called a **polarimeter**. The simplest polarimeter consists of monochromatic light source, a polarizer, a sample cell, a second polarizer

which is called the analyzer and a light detector. Polariser and analyzer are made up of Nicol prisms. When analyzer is oriented  $90^\circ$  to the polarizer no light reaches to the detector. The polarizer is placed near to the light source while analyzer is placed between sample cell and the detector. The sample cell of suitable size and capacity with outward projection at the centre, to trap the air bubble is usually used. When an optically active substance is placed in the sample cell and beam of light is passed through, it rotates the polarization of the light reaching the analyzer so that there is a component that reaches the detector. The angle that the analyzer must be rotated from the original position is the optical rotation.



**Figure: Schematic of Polarimeter**

For a pure substance in solution, if the colour and the path length are fixed and the specific rotation is known, then observed rotation can be used to calculate the concentration. Optical activity is useful in studying the structure of anisotropic substances, and for checking the purity and identifying chiral mixtures. Adulterations in the optically active substances can be determined from the optical rotation.

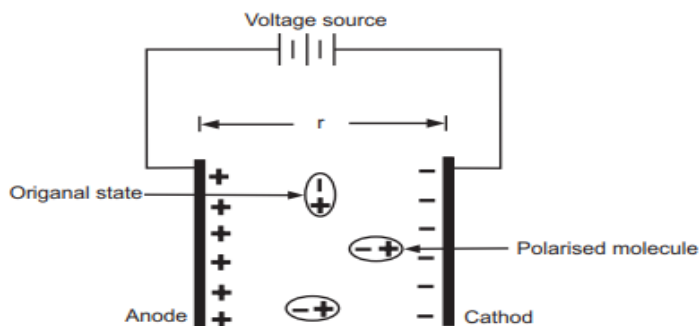
For example, optical rotation of honey is opposite to that of sugar due to the presence of fructose and glucose and hence can be determined from the optical

rotation. Chemical kinetic studies are also carried out by determining concentration at different time intervals as in case of sugar inversion.

Polarimetry is used in the analysis of various drugs and pharmaceutical formulations such as Adrenaline Bitartrate, anticoagulant Citrate Dextrose Solution, Dextran 40 Injection, Dextrose Injection, Sodium Chloride and Dextrose injection etc.

### Dielectric Constant:

A polar molecule can sustain a separation of electric charge either through the induction by an external electric field or by a permanent charge separation within a molecule. The separation of charge can be best understood from the concept called dielectric constant. Consider the example of parallel plate condenser.



**Figure: Parallel Plate Condenser**

The parallel plates are separated by some medium across a distance  $r$  and connected to voltage supply source. The electricity will flow across the plates from left to right through the battery until potential difference of the plates equals that of the battery which is supplying the initial potential difference. The capacitance,  $C$ ,

is equal to the amount of electric charge,  $q$ , stored on the plates, divided by  $V$ , the potential difference between the plates.

$$C = q / V$$

The capacitance of condenser depends on the type of thickness of the condenser separating the plates. The  $C_0$  is used as capacitance reference medium on which to compare other mediums. The  $C_0$  is the capacitance between the plates when a vacuum fills the space between the plates. The ratio of capacitance of test substance ( $C_x$ ) divided by the capacitance of reference substance is termed as dielectric constant.

$$\epsilon = C_x / C_0$$

where,  $\epsilon$  is dielectric constant and since it is ration of capacitance it is unit less quantity. The dielectric constants of some liquids are given in Table.

**Table: Dielectric Constants of Some Liquid at 20°C**

<b>Liquids</b>	<b>Dielectric constants</b>	<b>Liquids</b>	<b>Dielectric constants</b>
Acetone	21.4	CCl <sub>4</sub>	2.24
Benzene	2.28	hexane	5.0
Glycols	50.0	Formaldehyde	22.0
Chloroform	4.8	Methanol	33.7
Phenol	9.7	Ethanol	25.7

The polarity of the solvent depends on the dielectric constant as more is the polar solvent greater is the dielectric constant. Therefore dielectric constant of a substance affects the solubility of that substance. The highest solubility of caffeine at 25°C in dioxane – water mixture was found in the dielectric constant range of 20 to 40.

**Table: Dipole Moment and Polarizability of Some Gases**

Gas	Dipole moment	Polarizability
HCl	3.6	2.93
NH <sub>3</sub>	4.90	2.47
H <sub>2</sub> O	6.17	1.65
C <sub>6</sub> H <sub>5</sub>	0	11.6

If the polar molecules are placed between plates of charged capacitor, the molecules can undergo an induced polarization. This occurs because of the separation of the electric charge within the molecules as it is placed in the electric field between the plates. This polarization is usually temporary and is independent on the ease with which the molecules can be polarized. This temporary induced polarization is proportional to field strength of capacitor and induced polarizability,  $\alpha_p$ , which is characteristic property of the particular molecules. The ease with which a molecule is polarized by any external force (electric field, light or any other molecule) is known as polarizability.

The relation between concentration, dielectric constant and polarizability is given by Clausius-Mossotti equation as;

$$\frac{(\epsilon - 1)}{(\epsilon + 2)} = \left[ \frac{4}{3} \right] [\pi n \alpha_p]$$

In the above equation  $n$  is the number of molecules per unit volume. The total polarization is the sum of induced molar polarization and temporary polarization.

$$P = P_i + P_o$$

Since  $\pi = 0$ ,  $P_o$  is zero. To obtain an induced molar polarization ( $P_i$ ) the above equation can be multiplied by the  $M/\rho$  on both sides.

$$\frac{(\epsilon - 1)}{(\epsilon + 2)} = \left[ \frac{4}{3} \right] \times \frac{\pi n M \alpha_p}{\rho}$$

$$= \left[ \frac{4}{3} \right] \chi_{\pi} n n \alpha p$$

$$= p_i$$

A condition in which electric field strength of condenser (V/m) is unity,  $\pi$  represents the induced molar polarization.

### **Dipole moment:**

Dipole is a pair of separated opposite electric charges. Electric dipole is an assemblage of atoms or subatomic particles having equal electric charges of opposite sign separated by a finite distance. Dipoles are characterized by their dipole moment, a vector quantity with a magnitude equal to the product of charge or magnetic strength of one of the poles and the distance separating the two poles.

$$\mu = q \times r$$

where,  $\mu$  is dipole moment,  $q$  is charge on atom and  $r$  is distance of separation of charge. The direction of the dipole moment corresponds for electric dipoles, to the direction from the negative to the positive charge. The direction of an electric field is defined as the direction of the force on a positive charge, electric field lines away from a positive charge and toward a negative charge.

### **Molecular Dipoles:**

Many molecules have dipole moments due to non-uniform distributions of positive and negative charges on its various atoms. In the case of HCl, the bonding electron pair is not shared equally rather is attracted towards the more electronegative chlorine atom due to its higher electro-negativity which pulls the electrons towards it. It leads to development of positive charge to H atom and negative charge to chlorine atom. A molecule having positive and negative charges



at either terminal is referred as **electric dipoles or just dipole**. Dipole moments are often stated in Debyes. The SI unit is the **coulomb meter**.

Molecular dipoles are of three types;

1. **Permanent dipoles:** These occur when two atoms in a molecule have substantially different electro-negativity with one atom attracting electrons more than another becoming more electronegative, while other atom becomes more electropositive.

2. **Instantaneous dipoles:** These occur due to chance when electrons happen to be more concentrated in one place than another in a molecule, creating a temporary dipole.

3. **Induced dipole:** These occur when one molecule with a permanent dipole repels another molecule's electrons, inducing a dipole moment in that molecule. In a diatomic molecule, the dipole moment is a measure of the polar nature of the bond; i.e. the extent to which the average electron charges is displaced towards one atom. In a polyatomic molecule, the dipole moment is the vector sum of the dipole moments of the individual bonds. In a symmetrical molecule, such as tetrafluoromethane ( $\text{CF}_4$ ), there is no overall dipole moment though the individual C-F bonds are polar.

### **Molecular dipole moments:**

In most molecules even though the total charge is zero, the nature of chemical bond is such that the positive and negative charges do not overlap. These molecules are said to be polar because they possess a permanent dipole moment. The example of this type is water molecule. The molecules with mirror symmetry like oxygen, nitrogen carbon dioxide and carbon tetrachloride have no permanent dipole moments. Even if there is no permanent dipole moment, it is possible to induce a dipole moment by the application of an external electric field and is called

as **polarization**. The magnitude of the dipole moment induced in the molecules is a measure of the polarizability of that molecular species.

### **Permanent dipole moment:**

The permanent dipole moment differs from induced polarization in the sense that it is a permanent separation and it happens only to be in the polar but not in the non-polar molecules. These charges that separate balance out each other and therefore have a net charge of zero. The water is example of permanent dipole moment. The permanent dipole moment is defined as the vector sum of the individual charge moments within the molecules.

### **Applications:**

- The structure of the molecule can be confirmed from the dipole moment values, for example, chlorobenzene, benzene, carbon dioxide etc.
- The cis and trans isomers can be differentiated from dipole moment values, for example, cis and trans dichloroethylene.
- Dipole moments can be used to determine percent ionic characteristic of bond of the molecule, e.g. H-Cl a covalent bond, ionic characteristic is 17%.
- Permanent dipole moments can be correlated with the biological activities to obtain information about the physical parameters of molecules.
- The more soluble the molecule the easier it passes the lipoidal membrane of insects and attacks the insect's nervous system. Therefore, the lower is the dipole moment the greater is the insecticidal action.
- For example, p, m and o isomers of DDT show different insecticidal activities due to their differences in permanent dipole moment as p- isomer shows  $\mu=1.1$  and has predominant toxicity, o-isomer shows  $\mu = 1.5$  with intermediate toxicity while m-isomer shows  $\mu = 1.9$  with least toxicity. The

variations in activities of different isomers are due to the greater solubilities in non-polar solvents.

### **Dissociation constant:**

- The fraction of the amount of the electrolyte in solution present as free ions is called the degree of dissociation.
- Since majority of the drugs are either weakly acidic or weakly basic, their solutions in water contain ionised and unionised species.
- In other words the drug undergoes dissociation in their aqueous solutions.
- The extent of ionisation depends on the pH of the solution.
- For example a weak acid will ionize mostly in alkaline pH while a weakly basic will ionize in an acidic medium.
- The unionised drug is more lipid soluble and thus readily absorbed.
- Hence gastrointestinal absorption of a drug can be improved if the extent of ionisation is reduced.
- The dissociation constants are expressed for weak acids and weak bases.
- The absorption of weakly acidic or basic drugs can be influenced by the following factors:
  - pH at the site of absorption
  - Dissociation constant
  - Lipid solubility of unionised drugs
- At a particular pH the relative concentration of unionised and ionised species in a drug solution can be estimated with the help of Henderson-Hasselbalch equation;
  - For acidic drugs:  
$$\text{pH} = \text{pK}_a + \log \frac{[\text{ionised drug}]}{[\text{unionised drug}]}$$

- For basic drugs:

$$\text{pH} = \text{pK}_a + \log \left[ \frac{\text{unionised drug}}{\text{ionised drug}} \right]$$

- The contents of the stomach have pH ranging from 1-3.
- Weakly acidic drugs having a pKa value less than 4.3 were absorbed faster than those having pKa value within 2- 4.3, strongly acidic drugs were almost not absorbed.
- pH of intestinal fluid vary from 5-8.
- In case of basic drugs the absorption is faster for those having pKa values less than 8.5 than those having pKa values between 9 -12.
- Thus for prediction of the site of absorption of weakly acidic or basic drugs, knowledge of pKa is very important.

Factors influencing the degree of dissociation

- (1) Nature of solute.
- (2) Nature of the solvent.
- (3) Concentration.
- (4) Temperature.

The degree of dissociation of an electrolyte in solution depend on following factors

**Nature of solute:**

- Strong acids, strong bases and salts obtained by their interaction are almost completely dissociated.
- Weak acids, weak bases and their salts are feebly dissociated.

**Nature of solvent:**

- Dielectric constant: The capacity of solvent to weaken the force of attraction between the electrical charges immersed in the solvent.
- The higher the value of dielectric constant the greater is the dissociation of the electrolyte dissolved in it.

**Concentration:**

- Dissociation of an electrolyte is inversely proportional to the concentration of its solution.
- This is due to the fact that in a dilute solution the ratio of solvent molecules to the solute molecule is large and the greater number of solvent molecule will separate more molecules of the solute into ions.

**Temperature:**

- Higher the temperature greater is the dissociation.
- At high temperature the increased molecular velocities overcome the force of attraction between the ions and consequently the dissociation increases.

**Applications of dissociation constant:**

- Dissociation constant is incorporated in Henderson-Halssselbalch equation to calculate the extent of ionization or dissociation, i.e. percent unionized and ionized forms of drug.
- The absorption of drugs in GIT can be predicted using dissociation constant of the drug and pH at different sites of GIT.
- The concentration of preservatives such as benzoic acid required to preserve solutions and emulsions can be predicted.
- The pH of the solution can be calculated. When the concentration of acid is equal to the concentration of salt, the pH of the solutions is same as the  $pK_a$  of the drug.
- Ionization constant values can be used to obtain maximal yields in the extraction of drugs.

**Methods of determination of dissociation constant:**

- Potentiometric measurements

- NMR spectroscopy
- Capillary electrophoresis
- High performance liquid chromatography
- Hyper rayleigh scattering
- Displacement ELISA
- Calorimetry
- `Partition and distribution coefficient.
- UV spectroscopy [orthogonal method]
- Density functional theory [DFT]
- Isohydric solution principle
- Solvation model
- Solubility data
- Thermal lensing spectroscopy
- Surface tension and interfacial tension
- Voltametry
- Fluorometric method
- Fluorescence polarization method
- Mass spectroscopy
- Surface plasma resonance

**Potentiometric measurement:**

In a potentiometric titration, a sample is titrated with acid or base using a pH electrode to monitor the course of titration. The pKa value is calculated from the change in shape of the titration curve compared with that of blank titration without a sample present. Potentiometric titration are based on the quantitative relationship of the E.M.F. of a cell as given by the following equation

$$E_{\text{Cell}} = E_{\text{ref}} + E_{\text{indicator}} + E_{\text{junction}}$$

A Known volume of the acid to be titrated is kept in a beaker. The hydrogen half-cell was combined with the reference electrode, half-cell through a solution bridge. After each addition of titrant into beaker, the E.M.F. is measured. The potential  $[E_{\text{ref}}]$  reference electrode is first measured against the standard hydrogen electrode. The stable E.M.F. of the cell  $[E_{\text{cell}}]$  is measured two hours after the cell had been assembled and the hydrogen ion concentration calculated from the following equation

$$\text{pH} = [E_{\text{cell}} - E_{\text{ref}}] / 0.0591$$

The ionization constants for the acids in different solvents are then calculated from  $\text{pH} = \text{pKa}$  at half neutralization. The equivalence point gets on the titration curve in the part where there is a relatively large change in pH with a relatively small change in volume. Therefore it concluded that the change in electrode potentials of the cell is proportional to the change in pH during titration. The point where the E.M.F. increases rapidly gives the end point. The different methods used for calculation of pKa like potentiometry, conductometry have limited application for determination of second dissociation constant of very weak acid. The main advantage of this method is applicable for analysis of coloured and dilute solutions. The apparatus required is generally inexpensive, reliable and readily available. However, its disadvantages include the requirements to use a milligram of pure compounds and a mixture of aqueous buffers and to avoid errors, especially for measurements at neutral-to-high pH, carbonate-free solutions must be prepared laboriously.

### **Displacement ELISA**

ELISA method is valuable for the measurement of the dissociation constant of antigen-antibody complexes. ELISA used for determination of plasma HDL concentration in clinical specimens using monoclonal antibodies A-130 and A-14 reagent. Affinity constant has been measured for monoclonal Ab-Ag pairs using ELISA methods, but the results tends to be inconsistent.

ELISAs makes possible the determination of dissociation constants of complexes formed between immobilised antigens and the displacing molecules without knowing the precise concentration of the antigen. The indirect ELISA is an effective method for studying the interaction of Ab with Ag; the immobilized Ag captures the Ab from the test solution and the bound Ab is then revealed by the enzyme-labelled anti-IgG.

### **Calorimetry**

All calorimetric methods work by the same principle: a physical or chemical process takes place in a sample and the amount of heat evolved is measured. For the measurement of pKa values, in Isothermal Titration Calorimetry [ITC], a regular acid-base titration is carried out inside the calorimeter while the energy required to keep the temperature constant is measured. In recent years, the ITC-method used to calculate the dissociation constants of peptides and the influence of binding on the specific ionizable groups. This method also calculates the pKa indirectly from a measured enthalpy change  $\Delta H$ . By plotting the minima or maxima versus pH, a sigmoid curve is obtained from which the pKa can be determined from the inflection point.

In isothermal titration calorimetry [ITC], the label-free solution used to measurement, where the heat change associated with binding is the read-



out. ITC has been termed the “gold standard” for characterizing biomolecular interactions, even though its main drawback of large amount of sample required for a measurement. If a ligand binds to a protein with a single-digit nanomolar or tighter dissociation constant, it is difficult to measure the affinity accurately using ITC because the titration curve becomes too steep to fit accurately. In such cases, one can measure the displacement of a weakly binding ligand during titration with a strongly binding ligand of interest.

### **Partition and distribution coefficient**

The distribution constant or partition coefficient means if a third substance is added to a system of two immiscible liquids in equilibrium, the added component will distribute itself between the two liquid phases until the ratio of its concentrations in each phase attain a certain value.[58] Nernst concluded in 1891 that the partition coefficient [P] was only a constant if a single substance was considered. The partition coefficients determination of ionizable compounds requires the knowledge of the pKa value. W. R. Vezin determined dissociation constant of phenothiazine derivative based on the pH dependency of partition coefficient at a range of pH value. Although log P is a relatively simple quantity to determine, it has been proven that there are still many sources of errors, one of which is common miscibility of the two phases.