The Thermodynamic Overlapping pK\textsubscript{a} of the Antitumor Drug \textit{Ibrutinib} Using Multiwavelength UV/VIS-Spectroscopy and Potentiometry

**Abstract**

Potentiometric and spectrophotometric pH-titrations of the antitumor drug \textit{Ibrutinib} for dissociation constants determination were compared. \textit{Ibrutinib} is targeting B-cell malignancies, for treatment of chronic lymphocytic leukemia, and Waldenström’s macroglobulinemia. Chemometrics approach to the nonlinear regression of the pH-spectra (REACTLAB, SQUAD84) and pH-titration (ESAB) determines four dissociation constants. \textit{Ibrutinib} exhibits four protonatable centers in a pH range of 2 to 10, where only two pKa are well separated (ΔpK > 3), while the others are near dissociation constants of overlapping equilibria. The molecule LH\textsubscript{2} can protonate to sparingly soluble cations LH\textsubscript{3+} and LH\textsubscript{42+} and dissociate to anions LH\textsubscript{-} and L\textsubscript{2-}. The set of spectra for pH from 2 to 11 in 220 to 300nm exhibits chromophore sensitivity to a pH change. Since pH above 10 and pH below 5 occurs in a titrated solution of a very fine precipitate of \textit{Ibrutinib}, this part of the potentiometric titration curve pH over 10 and pH below 5 did not undergo regression analysis to estimate pK\textsubscript{a}s. Depending on ionic strength the thermodynamic dissociation constants were estimated at 25°C and 37°C: pK\textsubscript{a1,T} = 3.22 and 3.22, pK\textsubscript{a2,T} = 4.17 and 5.21, pK\textsubscript{a3,T} = 6.77 and 6.77, pK\textsubscript{a4,T} = 9.82 and 9.81.

**Keywords:** Dissociation constants; Ibrutinib; Spectrophotometric titration; REACTLAB; SQUAD84; ESAB

**Graphical Abstract**

\textit{Ibrutinib} (antitumor drug)

**Introduction**

\textit{Ibrutinib} (USAN, also known as PCI-32765 and marketed under the name \textit{Imbruvica}) (Figure 1) of formula C\textsubscript{25}H\textsubscript{24}N\textsubscript{6}O\textsubscript{2} and molar mass 440.497 is an anticancer drug targeting B-cell malignancies. It was approved by the US Food and Drug Administration (FDA) in November 2013 for the treatment of mantle cell lymphoma and in February 2014 for the treatment of chronic lymphocytic leukemia.

\textit{Ibrutinib} is currently under development by Pharmacyclics, Inc and Johnson & Johnson’s Janssen Pharmaceutical division for additional B-cell malignancies including diffuse large B-cell lymphoma and multiple myeloma [1, 2]. In January 2015, \textit{Ibrutinib} was approved by the FDA for treatment of Waldenström’s macroglobulinemia, a form of non-Hogkin’s lymphoma.

**Figure 1:** Structural formula of \textit{Ibrutinib}.
lymphocytic leukemia (CLL) cells. Ibrutinib has been reported to promote apoptosis, inhibit proliferation, and also prevent CLL cells from responding to survival stimuli provided by the microenvironment [1,2]. In early clinical studies, the activity of Ibrutinib has been described to include a rapid reduction in lymphadenopathy accompanied by transient lymphocytosis, suggesting that the drug might have direct effects on cell homing or migration to factors in tissue microenvironments [3].

Knowledge of the possible ionization states of a pharmaceutical substance, embodied in the logarithm of the mixed acid dissociation constant 
\[ pK_a \] is vital for understanding many properties essential to drug development [4]. As the majority of drugs are weak acids and/or bases, knowledge of the dissociation constant in each case helps in understanding the ionic form a molecule will take across a range of pH values and the level of general interest in such ionization phenomena is evident from the large number of recent publications on the topic [5-8]. 

\[ pK_a \] values can be either experimentally measured or theoretically predicted:

a. Many new substances are poorly soluble in aqueous solutions, conventional potentiometric determination of dissociation constants of these compounds can often be difficult [9]. Spectrophotometry is a convenient method to determine 
\[ pK_a \] in very diluted aqueous solutions (about \( 10^{-5} \) to \( 10^{-6} \) M), provided that the compound possesses pH-dependent light absorption due to the presence of a chromophore in proximity to the ionization centre [10-12]. In previous works [13-17] the authors have shown that the spectrophotometric method in combination with suitable chemometric tools can be used to determine dissociation constants 
\[ pK_a \] even for sparingly soluble drugs. The most relevant algorithms are SQUAD84 [11,18,19] and REACTLAB [20] or its previous version SPECFIT [21].

b. Nine commercially available or free programs for predicting ionization constants were compared [4]. Meloun et al. [22] used the REGDIA regression diagnostics algorithm written in S-Plus [23] to critically examine the accuracy of 
\[ pK_a \] predictions with four programs (ACD/pK [24-26], Marvin [25,27], PALLAS [25,28] and SPARC [29,30]) considered the best. Balogh et al. [25] found the most predictive and reliable predictors to be MARVIN and ACD/Percepta [26].

The aim of our study was to examine and verify the spectrophotometric analysis of the pH-absorbance matrix and to carry out a potentiometric determination of the protonation model to find suitable conditions for a reliable regression determination of dissociation constants from the spectra of the Ibrutinib antitumor drug. Here we are reporting our obtained experimental results that were also evaluated by two different LFER based 
\[ pK_a \] predictions tools, MARVIN and ACD/pK software.

Materials and Methods

Data analysis

The procedure of spectrophotometric study of the acid-base equilibria of Ibrutinib was described previously [16,17]. The general procedure used to build the protonation model with SPECFIT32, REACTLAB or SQUAD84 was described in [15,31,32]. Two programs for the numerical analysis of spectra were used, REACTLAB and SQUAD84, which compared the consensus found in numerical parametric estimates and in a fitness of the predicted absorbance spectra through measured absorbance data. The potentiometric determination with the use of ESAB program was described previously [33-35].

Reliability of 
\[ pK_a \] estimates obtained by the goodness-of-fit test

A detailed procedure of the graphical and numerical analysis of residuals is described in [13,16,17]. The vector of residuals in each spectrum and finally in the entire absorbance matrix is statistically analyzed and the closest fit of the data is proven. The adequacy of a proposed regression model with experimental data and the reliability of found parameter estimates, \( b, j = 1, ..., m \), may be examined by the goodness-of-fit test, cf. page 101 in ref. [36]. The statistics of residuals can be used for a numerical goodness-of-fit evaluation, cf. page 290 in ref. [37].

Chemicals and solutions

Ibrutinib donated by ZENTIVA GROUP, Ltd. (Prague) with declared purity checked by a HPLC method and alkalimetrically, was always >99%. This drug has been weighted straight to a reaction vessel to reach a resulting concentration of about 5.8×10^{-5} mol dm^{-3}. Other chemicals were described previously [14,38].

Apparatus and procedure

The apparatus used and both titration procedures have been described previously in detail [15,16,31]. The experimental and computation scheme to determine the dissociation constants of the multi-component system is taken from Meloun et al. [36] and the five steps are described in details [16].

Computation and software

Estimation of dissociation constants was performed by regression analysis of the UV/VIS spectra using the SQUAD84, REACTLAB and ESAB programs and the spectra interpretation with the use of the INDICES programme [39] aims to evaluate the quality of the dataset. Most graphs were plotted using ORIGIN 9 [40] and S-Plus. ACD/pK and MARVIN programs for predictions are based on the structural formulae of drug compounds.

Results

Computational prediction of 
\[ pK_a \]

Ibrutinib has six functional groups (denoted with letters A, B, C, D, E, F in the upper part of Figure 2) that can be associated with dissociation constants; two ionizations are associated with the oxygen and four ionization with the nitrogen atom.

The macrodissociation constants of Ibrutinib were predicted according to the chemical structure analyzed by two reliable 
\[ pK_a \] prediction tools. ACD/Percepta [26] was run using the GALAS model, which uses an internal training set of 31000 individual 
\[ pK_a \] values for approximately 16000 compounds in aqueous solutions, experimental data of 2000 molecules in nonaqueous solvents and gives more detailed information about macrospeciation. Marvin 
\[ pK_a \] predictions [41] are based on the calculated partial charge of the atoms located in the analyzed structure, using the Hammett-Taft approach (Figure 2).
Beside macro dissociation constants, the ionic species distribution diagram of Ibrutinib was also predicted using ACD/Percepta software (lower part of Figure 2). The most significant species (>10% in solution) were obtained for Ibrutinib. Once the solution pH increased, the following ionization sequence was obtained: LH₂⁺(F) means that center F is protonated; LH₂⁺(C, F) means that centers C, F are protonated or the centers B, F in tautomeric form are protonated; LH⁻³⁺(B, C, F) means that centers B, C, F are protonated or the centers A, B, F in tautomeric form are protonated; LH⁻²⁺(A, B, C, F) means that centers A, B, C, F are protonated. According to the Marvin-distribution diagram in Figure 2, Ibrutinib is supposed to behave mostly as a neutral molecule LH₄⁺ in biological relevant pH from 5 to 8. We also performed an ionic distribution analysis using Marvin and the obtained data were similar to the ones obtained with ACD/Percepta. The structural prediction of dissociation constants of Ibrutinib was performed using the MARVIN program to specify protonation locations, (Figure 2).

**Spectra analysis**

The strategy for efficient experimentation in dissociation constants determination followed by spectral data treatment was used according to the published Tutorial [16]. A qualitative interpretation of the spectra aims to evaluate the quality of the data set, remove spurious data, and estimate the minimum number of factors, that is contributing aqueous species, which are necessary to describe the experimental data.

**Determination of the number of light-absorbing species:** Ibrutinib contains a complicated molecular structure introduced in Figure 1 & 2 and several protonation equilibria were monitored spectrophotometrically to analyze a spectra set in two steps (Figure 3): first, the spectral data in the form of a data matrix were subjected to principal component analysis to determine the number of independent light absorbing species using the INDICES algorithm [39].

**Conditions:** Solution of 5.0×10⁻³ M Ibrutinib in phosphate buffer (pH=6.04 adjusted with KCl) at 25°C was titrated with 0.014M KOH or 1.044M HCl to get the absorbance matrix of Ibrutinib k=4 which leads to four light-absorbing species in the mixture, n=4, and the actual instrumental error s(k) of the spectrophotometer used s(k) = 0.4 mAU, (lower part).

The INDICES indicate the position of break points on the s(A) = f(k) curve in the screen plot using the most reliable approaches by Kankare’s s(A), [12,39]) and gives k = 4 with the corresponding co-ordinate s(A) = 0.4 mAU (Figure 3c). This value also represents the actual instrumental error sinst(A) = 0.40 mAU of the spectrophotometer CINTRA 5 (GBC, Australia). The number of light-absorbing species helps to establish a protonation model. This means that four dissociation constants will be preferred and five species LH⁺, LH⁺, LH⁻, LH⁻, LH⁻ and L²⁺ are supposed to be present. For the large variations in the indicator values, these latter graphs are plotted on a logarithmic scale and the number of

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light-absorbing species $p$ can be predicted from the index function by finding the point $p = k$ where the slope of index function $PC(k) = f(k)$ changes, or by comparing $PC(k)$ values with the instrumental error $s_{\text{inst}}(A) = 0.40$ mAU when $\log s_{\text{inst}}(A) = -3.4$, (Figure 3).

**Search for the protonation model:** The search for the best chemical model containing either 3, 4 or 5 dissociation constants is shown in Figure 4: The spectra set of useful analytical wavelengths ranges was examined to indicate the best wavelengths range in which the actual chromophore is active and reflects the protonation/dissociation in the molecule. The best regression model was sought by testing three working hypotheses about the protonation model: the first concerning three and the others with four or five dissociation constants. The criterion of reliability between both hypotheses was the goodness-of-fit test. At the same time the estimates of the dissociation constants using two regression programs were compared, i.e. SQUAD84 and REACTLAB, (Table 1). The standard deviation of residuals and the Hamilton $R$-factor of relative fitness generally showed that better fit of the calculated spectra was always achieved for the protonation model with 4 dissociation constants.

![Figure 4](typical_squad84_working_environment_testing_the_best_protonation_model_of_ibrutinib_in_the_ph_range_from_2_to_11_for_the_hypotheses_of_three_four_and_five_dissociation_constants.png)

The Thermodynamic Overlapping $pK_a$ of the Antitumor Drug Ibrutinib Using Multiwavelength UV/VIS-Spectroscopy and Potentiometry

Four dissociation constants $pK_a$, $pK_{a1}$, $pK_{a2}$ and five molar absorptivities of *Ibrutinib* $\varepsilon_r$, $\varepsilon_{1084}$, $\varepsilon_{589}$, $\varepsilon_{492}$ and $\varepsilon_{440}$ were estimated using SQUAD84 and REACTLAB in the first run. The reliability of the parameter estimates may be tested using the following diagnostics as was published [16] (Figure 4).

The first diagnostic value indicates whether all of the parametric estimates $pK_a$ and $\varepsilon$ have physical meaning and reach realistic values. As the standard deviations $s(\log pK_a)$ of parameters $\log pK_a$ and $s(\varepsilon)$ of parameters $\varepsilon$ are significantly smaller than their corresponding parameter estimates, all the variously protonated species are statistically significant at a significance level of $\alpha = 0.05$. Figure 4 shows the estimated molar absorptivities of all of the variously protonated species $\varepsilon_r$, $\varepsilon_{1084}$, $\varepsilon_{589}$, $\varepsilon_{492}$ and $\varepsilon_{440}$ of *Ibrutinib* with regard to wavelength. The curves of $\varepsilon_{1084}$ and $\varepsilon_{440}$ seemed to be the same and therefore the cation LH$^+$ was denoted as the computer species not having chemical sense.

The second diagnostic examines whether all of the calculated free concentrations of variously protonated species on the distribution diagram of the relative concentration expressed as a percentage have physical meaning, which proved to be the case (right panel of Figure 4). The distribution diagram shows the protonation equilibria of LH$^+$, LH$^2_-$, LH$^3_-$, LH$^4_-$ and LH$^5_-$ at neutral pH from 5 to 8 *Ibrutinib* prevails as the neutral molecule LH$^-$ and cation LH$^+$; and from pH 6 to pH 9 it does so in the form of anions LH$^2_-$, LH$^3_-$, LH$^4_-$ and LH$^5_-$. At neutral pH from 5 to 8 *Ibrutinib* prevails as the neutral molecule LH$^-$ and cation LH$^+$; and from pH 6 to pH 9 it does so in the form of anions LH$^2_-$, LH$^3_-$, LH$^4_-$ and LH$^5_-$. Acidification of the neutral molecule LH$^-$ first creates the cation LH$^+$, which in a solution of pH 2 to pH 4 predominates to 80% LH$^2_-$; further acidification of the cation LH$^2_-$ could create the cation LH$^3$; from pH 4 to pH 1. At concentrations of $10^{-4}$ to $10^{-5} M$ the *Ibrutinib* is sufficiently soluble and all its dissociation constants can therefore be spectrophotometrically determined.

The next diagnostic concerns the goodness-of-fit. Although this statistical analysis of residuals [16] gives the most rigorous test of the degree-of-fit, realistic empirical limits must be used. The statistical measures of all residuals $e$ prove that the minimum of the elliptic hyperparaboloid RSS has been reached (Table 1): the standard deviation of residuals $s(\varepsilon)$ and the mean residual $E[\varepsilon]$ always have sufficiently low values, lower than 2 mAU, which is less than 0.2% of measured absorbance value proving a good fitness (Figure 5).

Dissociation constants estimated with SQUAD84 and REACTLAB are in good agreement. The SQUAD approach has a great advantage in a rigorous goodness-of-fit test made by the statistical analysis of residuals. Reproducibility of four experimental spectra sets with the use of two regression programs shows that three from four dissociation constants $pK_a$ 3.61, $pK_{a2}$ 7.16 and $pK_{a3}$ 10.81 are well-conditioned in the regression model, and therefore their numerical evaluation is quite reliable. The second dissociation constant $pK_{a4}$ 5.43 is ill-conditioned in the regression model, the hyperparaboloid shape on this parameter is rather saucer-like without a distinctive minimum. Numerical enumeration of all coordinates of this minimum is more difficult, and the parameter estimates are therefore less reliable and $pK_{a4}$ is rather uncertain.

Figure 6 presents six following figures from pH=4.06 through pH=10.54 to show the consecutive deprotonation response in spectra, when each spectrum was decomposed into the spectrum

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of differently protonated species in a mixture of Ibrutinib. At pH = 4.06 the cation LH₃⁺ accompanying cation LH₄²⁺ predominates in the solution. At pH=5.34 together with the neutral molecule LH₂ one species LH₃⁺ exhibits absorption band at the same wavelength of the absorption maximum λₙₐₓ. At pH = 6.13 and 6.96 the experimental spectrum is decomposed to two absorption bands concerning the neutral molecule LH₂ which dissociates to anion LH. At pH=9.69 the anion LH⁻ occurs with anions LH₂⁻ and L²⁻, and concentration of L²⁻ in the solution increases up to pH=10.54.

**Table 1:** The search for the best protonation model of Ibrutinib in the pH range from 2 to 11 tested models up to five dissociation constants pKₐ, pKₐ', pKₐ'' with SQUAD84 and REACTLAB at 25°C. Solution of 5.8·10⁻⁵ M Ibrutinib at I=0.004 at 25°C, for n_s spectra measured at n_w wavelengths for n=2 basic components L and H forms variously protonated species. The standard deviations of the parameter estimates are in the last valid digits in brackets. The resolution criterion and reliability of parameter estimates found are proven with goodness-of-fit statistics such as the residual standard deviation by factor analysis s_k(A) [mAU], the mean residual |E| |ê| [mAU], the standard deviation of absorbance after termination of the regression process s(ê) [mAU], the sigma s(A) [mAU] from REACTLAB and the Hamilton R-factor of relative fitness [%] from SQUAD84.

| Protonation Model Contains | Five pKₐ's | Four pKₐ's | Three pKₐ's |
|----------------------------|------------|------------|-------------|
| Cattel’s Scree Plot Indicating the Rank of the Absorbance Matrix (INDICES) | | | |
| Number of spectra measured, n_s | 63 | | |
| Number of wavelengths, n_w | 70 | | |
| Number of light-absorbing species, k' | 4 or 5 | | |
| Residual standard deviation, s_k(A) [mAU] | 0.53 or 0.44 | | |

| Dissociation constant | Program | Value (s Standard) |
|-----------------------|---------|--------------------|
| pKₐ(s₁), LH₄²⁺=H⁺+LH₃⁺ | SQUAD84 | 2.08(774) XX XX |
|                      | ReactLab| 1.88(116) XX XX   |
| pKₐ'(s₂), LH₃⁺=H⁺+LH₂⁺ | SQUAD84 | 3.40(04) 3.42(02) XX |
|                      | ReactLab| 3.39(03) 3.42(00) XX |
| pKₐ''(s₃), LH₂⁺=H⁺+LH   | SQUAD84 | 6.04(02) 6.08(02) 3.46(01) |
|                      | ReactLab| 6.04(01) 6.07(01) 3.46(00) |
| pKₐ'''(s₄), LH=H⁺+L     | SQUAD84 | 7.61(01) 7.62(01) 6.76(01) |
|                      | ReactLab| 7.62(03) 7.62(01) 6.76(01) |
| pKₐ''''(s₅), LH=H⁺+L²⁻  | SQUAD84 | 10.30(01) 10.31(01) 10.26(01) |
|                      | ReactLab| 10.30(01) 10.31(01) 10.26(01) |

| Goodness-of-Fit Test with the Statistical Analysis of Residuals | | | |
|---------------------------------------------------------------|------------|------------|-------------|
| Mean residual | E | | | \[mAU]\ | SQUAD84 | 0.77 | 0.79 | 1.79 |
|                | ê | | | [mAU]   | ReactLab| 0.6 | 0.62 | 1.3  |
| Standard deviation of residuals \(s(ê)\) [mAU] | SQUAD84 | 1.23 | 1.27 | 2.51 |
|                | | ReactLab| 0.84 | 0.89 | 1.64 |
| Sigma from ReactLab [mAU] | SQUAD84 | --- | --- | --- |
| | ReactLab| 1.26 | 1.3 | 2.51 |
| Hamilton R-factor from SQUAD84 [%] | SQUAD84 | 0.28 | 0.3 | 0.6 |
| | ReactLab| XX | XX | XX |
| Hypothesis about the protonation model is | | | |
| Rejected | Accepted | Rejected |
Signal-to-Noise Ratio (SNR) and small changes in spectra:
To express small changes of absorbance in the spectral set, the absorbance differences for the \( j \)th wavelength of the \( i \)th spectrum \( \Delta_{ij} = A_{ij} - A_{i,\text{acid}} \) were calculated [17] (Figure 7). The plot of small absorbance changes in the spectrum of the drug studied means that the value of the absorbance difference for the \( j \)th wavelength of the \( i \)th spectrum \( \Delta_{ij} = A_{ij} - A_{i,\text{acid}} \) is divided by the instrumental standard deviation \( s_{\text{inst}}(A) \), and the resulting ratios \( \text{SER} = \Delta_{ij}/s_{\text{inst}}(A) \) are plotted according to wavelength \( \lambda \) for all absorbance matrix elements, where \( A_{i,\text{acid}} \) is the initial spectrum of the acid form of the drug being measured for the starting pH value of the pH range studied. This SER ratio is then, for example, compared to the limiting SER value to test if the absorbance changes are significantly larger than the instrumental noise. When the SER is larger than 10, a factor analysis is able to predict the correct number of light-absorbing components in the equilibrium mixture. To prove that non-linear regression can analyze such data the residuals set was compared to the instrumental noise \( s_{\text{inst}}(A) \). Figure 7 shows a comparison of the ratio \( e/s_{\text{inst}}(A) \) according to wavelength for the measured Ibrutinib. It is clear from the figure that most of the residuals are of the same magnitude as the instrumental noise.
Determinaton of the thermodynamic dissociation constants:

Examining the effect of the ionic strength on protonation of chromophore it is obvious from the dependence of the spectra shape on the ionic strength that at a low value of ionic strength the chromophore of three species LH₂, LH and L²⁻ is not significantly affected by the change of pH (Figure 8). Two cations LH⁺ and LH₂⁺ differ in spectra in pure water but with increasing ionic strength the difference between \( t_{\text{LH}} \) and \( t_{\text{LH}^+} \) decreases and for ionic strength \( I=0.2 \) both spectra are nearly the same (Figure 8).

\[
\begin{align*}
\text{Figure 8: The influence of ionic strength (KCl) on protonation of the chromophore in 5.8·10^{-4} \text{ M Ibrutinib at } 25^\circ \text{C leads to some changes of the molar absorption coefficients depending on the wavelength (nm) analysed with SQUAD84 and REACTLAB, Conditions: the same as in Figure 2, (SQUAD84).} \\
\text{The limited form of the Debye–Hückel equation to the data for aqueous solutions and } 25^\circ \text{C was applied so that the mixed dissociation constant } pK_a \text{ is a dependent variable while the ionic strength } I \text{ stands for the independent variable. One unknown parameter } pK_a^i \text{ is estimated by a minimization of the sum of squared residuals. The nonlinear estimation problem is simply a problem of optimization in the parameter space in which the } pK_a^i \text{ and } I \text{ are known and given values while the parameter } pK_a^i \text{ is an unknown variable to be estimated using extrapolation to zero value of ionic strength (Figure 9).} \\
\text{Potentiometric titration data analysis} \\
\text{The potentiometric titration of a mixture of HCl and Ibrutinib with potassium hydroxide was carried out at } 25^\circ \text{C and also } 37^\circ \text{C for the adjusted value of ionic strength (Figure 10). The initial tentative value of the dissociation constant of the Ibrutinib antitumor drug studied, corresponding to the midpoint value in each plateau of the potentiometric titration curve, was refined by the ESAB program.} \\
\text{Since Ibrutinib exhibits three close dissociation constants, their numerical estimation is rather difficult and even impossible without the use of a computer-assisted nonlinear regression. A regression analysis was employed by using a plateau of the middle part titration curve which concerned an alkalized Ibrutinib titrated with hydrochloric acid, followed by a subsequent retitration with potassium hydroxide. Also calculated on the assed point titration curve was the Bjerrum formation protonation function, which is shown in the graph in Figure 10. The estimates of three dissociation constants } pK_{a1}, pK_{a2}, pK_{a3} \text{ are plotted on the Bjerrum formation curves. Since } pH \text{ above } 9 \text{ and } pH \text{ below } 5 \text{ occurs in a titrated solution, a very fine precipitate of Ibrutinib which is initially forming a slight opalescence, this part of the titration curve } pH \text{ over } 9 \text{ and } pH \text{ below } 5 \text{ did not undergo regression analysis only for estimating } pK_{a1}, pK_{a2}, pK_{a3} \text{ (Figure 11).} \\
\end{align*}
\]

\[
\begin{align*}
\text{Figure 9: Dependence of the mixed dissociation constant } pK_a \text{ of Ibrutinib on the square-root of the ionic strength leading to the thermodynamic dissociation constant } pK_a^i \text{ at } 25^\circ \text{C.} \\
\text{Since it is a difficult task in regression analysis to estimate such close dissociation constants, the ESAB computer program was used and the resulting } pK_a \text{ estimates were compared. The ESAB residuals are defined as the difference between the experimental and calculated titrant volume. The goodness-of-fit test is performed with the statistical analysis of residuals. As further group parameters are refined, the fit is improved. A quite sensitive criteria of the reliability of the dissociation constants estimated is the mean of absolute values of residuals } E|\hat{e}|. \text{ Comparing residuals with the instrumental noise, } s_{\text{inst}}(y), \text{ represented here by either } s_{\text{inst}}(y) = s(V) = 0.001 \text{ mL, an excellent fit is confirmed since the mean } E|\hat{e}| \text{ and the residual standard deviation } s(\hat{e}) \text{ are nearly the same and are lower than the experimental noise } s_{\text{inst}}(y). \text{ Here, } E|\hat{e}| = 0.001 \text{ mL and } s(\hat{e}) = 0.0002 \text{ mL are similar and both are lower than the microburette error } s(V) = 0.0001 \text{ mL.} \\
\end{align*}
\]
All residuals oscillate between lower -0.0005mL and upper 0.0005mL of Hoaglin’s inner bounds and therefore no outlying residuals lay outside these bounds [42]. The estimates of the dissociation constants estimated by ESAB are reliable. The curve-fitting is significantly improved using the refinement of the group parameter \( L_0 \), the concentration of the titrated drug \( Ibrutinib \).

Discussion

Spectroscopic titration has been utilized as an alternative to determine \( pK_a \) values of substances with large molar absorptivities because of its high sensitivity at concentrations of substance as low as \( 10^{-6} \) M. However, the compound under investigation must possess chromophore(s) in proximity to the ionization center(s) so that the protonated and deprotonated species exhibit sufficient spectral dissimilarity. In UV titration, the spectral data of \( Ibrutinib \) measured are a series of spectra acquired at different pH values. Acidifying the solution of the neutral molecule \( LH_2 \) leading to cations \( LH^+ \), \( LH^2+ \) and \( LH^3+ \) may be disturbed by \( Ibrutinib \) precipitation which manifests itself especially at higher concentrations in potentiometric determination. For this reason, in potentiometric concentration 0.0005 M the dissociation constant \( pK_a \), is not available since \( Ibrutinib \) precipitates in an acidic solution. Both programs SQUAD84 [11,18,19] and REACTLAB [20] or its previous version SPECFIT [21] for spectra analysis produce for spectrophotometric concentration \( 5.8 \times 10^{-5} \) M \( Ibrutinib \) the same estimates of all four dissociation constants which exhibit identical goodness-of-fit test. The influence of temperature at 25°C and 37°C does not seem to be significant here.

The ESAB program [33-35], minimizing residuals \( e = (V_{\text{calculated}} - V_{\text{measured}}) \) reaches 0.1 or 0.2 microliters, thus proving an excellent fit. It may be concluded that the reliability of the dissociation constants of \( Ibrutinib \) was proven even when group parameters \( L_0, H \), were ill-conditioned in a model. The goodness-of-fit proved sufficient reliability of the parameter estimates for four dissociation constants of the drug \( Ibrutinib \) at 25°C and 37°C.

It was proven that the most reliable regression estimate of the dissociation constants comes from reliable experimental data. In the case of close dissociation constants, a higher degree of uncertainty in estimates should be expected and therefore two independent instrumental methods should usually be applied.
and the results calculated using several independent programs compared. All determined dissociation constants are not in a good agreement with the predicted values from program MARVIN [25,27] as stated in the results and conclusion chapter. This discrepancy might be caused by unclear resonance structure of the heterocyclic molecule core, and, consequently, different electrons distribution, which can further lead to different predicted values according to the proposed structure. Moreover, these values can differ from the experimentally determined dissociation constants. In such cases prediction programs MARVIN [25,27] and ACD/pK [24-26] may fail and experimental laboratory determination is needed. As both potentiometric and spectrophotometric results are similar and regarding the goodness of fit tests one can conclude the obtained experimental results are reliable and they show the real dissociation of the substance.

Conclusion

Spectrophotometric and potentiometric pH-titration allowed the measurement of four dissociation constants of Ibrutinib, but worse solubility at pH above 10 and also pH below 5 for the Ibrutinib concentration of micromoles leads to the conclusion that an estimation of the pKα higher than 10 and in potentiometry lower than 5 is not reliable enough.

a. The sparingly soluble form LH+ of Ibrutinib is capable of protonation to form the still soluble cations LH2+, LH3+ and anions LH−1, L2− occurs in pure water in the neutral pH. The graph of molar absorption coefficients of variously protonated species according to wavelength shows that the spectrum of species LH+ and L2− slightly vary in colour, while protonation of chromophore LH+ to LH2+ and LH3+ has greater influence on chromophores in Ibrutinib molecule (Figure 8).

b. We have proven that in the range of pH 2 to 10 four dissociation constants can be reliably estimated from the spectra when concentration of Ibrutinib is about 5 × 10−4 M. Although the change of pH somewhat less affected changes in the chromophore, four mixed dissociation constants at an ionic strength I=0.004 can be reliably determined with SQUAD(84) and REACTLAB reaching the similar values with both programs, pKα1=3.42(02), pKα2=6.07(02), pKα3=7.62(01) and pKα4=10.31(01) at 25°C and pKα1=3.12(02), pKα2=6.10(02), pKα3=7.61(02), pKα4=9.22(04) at 37°C. The standard deviations in the last valid unit number are in the brackets.

c. Only three dissociation constants of Ibrutinib in a potentiometric concentration of 5×10−4 mol/dm−3 were determined by the regression analysis of potentiometric titration curves with the ionic strength I=0.004 and using ESAB, pKα1=6.39(01), pKα2=7.60(01), pKα3=9.96(05) at 25°C and pKα1=6.02(02), pKα2=6.96(02), pKα3=9.65(04) at 37°C.

d. Prediction of the dissociation constants of Ibrutinib was performed using the MARVIN program to specify protonation locations and using the ACD/pK program. Comparing two predictive with two experimental techniques it may be concluded that the prediction programs often vary considerably in estimating pKα.

e. Applying the Debye-Hückel equation to the dependence of the mixed dissociation constant on an ionic strength coming from pH-potentiometric titration, the unknown thermodynamic dissociation constants pKα1 have been estimated at the two experimental temperatures 25°C and 37°C: pKα1=3.22 and 3.22, pKα2=4.17 and 5.21, pKα3=6.77 and 6.77, pKα4=9.82 and 9.81. (Figure 9 & 11).

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