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AN UPDATE ON VARIOUS ANALYTICAL TECHNIQUES BASED ON UV SPECTROSCOPY USED IN DETERMINATION OF DISSOCIATION CONSTANT

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ABSTRACT

It is well known that the dissociation constant is most important parameter in development and optimization of new compound for effective formulation development. Dissociation constant plays a crucial role in understanding chemical phenomenon of drug because it affects pharmacokinetic behavior like absorption, distribution, metabolism, and elimination (ADME). Solubility, permeability, protein binding of a compound is also influenced by its pKa. There are various techniques available for determination of dissociation constant. Among Photometric techniques available, ultraviolet-visible spectrophotometry is one of the most frequently employed technique in pharmaceutical analysis for determination of this parameter. In this review the most valuable UV spectroscopic methods such as derivative spectrophotometry, simultaneous equation method, orthogonal polynomial method, multi-wavelength method, molar ratio method, difference spectrophotometry for determining accurate pKa value are presented.

Keywords: Dissociation constant, pH, Spectrophotometer, Derivative spectrum, Deprotonation, pKa.

INTRODUCTION

Dissociation constant is most valuable 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.[1-9] The pKa of a compound is the pH at which the compound is 50 % protonated.[10] There are various methods for determination of pKa such as potentiometric titration where the pKa is determined from titration curve.[11-15] Using NMR, pKa is derived from data of chemical shift, coupling constant and pH.[16-19] Capillary electrophoresis is carried out in different buffer solutions and pKa is determined from relative mobility of ions at different pH.[3,20-25] ELISA, pKa is determined from linearized plot.[26-28] In HPLC, pKa is derived from data of retention factor of protonised and nonprotonised form of compound and pH of mobile phase.[29-33] Hyper-rayleigh scattering technique measures dissociation constant from data of first hyperpolarizability of neutral and ionic molecule.[15,34-35] UV spectroscopic methods, where a UV spectrum of the compound is recorded for each point of the titration and the change in UV absorbance is plotted against the pH.[36-38] The technique of ultraviolet-visible spectrophotometry is one of the most frequently employed technique in pharmaceutical analysis for determination of dissociation constant. 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.[39] The pH and pKa influence absorption of drug is given in Table 1. The higher the pKa of a basic drug, the stronger the base. Thus from the knowledge of pKa of the drug and pH at the absorption site, the relative amount of ionized and unionized drug in solution at a particular pH and the percent of drug ionized at this pH can be determined by Henderson – Hasselbach equations.[40]

For weak acids,
pH = pKa + log(ionized drug)/(unionized drug)

For weak bases,
pH = pKa + log(unionized drug)/(ionized drug)

The UV spectroscopic method is easy and simple method for calculation of dissociation constant. At pH = pKa, the ionized and unionized form of compound are equal. The different methods used for calculation of pKa like potentiometry, conductometry have limited application for determination of second dissociation constant of very weak acid.[41] There are various factors which affect determination of dissociation constant. The use of cosolvent yields higher pKa value for acids and lower value for bases than does pure water.[42] The temperature and surfactant also affect dissociation constant of compound.[2,4] The pH of the mobile phase affects retention of acidic and basic drug.[43] Hence the conditions used in the measurement should be critically analyzed.

UV-Visible spectroscopy involves the absorption of electromagnetic radiation from the 200–800 nm range and the subsequent excitation of electrons to higher energy states. The absorption of ultraviolet/visible light by organic molecules is restricted to certain functional groups (chromophores) that contain valence electrons of low excitation energy.[44]

In human body, different organ have different pH as given in Table 2. As pH changes dissociation constant also changes, so it is necessary to study dissociation constant at different pH to determine the extent of dissociation at the site of absorption.[45]

UV SPECTROSCOPIC METHODS OF DETERMINATION OF DISSOCIATION CONSTANT

2.1 Simultaneous equation method.
2.2 Second derivative method.
2.3 Orthogonal functional method.
2.4 Difference spectrophotometry.
2.5 UV spectroscopy using 96 – well microtiter plates.
2.6 Multi-peaks Gaussian fitting method
2.7 Molar ratio method.
2.8 Multiwavelegth spectroscopic method.

Simultaneous equation method: An alternative to potentiometric titration is UV–VIS spectrophotometry because it can handle compounds with lower solubility and lower sample concentrations. The main advantage is higher sensitivity to compounds with favourable molar absorption coefficients. However, in such a case, a compound must contain a UV-active chromophore close enough to the site of the acid–base function in the molecule. The limitation of this method is spectral difference between neutral and ionized formed are required.[1,3,46] A number of aromatic molecules show characteristic absorption in the ultraviolet region and can therefore are easily determined by UV spectroscopy.[47] The pKa value of compound determined from following equation,

$$pKa = pH - \log [MR^-]/[HMR]$$

where MR^- is the dissociated ion and HMR is the undissociated ion

$$pKa = pH - \log \frac{[A_{\lambda HMR}. \varepsilon_{HMR,\lambda MR} - A_{\lambda MR}. \varepsilon_{HMR,\lambda HMR}]}{[A_{\lambda MR}. \varepsilon_{MR,\lambda HMR} - A_{\lambda HMR}. \varepsilon_{MR,\lambda MR}]}$$

$A_{HMR, \lambda MR}$ is the absorbance of undissociated ion at wavelength of dissociated ion.

$A_{HMR ,\lambda HMR}$ is the absorbance of undissociated ion at wavelength of undissociated ion.

$A_{MR, \lambda MR}$ is the absorbance of dissociated ion at wavelength of dissociated ion.

$A_{MR, \lambda HMR}$ is the absorbance of dissociated ion at wavelength of undissociated ion.

$\varepsilon_{MR, \lambda MR}$ is the molar absorption coefficient of dissociated ion at wavelength of dissociated ion.

$\varepsilon_{MR, \lambda HMR}$ is the molar absorption coefficient of dissociated ion at wavelength of undissociated ion.

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If sample contains two absorbing drugs or two different form of of same drug each of which absorbs at the λmax of the other. The λmax of two different form of drug must be different.[48-49]

Second derivative method: A derivative spectrum shows better resolution of overlapping bands than the fundamental spectrum and permits accurate determination of the λ_{max} of the individual bands and discriminate narrow spectral bandwidth. This is because the derivative amplitude (D) i.e. the distance from a maximum to a minimum, is inversely proportional to the fundamental spectrum bandwidth (W) raised to the power (n) of the derivative order. Thus,[49]

$$D \alpha (1/W)^n$$

Compounds in which absorption spectra overlap and cannot be separated by conventional methods, are easily recorded in derivative spectrophotometry.[50] In derivative spectrophotometric method normal spectrum is converted to its first, second, or higher derivative spectrum. The first derivative spectrum is plot of rate of absorbance with wavelength against wavelength. The second derivative spectrum is plot

by taking the curvature of the normal spectrum (zero order) against wavelength or plot of $d^2A/d\lambda^2$ vs λ.
In this method sample solution is prepared by dissolving an excess amount of sample to show turbidity due to precipitation of the undissolved species. The concentration of solubalised species in a turbid sample solution is measured by taking the absorbance of its supernatant. The effect of the background scattering signal caused by turbidity of sample solutions is easily eliminated by second derivative or higher derivative spectroscopy.

Suppose AH^+X^- is the salt of strong acid and weak base. When the neutral species A is sparingly soluble, addition of excess amount of salt to an alkaline buffer causes precipitation of A, therefore the turbid solution is formed and when A is saturated, the dissociation constant (pKa) of weak acid AH^+ is calculated by

$pKa=S_o [H^+]/[AH^+]$ ……..a

where So is the concentration of saturated B, i.e., the solubility of B. If S denotes the sum of S_o and $[AH^+]$, i.e.,

$S = S_o + [AH^+]$ ……b

$[AH^+] = S - S_o$ ……c

$pKa = S_o [H^+] / (S – S_o)$ ……..d

Equation d can be rearranged to give

$S = S_o [H^+] / pKa + S_o$ …….e

From above equation 'e' pKa value is obtained by a plot of S against $[H^+]$ should give a straight line with a slope S_o/pKa and an intercept S_o. As the signal amplitude of a derivative spectrum is proportional to the concentration of dissolved species, and the spectra of A and AH^+ of these sparingly soluble derivatives are identical, S = mD, Then, the previous equation becomes

$D = Do [H^+]/Ka + Do$ ……(f)

where Do is the corresponding amplitude for saturated molecule. D is the derivative signal amplitude at a particular wavelength and m is a constant. The pKa value is determine from slope of plot of D (instead of S) against $[H^+]$(shown in fig.1).
Spectrophotometric methods requires different spectra for different species and reagents must be pure.[51-52] When amount of substances is very less to show a measurable absorbance second derivative spectrum is used.[53]

Orthogonal method: The essential feature of a difference spectrometric assay is that the measured value is the difference absorbance (ΔA) between two equimolar solutions of the analyte in different chemical forms which exhibit different spectral characteristics.[49] ΔA method is suitable for compound in which absorption affects with pH change. But some compound shows subsidiary peaks without any large change in intensity or the ΔA value may be small. Such compounds have limited the application of the ΔA method. In these situations, application of the orthogonal function (ΔP_j) method gives solution for the analysis of such compounds. When the absorption spectra of the different species in solution overlap, the use of the A_{max}, method may give incorrect results. In such cases, the application of the orthogonal function (ΔP_j) method helps in the resolution of the spectral overlapping which make easy the determination of the ionised species at any pH value. For determination of pKa value the drug should be dissolved in solvent in which sample is fully dissociated. The determination of pKa of drug is based upon recording the absorption spectra in strong acid, strong alkali and in series of buffer solution of different pH values. The pH can be adjusted in such way that there will be a great preponderance of one form in the solution and observed spectrum will be that of the predominant form. At pH values intermediate between the extremes, both forms are present and the observed spectrum is a linear combination of both components providing that Beer's law is followed. According to general rules the order of the polynomial, P_j is chosen such that it makes a large contribution to a certain segment in the absorption curves of the compound at a given pH value and a small contribution over the same segments at another pH value. So, the coefficient difference, ΔP_j, will correspond with the maximum in the convoluted curve.

The pKa value of acidic and basic drugs are determined from orthogonal function by using equation a and b

$pKa = pH + \log (P_{jd} – P_{jb})/(P_{jb} – P_{ju})$ …….(a)

$pKa = pH + \log (P_{jb} – P_{ju})/(P_{jd} – P_{jb})$ ……(b)

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 proposed method is applied for the determination of the pKa, value(s) of drugs with single, double and triple pKa, values. To obtain precise values of Δpj the comparative coefficients, ΔQj, have been applied where $\Delta Q_j = \Delta p_j N_j^{1/2}$ and N_j is the normalizing factor.

$pKa = pH + \log(Q_{jd}–Q_{jb})/(Q_{jb}–Q_{ju})$ ….(c)

$pKa = pH + \log(Q_{jb}–Q_{ju})/(Q_{jd}–Q_{jb})$ .....(d)

where Q_{jb}, Q_{jd} and Q_{ju}, are the comparative coefficient of the buffered, dissociated and un

dissociated drug solution, respectively. The pKa value of drug is also determined by graphical method (plot of Q_j vs pH). The pKa is obtained from pH value at which concentration of dissociated and undissociated form of the compound are equal. $Q_j^{1/2}$, (at half neutralization) can be calculated using (Q_{ja} + Q_{jb} / 2, where a and b are the fully acidic and fully basic solutions for singly dissociated compounds. The pH which corresponds to $Q_j^{1/2}$ is taken as pKa.[54] The method eliminates the effect of interferences during analysis.[55]

UV spectroscopy using 96 – well microtiter plates: This method is useful for poor water soluble drug. The advantage of this method is sensitivity of UV spectroscopy with rapid measurement of pKa value. In this method UV spectrophotometer is equipped with pH meter (use to preparation of buffer solutions) and microplate reader. The pKa determination by UV spectrophotometry require chromophore group near the ionization centre. If the compound is stable with DMSO solvent then stock solution is prepared in this solvent. The different buffer solution of constant ionic strength is filled in 96 well microtiter plates and then amount of stock solution is added to each well, leaving a series of blank buffers as correction factor. The data of UV spectrum which is taken at each wavelength as function of pH and pKa are processed with prism program. The pKa value work out by nonlinear regression (prism program) using following equation.

$$\text{Absorbance total} = \frac{\varepsilon_{HA} - \varepsilon_{A^-} * [10^{(pH - pKa)}]}{1 + 10^{(pH - pKa)}} * [St]$$

Where ε_{HA} and ε_{A^-} are the extinction coefficient of the acid and base forms of the compound respectively, [St] is the total compound concentration. The plot of absorbance difference against pH also give pKa value at inflection point.[10,56]

Difference spectrophotometry: The selectivity and accuracy of spectrophotometric analysis of samples containing absorbing interferences may be markedly improved by the technique of difference spectrophotometry. The essential feature of a difference spectrophotometric assay is that the measured value is the difference absorbance (ΔA) between two equimolar solutions of the analyte in different chemical forms which exhibit different spectral characteristics. The simplest and most commonly used technique for altering the spectral properties of the analyte is the adjustment of the pH by means of aqueous solutions of acid, alkali or buffers. The ultraviolet- visible absorption spectra of many substances like aromatic carboxylic acid, phenol and amines contain ionizable functional groups. Their absorption spectra are dependent on the pH of the solution. The selectivity of the ΔA procedure depends on the correct choice of the pH values to induce the spectral change of the analyte.[49] The plot of absorbance difference against pH give pKa value at inflection point.[56]

The pKa of the drug is also determined by following equation,

$$pKa = pH - \text{Log } (A - Aa) / (Ab - A)$$

where A is the absorbance of the solution at particular pH, Aa and Ab is the absorbance at the same concentration in acid and alkaline solution respectively. The use of 0.1 M hydrochloric acid and 0.1 M sodium hydroxide to induce the ΔA of the analyte is convenient.[57-58]

Multi-peaks Gaussian fitting method: UV-visible absorption spectra of compound (suppose HMR) are measured at different pH values. A multi-peaks Gaussian is used to interpret the spectra. The multi-peaks Gaussian fitting calculation on the overlap peaks gives the integrated absorbance ratio A1/A2, then the pKa of compound can be obtained.

Theory of multi-peaks Gaussian fitting method:

Ionization equilibrium of compound in aqueous solution is express by following equation

$$HMR \leftrightarrow MR^- + H^+$$

When HMR and MR^- exist simultaneously to give a spectra with two peaks at particular pH value. Multi-peaks Gaussian fitting on the spectra which have two peaks gives following equation

$$y = y_o + \frac{A_1}{w_2\sqrt{\pi/2}} \exp\left[-2((x-\lambda_{max1})/w_1)^2\right] + \frac{A_2}{w_2\sqrt{\pi/2}} \exp\left[-2((x-\lambda_{max2})/w_2)^2\right]$$

where,

y_o = Baseline,

λ_{max1} and λ_{max2} = Maximum absorption wavelengths for MR^- and HMR,

w_1 and w_2 = Half peak widths for MR^- and HMR,

A_1 and A_2 = Absorbance of the two peaks for MR^- and HMR.

From spectra Multi-peaks Guassian fitting method gives the λmax_1, λmax_2, w_1, w_2, A_1, A_2. The pKa value at different pH is calculated by following equation.

$$pKa = pH - \log [MR^-] / [HMR]$$
$$= pH - \log\ \varepsilon_2 A_1 / \varepsilon_1 A_2$$
$$= pH + \log\varepsilon - \log A_1/A_2$$

where, $A_1/A_2 = \varepsilon_1[MR-] / \varepsilon_2[HMR]$

ε_1 is the molar absorption coefficients of MR

ε_2 is the molar absorption coefficients of HMR.
Based on absorption spectra of compound multi-peaks Gaussian fitting gives the integrated absorbance of MR^- and HMR absorption peaks which is used to calculate concentration of MR^- and HMR. Hence the pKa measurement results are more reliable and repeatable.[59]

Molar ratio method: The dissociation constant of polymer metal complex is determined using molar ratio spectroscopic method. Absorption spectra of polymer, metal ion and polymer - metal ion complex solutions are recorded and λmax values for each system are determined.

$Me^+ + nPL \longleftrightarrow Me(PL)_n^+$

Where Me^+ is the metal ion, PL is the chelating unit of the polymer ligand, and n is the coordination number of the metal ion.
The equilibrium constant, Kf, can be calculate by the following equation

$$Kf = \frac{[Me^+(nPL)]}{[Me^+][PL]^n} = \frac{1-\alpha}{\alpha(\alpha\, n\, C)^n}$$

where α is the dissociation constant which is calculated as the ratio of the absorbance for a defined coordination number to the theoretical absorbance value for maximum coordination, and C is the complex concentration.[60]

Multi-wavelength spectroscopic method : The multi-wavelengths spectrophotometric method used to determine the pKa value of sparingly soluble drugs. There are various methods like target factor analysis, rank annihilation factor analysis, principal component analysis used to derive the pKa values from the multi-wavelengths spectrophotometric data obtained at different pH. UV absorption spectra of the drug solution are acquired using a flexible device based on a fiber optics dip probe, a light source and a photodiode array (PDA) detector. The multi-wavelength spectroscopic method also refer as WApH technique because in this system the pKa value is determined based on changes in absorbance and wavelength as function of pH. The advantages of this method over the other methods are that it enables the pKa values independent of light absorbing species present in the chemical system. Their absorption spectra are determined from a single titration, without prior knowledge of their optical properties. [9,45,61-62]

CONCLUSION

The pKa is the very important physico-chemical property of the newly synthesized compound. It provides useful information about pharmacokinetic parameters and biological effects. So it is necessary to find method by which accurate and precise determination of pKa is possible. At lower concentration the pKa determination by potentiometry is not possible while by use of UV spectroscopy give accurate results. Limitation of UV spectroscopic method is the requirement of presence of chromophore near the ionization site and absorption spectra of dissociated and undissociated forms of compounds should be different.

Table 1. Influence of drug pKa and gastrointestinal pH on drug absorption [40]

Sr.no.	Acid/ base strength	pKa	Site of absorption
1.	Very weak acids	> 8.0	Unionized at all pH values; absorbed along the entire length of GIT.
2.	Moderately weak acids	2.5 to 7.5	Unionized in gastric pH and ionized in intestinal pH, better absorbed from stomach
3.	Stronger acids	< 2.5	Ionized at all pH values; poorly absorbed from GIT.
4.	Very weak bases	< 5.0	Unionized at all pH values; absorbed along the entire length of GIT.
5.	Moderately weak bases	5 - 11	Ionized at gastric pH, relatively unionized at intestinal pH; better absorbed from intestine.
6.	Stronger bases	> 11.0	Ionized at all pH values; poorly absorbed from GIT.

Table 2. The pH ranges of different body fluids in the mammals.[45]

Blood plasma	7.4
Cell lysosomes	<5
Pancreatic juice	1.5 -3
Urine	5-8
Cell cytosol (liver cells)	6.9
Gastric juice	1.5-3
Saliva	6.4-7

Table 3. Application of various UV Spectroscopic methods for pKa determination.

	Method	Drug	Ref
1.	Simultaneous method	Bromothymol blue	48
2.	Second derivative method	Promazine Chlorpromazine	50
3.	Orthogonal functional method	Benzoic acid Paracetamol Phenobarbitone	53
4.	Difference spectrophotometry	Trandolapril	56
5.	UV spectroscopy using 96 – well microtiter plates	4-nitro phenol	55
6.	Multi peaks Gaussian fitting method	Methyl red	58
7.	Multi-wavelength spectroscopic method	Niflumic acid Nitrazepam Quinine	61

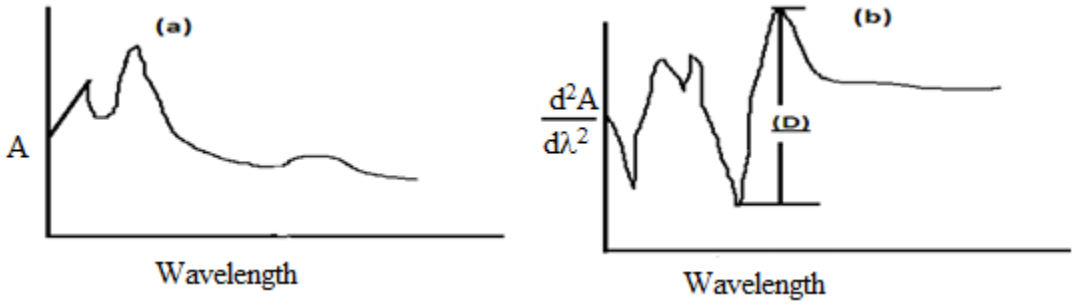

Fig 1 (a) Absorption and (b) second derivative spectra of a turbid sample solution prepared by adding drug above saturated concentration.

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