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Title: **Overview on chromatographic and potentiometric based approaches for  $pK_a$  determination of sparingly soluble substances**

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## Overview on chromatographic and potentiometric based approaches for $pK_a$ determination of sparingly soluble substances

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### Abstract

The dissociation constant ( $pK_a$ ) is one of the most important physicochemical property of the compounds and it is of great importance for the pharmaceutical industry. Many of the active pharmaceutical ingredients are sparingly soluble in water or have no chromophore, thus the conventional *UV/Vis* spectrometry and potentiometry methods are not applicable. This article presents an overview of several approaches based on RP-HPLC and cosolvent/surfactant potentiometry that have been recently published for the  $pK_a$  determination of sparingly soluble active pharmaceutical ingredients. Additionally, different calculation methods that have been used for the estimation of the aqueous dissociation constant are summarized.

**Keywords:** dissociation constant;  $pK_a$ , sparingly soluble active pharmaceutical ingredients, determination, reverse-phase liquid chromatography, potentiometry, surfactants

### Introduction

The dissociation constant ( $pK_a$ ) is one of the primary physicochemical property of active pharmaceutical ingredients. The accurate measurement of the  $pK_a$  value is of great importance to a wide range of research fields. The pharmacokinetics (ADMET: adsorption, distribution, metabolism, excretion and toxicity) of active pharmaceutical ingredients depend on their  $pK_a$

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value. The determination of the  $pK_a$  value during the early stage studies could provide proper selection of a candidate for active pharmaceutical ingredient among the large number of compounds produced by combinatorial chemistry. The knowledge of the  $pK_a$  value for the analyte and its related impurities is essential for analytical method development; providing ruggedness and faster method optimization (Avdeef, 2001; Kerns & Di, 2004; Manallack, 2007).

Several techniques are used for determination of the dissociation constant. Computational methods are valuable tool for  $pK_a$  calculation, especially for large set of compounds present in the screening stage of the drug candidate. There are several computer programs (such as  $pK_a$  Pallas program, ACD/ $pK_a$ , CSpKa Predictor, ADME/Tox WEB, etc.) which are based on different theoretical approaches for predicting the  $pK_a$  value. It should be noted that the accuracy of the computational methods depends only on the model used to perform the calculations. However, computational methods could give unreliable results for sparingly soluble compounds (Babic et al., 2007; Špirtović-Halilović & Završnik, 2010; Reijenga et al., 2013).

Traditionally,  $UV/Vis$  spectrometry and potentiometry have been standard methods for the  $pK_a$  determination (Erdemgli et al., 2007). The  $UV/Vis$  spectrometric method offers great precision, but it hinges on a pH dependent spectrum of the compound in question. It means that a presence of a chromophore close to the ionization site in the molecule is needed. Additional requirement of this technique is that the tested compound should be pure or its impurities should not absorb in the  $UV/Vis$  range, since overlapping spectra could be obtained (Babic et al., 2007; Demiray et al., 2012; Pandey, 2013; Reijenga et al., 2013). Potentiometry is the method of choice for the  $pK_a$  determination of water soluble compounds. This technique offers great precision, accuracy and fast determination. The major drawback is the requirement of larger quantity of pure compound having a water solubility of at least  $10^{-4}M$  (Babic et al., 2007; Narasimham et al., 2011; Reijenga et al., 2013).

In the last twenty years, there is a growing interest for the reverse-phase high performance liquid chromatography (RP-HPLC) as a method for the  $pK_a$  determination, based on the relationship between the capacity factor and the pH value of the mobile phase (Babic et al., 2007; Manderscheid & Eichinger, 2003; Uhrova et al., 1997; Wiczling et al., 2004). The increased application of this technique for the  $pK_a$  determination is due to the several advantages, including

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very small quantity of sample is required, the solubility is not a concern and the purity of the compound is not a question.

Many active pharmaceutical ingredients are sparingly soluble in water, thus the  $pK_a$  determination could not be performed in pure water, and thus water-organic mixtures are needed. It is well established that the increase of the organic content in water-organic mixtures leads to suppression of the basic analyte  $pK_a$  and to an increase in the acidic analyte  $pK_a$  (Kazakevich & Lobrutto, 2007). In such case, the obtained  $pK_a$  values are referred as apparent  $pK_a$  ( $^s pK_a$ ) values and consequently an estimation of the aqueous  $pK_a$  ( $^w pK_a$ ) value is needed (Babic et al., 2007; Manderscheid & Eichinger, 2003; Uhrova et al., 1997).

Generally, the RP-HPLC technique and modifications of the potentiometric titration are the methodological approaches of choice for the  $pK_a$  determination of sparingly soluble compounds. This review article, describes the principles, advantages and drawbacks on several approaches based on RP-HPLC and potentiometry which are recently used for this purpose. Additionally, an overview of different approaches for estimation of the aqueous  $pK_a$  values is given.

## **RP-HPLC approaches for $pK_a$ determination**

### *Isocratic RP-HPLC approach*

The HPLC methodological approach of the  $pK_a$  determination is based on the assumption that the neutral and ionic forms of the compound have different chromatographic behavior. The determination of  $pK_a$  value by means of HPLC was first reported by Horvath et al. (1977) and by Van de Venne et al. (1978). The most widely used HPLC procedure for the  $pK_a$  determination is the isocratic HPLC approach (Babic et al., 2007; Manderscheid & Eichinger, 2003; Uhrova et al., 1997). This approach involves a series of isocratic experiments at different pH value of the mobile phase, using at least three different concentration of the organic modifier (acetonitrile or methanol) in the mobile phase (Demiray et al., 2012; Erdemgli et al., 2007; Gumustas et al., 2010; Huo et al., 2013; Kazakevich & Lobrutto, 2007). More accurate results could be obtained if lower organic modifier concentration is used, thus the organic modifier concentration in the mobile phase should not be more than 40%. The selected organic modifier concentration in the mobile phase should be able to elute the neutral form in a reasonable time, while providing a value for the retention factor

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( $k'$ ) of the ionized form of at least one. The  ${}^s_w pK_a$  value could be determined using nonlinear regression analysis software, such as NLREG program (Demiralay et al., 2009, Gumustas et al., 2010; Şanlı et al., 2010) where the plot  $k'$  versus pH value at a particular organic content concentration is fitted to a nonlinear symmetrical sigmoidal model. The inflection point of the curve corresponds to the  ${}^s_w pK_a$  value of the compound.

The drawback of the isocratic concept is that long retention times could be observed using low organic content mobile phases. Additionally, 8 to 10 experiments should be performed at each organic content composition, which makes this concept a time-consuming (Babic et al., 2007; Wiczling et al., 2004). Beforehand the column stability was the limiting factor for the range of the  $pK_a$  values that could be determined. However, nowadays there is variety of stationary phases with wide pH stability range that could fit this purpose, so the chromatographic column stability is not an issue anymore. The isocratic RP-HPLC approach is applicable for simultaneous  $pK_a$  determination of small number of compounds, thus this approach is not a method of choice for early stage screening of drug candidates.

#### *Gradient RP-HPLC approach*

The methodology for the gradient RP-HPLC determination of  $pK_a$  is based on two organic solvent gradient runs and one pH gradient RP-HPLC run (Kaliszan & Wiczling, 2010; Wiczling et al., 2004; Wiczling et al., 2006, Wiczling et al., 2008). The initial organic solvent gradient experiments at two different gradient times should be carried out at fixed pH value, where the ionization of the compound is suppressed (pH 3.0 for acidic and pH 10.5 for basic compounds). These organic solvent gradient runs are intended to define the organic solvent concentration, needed for providing appropriate retention time for completing the pH gradient experiment. Afterwards, a programmed pH gradient experiment is carried out at the chosen organic solvent concentration in the mobile phase. The pH gradient should be started at pH value that ensures complete suppression of the ionization of the compounds and it ends at a pH that provides their full ionization. This means that the pH gradient experiment for acidic compound should be started at pH 3.0 and finished at pH 10.5. At the end, one isocratic experiment at pH value that ensures a complete ionization of the compound is needed in order to obtain the retention of the ionized form of the compound.

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The described procedure has the advantage of fast  $pK_a$  determination of large set of substances in a mixture. Additional increase of the number of analyzed compounds in individual run could be gained with the replacement of the UV detector with time-of-flight mass spectrometry (TOF-MS) detector. Recently, a gradient RP-HPLC-TOF-MS method has been published for determination of the hydrophobicity and dissociation constant of mixture of 40 compounds (Wiczling et al., 2014). The same research team expands the applicability of the proposed method on a diverse group of 161 chemical compounds (Wiczling et al., 2015). Some of the  $pK_a$  values derived with this methodology differ from the literature data; therefore, the gradient RP-HPLC approach is useful for a rough, preliminary estimation of the  $pK_a$  values of drug candidates.

#### *Solely aqueous RP-HPLC approach*

Recently, a new RP-HPLC approach based on solely aqueous mobile phase was published (Volna et al., 2017). The mobile phase is composed of 100% modified Britton-Robinson buffer, where the pH of the eluent is set by mixing the Britton-Robinson buffer with diethylamine (0.1% v/v) in the HPLC pump. In case of very lipophilic compounds, 5% methanol could be added to the mobile phase. This methodology is applicable for compounds having wide range of lipophilicity (log P from -2.26 to 2.26). The use of the solely aqueous mobile phase avoids the need of  $pK_a$  determination at several sets of measurements in eluent at different organic solvent content, which speeds up the procedure. Another advantage of this methodology is that there is no need to use more complex calculation, such as Yasuda-Shedlovsky extrapolation (Shedlovsky, 1962), for the estimation of the  ${}^w pK_a$  value. Also, it should be emphasized that good agreement between the  $pK_a$  values derived with this procedure and literature values was encountered for acidic compounds. Hence, the solely aqueous RP-HPLC approach presents a promising approach for fast and relatively simple approach for  $pK_a$  determination of large number of compounds.

#### **Potentiometric approaches for $pK_a$ determination**

The potentiometry is a well-established method for  $pK_a$  determination. The simplicity and the low cost of the potentiometric titrations have made it one of the most commonly used methods

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for  $pK_a$  determination. However, the poor solubility of the compounds impedes the traditional potentiometric determination, thus alternative approaches are needed.

#### *Cosolvent (mixed-solvent) approach*

The cosolvent approach is based on the measurements of the apparent dissociation constant in different ratios of organic solvent/water mixtures where the  ${}^w pK_a$  value is obtained by extrapolation (Avdeef et al., 1999; Fallavena & Schapoval, 1997; Takacs-Novak et al., 1997). According to the reported literature data, the potentiometric titrations could be performed by use of different organic solvents, such as: methanol (Erdemly et al., 2007; Fallavena & Schapoval, 1997; Herrero-Martinez et al., 2008; Takacs-Novak et al., 1997), dioxane (Fallavena & Schapoval, 1997), acetone (Fallavena & Schapoval, 1997; Takacs-Novak et al., 1997), isopropyl alcohol (Rafols et al., 1997). However, methanol has the widest application for the purpose because the solvation effect of this solvent is closest to water (Avdeef, 1993a; Avdeef, 1993b; Takacs-Novak et al., 1997; Völgyi et al., 2007).

However, some compounds could not be dissolved in single organic solvent/water mixture. To overcome this problem, Völgyi and coworkers (2007) proposed a multicomponent cosolvent (MDM-water mixture) approach. The MDM-water mixture consists of 60%  $v/v$  MDM stock solution (equal volumes of methanol, dioxane and acetonitrile) and 40%  $v/v$  water (and 0.15M KCl, for ionic strength adjustment), and this mixture improves the solubility of the compounds. Consequently, the  $pK_a$  measurements could be performed using lower proportions of organic solvent which allows sidestep of the long-distance extrapolations to zero organic solvent. Hence, more reliable results could be obtained. The applicability of the MDM-water mixture approach was confirmed through the  $pK_a$  determination of compounds having diverse structure (Völgyi et al., 2007; Zapala, 2011).

The apparent ionization constant obtained with the cosolvent approach could be deduced graphically from the Bjerrum plots,  $n_H^-$  vs pH (Avdeef, 2003; Kraft, 2003), where the Bjerrum function,  $n_H^-$ , is the average number of bound protons at particular pH and the  $pK_a$  value is read from the Bjerrum plot at  $n_H^- = 0.5$ .

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### Surfactant based approach

Generally, the cosolvent concept is more time-consuming. Recently, an alternative approach using nonionic surfactants is proposed (Ravichandiran et al., 2011). In this study, the desired solubility of the poorly soluble compounds was achieved using low concentrations (2-5%) of Tween 80, Chemophol and Labrasol. The authors of this concept demonstrated that the ionization behavior of several compounds remains unaffected by the use of the nonionic surfactants, with the same level of accuracy without a need of extrapolations to pure water.

However, several literature reports revealed that cationic (cetyltrimethylammonium bromide), anionic (sodium dodecyl sulfate) and non-ionic (Triton X100) micellar solutions shift the dissociation equilibrium as compared to those in pure water (Jaiswal et al., 2005; Nemcova et al., 2009; Yuangin et al., 2002). In case of cationic and anionic surfactant solution, electrostatic effect and polarity effect have an impact on dissociation equilibrium, as for non-ionic surfactants the polarity is the dominant factor. Generally, the  $pK_a$  shifts are smaller when nonionic surfactants are used. This suggests that measurements using different concentration of nonionic surfactant should be performed in order to perceive their effect on the dissociation constant.

### Estimation of aqueous $pK_a$ value

The literature data suggest that several approaches could be used for the estimation of the  ${}^w pK_a$  value. One of the most commonly used approach for the  ${}^w pK_a$  value estimation is based on the linear relationship between the  ${}^s pK_a$  values and the volume fraction of the organic solvent used for the determination. The intercept of the linear equation (extrapolation to pure water mobile phase) presents the  ${}^w pK_a$  value of the compound.

The second method known as Yasuda-Shedlovsky extrapolation establishes linear relationship between the apparent  $pK_a$  and the solvent dielectric constant (Sheldovsky, 1962), following the equation:

$${}^s pK_a + \log[H_2O] = \frac{\alpha}{\epsilon} + \beta \quad \text{Eq.1}$$

where  ${}^s \epsilon$  denotes the solvent dielectric constant,  $[H_2O]$  is the molar concentration of water in the mobile phase, and  $\alpha$  and  $\beta$  are constants.

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The plot of  ${}^s pK_a$  value + log [H<sub>2</sub>O] versus 1/ $\epsilon$  is extrapolated to a value corresponding to pure water ( $\epsilon=78.3$ , [H<sub>2</sub>O] = 55.5). The  ${}^w pK_a$  value could be calculated from the equation 1, using the obtained value for slope and intercept as  $\alpha$  and  $\beta$  constants, respectively. This method is most widely used procedure for cosolvent techniques, such as isocratic RP-HPLC and cosolvent potentiometry procedure (Demiray et al., 2012; Herrero-Martinez et al., 2008; Völgyi et al., 2007; Wiczling et al., 2004; Zapala, 2011).

The  ${}^w pK_a$  value could be calculated using the empirical equation (Eq.2 - Eq. 4) presented by Algelov et al. (2008).

$$k_{\text{obs}} = \frac{k_{\text{HA}} + k_{\text{A}^-} \cdot 10^{pH - pK_a}}{1 + 10^{pH - pK_a}} \quad (\text{for acidic compounds}) \quad \text{Eq. 2}$$

$$k_{\text{obs}} = \frac{k_{\text{B}} + k_{\text{BH}^+} \cdot 10^{pK_a - pH}}{1 + 10^{pK_a - pH}} \quad (\text{for basic compounds}) \quad \text{Eq. 3}$$

where  $k_{\text{HA}}$ ,  $k_{\text{B}}$  and  $k_{\text{A}^-}$ ,  $k_{\text{BH}^+}$  are the neutral and ionic forms of the compound, respectively.

The conversion formula is as follows:

$$pK_a = pH - \frac{\log(k_{\text{HA}} - k)}{k - k_{\text{A}^-}} \quad \text{Eq. 4}$$

where  $k_{\text{HA}}$  and  $k_{\text{A}^-}$  are  $k'$  of the neutral and ionic form of the compound, respectively; the  $k_{\text{HA}}$  is the minimum value of  $k'$  and  $k_{\text{A}^-}$  is the maximum one. The pH and  $k$  values are defined as points at maximum slope of the pH- $k'$  plot. Huo et al. (2013) applied this method to determine the  $pK_a$  value of oxysophocarpine. This procedure was also used to calculate the  $pK_a$  value of compound with wide range of lipophilicity using the solely aqueous RP-HPLC approach (Volna et al., 2017).

Another empirically based approach takes into account the type (acetonitrile or methanol) and concentration of the organic modifier. For acetonitrile/water mobile phase the aqueous  $pK_a$  could be calculated by the following equations:

$${}^w pK_a = {}^s pK_a + (x\% \text{ organic}) * A \quad \text{Eq. 6}$$

$${}^w pK_a = {}^s pK_a + (x\% \text{ organic}) * B \quad \text{Eq. 7}$$

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The value of A is 0.03 and the value of B is 0.02 and they correspond to acidic and basic  $pK_a$  shift per 10% ACN, respectively (Kazakevich & Lobrutto, 2007). This calculation is applicable only for the isocratic RP-HPLC approach.

## Conclusion

Several RP-HPLC and potentiometric based approaches are available for the determination of the  $pK_a$  value of sparingly soluble active pharmaceutical ingredients.

The isocratic RP-HPLC is simple and easy approach for the  $pK_a$  determination of compound in simple mixtures. Although it is a time-consuming, this approach gives very accurate results. Recently solely aqueous RP-HPLC has emerged as a simple alternative approach. However more literature data are required to evaluate the reliability of the  $pK_a$  data obtained using this approach. The gradient RP-HPLC concept is more applicable for rough estimation of the  $pK_a$  value. The gradient RP-HPLC/MS methodological approach is useful for a preliminary evaluation of the drug candidates, considering the short analysis time.

The cosolvent potentiometric approach is a well-established procedure for the  $pK_a$  determination of pure, sparingly soluble compounds. The major disadvantage of this concept could be seen in the large number of experiments needed for the determination and in the complexity of calculations for estimation of the aqueous  $pK_a$  value. The surfactant concept appears as a more simple approach and it has a great potential, but additional investigation that include a larger number of compounds is needed.

This overview contributes to the enrichment of the scientific data related to the alternative chromatographic and potentiometric based approaches for determination of sparingly soluble active pharmaceutical ingredients.

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## Резиме

### Преглед на хроматографски и потенциометриски методи за определување на $pK_a$ вредност на слабо растворливи супстанции

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**Клучни зборови:** константа на дисоцијација,  $pK_a$ , слабо растворливи супстанции, определување, реверзно-фазна течна хроматографија, потенциометрија, сурфактанти

Константата на дисоцијација ( $pK_a$ ), како една од најважните физичко-хемиски својства на активните супстанции, има големо значење за фармацевтска индустрија од повеќе аспекти. Повеќето активни супстанции се нерастворливи во вода или немаат хромофор, поради што не се применливи конвенцијалните техники ( $UV/Vis$  спектрометрија и потенциометрија) за определување на константата на дисоцијација. Во овој ревијален труд даден е преглед на новите пристапи што се користат за определување на  $pK_a$  вредноста на слабо растворливите супстанции, со посебен осврт на реверзно-фазната течна хроматографија и на потенциометриски титрации во присуство на корастворувач/сурфактант. Дополнително, даден е осврт на различни пристапи што се применуваат за пресметување на константата на дисоцијација.

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