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The dissociation constants of the cytostatic bosutinib by nonlinear least-squares regression of multiwavelength spectrophotometric and potentiometric pH-titration data

Milan Meloun[a,*], Veronika Nečasová[a], Milan Javůrek[b], Tomáš Pekárek[c]

[a] Department of Analytical Chemistry, University of Pardubice, CZ 532 10 Pardubice, Czech Republic
[b] Department of Process Control, University of Pardubice, CZ 532 10 Pardubice, Czech Republic
[c] Zentiva k.s., U kabelovny 130, CZ 102 37 Prague, Czech Republic

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ABSTRACT

Potentiometric and spectrophotometric pH-titration of the multiprotic cytostatics bosutinib for dissociation constants determination were compared. Bosutinib treats patients with positive chronic myeloid leukemia. Bosutinib exhibits four protonatable sites in a pH range from 2 to 11, where two pK are well separated ($\Delta pK > 3$), while the other two are near dissociation constants. In the neutral medium, bosutinib occurs in the slightly water soluble form LH that can be protonated to the soluble cation LH_4^{3+}. The molecule LH can be dissociated to still difficultly soluble anion L^-. The set of spectra upon pH from 2 to 11 in the 239.3–375.0 nm was divided into two absorption bands: the first one from 239.3 to 290.5 nm and the second from 312.3 to 375.0 nm, which differ in sensitivity of chromophores to a pH change. Estimates of pK of the entire set of spectra were compared with those of both absorption bands. Due to limited solubility of bosutinib the protonation in a mixed aqueous-methanolic medium was studied. In low methanol content of 3–6% three dissociation constants can be reliably determined with SPECFIT/32 and SQUAD(84) and after extrapolation to zero content of methanol they lead to $pK_{c1} = 3.43(12)$, $pK_{c2} = 4.54(10)$, $pK_{c3} = 7.56(07)$ and $pK_{c4} = 11.04(05)$ at 25 °C and $pK_{c1} = 3.44(06)$, $pK_{c2} = 5.03(08)$ $pK_{c3} = 7.33(05)$ and $pK_{c4} = 10.92(06)$ at 37 °C. With an increasing content of methanol in solvent the dissociation of bosutinib is suppressed and the percentage of LH_3^{2+} decreases and LH prevails. From the potentiometric pH-titration at 25 °C the concentration dissociation constants were estimated with ESAB $pK_{c1} = 3.51(02)$, $pK_{c2} = 4.37(02)$, $pK_{c3} = 7.97(02)$ and $pK_{c4} = 11.05(03)$ and with HYPERQUAD: $pK_{c1} = 3.29(12)$, $pK_{c2} = 4.24(10)$, $pK_{c3} = 7.95(07)$ and $pK_{c4} = 11.29(05)$.

1. Introduction

Perhaps the most important physicochemical characteristics of drugs and excipients are their acidity expressed by their pK_a values, their hydrophobicity and their dependence on pH in the field of industrial pharmacy and pharmaceutical development [1–4]. Acid–base behavior determines whether the drug in the organism is in dissociated ionic or nonionic form, which affects its solubility and dissolution rate mainly in the gastrointestinal tract and in permeability through biological membranes. The bioavailability of the drug is determined by the prevailing state of protonation at biological pH [5].

Previous work [1,4,5] has shown that the spectrophotometric method can be used in combination with suitable chemometric tools to determine acid dissociation constants pK_a even of barely soluble drugs. When the components involved in the protonation equilibrium have distinct spectral responses, their concentrations can be measured directly and determination of the protonation constant is trivial. In a molecule with overlapping log K (Δ <3 pH units) these constants acid–base balance are characterized as a whole and information on specific protonable sites in the molecule is not provided [6–13].

For poorly water soluble compounds this problem is solved by adding an organic solvent. The obtained log K value is then related to the particular solvent used and should be used to extrapolate calculate log K at zero content of the organic solvent according to Yasueda et al. [14].

Bosutinib has a systematic title based (IUPAC) 4-[(2,4-dichloro-5-metoxyfenyl) amino]-6-metoxy-7-[(3-(4-methylpipe-

* Corresponding author. Fax: +42 0466037068.
E-mail address: milan.meloun@upce.cz (M. Meloun).

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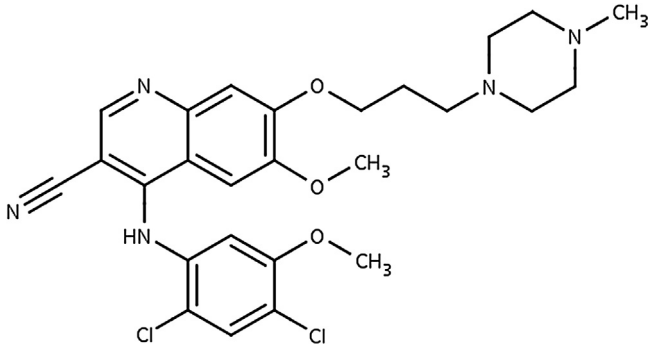

Fig. 1. The structure of bosutinib.

razin-1-yl) propoxy)]chinolin-3-carbonitril. The bosutinib structure is shown in Fig. 1. Bosutinib belongs to the pharmacological class of drugs, referred to as cytostatics or anti-tumor drugs. These substances retard or arrest cell growth or even cause their disintegration. Bosutinib is designated for the treatment of adult patients with chronic, accelerated and blast phase Philadelphia chromosome positive chronic myeloid leukemia (Ph + CML) [15]. The term for this type of chronic leukemia means that the cancer progresses more slowly than acute forms of myeloid leukemia and refers to the type of cells affected by the type of cancer [16–18]. Bosutinib can be found under the trade name SKI-606 or Bosufil. It is a white to tan powder slightly soluble in water but soluble in methanol and DMSO dimethylsulphoxide. Its molecular weight is 530.446 g/mol, density 1.36 g/cm^3 and a melting point in the range 116–120 °C [15]. Bosutinib molecule has in its structure 4 protonation sites in the pH range from 1 to 12, wherein the two are distinguishable from each well and the other two are near the dissociation constant, which overlap to form diprotonated system. A detailed sub-molecular protonation bosutinib diagram has been proposed by the authors [18]. The aim of our study was to examine and verify a potentiometric determination of the protonation model, and carried out the spectrophotometric analysis of the pH-absorbance matrix to find suitable conditions for a reliable regression determination of four dissociation constants from spectra.

2. Theoretical

2.1. Determination of protonation model

The acid–base equilibrium of bosutinib studied is described in terms of the protonation of the Brönstedt base L^{z-1} according to the equation $L^{z-1} + H^+ \rightleftarrows HL^z$. The protonation equilibria between the anion L (the charges are omitted for the sake of simplicity) of a drug and a proton H are considered to form a set of variously protonated species L, HL, H_2L, H_3L . . .etc., with the general formula H_rL in a particular chemical model and which are represented by n_c (the number of species), r_i, $i = 1, \ldots, n_c$ where index i indicates their particular stoichiometry; the overall protonation (stability) constant of the protonated species, β_r, may then be expressed as

$$\beta_r = \frac{[H_rL]}{[L][H]^r} = \frac{c}{lh^r} \quad (1)$$

where the free concentration $[L] = l$, $[H] = h$ and $[H_rL] = c$. For dissociation reactions realized at constant ionic strength the "mixed dissociation constants" are defined as

$$K_a = \frac{[H_{j-l}L]a_{H^+}}{[H_jL]} \quad (2)$$

As each aqueous species is characterized by its own spectrum, for UV–vis experiments and the ith solution measured at the jth wavelength, the Lambert–Beer law relates the absorbance, $A_{i,j}$, being defined as

$$A_{i,j} = \sum_{n=1}^{n_c} \epsilon_{j,n} C_n = \sum_{n=1}^{n_c} (\epsilon_{r,j} \beta_r l h^r)_n \quad (3)$$

where $\varepsilon_{r,j}$ is the molar absorption coefficient of the H_rL species with the stoichiometric coefficient r measured at the jth wavelength. The absorbance $A_{i,j}$ is an element of the absorbance matrix A of size $(n_s \times n_w)$ being measured for n_s solutions with known total concentrations of $n_z = 2$ basic components, c_L and c_H, at n_w wavelengths. The general procedure used to build the protonation model with SPECFIT32 was described in [19,20]. Determining the chemical model of the drugs protonation equilibria by a regression analysis of potentiometric titration data or by spectra seems to be dependent on user experience and the software used. A significant role is played by resolution hypotheses of the proposed regression model and distinguishability of spectra concerning differently protonated chromophores in the molecule.

The accuracy of estimated dissociation constants is influenced by both, experimental error and mathematical deviation from the correct value. Experimental error is due to systematic errors in the experiment, which should always be found and removed. These errors arise from the incomplete adjustment of experimental variables such as pH, from the uncertain drug concentration c_L, from a non-constant ionic strength I, from varying temperature T, etc. Mathematical deviation represents a bias that arises with imperfect mathematical evaluation, which may occur in a nonlinear regression procedure when no global minimum of an elliptical hyperparaboloid of the residuals-square-sum function was reached. Primarily, it often depends on the option of the appropriate software or the reliable minimization procedure with some criteria for terminating the minimization process before reaching a minimum.

Two different programs for the numerical analysis of spectra were used, SPECFIT/32 and SQUAD(84), which compared the consensus found in numerical parametric estimates and in a fitness of the predicted absorbance spectra through measured absorbance data [2]. Both programs reached the same results usually obtained at the dataís sufficient goodness-of-fit.

2.2. Reliability of β_r and $K_{a,j}$ estimates obtained by the goodness-of-fit test

The detailed procedure of the graphical and numerical analysis of residuals is described in [20]. 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 vector of residuals should exhibit a Gaussian distribution and the average of absolute values of residuals should have a magnitude similar to the signal noise or instrumental standard deviation of absorbance $s_{inst}(A)$.

Another important result of the spectra regression analysis consists of the numerical estimates of the molar absorption coefficients of differently protonated light-absorbing species in an equilibrium mixture according to the wavelength λ. In addition to the point estimates of molar absorption coefficients, their standard deviations are also calculated.

The distribution diagram presents the relative concentration of differently protonated light-absorbing species in the protonation equilibria and provides a specific image on the protonation model. It allows for the chemical interpretation of a proposed regression model, to perform its correction, to comment on the presentation of major and minor species in an equilibrium mixture, and to reveal which protonated species are present in the solution at a given pH. It represents the culmination of an interpretation of regression anal-

ysis of the spectra. The detailed procedure of the determination of the number of light/absorbing species is described in [6]. To determine the number of light-absorbing species the factor analysis may be applied by using a rank of the second moment of the absorbance matrix A obtained by pH-spectrophotometric titration [1].

2.3. Computation and software used

Computation relating to the determination of dissociation constants were performed by regression analysis of the UV/VIS spectra using the SQUAD(84) [19] and SPECFIT/32 [21] programs. Computation relating to the determination of the dissociation constants also was performed by regression analysis of the potentiometric titration curve using ESAB2M [22,23] and HYPERQUAD2008 programs [7]. Most graphs were plotted using ORIGIN 9 [24] and pK_a predicted with PALLAS [25].

3. Experimental

3.1. Chemicals and apparatus

Bosutinib was donated by ZENTIVA k.s., with declared purity by a HPLC. Other chemicals and solutions, the apparatus used and the pH-spectrophotometric titration procedure have been described previously in detail [19,20]. The experimental and computation scheme to determine the protonation constants of the multi-component system are described in detail elsewhere [19,20]. The apparatus used and the pH-potentiometric titration procedure have been described previously in detail [26].

3.2. Correction of pH on the aqueous-methanolic medium

Measurement of pH in aqueous-methanolic medium has been performed by transforming the measured values pH_{read} using the glass electrode into the concentration Sőrensen scale $pH_c = -\log[H^+]$. Standardization titration 0.25 ml 1.096HCl was carried out with 0.897 M KOH in an aqueous-methanolic medium with the adjusted ionic strength of 0.1 M KCl. Creating a correction equation for transforming, the read values pH_{read} in the mixed aqueous-methanolic solvent to Sőrensen concentration value pH_c in the environment 3%, 4%, 6%, 12%, 18%, 24%, 36% and 48% methanol set at an ionic strength of 0.1 M KCl is described by the 5th order polynomial.

$$pH_c = \beta_0 + \beta_1 pH_{read} + \beta_2 pH_{read}^2 + \beta_3 pH_{read}^3 + \beta_4 pH_{read}^4 + \beta_5 pH_{read}^5 \quad (4)$$

While on the y-axis the measured values pH_{read} are plotted, on the x-axis the values are $pH_c = (v_{HCl} \times c_{HCL} - v_{KOH} \times c_{KOH})/V_0$ where $v_{HCl} = 0.25$ ml, $c_{HCL} = 1.096$ M, $c_{KOH} = 0.897$ M, $V_0 = 15.25$ ml for the first part of the titration curve HCl–KOH before the inflexion point in aqueous-methanolic medium, for the second part of the titration curve HCl–KOH after inflexion point the values are $pH_c = 14 - 10^{(v_{KOH} - c_{KOH})/V_0}$.

4. Results and discussion

4.1. pH-spectrophotometric titration

In their publication, Box et al. [18] showed that while all four protonation constants can be determined by potentiometry in the range pH 2–12, the spectrophotometric pH-titration could monitor protonation only of two places in a chromophore, i.e., $LH = L + H^+$ with a log $K_{a3} = 11.2$ and $LH_2^+ + H^+ = LH_3^{2+}$ with log $K_{a1} = 4.75$ in UV-spectrum. The authors proposed a macroscopic protonation system showing that the first two protonation constants log K_1 and log K_2 are far and well separated equilibrium, since $|\log K_1 - \log K_2| = |11.2 - 7.93| > 3$, while the two remaining constants log K_3 and log K_4 express the near equilibrium since $|\log K_3 - \log K_4| = |4.75 - 3.79| < 3$ and thus they form a dibasic protonation system.

The pH-absorbance response surface was recorded with the acid–base titration of 2.3×10^{-5} M bosutinib acidified with HCl in an aqueous-methanolic medium of phosphate, borax and acetate buffer with an ionic strength adjusted to 0.1 M with KCl and inerting with argon at 25 °C and 37 °C. Then an acidified solution of bosutinib was titrated with KOH to pH 11. Spectrophotometric titration was also applied at 37 °C. A set of recorded bosutinib spectra according to pH from 2 to 11 covering a wavelength range from 239.3 to 375.0 nm was identified as the entire spectrum and labeled P (Fig. 2a) . This spectrum was further divided into two absorption bands, wherein the first one covering the region from 239.3 to 290.5 nm was labeled P1 (Fig. 2b) and the second one covering the region of 312.3–375.0 nm was labeled P2 (Fig. 2c). Both absorption bands P1 and P2 differ in sensitive chromophores to pH change, so it was necessary to compare the dissociation constants estimated from the entire band P with those estimated from the two separate bands P1 and P2.

The change of pH does not cause significant changes in the bosutinib spectrum because some chromophores are only slightly affected by a pH change. Therefore, a number of light-absorbing species were estimated by three different modifications of the Cattel's scree plot to indicate a rank of the absorbance matrix using the INDICES program. Since an extrapolation of tangents in hyperbolic curve s_k $(A) = f(k)$ was uncertain, the logarithmic transformation of y-axis was preferred (Fig. 3). Wernimont–Kankare's modification (Fig. 3a) was found reliable enough in determining the matrix rank $k = 5$ and the residual standard deviation s_k $(A) = 0.63$ mA U. This means that the protonation equilibria of bosutinib in pH range 2–11 exhibit five light-absorbing species $n_c = 5$ and the instrumental error of spectrophotometer used is close to $s_{inst}(A) = 0.63$ mA U. When a regression model exhibits the estimated standard deviation of absorbance after regression s (A), which is close to the value $s_{inst}(A) = 0.63$ mA U, then the protonation model is accepted as the best and most reliable one.

As the bosutinib is difficult to dissolve in aqueous solvents, an aqueous-methanolic medium was applied. In the first step of this equilibria study and on the suspicion that bosutinib could form in certain medium of buffers some adducts, oligomers or isomers, the simplest composition of a solution containing only 0.1 M KCl to adjust ionic strength and under intensive argon inerting was used. No kinetic changes occurred within 24 h of dissolving bosutinib were detected. Titration curves were monitored by an acid–base titration of the neutral molecule of bosutinib LH (having pH about 8) with HCl from pH 8.5 to pH 2 and then retitrated with KOH from pH 2 to pH 7. In an unbuffered medium it was difficult to measure pH in the range from 5 to 8, and thus in this region the titration curve contains less experimental points. Authors Box et al. [18] found a difficult pH range from 3 to 6, where two close protonation equilibria of two near dissociation constants exist and bosutinib acts then as a dibasic acid. Therefore a reproducibility and hysteresis effect of a titration curve were examined during an acid–base titration with HCl and retitration with KOH.

Dissociation constants were determined initially only in the pH range from 2 to 7, wherein it was investigated whether the protonation of molecules takes place in two or three protonation steps. The protonation model containing three dissociation constants was found as the best one using two different regression programs SPECFIT/32 and SQUAD(84) (Fig. 4). Bosutinib exhibited a spectrum in the UV range of 240–375 nm and data are denoted G, and this spectral band can be distinguished into two absorption bands,

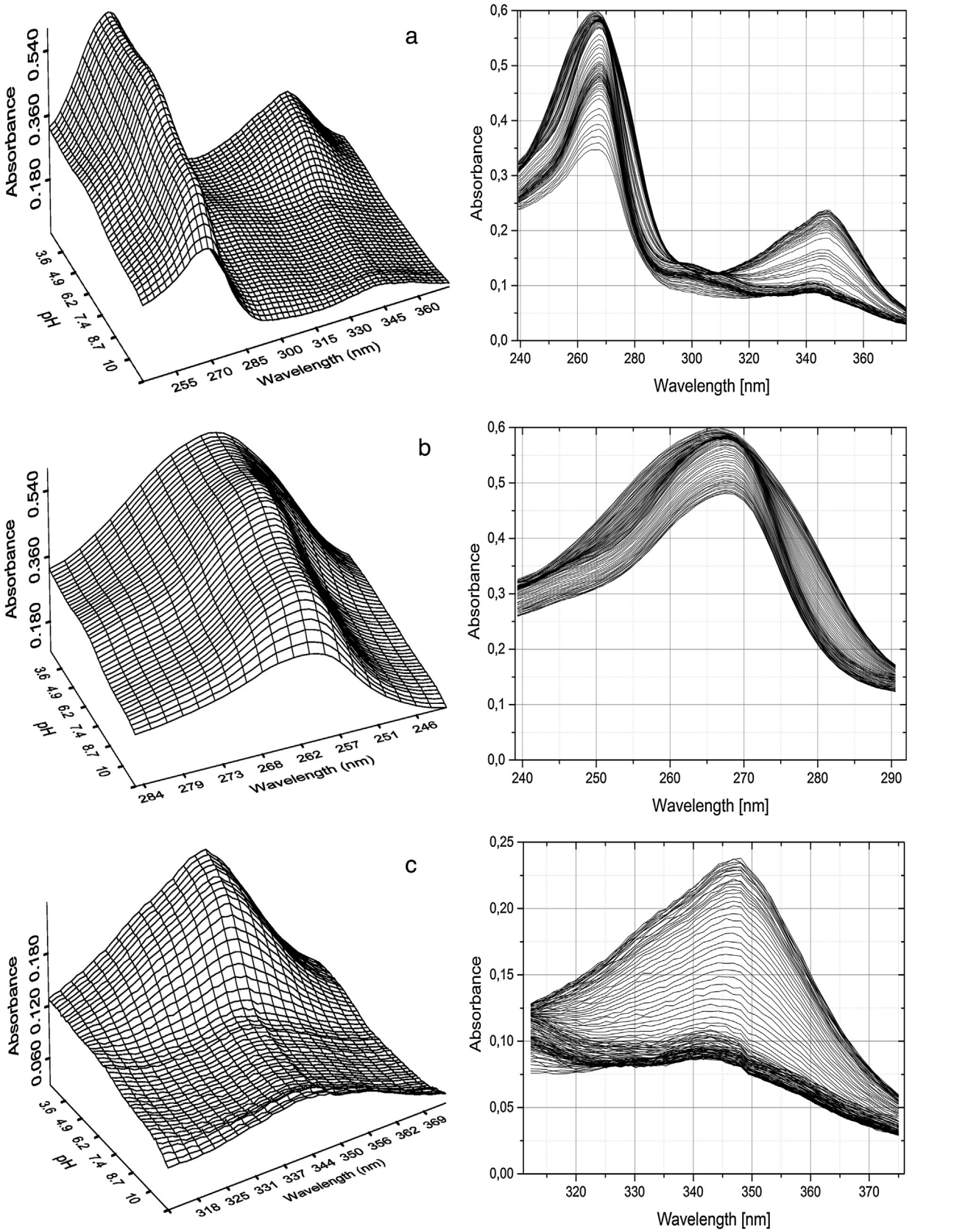

Fig. 2. The 3D-absorbance-response-surface (left) and 2D-graph (right) representing the measured multiwavelength absorption spectra for 2.3×10^{-5} M bosutinib according to pH. Bosutinib was acidified with HCl to pH 2 by aqueous methanolic medium of phosphate, borax and acetate buffer with an adjusted ionic strength 0.1 M (KCl) and made inert with argon at 25 °C and then titrated with KOH: (a) Spectrum range P of 239.3–375.0 nm was divided into (b) the first absorption band P1 of a range of 239.3–290.5 nm and (c) the second absorption band P2 of 312.3–375.0 nm prior further regression analysis, (S-PLUS).

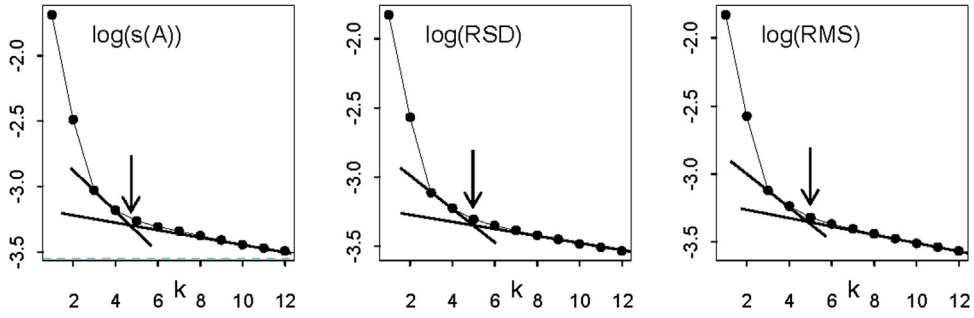

Fig. 3. Cattel's scree plot of the Wernimont–Kankare procedure for the determination of the rank of the absorbance matrix $k^* = 5$ leads to five light-absorbing species in the mixture $n_c = 5$ and the actual instrumental error of the spectrophotometer used $s_{inst}(A) = s_k(A) = 0.93$ mA U when three graphical modifications were used: (a) Kankareís method log($s(A)$), (b) Real error method log (RSD), (c) Extracted error method log (RMS), (INDICES in S-PLUS).

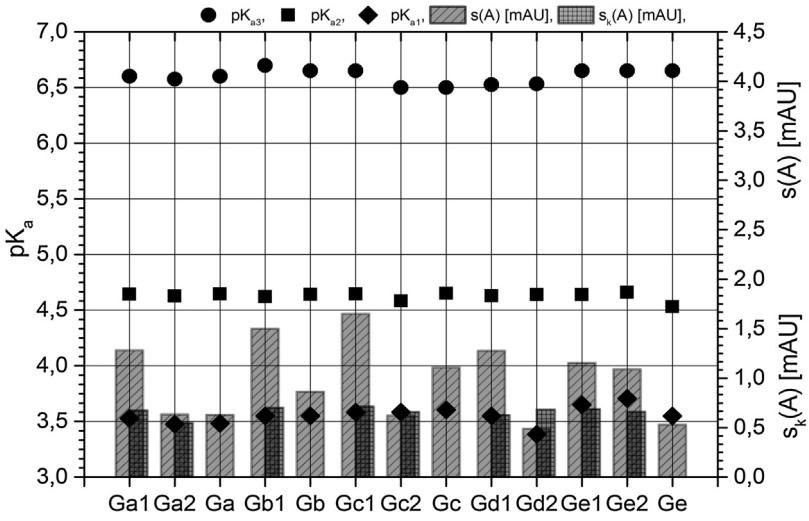

Fig. 4. Reproducibility of the estimated dissociation constants of bosutinib titrated by HCl to pH 2 and after a subsequent retitration with KOH to pH 7 at 25 °C with three dissociation constants model. The goodness-of-fit is expressed on the right axis with the standard deviation of absorbance after regression $s(A)$ [mA U] versus $s_k(A)$ [mA U], (SPECFIT, ORIGIN).

the first one in 239.3–290.5 nm denoted G1 and the second one in 312.3–375.0 nm denoted G2. A protonation was monitored within a pH-spectrophotometric titration with HCl from a neutral solution of LH from pH 7 to 2 and achieving protonated species LH_4^{3+} (data Ga, Gc, Ge) and subsequently within a retitration with KOH from pH 2 to 7 (data Gb, Gd). Estimated dissociation constants obtained from sets Gb, Gd are consistent with those from the previous sets of Ga, Gc and Ge. The titration process was repeated, i.e., Ga against Gb, Gc against Gd, to investigate the hysteresis of the first three dissociation constants pK_{a1}, pK_{a2}, pK_{a3} of bosutinib.

The first diagnostic value in the regression model building indicates whether all parametric estimates pK_{a1}, pK_{a2}, pK_{a3} and ε_L, ε_{LH}, ε_{LH2}, ε_{LH3} and ε_{LH4} have physical meaning and obtain realistic values. Attention was paid to the selection of an efficient spectrum band in which dissociation of bosutinib affects the chromophore and repeatability: the mean value of reproduced dissociation constants for the different wavelengths evaluated with SPECFIT:

(a.) The first dissociation constant pK_{a1} of the entire spectrum G is pK_{a1} = 3.49(09), and of G1 is pK_{a1} = 3.53(116), and of G2 is pK_{a1} = 3.51(12), whereas the mean of all three bands G, G1, G2 gives pK_{a1} = 3.51(13).

(b.) The second dissociation constant pK_{a2} of the entire spectrum G is pK_{a2} = 4.62(04), and of G1 is pK_{a2} = 4.63(01) and of G2 is pK_{a2} = 4.63(03), whereas the mean of all three bands G, G1, G2 gives pK_{a2} = 4.63(01).

(c.) The third dissociation constant pK_{a3} of the entire spectrum G is pK_{a3} = 6.57(09), and of G1 is pK_{a3} = 6.63(05) and of G2 is pK_{a3} = 6.61(13), whereas the mean of all three bands G, G1, G2 gives pK_{a3} = 6.60(00).

Standard deviation s (p$K_{a,i}$) of parameters p$K_{a,i}$ estimated in the last valid digits are in brackets. As they are significantly smaller than their corresponding parameter estimates, all the variously protonated species are statistically significant at a significance level $\alpha = 0.05$. The option of a chromophore in the absorption band G, G1 and G2 exhibited the same values of dissociation constants and the difference was statistically not significant.

A search for the best protonation model in Tables 1 and 2 proved that, according to the goodness-of-fit test, the model with three dissociation constants pK_{a1}, pK_{a2}, pK_{a3} results in a better fit. Estimates of four dissociation constants along with standard deviations $s(A)$ [mA U] of fitness yields the graphical overview on Fig. 4. To measure the reliability of regression estimation the goodness-of-fit is considered with the use of the calculated standard deviation of absorbance after performed regression $s(A)$ [mA U] with the instrumental standard deviation $s_{inst}(A)$ [mA U]. Poor conditioned parameters in a regression model or a small change in a chromophore influence caused by the pH change resulted in the parameters exhibiting a somewhat greater spread. The achieved goodness-of-fit was easily seen by an examination of the residuals between the experimental and calculated values of absorbance, $e_i = A_{exp,i,j} - A_{calc,i,j}$. Examination of the predicted

Table 1
The search of the best protonation model of bosutinib in the pH range from 2–11 lead to three dissociation constants pK_{c1}, pK_{c2}, pK_{c3} with SQUAD(84) and SPECFIT/32. Solution of $2{,}4 \times 10^{-5}$ M bosutinib in 6% methanol at $I = 0.10$ M KCl at 25 °C, for $n_s = 87$ (and 43 after data analysis) spectra measured at $n_w = 87$ (and 87 after data analysis) wavelengths for $n_z = 2$ basic components L and H forming $n_c = 5$ variously protonated species. The charges of the ions are omitted for the sake of simplicity and the standard deviations of the parameter estimates are in the last valid digits in brackets. The resolution criterion and reliability of parameter estimates found is proven with goodness-of-fit statistics.

Data set	Unprocessed spectra before data criticism		Processed spectra without outliers	
Software	SPECFIT/32	SQUAD(84)	SPECFIT/32	SQUAD(84)
Determination of the number of light-absorbing species by factor analysis				
Number of spectra measured n_s	87		43	
Number of wavelengths n_w	87		87	
Number of light-absorbing species k^*	5	5	5	5
Residual standard deviation $s_k^*(A)$[mA U]	0.93	0.94	0.52	0.47
Estimated dissociation constants				
$pK_{c1}(s_1)$	3.57(17)	3.60(05)	3.42(04)	3.39(02)
$pK_{c2}(s_2)$	4.60(04)	4.61(01)	4.58(01)	4.56(02)
$pK_{c3}(s_3)$	6.84(05)	6.84(02)	7.23(04)	8.32(02)
$pK_{c4}(s_4)$	10.73(01)	10.73(00)	10.69	10.69
Goodnes-of-fit test by the statistical analysis of residuals				
Residual square sum RSS	1.51E-01	1.53E-01	1.68E-03	1.93E-03
Mean residual $\|e\|$ [mA U]	Not estimated	2.49	Not estimated	0.56
Standard deviation of residuals $s(e)$[mA U]	4.50	4.66	0.67	0.76
Residual skewness $g_1(e)$	Not estimated	−0.71	Not estimated	−0.09
Residual kurtosis $g_2(e)$	Not estimated	12.84	Not estimated	2.84
Hamilton R-factor[%]	Not estimated	0.9	Not estimated	0.13
ε (all species) versusλ are	Realistic	Realistic	Realistic	Realistic

Table 2
The search of the best protonation model of bosutinib in the pH range from 2 to 11 lead to three dissociation constants pK_{c1}, pK_{c2}, pK_{c3} with SPECFIT/32. Corrected values of pK_c are for zero content of methanol according to Yasuda and Shedlovsky. Model of three dissociation constants pK_{c1}, pK_{c2}, pK_{c3}. *Experimental conditions:* 2.4×10^{-5} M bosutinib in 6% methanol at $I = 0.10$ M KCl at 25 °C and pH-spectrophotometric titration with HCl from pH 7 to 2 (data Ga, Gc, Ge) and subsequent retitration with KOH (data Gb, Gd, Gf). Reproducibility of six spectra bands Ga to Gf were analyzed for each entire spectrum Ga to Gf, for the first band Ga1 to Gf1 and for the second band Ga2 to Gf2. The charges were omitted for the sake of simplicity. Standard deviations of parameter estimated in last valid digits are in brackets.

	Ga1	Ga2	Ga	Gb1	Gb2	Gb	Gc1	Gc2	Gc
Spectrum range	239.3–290.5	312.3–375.0	239.3–375.0	239.3–290.5	312.3–375.0	239.3–375.0	239.3–290.5	312.3–375.0	239.3–375.0
Number of spectra n	34	34	28	39	36	38	40	40	26
Number of wavelength m	41	50	107	41	50	107	41	50	107
k, $s_k(A)$[mA U]	3. 0.67	2. 0.64	3. 0.61	3. 0.70	2. 0.66	3. 0.68	3. 0.71	2. 0.66	3. 0.60
$pK_{c1}(s)$	3.91(03)	3.87(06)	3.87(03)	3.93(05)	3.93(05)	3.93(04)	3.95(04)	3.95(06)	3.97(02)
$pK_{c2}(s)$	4.93(04)	4.92(05)	4.94(04)	4.91(01)	4.95(06)	4.93(01)	4.94(02)	4.87(04)	4.94(01)
$pK_{c3}(s)$	7.07(41)	7.03(32)	7.07(43)	7.18(05)	7.39(10)	7.12(04)	7.12(11)	6.95(18)	6.95(06)
$s(A)$[mA U]	1.26	0.63	0.64	1.39	0.51	0.75	1.48	0.59	0.45

	Gd1	Gd2	Gd	Ge1	Ge2	Ge	Gf1	Gf2	Gf
Spectrum range	239.3–290.5	312.3–375.0	239.3–375.0	239.3–290.5	312.3–375.0	239.3–375.0	239.3–290.5	312.3–375.0	239.3–375.0
Number of spectra n	45	45	45	44	44	29	42	42	42
Number of wavelength m	41	50	107	41	50	107	41	50	107
k, $s_k(A)$[mA U]	3. 0.63	02. .63	3. 0.62	3. 0.69	2. 0.66	3. 0.61	3. 0.72	2. 0.63	3. 0.66
$pK_{c1}(s)$	3.93(03)	3.79(04)	3.79(03)	4.01(08)	4.06(11)	3.93(03)	3.71(04)	3.75(04)	3.75(04)
$pK_{c2}(s)$	4.92(01)	4.93(01)	4.92(01)	4.94(05)	4.95(11)	4.82(01)	4.91(01)	4.93(01)	4.93(01)
$pK_{c3}(s)$	6.99(03)	6.99(05)	6.95(03)	7.12(18)	7.12(19)	7.12(07)	7.12(03)	6.96(04)	7.07(03)
$s(A)$[mA U]	1.13	0.47	0.75	3.06	0.97	0.73	1.22	0.58	0.86

absorbance response-surface graph through all the experimental points should reveal whether the results calculated are consistent and whether any gross experimental errors have been found in the spectra. One of the most important statistics calculated seems to be the standard deviation of absorbance, $s(A)$, calculated from a set of refined parameters at the end of the minimization process. This is usually compared to the residual standard deviation of absorbance calculated by the INDICES program, $s_k(A)$, and if $s(A) \le s_k(A)$, or $s(A) \le s_{inst}(A)$, the fit is considered to be statistically acceptable. Although this statistical analysis of residuals gives the most rigorous degree-of-fit test, realistic empirical limits must be used (Table 1). The statistical measures of all residuals e prove that the minimum of the elliptic hyperparaboloid U is reached with SQUAD(84): the mean residual $|\bar{e}| = 0.56$ mAU and the residual standard deviation $s(e) = 0.76$ mA U have sufficiently low values. The skewness $g_1(e) = -0.09$ was close to zero and proved the symmetric Gaussian distribution of the residuals set, while the curtosis $g_2(e) = 2.84$ was close to 3 proving a Gaussian distribution. The Hamilton R-factor of relative fitness was 0.13% calculated with SQUAD(84) only, proving an excellent achieved fitness, and the parameter estimates may therefore be considered reliable. If the Hamilton R-factor of a relative fit, expressed as a percentage, is lower than 0.5% the fit is considered as an excellent one. But if it is greater than 2%, the fit is considered as poor.

Protonation of the neutral bosutinib molecule LH in a titration with HCl leads to the second dissociation constant $pK_{c2} = 4.92(04)$, which is not significantly different from values $pK_{c2} = 4.93(01)$ from deprotonation of species LH_3^{2+} in a retitration with KOH. The determination is more reliable than the determination of the previous estimate pK_{c1} and the estimate $pK_{c2} = 4.92(04)$ from a HCl titration is more precise and, moreover, in good agreement with the estimate of 4.93(01) obtained from a retitration with KOH. Protonation of the neutral bosutinib molecule LH with HCl leads to the third dissociation constant $pK_{c3} = 7.07(06)$ which does not significantly

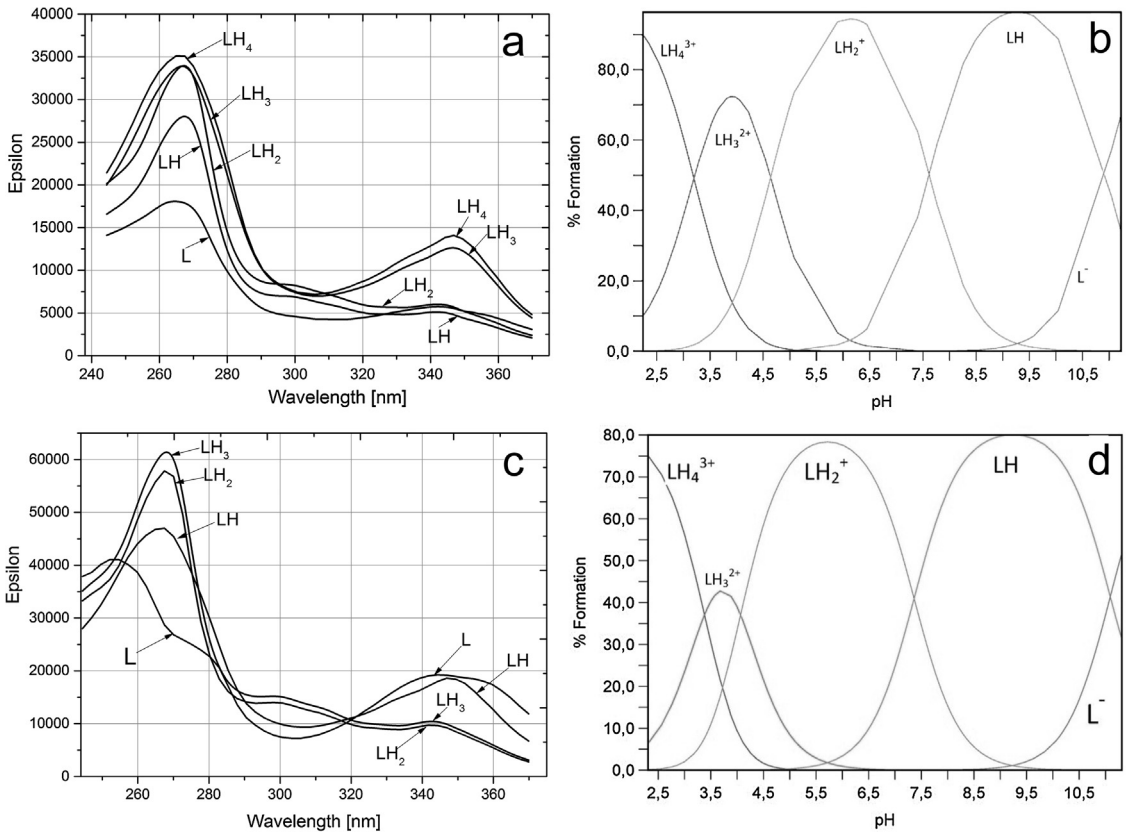

Fig. 5. The graph of molar absorption coefficients differently protonated particles bosutinib for two different contents of methanol solvent: (a) 6% methanol and (c) 48% methanol and corresponding distribution diagram of relative concentration of variously protonated species for (b) 6% methanol and (d) 48% methanol. The charges of species are omitted for the sake of simplicity, (SPECFIT, S-PLUS, ORIGIN).

differ from the values $pK_{c3} = 7.08(13)$ coming from species LH^{2+} in a retitration with KOH. As the pH range 5–7 was not buffered, the measured values of pH apparently exhibited greater uncertainty. The third dissociation constant pK_{c3} is also poorly conditioned in the regression model and its elliptical hyperparaboloid exhibits rather plate pit, which is therefore more difficult to reach. This pK_{c3} is inaccurately estimated with considerable uncertainty. It is necessary to use a buffered medium around pH 5–7 and a precise measurement of pH and to strive for a measurement of a larger number of spectra in this pH region. It can be concluded that a significant hysteresis in the protonation of bosutinib molecules can be observed only for the species LH_4^{3+} while rather uncertainty of pH measurement is applied to the species LH_2^{+}.

Fig. 5 shows that the spectra of species LH_4^{3+} and LH_3^{2+} differ very little, and a protonation of the chromophore of LH_4^{3+} and LH_3^{2+} has little influence on the spectral shape. The same conclusion also applies to the proton chromophore of LH and LH_2^{+} while a protonation of the chromophore of LH_2^{+} to LH_3^{2+} greatly affects the chromophore molecule of bosutinib, and the resulting change of a spectrum is therefore considerable. This effect is apparent in the second absorption band (around 340 nm). Fig. 5a shows the spectra for a minimum methanol content of 6% methanol in solvent and for the greatest content of 48% methanol Fig. 5c. The important result of interpretation of the pH-absorbance response surface for the chemist is primarily the relative concentration diagram of differently protonated species according to pH. The increasing methanol content in the solvent suppresses a dissociation and therefore decreases the percentage presence of species LH_3^{2+}. The species LH the prevails in the solution.

The 2nd diagnostic in the regression model building showed that all calculated free concentrations of the five variously protonated species in a distribution diagram have physical meaning. The calculated free concentration of the basic components and variously protonated species of the protonation equilibria model should show higher molarities than 10^{-8} M. Expressed in percentage terms, a species present at about 1% relative concentration or less in the equilibrium behaves as numerical noise in a regression analysis. Since the molar absorptivities will generally be in the range 10^3–10^5 l mol^{-1} cm^{-1}, species present at less than ca. 0.1% relative concentration will affect the absorbance significantly only if their ε is extremely high.

The aim of the nonlinear regression of the pH-absorbance response surface is to achieve the best estimates of the dissociation constants and the curves of the molar absorption coefficients according to the wavelength, and then a distribution diagram of a relative concentration of differently protonated species according to on pH. While SPECFIT/32 is based on the nonlinear regression of pH-absorbance response surface using the Levenberg–Marquardt method the SQUAD(84) program uses the classical Newton–Raphson algorithm. SQUAD(84) was not used solely for comparison of the algorithmic process (SPECFIT/32) in relation to a heuristic (SQUAD(84)) one in a non-linear regression, but especially for its advanced statistical analysis of residuals, which is an indispensable tool in seeking the best regression model from a choice of several proposed ones. The graph of reproducibility shows that for ill-conditioned parameters in the regression model (mainly pK_{a3} and pK_{a1}) SPECFIT/32 and SQUAD(84) were not always able to find the global minimum of an elliptical hyperparaboloid. The parameter pK_{a2} was well-conditioned in the model and its estimate was therefore sufficiently reliable and easy to estimate. Still we managed to distinguish that the protonation model of bosutinib with four dissociation constants exhibits markedly better fit

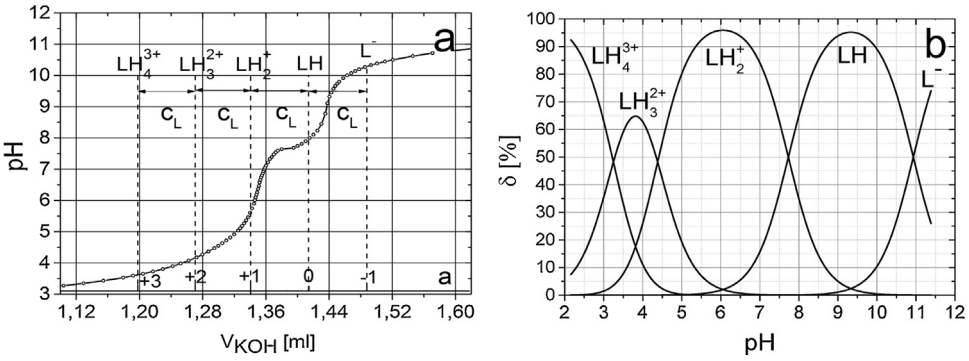

Fig. 6. Protonation equilibria of bosutinib analyzed with ESAB: (a) Potentiometric titration curve of bosutinib with KOH in 3% methanol, (b) distribution diagram of relative presentation of variously pronated species of all variously protonated species L^-, HL, HL_2^+, LH_3^{2+} and LH_4^{3+} of bosutinib according to pH at 25 °C, Estimates of parameters: $pK_{a1} = 3.25(12)$, $pK_{a2} = 4.38(10)$, $pK_{a3} = 7.73(07)$, $pK_{a4} = 10.94(03)$, $V_0 = 15.12$ ml, $c_L = 0.0080(0028)$ mM, $H_0 = 0.137(001)$ mM. Statistical analysis of residuals: $E(e) = -0.025$ j.pH, $|e| = 0.082$ j pH, $s(e) = 0.120$ j pH, $M = 0.001$ j pH, (ESAB, HYPERQUAD, ORIGIN).

of calculated spectra through experimental points than the model with only three constants. The standard deviation of absorbance $s(A)$ was used for the reliability criterion of the found estimates of dissociation constants. Since $s(A)$ reaches in all three spectra bands a value of 1 mA U and smaller, it can be stated that the estimates of dissociation constants are sufficiently reliable (Table 2).

4.2. pH-potentiometric titration

Bosutinib in a neutral medium of pH 7 occurs in a sparingly water-soluble form LH, which is capable of protonation to form a better soluble cation LH_4^{3+} or may dissociate to a difficultly soluble anion L^-. Acid–base titration of the tetraprotic cation LH_4^{3+} with the strong base KOH includes a mixture of eight various species H_3O^+, OH^-, LH_4^{3+}, LH_3^{2+}, LH^{2+}, LH, L^- and potassium K^+ cation. Other variables in the equation of the titration curve are the initial concentration of bosutinib (L_0), the initial concentration of the strong base KOH (H_T), the initial volume of titrand (V_0) and the volume of the addition of the strong base KOH (V_i). The mathematical description of the titration curve of the tetraprotic cation LH_4^{3+} with the strong base KOH (neglecting activities a) is specifically characterized by four steps of dissociation (Fig. 6) that can be expressed with the mixed dissociation constants pK_{a1}, pK_{a2}, pK_{a3}, pK_{a4}, the equation for the ionization of water pK_w, and further equations of the mass and charge balance, as well as two equations expressing the dilute concentration of a weak acid and a strong base within titration. Adjusting the ionic strength of the titrand, mixtures of HCl and drug prior to potentiometric titration was made with by the KCl solution. To determine precise estimates of the dissociation constants of bosutinib the potentiometric titration data were analysed by a non-linear regression using two different programs: ESAB and HYPERQUAD. Both programs enumerated values pK_{a1}, pK_{a2}, pK_{a3}, pK_{a4} of bosutinib.

Fig. 6a and b shows a graphical presentation of (a) the potentiometric titration curve of the bosutinib in 3% methanol solution with HCl titrated with KOH at 25 °C, (b) a distribution diagram of relative concentrations of all variously protonated species within titration. The intersections of both curves give a value of dissociation constant on the pH axis.

Table 3 shows the results obtained from regression analysis with ESAB. Four common parameters were estimated pK_{a1}, pK_{a2}, pK_{a3}, pK_{a4}, and two *group parameters* L_0 and H_T. Its statistical analysis of the residuals was examined to ascertain the best fitting of a titration curve. The goodness-of-fit of the experimental data can be evaluated by comparing the values of residuals with the instrumental noise $s_{inst}(V)$, which are represented by the standard deviation calculated either from the added volume of titrant to

Table 3
The ESAB analysis of titration concerning the acidified bosutinib solution with KOH in 3% MeOH medium. The standard deviations of the parameter estimates in units of the last significant digits are shown in parentheses. Common parameters: $pK_{c1} = 3.65(02)$, $pK_{c2} = 4.61(02)$, $pK_{c3} = 7.79(03)$ and $pK_{c4} = 10.30(03)$. Group parameters: $L_0 = 4.161 \times 10^{-4}$ mol/dm^3, $H_T = -0.08984$ mol/dm^3. Constants: $H_0 = 7.180 \times 10^{-3}$ mol/dm^3, $T = 25.0$ °C, $pK_w = 13.9799$, $V_0 = 15.12$ cm^3, with $s(V) = 0.0001$ cm^3, $I_0 = 0.0$ (in vessel), $I_T = -0.08984$ (in burette).

Arithmetic mean of residuals, $E(\hat{e})$	8.80E-05 cm^3
Mean of absolute value of residuals, $E\|\hat{e}\|$	0.0014 cm^3
Variance, $S^2(\hat{e})$	4.08E-06
Standard deviation, $S(\hat{e})$	0.0020 cm^3
Residual sum of squares, $U(b)$	2.41E-04

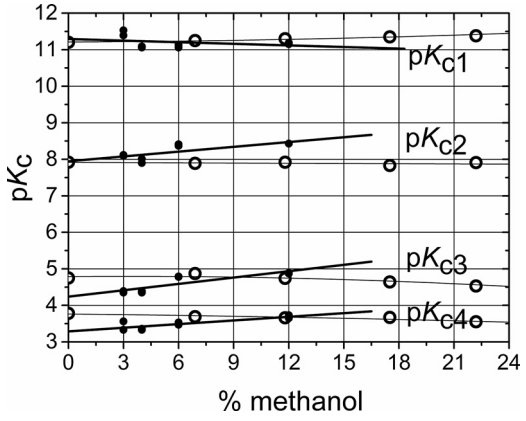

Fig. 7. Comparison of the four concentration dissociation constants by potentiometric titration bosutinib (ESAB) with literature values: open circles are values according to Box [18] and closed circles are values found in this work.

$s(V) = 0.002$ cm^3 (ESAB) or from measured values of the pH value $s(pH) = 0.01$ (HYPERQUAD). The measurements were considered of a good fitness when both, the arithmetic mean of the absolute values of residuals $E|\hat{e}|$ and the residual standard deviation $s(e)$ were almost of the same size or smaller than the value of the noise $s_{inst}(V)$, resp. $s_{inst}(pH)$. The above mentioned titration curves exhibited $E|\hat{e}| = 0.0014$ cm^3 and $s(\hat{e}) = 0.0020$ cm^3, which are lower than or similar to the instrumental error $s(V) = 0.0020$ cm^3. Given that $E(\hat{e})$ is equal to the value 8.8×10^{-5}, being considered as a zero, no systematic errors can be expected during titration curve fit. Outliers or certain points of the titration curve deemed to be ill-conditioned points in the regression model, were gradually deleted, the goodness-of-fit of the calculated titration curve was significantly improved, and thus the reliability of the estimates of the dissociation constants increased Fig. 7.

Table 4
Overview of extrapolated estimates of the concentration of dissociation constants bosutinib to zero content of methanol.

Estimates of pK_c programs SPECFIT (spectrophotometry)			
	25 °C	37 °C	Box et al. [30]
pK_{c4}	11.04(02)	10.92(06)	11.20
pK_{c3}	7.56(02)	7.33(05)	7.92
pK_{c2}	4.54(06)	5.03(08)	4.75
pK_{c1}	3.43(08)	3.44(06)	3.78

Estimates of pK_c programs ESAB and HYPERQUAD (potentiometry)			
	25 °C (ESAB)	25 °C (HYPERQUAD)	Box et al. [30]
pK_{c4}	11.05(03)	11.29(05)	11.20
pK_{c3}	7.97(02)	7.95(07)	7.92
pK_{c2}	4.37(02)	4.24(10)	4.75
pK_{c1}	3.51(02)	3.29(12)	3.78

Table 4 summarizes the results of the dissociation constants estimated for various content of methanol evaluated with ESAB and with HYPERQUAD. The ESAB program minimizing residuals $e_i = (V_{exp,i} - V_{calc,i})$ reaches 0.1–0.3 μl and HYPERQUAD minimizing $e_i = (pa_{H+,exp,i} - pa_{H+,calc,i})$ reaches a SIGMA value of about 2 or less, thus proving an excellent fit.

5. Conclusions

Spectrophotometric and potentiometric pH-titration allowed the measurement of four dissociation constants of bosutinib, but worse solubility required a ratio of methanol co-solvents for determining namely the first constant pK_{c1}. Our experience in profiling acid-base properties of bosutinib reinforces the belief that it is necessary to use a combination of several techniques to profile complex acid-base properties of drug molecules. The following conclusions were reached:

1) In the neutral pH 7 bosutinib occurs in water sparingly soluble form LH, which is capable of protonation to form better soluble cation LH_4^{3+}. The neutral molecule LH can be dissociated into still hardly water soluble anion L^-. Acid–base titration of the tetraprotic cation LH_4^{3+} with KOH leads to a mixture of eight species H_3O^+, OH^-, LH_4^{3+}, LH_3^{2+}, LH_2^+, LH, L^- and the potassium cation K^+. The graph of molar absorption coefficients of variously protonated species according to wavelength shows that the spectrum of cations LH_4^{3+} and LH_3^{2+} are of only a little different color and dissociation of the chromophore LH_4^{3+} to LH_3^{2+} has little influence on the shape of the spectrum. The same is true for the proton chromophore at LH to LH_2^+, while protonation of chromophore LH_2^+ to LH_3^{2+} has greater influence on chromophores in bosutinib and the results on the spectral change are therefore considerable. With increasing methanol content in solvent the dissociation apparently lessens and therefore the percentage of species LH_3^{2+} decreases and in solution the species LH prevails.
2) Despite of Box et al. [18] argue that in the acidic pH region of 2–7 the pH change does not affect enough bosutinib chromophores in molecule, and therefore the chromophore is unable to distinguish dissociation into two stages, we have proven that with a low methanol content of the solvent 3–6% in the range of pH 2–7 three dissociation constants can be reliably estimated from the spectra. Although the change of pH somewhat less affected changes in the chromophore, three concentration dissociation constants pK_{c1} = 3.41, pK_{c2} = 4.69 and pK_{c3} = 6.4 could be distinguished.
3) Dissociation constants of bosutinib were determined by regression analysis of potentiometric titration curves with the ESAB2M and HYPERQUAD programs and at 25 °C without adjusting the ionic strength and for various content of methanol. Dissociation constants were pK_{c1} = 3.29(12), pK_{c2} = 4.24(10), pK_{c3} = 7.95(07), pK_{c4} = 11.29(05). In brackets are the standard deviations in the last valid unit number.
4) Structural prediction of dissociation constants of bosutinib was performed using the PALLAS program [25] to give values pK_1 = 4.43, pK_2 = 6.30, pK_3 = 7.88, pK_4 = 9.02.

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