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Review Article

A REVIEW ON VARIOUS ANALYTICAL METHODS USED IN DETERMINATION OF DISSOCIATION CONSTANT

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ABSTRACT

Determination of dissociation constant for a drug capable of ionization within a pH range of 1 to 10 is important since solubility and consequently absorption, can be altered by orders of magnitude with changing pH. The dissociation constant [pKa] value, are essential for understanding many fundamental reactions in chemistry. These values reveal the deprotonation state of a molecule in a particular solvent. There is great interest in using experimental methods to calculate the pKa values for many different types of molecules. In this review the most valuable methods for determining accurate pKa values are presented. There are many diverse strategies for determining dissociation constant, so the main aim of the review is to discuss the available analytical methods.

Keywords: Dissociation constant, pH, potentiometric measurement, capillary electrophoresis, Deprotonation.

INTRODUCTION

The knowledge of ionization constants is important in the understanding of certain chemical phenomena particularly for developing new APIs, the pKa has become of great importance because the transport of drugs into cells and across other membranes is a function of physicochemical properties, and of the pKa of the drugs. Dissociation in chemistry and biochemistry is a general process in which ionic compounds [complexes, or salts] separate or split into smaller particles, ions, or radical, usually in a reversible manner.[1]

Dissociation constant is most important parameter to understand chemical phenomenon such as biological activity, absorption and extent of ionization of compound in different pH, so is the key parameter in drug development and optimization.[2-11] The pKa of a compound is the pH at which the compound is 50 % protonated.[12]

The pH partition theory states that for drug compounds of molecular weight greater than 100, which are primarily transported across the biomembranes by passive diffusion, the process of absorption is governed by: 1] The dissociation constant [pKa] of the drug, 2]The lipid solubility of the unionized drug, 3]The pH at the absorption site. Since most drugs are weak electrolytes [weak acids or weak bases], their degree of ionisation depends upon the pH of the biological fluid. The amount of drug that exists in unionized form is a function of dissociation constant [pKa] of the fluid at the absorption site. The Henderson- Hasselbatch equation provides an estimate of the ionized and unionized drug concentration at a particular pH.

For weak acids,

pH = pKa + log[ionized drug]/[unionized drug]……[1]

For weak bases,

pH = pKa + log[unionized drug]/[ionized drug]……[2]

The pKa is the pH at which concentrations of ionized and un-ionized forms are equal. When the pH is lower than the pKa, the un-ionized form of a weak acid predominates, but the ionized form of a weak base predominates.[13, 14] For a weakly acidic drug with pKa value greater than three, the unionized form is present within acidic contents of the stomach, but the drug is ionized predominantly in the neutral media of the intestine. For basic drug [pKa 8-9], the ionized form is predominant in both the stomach and intestine.[15] In many experimental methods to determine pKa values, a certain parameter is measured as a function of pH. This results in a characteristic sigmoid curve from which the pKa may be determined by locating the inflection point. [16] It is customary to express the dissociation constant of both acidic and basic drug by pKa values. The lower the pKa of an acidic drug, stronger the acid.[17]

Ionization constant [pKa] is one of the important physicochemical properties of drugs to understand their site of absorption, distribution to various organs and excretion. Dissociation constant is also helpful in screening salts, developing pre-clinical and clinical formulation. The pKa is the negative logarithm of the equilibrium constant of the acid-base reaction of the compound of interest.[18] In order to have a clear focus and keep this review to a manageable size, the following discussion deals only with the methods useful in finding dissociation constant.

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

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

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_{ref}] reference electrode is first measured against the standard hydrogen electrode. The stable E.M.F. of the cell [E_{cell}] is measured two hours after the cell had been assembled and the hydrogen ion concentration calculated from the following equation

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

The ionization constants for the acids in different solvents are then calculated from pH = 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.[19-24]

NMR spectroscopy

It works in highly basic and highly acidic media. A major advantage of the NMR technique is associated with the possibility of titrating a mixture of ligands, including impurities, if the total concentration of the ligands [and therefore of impurities associated with the ligands] is much less than the base [acid] concentration.[25]NMR spectroscopy can focus on either the proteins or the ligands by exploiting one of many possible probes, such as chemical shifts, diffusion constants, relaxation rates, and magnetization transfer or saturation transfer rates. Some NMR methods can provide information with atomic resolution about the region [epitope] of the protein that is involved in binding.[26]Nuclear magnetic resonance [NMR] spectroscopy is a unique tool to study interactions of small ligands with biologically relevant macromolecules.[27,28]NMR-based screening, although its low intrinsic sensitivity, offers the largest dynamic range and is capable of capturing very weak interactions at the detailed molecular level, thereby facilitating a thorough view of how proteins function in living cells.[29-30]

In order to measure the dissociation constant of a protein–ligand complex, measure the equilibrium concentrations of free and bound species.

For the single site protein [1:1 complex], the solution composition is defined as:

$$\mathbf{[P]_0 = [P] + [LP]}$$

$$\mathbf{[L]_0 = [L] + [LP]}$$

Where $[L]_0$ and $[P]_0$ are the total concentrations of protein and ligand, respectively. In graphical method the fraction of ligand bound protein is obtained from the 1H NMR frequency of the reporter proton in free protein [δ_{free}], the fully saturated state [δ_{bound}], and at the equilibrium condition [δ_{obs}] according to:

$$\mathbf{X_{P[bound]} = [PL]/[P]_0 = [\delta_{obs} - \delta_{free}]/[\delta_{bound} - \delta_{free}]}$$

Assuming single site binding and fast exchange, it can be shown that:

$$\mathbf{1 / X_{P[bound]} = 1 + K_D\{1/[[L]_0 - X_{P[bound]}[P]_0]\}}$$

Thus a plot of $1/X_{P[bound]}$ versus $[[L]_0 - X_{P[bound]}[P]_0]$ is linear with slope pKa and intercept 1.[31]

High-Throughput Screening with NMR method used to determine the relative ranking of binding affinities. An advantage of using NMR to measure protein–ligand interaction is that the NMR method extends the range of measurable interactions into the mM range.[32] Proton, carbon-13, nitrogen -15 and sulfur-33 NMR spectrometry, successfully utilized for determining pKa, values. But natural isotopic abundances of [13]C and [15]N [1.01 and 0.37%. respectively] and low inherent sensitivity limited their usefulness.[33] Determination of pKa of sugars by variations of ^{1}H or [13]C NMR shifts with pH does not requires isolation of the individual anomers and is unaffected by their interconversion or decomposition.[34] The use of [15]N-relaxation data for determination of the dissociation constant of a protein–protein complex is proposed for the situation where a 15N-labeled protein is bound to an unlabeled protein of high molecular weight, and the chemical exchange between bound and free protein is fast on the NMR time scale.[35] To avoid an overwhelming H2O peak and the concomitant dynamic range problems in 1H NMR spectroscopy, the use of deuterium oxide as solvent has long been the only reasonable solution with all its known drawbacks such as losing resonances of exchanging amide protons in proteins, small deuterium isotope effect on chemical shift.[36-37]

Capillary Electrophoresis

Capillary zone electrophoresis [CZE] is a useful tool for determination of physicochemical parameters e.g. pKa values and/ or ionic mobilities. [38] To determine pKa values of compound, CE offers several advantages over potentiometric and spectroscopic methods: a slight consumption of the analyte is required, impurities do not disturb the measurements and the exact solute concentration is not necessary, but only migration times.

For basic compounds, acid–base dissociation constant [pKa] is defined as:

$$\mathbf{pKa = [B]\ [H^+]\ /\ [BH^+] = \gamma_B\ [B][H^+]\ /\ \gamma_{BH^+}[BH^+]} \quad \text{.....[1]}$$

Where [H^+], [BH^+] and [B] are, respectively, the activities of proton, protonated and neutral base. [BH+] and [B] are the concentration of protonated and neutral forms. γ is the activity coefficient of ionized [γ_{BH^+}] and neutral [γ_B] species

Usually, the activity coefficient of the neutral species [γ_B] is assumed to be 1. So relation [1] can be written as:

$$\mathbf{pKa = [B][H^+]\ /\ \gamma_{BH^+}[BH^+]} \quad \text{.....[2]}$$

Since the pH influences the electrophoretic behaviour of the substances, can be established a relation between pH, pKa and the electrophoretic mobility of compounds [μ_{ep}]. The electrophoretic mobilities were calculated using the following formula:

$$\mathbf{\mu_{ep} = \mu_{app} - \mu_{eof} = L_d L_t / V\ [1/t_m - 1/t_o]} \quad \text{.....[3]}$$

Where μ_{app} is the apparent electrophoretic mobility of the solute. μ_{eof} is the electroosmotic mobility of a neutral marker. A μ_{ep}, μ_{eof}, μ_{app} are in cm^2/Vs. Ld is the distance from the injection point to the detector [cm]. L_t is the total length of capillary [cm]; V is the applied voltage [volt]. t_m and t_o are the migration times [s] of the analyte and neutral marker, respectively.

$$\mathbf{1 / \mu_{ep} = pKa.\ \gamma_{BH^+} / \mu_{BH^+}[H^+] + 1 / \mu_{BH^+}} \quad \text{.....[4]}$$

This equation is used for linear regression analysis between $1/\mu_{ep}$ and $\gamma_{BH^+} / \mu_{BH^+}[H^+]$. The pKa values were obtained from slope of the plots of reciprocal effective mobility against inverse concentrations of protons.[39] The pKa value also obtained by the plot of μ_{eff} against pH. The limitation of method is increased ionic strength leads to increased current and thereby Joule heating which can distort peak shape and diminish separation efficiency.[40]

High Performance Liquid Chromatography

High-performance liquid chromatography is valuable technique for the separation of weak acids and bases. A general equation which relates the observed retention factor to the pH of the mobile phase, the dissociation constants, and the retention factors of the different ionic species has been used to determine dissociation constant of polyprotic weak acid and base. It is written as: [41]

$$\mathbf{K_D = \frac{ko + \Sigma^n_{r=1}\ kr.ka.\ [r]\ e^{rx}}{1 + \Sigma^n_{r=1}\ ka.\ [r]\ e^{rx}}}$$

- ko retention factor of undissociated species
- kr value are the retension factor of dissociated species,
- ka[r], product of the first r- dissociation constants,
- n is the ionisation constant
- X is related to pH of the mobile phase: x=2.303.ph

The LC method is based on the different retention behavior of the protonized and the nonprotonized form of the test material. The retention time is determined in relationship to the pH-value of the mobile phase by reversed - phase HPLC.[42] The high performance liquid chromatography method useful to determine pKa value of low water soluble drug,[43-44] with low sample consumption, rapid sample analysis, high sensitivity and precision.[45] The huge success of HPLC can be attributed to a number of intrinsic features associated with reproducibility, simple, ease of selectivity manipulation, and generally high recoveries. The most important feature is the excellent resolution that can be achieved under a wide range of conditions for very closely related molecules, as well as structurally relatively distinct molecules.[46- 47]Dissociation constant greatly affected by changing in pH, ionic strength, and temperature.[48]The pH of the mobile phase affects retention of acidic and basic drug.[49]

Hyper Rayleigh Scattering

Applications of the interface-selective methods of second harmonic and sum frequency spectroscopy to the investigation of equilibrium and time-dependent properties of interfaces are increasing at an explosive rate. [50] Hyper-Rayleigh Scattering [HRS] is second-harmonic light scattering mediated by the molecular first hyperpolarizability [*β]*, and HRS is widely used to measure hyperpolarizability of organic chromophores in solution. [51]Any molecule which has an anisotropic electronic charge distribution over its length is likely to show second harmonic scattering in solution and its intensity depends on its structure and electronic charge distribution over the whole backbone.[52]

Hyper-Rayleigh scattering technique used to obtain the dissociation constants of weak organic acids in protic solvents. The degree of dissociation of the acid [α] can be varied by altering the initial concentration of the acid in solution. From the linear plot of β^2 against α at different initial concentrations [C], the dissociation constant of the acid is obtained.

$$K_D = \alpha^2 C / 1 - \alpha \quad \text{..... [1]}$$

Since the HRS technique is based on a two-photon scattering process and powerful IR lasers are used in the measurements in conjunction with detection of light signal by efficient visible photomultiplier tubes, it is highly sensitive. Concentrations in the range 10^{-5}-10^{-8}M are routinely used for measurements.[24]

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.[53]Affinity constant has been measured for monoclonal Ab-Ag pairs using ELISA methods, but the results tends to be inconsistent.[54]

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.[55]

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 ΔH. By plotting the minima or maxima versus pH, a sigmoid curve is obtained from which the pKa can be determined from the inflection point.[16]

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. [56] 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.[57]

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.[59] The partition coefficients determination of ionizable compounds requires the knowledge of the pKa value.[60]W. R. Vezin determined dissociation constant of phenothiazine derivative based on the pH dependency of partition coefficient at a range of pH value.[61] 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.[16]

UV visible spectroscopy

The principle of UV-Visible spectroscopy is the absorption of electromagnetic radiation from the 200–800 nm range and the following excitation of electrons to higher energy states.[62] UV–VIS spectrophotometry handle compounds with lower solubility and lower sample concentrations.[63]A number of aromatic molecules are easily determined by UV spectroscopy.[64]The λmax of two different form of drug must be different.[65]

There are various UV spectroscopic methods such as Simultaneous equation method[66], 96 well microtiter plates method [67], Multi-peaks Gaussian fitting method [68], Molar ratio method[69], least squares nonlinear regression of Multi-wavelength spectroscopic method[70-74], Second derivative method[75-77], Orthogonal method, are used to dissociation constant. In this we describe orthogonal method in detail.

Orthogonal method:

The *ΔA* method applicable to analysis of compound which absorption spectra are affect with changes in pH. But peak of some compounds may split into secondary peaks without any appreciable change in intensity or the *ΔA* value may be small. The performance of these compounds restricted the application of the *ΔA* method. In these situation, application of the orthogonal function *[ΔP$_j$]* method existing a solution for the analysis of such compounds. The A_{max} method may give incorrect result if absorption spectra of the different species in solution overlap. Thus, the application of the orthogonal function *[ΔP$_j$]* method helps in the resolution of the spectral overlapping which facilitates the determination of the ionized species at any pH value.

$$pka = pH + \log [P_{jd} - P_{jb}] / [P_{jb} - P_{ju}]$$

$$pka = pH + \log [P_{jb} - P_{ju}] / [P_{jd} - P_{jb}]$$

Where P_{jb}, P_{jd} and P_{ju} are the coefficients of the polynomial, P_j, of the buffered, dissociated and undissociated drug solution, respectively. The orthogonal function *[ΔP$_j$]* method applicable for the determination of the pKa, value[s] of drugs with single, double

and triple pKa, values. The ΔP_j method applicable for compound which do not show significant change in their spectra over a suitable pH range.[78-79]

Density functional theory [DFT] method

The density functional theory is the computerized method. Density functional theory [DFT] is nowadays one of the most popular methods for ground state electronic structure calculations in quantum chemistry and solid state physics. Compared to traditional ab initio and semi-empirical approaches, current density functional methods show a favorable balance between accuracy and computational efficiency.[80]

Eduardo J. Delgado determine dissociation constant of dimethoxy pyrimidinylsalicylic based herbicides using DFT method at B3LYP/6-31G [d,p] level of theory. Gas –phase molecular geometries and electronic energies computed at DFT B3LYP/6-31G [d,p] level of theory. Free energies of solvation in water were computed by single point calculations on the gas phase optimized geometry using the Poison-Boltzmann Solvation model [PB]. The pKa values were calculated with the jaguar pKa prediction module using the thermodynamic cycle and following equation. Where D is the free energy changed[81]

$$pKa = 1 / 2.3RT * D$$

Yu. E. Zevatskii et al calculate dissociation constants of 15 carboxylic acids in water, methanol, and DMSO by empirical and quantum-chemical DFT methods.[82] Practical applicability of this method for all types of molecules is limited. It predict pKa of small molecules within the pKa range 4-5.[83]

Isohydric solution principle

This method is very sensitive method, useful for weak acids characterized by low pKa values. We can test the effect of contamination of a weak acid or its salt by a strong acid or base or carbonate. The special validity of this method results from the unique property of ionic strength constancy during the titration.

The term "isohydric" means solution of the same hydrogen ion concentration.[84] Concept of isohydric solution introduce by Arrhenius who stated that "if two isohydric solutions are mixed, pH of the resulting solution is unchanged regardless of the composition of the solutions". This statement is not valid when referred on any pair of electrolytic system. The isohydric solutions concept [isohydricity] should be limited to the systems where only acid-base equilibria occure. Two solutions are said to be isohydric, when pH is the same in both solutions and does not change after mixing. According to Chatelier-Brown;s principle addition of a strong acid [HB, C mol/L] into a weak acid [HX, C_0 mol/L] decreases pH value of the resulting mixture and consequently, shifts the HX dissociation. The pKa determine by isohydric principle method by following equation.[85]

$$C_0 = C + C^2 . 10^{pK_1 T}$$

Solvation models:

In this method dissociation constant measure by calculating the Gibbs energy change for the deprotonation in the gas phase [ΔGgas] and then using the salvation energies [ΔGs] of the molecule, anion and the proton to estimate the Gibbs free energy change of salvation [ΔΔGs]. The pKa value of solution calculated by using following equation

$$pKa = [\Delta Ggas + \Delta\Delta Gs - 269.0] / 1.3644$$

The above equation includes correction for conversion of the standard state in gas [1 atm] to solution [1M] and – 264.61 kcal mol^{-1} used as the hydration energy of proton and value of – 6.28 kcal mol^{-1} used as the Gibbs free energy of the proton in gas phase.[86]

Solubility data [mat-pKa calculations]

Lionel Vidaud et al developed mat-pKa tool for evaluation of acidity constants from solubility data of compound in different media. The Mat-pKa software based on three principal parts: first is the preparation of the initial matrix of data [pH and solubility], second is the resolution of the matrix system and third is the calculation of the constants and the intrinsic solubility of the predominant entity involved or driving the solubility of the product.

In some cases Mat- pKa can provide pKa that is not experimentally determined. This program can suggest the possibility to verify which predominant entity is responsible for the solubility of the compound. The solubility/pH couples of data used for the calculations should avoid the influence of the ionic strength of the solutions and the precipitations of the analyzed compound.[87] Following equation[which applies to weak base] are used to determine dissociation constant.

$$S = S_0 [1 + 10^{-pH}/Ka]$$

S_0 is the intercept. The limiting solubility of the molecule may be determined by unweighted linear regression. The main problem in this method is the accurate determination of the intercept So. The slope S_0 /Ka, is easily determined with reasonable accuracy, but if the solubility data have been determined over a wide pH range, the intercept will be close to zero and the precision of the determination will be low. The most general solution to this problem is to modify the above treatment by using weighted linear regression. Each value of the solubility is weighted by a factor $W = 1/\sigma^2$ where σ is the standard deviation determined either directly at each concentration, or as a function of concentration determined by extended least-squares.[88]

The determination of pKa of poorly soluble drugs using the surfactants found to be simple, accurate and less time consuming. Simple, traditional potentiometer without any additional feature like special calibration procedure and software is sufficient for pKa determination.[18]

Thermal lensing spectroscopy

Sung Ho Kim et al measure dissociation constant of thymol blue by using a portable Diode-laser/ fibre-optic thermal lensing spectroscopy, which consisted of a visible diode laser, a photodiode, and an optical fibre by using following equation:

$$pH = pKa + \log [TS_a / TS_b - TS_a]$$

Where TS_a is the thermal lensing signal of a solution at certain pH and TS_b is that of the most basic solution. One can determine pKa from the ratio of thrmal lensing signal of the acidic and basic forms of the indicator. But because of complexicity in operation and requires a large dimension, sophisticated experimental alignment skill, it still has a restriction to be a popular measurement method for dissociation constant.[89]

Surface and Interfacial dissociation constant:

Numerous amphipathic compounds - acids, bases and ampholytes - whose surface and interfacial behaviour is highly dependent on the pH of the aqueous subphase can be characterized quantitatively in terms of acid-base reactions, the activities of species arising as a result of these reactions, and their apparent surface/interfacial dissociation constants.

Surface/interfacial dissociation constants determined from the quantitative effects of pH on "two-dimensional" properties are what may best be described as apparent values.

Interfacial dissociation constant determine by following equation:

$$\frac{\gamma}{\gamma_o} = \frac{1}{1 + Ka / [H^+]_{aq}}$$

γ is the interfacial tension, γ_o is the interfacial tension at its highest value, $[H^+]$ is the interfacial hydrogen ion concentration. Plot of γ/γ_o vs pH gives dissociation constant by taking pH_{aq} = pKa. Dissociation constant also determine by following equation by using surface pressure data:

$$\frac{A}{A_o} = \frac{1}{1 + Ka / [H^+]_{aq}}$$

Plot of A/A_0 vs pH gives pKa by taking pH_{aq} = pKa. Apparent dissociation constant are determined from above equation. Absolute values are not determined from surface pressure data alone. So for determination of absolute pKa value require surface potential data. Partitioning of hydrogen ion between the bulk and surface will give rise to potential difference, so it is at this point that we must utilize the contribution of Gouy:

$$[H^+]_S/[H^+]_{aq} = \exp[-e\psi/Kt]$$

Where e is the electronic charge, ψ is the surface potential, k is the Boltzmann's constant, and T is the Kelvin temperature. This method applicable to any system whose surface/ interfacial properties may be quantitatively characterized as function of pH.[90]

Abhilash Thakur used Surface Tension [ST] property successfully for modeling dissociation constant pKa of the sulfonamides. The physicochemical parameter surface tension [ST] is found better than the widely used physicochemical parameters as Molar Refractivity [MR] for modeling the pKa.[91]

Voltametric method

Different number of protons are necessary for the electrochemical reduction of protonized and deprotonized forms of a compound. Therefore, the potential of its electrochemical reaction [half wave potential $E_{1/2}$ or peak potential E_p] is pH dependent in accordance with below equation

$$E_{1/2} = E^0_{1/2} + RT/\alpha zf * \ln [H^+]^p$$

Where $E^0_{1/2\,is}$ the half wave potential at standard conditions, α is the transfer coefficient; z and p are the numbers of exchanged electrons and protons respectively. The slope of the half wave potential [or peak potential] versus pH dependence is changing in consequence of dissociation in the vicinity of pH = pKa. The differential pulse voltametry [DPV] method gives approximate values of pKa due to the possibility of partially irreversible course of the electrode reaction. [92]

Fluorimetric method

Fluorimetry is a relatively fast and accurate means of determining the dissociation constants of sparingly soluble arylamines and hydroxy aromatic compounds. Fluorimetry is often considerably more sensitive than most analytical methods and used in cases where the ionizing group of an aromatic acid or base leads to a change in the fluorescence spectrum it might be expected to be a method of choice for the determination of pKa, values. This method used only for ionizing group exhibiting fluorescence spectrum.[93] Fluorescence-based assays are important new tools to examine Systematic binding interactions between small-molecule ligands and the RRE [*Rev response element]*.[94]

Fluorescence polarization method

Linda Prystay determine equilibrium dissociation constant of peptides size range from a 7-mer at about 1,400 Da [dermorphin] to a 22-mer at about 3,200 Da [3,200] by using fluorescence polarization saturation curve data. It is important to keep in mind the difference between classical and fluorescence polarization [FP] saturation curve data for fluorescence polarization equilibrium constant model formulation. Classical saturation curve data consist of "total binding" data corresponding to the signal from specifically and nonspecifically bound ligand and "nonspecific binding" data corresponding to signal from nonspecifically bound ligand. Fluorescence polarization saturation curve data consist of "total binding" data which corresponding to signal from specifically and nonspecifically bound ligand as well as remaining free ligand; "displaced ligand" data, which corresponds to signal from nonspecifically bound ligand; and free fluorescent ligand" data, which is the minimum signal attainable for fluorophore in assay medium.[95]

Mass spectroscopy

The chromatography together with mass spectroscopy methods allowed for the quick determination of dissociation constant and mainly applicable for complex mixtures. The use of ESI-TOF-MS detection technique allowed to achieve the medium-throughput screening rate [100 compounds/day] and provided a trouble-free approach to assess pharmacokinetically important physicochemical properties of drugs.[96]

Studying the noncovalent complexes with electrospray ionization mass spectrometry [ESI-MS] is dynamic field of research for quantitative determination of binding strengths. The speed of the ESI-MS method and the low sample consumption make it a powerful tool for examining the stoichiometry of a protein– ligand binding system and an easy to use technique for comparison with other established dissociation constant determination methods. [56]Measuring the m/z of complex ions by MS is the only method allowing for the direct measurement of a fundamental physical dimension of the complex, in this case its mass.[97]

Advantages of mass spectroscopy are high resolution, high sensitivity, fast speed of analysis, absence of unknown response factors, determined dissociation constants lower than picomolar with good accuracy by electrospray ionization mass spectrometry. [98-99]The ability to obtain stoichiometric information, and the mass measurement accuracy, determine dissociation constants [Kd] of non-covalent weakly bound complexes.[100-101]

Surface plasmon resonance

Surface plasmon resonance [SPR] is a generally accepted technique for the investigation of binding kinetics and binding strengths. SPR is most frequently used for studying biomolecular interaction. The principle of SPR measurements is relatively simple: a "bait" ligand is immobilized on the gold surface of the SPR chip.

A solution containing the 'prey'' receptor is made to flow over the bait layer in a microfluidic system. The incident light of the instrument is electromagnetically ''coupled'' to a propagating surface plasmon of the gold layer. When the surface plasmon is affected by a binding event, the reflection angle of the incident light changes proportionally to the mass differences of the bait and the prey. This change of reflection angle as a function of time is the read-out of the SPR technique.[56]

SPR imaging can be used to quantitate the strength of lectin-carbohydrate interactions by determining the adsorption coefficients and the solution dissociation constants for the lectins ConA and jacalin.[101] The SPR provides an excellent instrumentation for a label-free, real-time investigation of biomolecular interactions by using biosensors.[102] Drawbacks to SPR include a molecular weight limitation of around 180–200 Da, and immobilization may modify the protein; that modification can lead to inactivation.[103].

Table 1: Application of dissociation constant method

S. No.	Method	Drug	Advantages	Disadvantages	Ref.
1	Potentiometry method	Salicylic acid	Applicable for analysis of coloured and dilute solutions.	Requires to use a milligram of pure compounds.	20
2	NMR	clindamycin 2-phosphate, protein	titrating a mixture of ligands, including impurities.	low intrinsic sensitivity.	25,33,35,29,30
3	Capillary Electrophoresis	3-nitro-tyrosine, 2-amino-2-oxazolines	minor consumption of analyte, impurities do not disturb the measurements and the exact	Increased ionic strength leads to increased current and thereby Joule heating which can distort peak shape and	38,39,40

			solute concentration is not necessary.	diminish separation efficiency.	
4	HPLC	Leukotrienes	Determine pKa value of low water soluble drug, low sample consumption, rapid sample analysis, high sensitivity and precision	Dissociation constant greatly affected by changing in pH, ionic strength, and temperature. The pH of the mobile phase affects retention of acidic and basic drug	42,43,44, 45, 48, 49
5	Hyper-rayleigh scattering	Bilirubin-Human Serum Albumin	Determination of dissociation constants of weak organic acids in protic solvents, highly sensitive method, Concentrations in range 10^{-5}-10^{-8}M are routinely used for measurements.	-	52
6	Elisa	Antigen-antibody complex	Determination of pKa of complexes formed between immobilised antigens and the displacing molecules without knowing the explicit concentration of the antigen.	Results may be inconsistent.	53,54, 55
7	Calorimetry	Protein-ligand interaction	The label-free solution measurement are possible.	Large amount of sample required.	56
8	Partition and distribution coefficient	Phenothiazine derivative	In some specific cases, the technique used to determine pKa by P is largely unaffected by the choice of solvent.	Mutual miscibility of the two phases affect accuracy of result.	61, 16
9	UV spectroscopy (orthogonal method)	Benzoic acid, Paracetamol, Methyl paraben.	The *Δpj* method is useful for compounds which do not show appreciable change in their spectra over a suitable pH range.	-	78-79
10	DFT	dimethoxypyrimidinyl salicylic based herbicides, carboxylic acid	This method show a favorable balance between accuracy and computational efficiency.	Practical applicability for all types of molecules is limited. It predicts pKa of small molecules within the pKa range 4-5.	81,82
11	Isohydric solution principle method	Acetic acid, chloroacetic acid	Sensitive method, useful for weak acids characterized by low pKa values, one can test the effect of contamination of a weak acid or its salt by a strong acid or base or carbonate,	Not valid when referred on any pair of electrolytic system, The isohydricity concept should be limited to the systems where only acid-base equilibria occure.	85
12	Solubility data	Sulfadiazine, Butaperazine	Mat- pKa can provide pKa that were not experimentally determined, verify which predominant entity is responsible of the solubility of the compound, avoid the influence of the ionic strength of the solutions.	-	87
13	Surface and interfacial tension	benzenesulfonamide	This method applicable to any system whose surface/ interfacial properties may be quantitatively characterized as function of pH.	-	90,91
14	Thermal lensing spectroscoy	Thymol blue	Simple and convenient method.	Requires large dimension, complexicity in operation, and sophisticated experimental alignment skill.	89
15	Fluorometric method	amino and hydroxy aromatic compounds	Fluorimetry is a relatively fast and accurate method of determining the pKa of sparingly soluble compounds.	-	93
16	Fluorescencepolarization method	G protein-coupled receptor-ligand	Detection of both bound and free labled ligand.	Signal decreases as free ligand concentration increases, 0.4 nm or higher concentration required for analysis.	95
17	Mass spectroscopy	Protein-ligand, Lysozyme -NAG3 Complex	Low sample consumption, high resolution, high sensitivity, fast speed of analysis, absence of unknown response factors	Continuous efforts are made to enhance the reliability of ESI-MS methods by justifying a few complex issues addressing response factor of different species	96,98, 99
18	Surface Plasmon resonance	Protein carbohydrate complex	study characteristically weak protein-carbohydrate interactions, label-free, real-time investigation of biomolecular interactions	immobilization may modify the protein; which lead to inactivation, The sensitivity of an SPR instrument can be a problem since the angle of reflection is detected which is proportional to the mass of the analyte and can generate poor signals for small molecules	101, 102

CONCLUSION

Dissociation constant is the most important parameter in drug development process. All methods which are useful for determination of dissociation constant have its own advantages and disadvantages. So method selection is the important parameter and it requires knowledge of various methods useful for determination of dissociation constant. The advantage of potentiometric method is applicable for analysis of colored and dilute solutions. NMR and capillary electrophoresis methods are useful to determine accurate dissociation constant of sample even though it contain impurity. NMR and ELISA method used to study biological samples. HPLC and mass spectroscopic method useful to determine pKa value of low water soluble drug with low sample consumption, rapid sample analysis, high sensitivity, resolution and precision.

CONFLICT OF INTERESTS

Declared None

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