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Overlapping pKa of the multiprotic hemostyptic Eltrombopag using UV/VIS multiwavelength spectroscopy and potentiometry

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Abstract:	<p>pH-potentiometric and WAPh-spectrophotometric titrations of the multiprotic hemostyptic Eltrombopag for dissociation constants determination were compared. Hemostyptic and hemostatic Eltrombopag treats low blood platelet counts in adults with chronic immune idiopathic thrombocytopenia ITP. Eltrombopag exhibits five protonatable sites in a pH range of 2 to 10, where only two pK are well separated ($\Delta pK > 3$), while the other three are near dissociation constants of overlapping equilibria. According to MARVIN prediction, in the neutral medium Eltrombopag occurs in the slightly water soluble form LH3 that can be protonated to the soluble species LH4 and LH5. The molecule LH3 can be dissociated to still difficultly soluble species LH2, LH and L. Due to limited solubility of Eltrombopag above pH 9.5 the protonation was studied up to pH 10. Five dissociation constants can be reliably determined with REACTLAB and SQUAD84 leading to the same value. From a dependence on ionic strength the thermodynamic dissociation constants were estimated at 25°C: $pK_{a1T} = 2.69$, $pK_{a2T} = 6.97$, $pK_{a3T} = 7.13$, $pK_{a4T} = 7.65$, $pK_{a5T} = 8.30$. Since pH above 10 and pH down 5 occurs in a titrated solution the very fine precipitate of Eltrombopag which is initially forming a slight opalescence, this part of the potentiometric titration curve pH over 9 and pH below 5 was not taken into regression analysis to estimate $pK_{a2} = 6.59(01)$, $pK_{a3} = 7.56(04)$, $pK_{a4} = 8.48(59)$, $pK_{a5} = 9.29(34)$ at 25°C with ESAB.</p>	
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Overlapping pK_a of the multiprotic hemostyptic Eltrombopag using UV/VIS multiwavelength spectroscopy and potentiometry

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Abstract:

pH-potentiometric and WApH-spectrophotometric titrations of the multiprotic hemostyptic Eltrombopag for dissociation constants determination were compared. Hemostyptic and hemostatic Eltrombopag treats low blood platelet counts in adults with chronic immune idiopathic thrombocytopenia ITP. Eltrombopag exhibits five protonatable sites in a pH range of 2 to 10, where only two pK are well separated ($\Delta pK > 3$), while the other three are near dissociation constants of overlapping equilibria. According to MARVIN prediction, in the neutral medium Eltrombopag occurs in the slightly water soluble form LH_3 that can be protonated to the soluble species LH_4 and LH_5 . The molecule LH_3 can be dissociated to still difficultly soluble species LH_2 , LH and L . Due to limited solubility of Eltrombopag above pH 9.5 the protonation was studied up to pH 10. Five dissociation constants can be reliably determined with REACTLAB and SQUAD84 leading to the same value. From a dependence on ionic strength the thermodynamic dissociation constants were estimated at 25°C: $pK_{a1}^T = 2.69$, $pK_{a2}^T = 6.97$, $pK_{a3}^T = 7.13$, $pK_{a4}^T = 7.65$, $pK_{a5}^T = 8.30$. Since pH above 10 and pH down 5 occurs in a titrated solution the very fine precipitate of Eltrombopag which is initially forming a slight opalescence, this part of the potentiometric titration curve pH over 9 and pH below 5 was not taken into regression analysis to estimate $pK_{a2} = 6.59(01)$, $pK_{a3} = 7.56(04)$, $pK_{a4} = 8.48(59)$, $pK_{a5} = 9.29(34)$ at 25°C with ESAB.

Keywords: Dissociation constants; Eltrombopag; spectrophotometric titration; potentiometric titration; SQUAD84; REACTLAB; ESAB;

Highlights:

- First experimentally determined UV/VIS spectra and pK_a value of Eltrombopag.
- Eltrombopag treats low blood platelet counts in adults with chronic immune idiopathic thrombocytopenia.
- Five thermodynamic dissociation constants of Eltrombopag were determined at 25°C.

- Acid-base equilibria were studied with spectrophotometric and potentiometric titrations.
- The number of light-absorbing species of all protonation equilibria was estimated from the rank of the absorbance matrix.

Introduction

Eltrombopag of formula $C_{25}H_{22}N_4O_4$ (Fig. 1) and molar mass 442.467 g/mol is hemostyptic and hemostatic that has been developed for conditions that lead to thrombocytopenia i.e. abnormally low platelet counts. This hemostatic was discovered as a result of research collaboration between GlaxoSmithKline and Ligand Pharmaceuticals [1]. *Eltrombopag* (codenamed SB-497115-GR, CAS Number 496775-61-2, 496775-62-3, ATC code B02BX05, PubChem CID 9846180, ChemSpider 21106301) of the IUPAC name 3'-[(2Z)-2-[1-(3,4-dimethylphenyl)-3-methyl-5-oxo-1,5-dihydro-4H-pyrazol-4-ylidene]hydrazino]-2'-hydroxy-3-biphenylcarboxylic acid is used to treat low blood platelet counts in adults with chronic immune idiopathic thrombocytopenia ITP, when other medicines, or the spleen removal surgery, have not worked well enough. It works by causing the cells in the bone marrow to produce more platelets, [2]. *Eltrombopag* also increases the number of platelets in people who have hepatitis C a viral infection [3] that may damage the liver so that they can begin and continue treatment with interferon (Peginterferon, Pegintron, others) and ribavirin (Rebetol).

Fig. 1

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 [5-11]. pK_a values can be either experimentally measured or theoretically predicted:

1. Many new substances are poorly soluble in aqueous solutions, conventional potentiometric determination of dissociation constants of these compounds can often be difficult [12]. Spectrophotometric pK_a determination is an alternative method to potentiometry provided that the compound is water soluble to the extent of $10^{-6}M$ and provided the compound possesses pH-dependent light absorption due to the presence of a chromophore in proximity to the ionization centre *cf. ref.* [13-17]. In previous work [18-27] the authors have shown that the multiwavelength spectrophotometric pH-titration method in combination with suitable chemometric tools (called as the WApH-metod by Tam [28]) can be used for the determination of dissociation constants pK_a even for barely soluble drugs. Spectra are generally a superposition of spectra of the numerous compounds or components present. In many cases, however, the spectral responses of two and sometimes even more components overlap considerably, and analysis is no longer straightforward. Hard modelling methods, *e.g.* SQUAD84 [29,30,16], include traditional least-squares curve fitting approaches, based on a previous postulation of a chemical model, *i.e.* a set of species defined by their stoichiometric coefficients and formation

1 constants, which are then refined by the least-squares minimization. The most relevant algorithms are
2 SQUAD84 and REACTLAB [31] or its previous version SPECFIT32 [32]. Soft modelling techniques,
3 as for example REACTLAB, such as multivariate curve resolution methods based on factor analysis,
4 work without any assumption of a chemical model, and do not need to comply with the mass-action law.
5 The molar absorptivities are usually not required for analysis. However, explicit equations for the
6 equilibrium expression are necessary to rotate the eigenvectors to give the correct concentration profiles.
7 It may be difficult to generalize these explicit equations for multistep ionization system [28].
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10 2. Nine commercially available or free programs for predicting ionization constants were
11 compared [4]. Meloun et al. [27] used the REGDIA regression diagnostics algorithm written in S-Plus
12 [33] critically examine an accuracy of pK_a predictions with four programs ACD/pK [34,35], Marvin
13 Sketch [36,37], PALLAS, and SPARC [38,39] and the best was considered ACD/Labs™ [40], pK_a
14 Predictor 3.0 [41]. Balogh et al. [42] found the most predictive and reliable predictors MARVIN-Sketch
15 and ACD/Percepta [43,34,44].
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20 There are no systematic reports about the drug Eltrombopag concerning its UV-VIS spectra, pK_a
21 values and the distribution diagram of variously protonated species. The aim of our study was to examine
22 and verify the UV-metric pK_a determination (WApH-spectrophotometric titration) of the pH-absorbance
23 matrix and to carry out the pH-metric pK_a determination (pH-potentiometric titration) of the protonation
24 model to find suitable conditions for a reliable regression determination of dissociation constants.
25 Considering the role of pK_a predictions in early phase discovery, we concluded that the selection of
26 appropriate prediction tools for regular pharmaceutical chemistry use requires solid benchmarking
27 studies. We are reporting our obtained results that were evaluated by two different LFER based pK_a
28 predictions tools, MARVIN-Sketch and ACD/pK software [42].
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37 2. Theoretical

38 2.1 UV-metric pK_a determination (WApH-spectrophotometric titration)

39 The acid-base equilibrium of Eltrombopag studied is described in terms of the protonation of the
40 Brönsted base L^{z-1} according to the equation L^{z-1} + H⁺ ⇌ HL^z, [21]. The protonation equilibria between
41 the species L (the charges are omitted for the sake of simplicity) of a drug and a proton H are considered
42 to form a set of variously protonated species L, LH, LH₂, LH₃ ...etc., with the general formula LH_r in a
43 particular chemical model and which are represented by n_c (the number of species), r_i, i = 1, ..., n_c where
44 index i indicates their particular stoichiometry; the overall protonation (stability) constant of the
45 protonated species, β_r, may then be expressed as
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$$52 \beta_r = [\text{LH}_r] / ([\text{L}] [\text{H}]^r) = c / (l h^r) \quad (1)$$

53 where the free concentration [L] = l, [H] = h and [LH_r] = c, [21]. For dissociation realized at constant
54 ionic strength the “mixed dissociation constants” are defined as
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$$58 K_{a,j} = \frac{[\text{LH}_{j-1}] a_{\text{H}^+}}{[\text{LH}_j]} \quad (2)$$

As each species is characterized by its own spectrum, for UV/VIS experiments and the i th solution measured at the j th wavelength, the Lambert-Beer law relates the absorbance, $A_{i,j}$, being defined as

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

where $\varepsilon_{r,j}$ is the molar absorption coefficient of the LH_r species with the stoichiometric coefficient r measured at the j th 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 [19]. The general procedure used to build the protonation model with SPECFIT32, REACTLAB or SQUAD84 was described in [21,25,13,45]. Determining the chemical model of the drug 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. Two different 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.

2.2 pH-metric pK_a determination (pH-potentiometric titration)

The overall protonation constant of the protonated species, β_{qr} , eq. (1), and the mixed dissociation constants K_a , eq. (2), are used whereas the mass balance equations are $L = l + \sum_{j=1}^J \beta_{H_j} l h^j$ and

$$H = h - \frac{K_w}{h} + j \sum_{j=1}^J \beta_{H_j} l h^j. \text{ Potentiometric readings obtained with the proton-sensitive glass and}$$

reference electrodes cell can be described by the equation

$$E_{cell} = E^0 + \frac{f \cdot RT \ln 10}{F} \log a_{H^+} + j_a a_{H^+} - \frac{j_b K_w}{a_{H^+}} - E_{ref} = E^0 + S \log h \quad (3)$$

where E^0 is the standard potential of a glass electrode cell containing some other constants of the glass electrode as the asymmetry potential, etc., and $a_{H^+} = [H^+] y_{H^+} = h y_{H^+}$, a liquid-junction potential E_j is expressed by the term $E_j = j_a a_{H^+} - j_b K_w / a_{H^+}$, and $S = (f \cdot RT \ln 10) / F$ is the slope of glass electrode for a Nernstian response, K_w is the operational ion product of water at temperature T [K], the correction factor f , is taken as an adjustable parameter.

An explicit equation for the titration curve under a constant ionic strength expresses a dependence between the volume of the *added titrant* from burette V_i and the monitored *emf* $E_{cell,i}$ or pa_{H^+} with the vector of unknown parameters (\mathbf{b}) being separated into the vector of *common parameters* (\mathbf{K}_a)

and the vector of *group parameters* (\mathbf{p}), i. e. $V_i = f(E_{cell,i}; \mathbf{b}) = f(E_{cell,i}; \mathbf{K}_a, \mathbf{p})$. Here the vector of common parameters $\mathbf{K}_a = (K_{a,1}, \dots, K_{a,m})$ contains m dissociation constants of the acid LH_j while a vector of group parameters $\mathbf{p} = (E^0, S, K_w, j_a, j_b, L_0, L_T, H_0, H_T)$ containing besides two constants of Nernstian equation, E^0 and S , the total ligand concentration, L_0 , and the hydrogen ion concentration, H_0 of titrand in vessel, and the corresponding quantities of titrant, L_T and H_T in burette [46-48]. Group parameters \mathbf{p} can be refined simultaneously with the common parameters \mathbf{K}_a . Two independent regression approaches to a minimization of the sum of squared residuals have been applied:

(1) The program ESAB [46,47] uses this strategy for treating pa_{H^+} data to find dissociation constants that give the “best” fit to experimental data. As primary data contains the total concentration H_T of proton from burette and the measured pa_{H^+} , one could trust pa_{H^+} and minimize the residual sum of squares $(V_{exp} - V_{calc})^2$. The residual e is formulated with the volume of *added titrant* V from burette so that $e_i = (V_{exp,i} - V_{calc,i})$ and the resulting residual sum of squares $U(\mathbf{b})$ is defined

$$U(\mathbf{b}) = \sum_{i=1}^n w_i (V_{exp,i} - V_{calc,i})^2 = \sum_{i=1}^n w_i e_i^2 \quad (4)$$

where w_i is the statistical weight usually set equal to unity while in ESAB it may be equal to

$$\frac{1}{w_i} = s_i^2 = s_E^2 + \left[\frac{dE_i}{dV_i} \right]^2 s_V^2 \quad (5)$$

and with a good equipment, we have generally $s_E = 0.01$ pH units and $s_V = 0.0001 - 0.0005$ cm³.

(2) In the program HYPERQUAD [49] the objective function is given in matrix notation $U = \mathbf{e}^T \mathbf{W} \mathbf{e}$, where \mathbf{e} is a vector of residuals measured in pH and \mathbf{W} is a matrix of weights. To minimize the objective function, the Gauss-Newton-Marquardt method is used. The SIGMA criterion of a goodness-

of-fit is defined as $SIGMA = \sqrt{\frac{\sum_{i=1}^n w_i e_i^2}{n-m}}$ where the weights w are calculated from estimates of the error

in pH and titre, the latter only being important in regions where the titration curve slopes more steeply. Sigma squared is also a chi-squared statistic.

2.3 Reliability of pK_a estimates obtained

The detailed procedure of the graphical and numerical analysis of residuals is described in [21,25]. 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 $s_{inst}(A)$. The adequacy of a proposed regression model

with experimental data and a reliability of found parameter estimates, $b_j, j = 1, \dots, m$, may be examined by the goodness-of-fit test, cf. page 101 in ref. [33].

(1) *The quality of found parameter estimates* $b_j, j = 1, \dots, m$, [21] is considered according to their confidence intervals or according to their variances $D(b_j)$. Often an empirical rule is used: parameter b_j is considered to be significantly differing from zero when its estimate is greater than 3 standard deviations, $3\sqrt{D(b_j)} < |b_j|, j = 1, \dots, m$. Higher parameter variances are also caused by termination of a minimization process before reaching a minimum [33].

(2) *The quality of achieved curve fitting* [21]: the adequacy of a proposed model and m parameter estimates found with n values of experimental data is examined by the goodness-of-fit test based on the statistical analysis of classical residuals. If proposed model represents the data adequately, the residuals should form random pattern having a normal distribution $N(0, s^2)$ with the residual mean equal to zero, $E(\hat{e}) = 0$, and the standard deviation of residuals $s(\hat{e})$ being near to noise i.e. experimental error ε . Systematic departures from randomness indicate that the model and parameter estimates are not satisfactory. The following statistics of residuals can be used for a numerical goodness-of-fit evaluation, cf. page 290 in ref. [50]: (1) The *residual bias* being the arithmetic mean of residuals $E(\hat{e})$ should be equal to zero. (2) The *mean of absolute values of residuals* $E|\hat{e}|$, and the square-root of the residuals variance $s^2(\hat{e}) = U(\mathbf{b}) / (n - m)$ known as the estimate of the *residual standard deviation*, $s(\hat{e})$, should be both of the same magnitude as the instrumental error of regressed variable absorbance A , $s_{\text{inst}}(A)$. Obviously, it should be also valid that $s(\hat{e}) \approx s_{\text{inst}}(A)$. (3) The *residual skewness*, $g_1(\hat{e})$, for symmetric distribution of residuals should be equal to zero; (4) The *kurtosis*, $g_2(\hat{e})$, for normal distribution should be equal to 3.

(3) *The numerical estimates of the molar absorption coefficients* [21] of differently protonated light-absorbing species in an equilibrium mixture according to the wavelength λ represent another important result of the spectra regression analysis.

(4) *The distribution diagram* [21] 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 analysis of the spectra [51].

(4) *The deconvolution of each experimental spectrum* [21] into the spectra for the individual species shows whether the experimental design was efficient. If for a particular concentration range the spectrum consists of just a single component, further spectra for that range would be redundant though they should improve the precision. In ranges where many components contribute significantly to the experimental spectrum, several spectra should be measured.

3. Material and methods

3.1 Chemicals and Solutions

Eltrombopag was 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 0.001 mol. dm⁻³. *Hydrochlorid acid*, 1,044 mol. dm⁻³, was prepared by diluting a concentrated HCl (p. a., Lachema Brno) with redistilled water and standardization against HgO and KI with a reproducibility better than 0.2% according to the equation $\text{HgO} + 4 \text{KI} + \text{H}_2\text{O} \rightleftharpoons 2 \text{KOH} + \text{K}_2[\text{HgI}_4]$ and $\text{KOH} + \text{HCl} \rightleftharpoons \text{KCl} + \text{H}_2\text{O}$. *Potassium hydroxide*, 0.876 mol. dm⁻³, was prepared from the exact weight of pellets p. a., Aldrich Chemical Company with carbon-dioxide free redistilled water. The solution was stored for several days in a polyethylene bottle in argon atmosphere. This solution was standardized against a solution of potassium bi-phthalate using the derivative method with reproducibility 0.1%. All solutions were preserved from atmospheric CO₂ by means of soda lime traps. *Mercury oxide*, *potassium iodide* and *potassium chloride*, p. a. Lachema Brno were not extra purified. *Grade A glassware* and *twice-redistilled water* were employed in the preparation of all the solutions.

3.2 Apparatus and procedure

The apparatus used and the WApH-spectrophotometric titration procedure have been described previously in details [13,25,26]. The experimental and computation scheme to determine of the protonation constants of the multi-component system is taken from Meloun *et al*, *cf.* page 226 in *ref.* [33] and the five steps are described in details elsewhere [26].

The free hydrogen-ion concentration *h* was measured on the digital voltmeter Hanna HI 3220 with a precision of ±0.002 pH using the combined glass electrode Theta HC 103-VFR. Titrations were performed in a water-jacketed double-walled glass vessel of 100 ml, closed with a Teflon bung containing the electrodes, an argon inlet, a thermometer, a propeller stirrer and a capillary tip from a micro-burette. All pH measurements were carried out at (25.0 ± 0.1) °C. During the titrations, a stream of argon gas was bubbled through the solution both for stirring and for maintaining an inert atmosphere. The argon was passed through an aqueous ionic medium by prior passage through two vessels also containing the titrand medium before entering the corresponding titrant solution. All titrations were performed in solutions under argon atmosphere at (25.0 ± 0.1)°C using standardized 1M HCl or 1M KOH titrants. In general, sample solutions of 20 mL volumes were preacidified to a relatively low pH value (ca. 2 - 3) and then titrated alkalimetrically to an appropriate high pH value (ca. 10 - 11). The burettes used were syringe micro-burettes of 1250 µl capacity (META, Brno) with a 25.00 cm micrometer screw [52]. The potentiometric titrations of drugs with potassium hydroxide were performed using a hydrogen activity scale. Standardization of the pH meter was performed using WTW standard buffers values 4.006, 6.865 and 9.180 at 25°C.

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When the ESAB programme [46,47] estimated the total proton concentration in a burette H_T and the total concentration of the drug in the titration vessel L_0 from the actual titration of a mixture of the drug and hydrochloric acid with potassium hydroxide, some group parameters are given in the input data for ESAB such as the Nernstian slope and pK_w , which are both accessible from the literature [53]. With ESAB two group parameters, L_0 and H_T , were refined to give the best fit, while the fitness may be examined by the goodness-of-fit criteria.

3.3 Computation and Software

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Computation relating to the determination of dissociation constants was performed by regression analysis of the UV/VIS spectra using the SQUAD84 and REACTLAB programs. A qualitative interpretation of the spectra with the use of the INDICES programme [51] aims to evaluate the quality of the dataset and remove spurious data, and to estimate the minimum number of *factors*, i.e. contributing aqueous species, which are necessary to describe the experimental data and determine the number of dominant species present in the equilibrium mixture. Computation relating to determining the dissociation constants was performed by regression analysis of titration curve using the ESAB program and HYPERQUAD program [49]. Most graphs were plotted using ORIGIN 8 [50] and S-Plus [33]. ACD/pK [46] and MARVIN [54,36] are programs for predictions based on the structural formulae of drug compounds. Entering the compound topological structure descriptors graphically, pK_a values of organic compound are predicted using approximately hundreds of Hammett and Taft equations and quantum chemistry calculus.

4. Results and Discussion

4.1 Computational prediction of the protonation scheme

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The protonation scheme of the Eltrombopag has six functional groups (denoted with letters A, B, C, D, E, F in Fig. 2) that can be associated to dissociation constants; two ionization are associated to hydroxyl and other ionization to nitrogen atom. In following text the charges of ions are omitted for the sake of simplicity and ions are denoted as variously protonated species.

Fig. 2

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The macro-dissociation constants of Eltrombopag were predicted according to the chemical structure analyzed with the use of two reliable pK_a prediction tools [42]: ACD/percepta [43,34,44] was run using GALAS model, which uses an internal training set of > 31000 individual pK_a values for approximately 16000 compounds in aqueous solutions [55]. Marvin pK_a predictions are based on the calculated partial charge of the atoms located in the analyzed structure, using Hammett-Taft approach. MARVIN and ACD/Percepta [34,43] showed similar performance on the dataset and provide pK_a data for all the recognized ionization sites. Predicted pK_a values are assigned to the corresponding ionization sites by both tools that are essential for compounds with multiple ionization sites (Fig. 2). The whole molecule of Eltrombopag was further subdivided into four auxiliary fragments containing functional groups on which protonation occurred (Fig. 2). These predicted pK_a values served to compare with predicted values

throughout the structure of the Eltrombopag molecule. In the ionization site B the predicted pK_a of the Eltrombopag molecule is close to the predicted pK_a values of its auxiliary fragment. Other ionization sites (A, C, D, E, F) are located at the site of the molecule that forms a rather complex conformation. The fragments A, C, D, E contain sites which are not affected by the strong electron field of the rest of Eltrombopag molecule, and therefore their predicted pK_a values differ significantly from pK_a predicted for the whole Eltrombopag molecule (Table 1).

Table 1

4.2 UV-metric pK_a determination

Many drug compounds are sparingly soluble in water and a precise determination of their pK_a values poses a challenging problem for potentiometric titration since the accuracy of this method is restricted by its detection limit of about 10^{-4} M [56]. 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 [56]. 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 a UV titration, the spectral data of Eltrombopag measured are a series of spectra acquired at different pH values. The strategy for an efficient experimentation in dissociation constants determination followed by spectral data treatment was used according to published Tutorial [26]. Eltrombopag contains a complicated molecular structure introduced on Fig. 1 and 2 and several protonation equilibria were monitored spectrophotometrically to analyze a spectra set in two steps: 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 [51], (Fig. 3).

Fig. 3

The INDICES indicates the position of a break points on the $s_k(A) = f(k)$ curve in the screen plot using the three most reliable approaches (Kankare's $s(A)$, RSD and RSM, *cf. ref.* [51]) and gives $k^* = 6$ with corresponding co-ordinate $s_6^*(A) = 0.4$ mAU. This value also represents the actual instrumental error $s_{inst}^*(A) = 0.4$ mAU and $\log(s_{inst}^*(A)) = -3.4$ of the spectrophotometer CINTRA 5 (GBC, Australia). The number of light-absorbing species is an aid to the establishment of an protonation model. It means that five dissociation constants will be preferred and six species LH₅, LH₄, LH₃, LH₂, LH and L are supposed to be present. Due to the large variations in the indicator values, these latter graphs are plotted on a logarithmic scale (Fig. 3) and the number of 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 $s_{inst}^*(A) = 0.4$ mAU when $\log s_{inst}^*(A) = -3.4$. This is common criterion for determining p . Low values of $s_{inst}^*(A)$ prove that reliable spectrophotometer with convenient experimental technique was used [26].

Fig. 4

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In a spectra set on Fig. 4 the five analytical wavelengths *a* through *e* were optioned at which the absorbance-pH curves were analysed. Six following graphs on Fig. 4 show the consecutive deprotonation response in spectra, when each spectrum was deconvoluted on the spectrum of differently protonated species in particle mixture of Eltrombopag. At pH = 3.10 the species LH₅ accompanied species LH₄ predominates in the solution. At pH = 6.14 together with the molecule LH₃ two species LH₂, LH₄ exhibit absorption bands at the same wavelength of absorption maximum λ_{\max} . At pH = 7.61 the experimental spectrum is decomposed to three absorption bands concerning the species LH₃ which dissociate to species LH₂ and LH. At pH = 8.85 and 9.16 the species L occurs with species LH, and L while concentration of L in the solution increases up to pH = 10.02.

Fig. 5

Attention should be paid to a sensitivity of analytical wavelengths of spectra which should be chosen to determine the p*K*_a values namely when overlapped p*K*_a are observed in compounds in which the ionisable groups are in symmetrical positions and, do not interact. Fig. 5 brings four pH-spectrophotometric titration sets of spectra monitored at various wavelengths in which chromophore(s) are sensitive on pH change. The search of the best chemical model containing either 4 or 5 dissociation constants bring Table 2: Four various spectra ranges of useful analytical wavelengths ranges and the final one were examined to indicate the best spectra range in which the actual chromophore is active and reflect on protonation/dissociation in molecule. The best regression model was searched by testing two working hypotheses about a protonation model: the first concerning four and the other with five dissociation constants. 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. The standard deviation of residuals and Hamilton *R*-factor of a relative fitness generally shown that better fit of the calculated spectra through measuring points was always achieved for the protonation model with five dissociation constants.

Table 2

Fig. 6

Five dissociation constants p*K*_{a1}, p*K*_{a2}, p*K*_{a3}, p*K*_{a4}, p*K*_{a5} and six molar absorptivities of Eltrombopag ϵ_L , ϵ_{LH} , ϵ_{LH2} , ϵ_{LH3} , ϵ_{LH4} , ϵ_{LH5} were estimated using SQUAD84 and REACTLAB in the first run. The reliability of the parameter estimates may be tested with the use of following diagnostics:

The first diagnostic value indicates whether all of the parametric estimates β_r and ϵ_r have physical meaning and reach realistic values. As the standard deviations $s(\log \beta_r)$ of parameters $\log \beta_r$ and $s(\epsilon_r)$ of parameters ϵ_r are significantly smaller than their corresponding parameter estimates, all the variously protonated species are statistically significant at a significance level $\alpha = 0.05$. The absolute values of $s(\beta_r)$, $s(\epsilon_r)$ gives information about the last *RSS*-contour of the hyperparaboloid in the neighbourhood of the pit, *RSS*_{min}. For well-conditioned parameters, the last *RSS*-contour is a regular ellipsoid, and the standard deviations are reasonably low. High *s* values are found with ill-conditioned parameters and a “saucer”-shaped pit. The relation $s(\beta_j) \times F_\sigma < \beta_j$ should be met where *F*_σ is equal to 3. The set of standard deviations of ϵ_r for various wavelengths, $s(\epsilon_r) = f(\lambda)$, should have a Gaussian

1 distribution; otherwise, erroneous estimates of ε_r are obtained. Upper part of Fig. 6 shows the estimated
2 molar absorptivities of all of the variously protonated species ε_L , ε_{LH} , ε_{LH_2} , ε_{LH_3} , ε_{LH_4} , ε_{LH_5} of Eltrombopag
3 in dependence on wavelength. Three species LH_2 , LH and L exhibit similar spectra at $\lambda_{\max} = 440$ nm,
4 which within a consecutive protonization decline. The species L exhibits the highest value ε_{\max} at $\lambda_{\max} =$
5 440 while the lowest value ε_{\max} has the species LH_2 . The species LH_3 exhibits in comparison to the
6 previous species LH_2 due to a hypsochromic effect the lower value of the wavelength $\lambda_{\max} = 420$ nm,
7 which is the result of a consecutive protonation of molecule. Within a further protonation of molecule
8 LH_3 to the species LH_4 the spectrum and ε_{\max} significantly decreases keeping the same λ_{\max} as has LH_3 .
9 Further acidification of species LH_4 to pH 2 or 1 leads to species LH_5 whose spectrum increases.
10 Interestingly, the spectra LH_2 , LH and L are similar with the common wavelength $\lambda_{\max} = 440$ nm and
11 spectra LH_3 and species LH_4 and LH_5 are similar in a shape with the common $\lambda_{\max} = 420$ nm.

12 The second diagnostic examines whether all of the calculated free concentrations of variously
13 protonated species on the distribution diagram of the relative concentration expressed as a percentage
14 have physical meaning, which proved to be the case (lower part of Fig. 6) [13]. The calculated free
15 concentration of the basic components and variously protonated species of the chemical model should
16 show molarities down to about 10^{-8} M. Expressed in percentage terms, a species present at about 1%
17 relative concentration or less in an equilibrium behaves as numerical noise in a regression analysis. A
18 distribution diagram on Fig. 6 makes it easier to judge the contributions of individual species to the total
19 concentration quickly. Since the molar absorptivities will generally be in the range $10^3 - 10^5$ l. mol^{-1} . cm^{-1} ,
20 species present at less than *ca.* 0.1% relative concentration will affect the absorbance significantly
21 only if their ε is extremely high. The distribution diagram shows the protonation equilibria of LH_5 , LH_4 ,
22 LH_3 , LH_2 , LH and L . At neutral pH 5 - 8 prevails Eltrombopag as the species LH_3 and from pH 6 to pH
23 9, then in the form of species LH_2^- . In the pH range of 6 to 10 the species LH_3 deprotonates to the species
24 LH_2 and LH and finally into L . Acidification of the species LH_3 arises firstly LH_4 that in solution of pH
25 3 to pH 7 predominates to 90% LH_4 . Another acidification of the species LH_4 arises from pH 4 to pH 1
26 the species LH_5 . At concentrations of 10^{-4} to 10^{-6} M the Eltrombopag is sufficiently soluble and all its
27 dissociation constants can therefore be spectrophotometrically determined.

28 The next diagnostic concerns the goodness-of-fit [20]. The goodness-of-fit achieved is easily
29 seen by examination of the differences between the experimental and calculated values of absorbance,
30 $e_i = A_{\text{exp},ij} - A_{\text{calc},ij}$. Examination of the spectra and of the graph of the predicted absorbance response-
31 surface through all the experimental points should reveal whether the results calculated are consistent
32 and whether any gross experimental errors have been made in the measurement of the spectra. One of
33 the most important statistics calculated is the standard deviation of absorbance $s(A)$, calculated from a
34 set of refined parameters at the termination of the minimization process. Although this statistical analysis
35 of residuals [26] gives the most rigorous test of the degree-of-fit, realistic empirical limits must be used.
36 The statistical measures of all residuals e prove that the minimum of the elliptic hyperparaboloid RSS is
37 reached (Table 2): the residual standard deviation $s(\hat{e})$ always has sufficiently low values, lower than 3
38 mAU, which is less than 0,2% of measured absorbance value.

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Dissociation constants estimated with SQUAD84 and REACTLAB are in a good agreement. The SQUAD approach has a great advantage in 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 pK_{a3} 7 pK_{a4} 7.5 and pK_{a5} 9.3 are well-conditioned in regression model, and therefore their numerical evaluation is quite reliable. The first two dissociation constants pK_{a1} 3.1 and pK_{a2} 6.5 are ill-conditioned in regression model, a shape of the hyperparaboloid on these two parameters is rather saucer without a distinctive minimum. Numerical enumeration of all coordinates of this minimum is more difficult, and the parameter estimates are therefore less reliable. Acidifying the solution of a species LH_3 leading to species LH_4 and LH_5 may be disturbed with a precipitation of Eltrombopag, which manifests itself especially at higher concentrations in potentiometric determination. For this reason, not be at a concentration of 0.0001 M provide pK_{a1} because Eltrombopag in acidic solution precipitates. Both programs come to the same estimates of all five dissociation constants and identical to the goodness-of-fit test.

4.2 Potentiometric titration data analysis

For the adjusted value of an ionic strength the potentiometric titration of a mixture of HCl and Eltrombopag with potassium hydroxide was carried out at 25°C (Fig. 7). The initial tentative value of the dissociation constant of the drug studied, corresponding to the midpoint value in each plateau of the potentiometric titration curve, was refined by the ESAB and the HYPERQUAD programs.

Fig. 7

Because Eltrombopag exhibits four close dissociation constants, their numerical estimation is rather difficult and impossible without the use of a computer-assisted nonlinear regression. A regression analysis was employed with the use of a plateau of the middle part titration curve which concerned an alkalized Eltrombopag titrated with hydrochloric acid and then followed a subsequent retitration with potassium hydroxide. Of the assessed point titration curve was calculated as well the Bjerrum formation protonation curve function, which is shown in the graph in Fig. 7. On the Bjerrum formation curves the estimates of three or four dissociation constants pK_{a2} , pK_{a3} , pK_{a4} , pK_{a5} are plotted. Since pH above 9 and pH down 5 occurs in a titrated solution the very fine precipitate of Eltrombopag which is initially forming a slight opalescence, this part of the titration curve pH over 9 and pH down 5 was not taken into regression analysis to estimate pK_{a2} , pK_{a3} , pK_{a4} , pK_{a5} only.

Because it is a difficult task in regression analysis to estimate such close overlapping dissociation constants, two computer programs, ESAB and HYPERQUAD, were used and the resulting pK estimates were compared. Both programs differ in the definition of the sum of the squares of residuals. While in the ESAB the residuals are defined as the difference between the experimental and calculated titrant volume, in the HYPERQUAD the residuals are defined as differences between the experimental and calculated values of pH. Assumptions of the least squares method requires that the independent variable was not loaded by random errors and subject to regression analysis is the dependent

variable, which is loaded with a random experimental error. This assumption is met only with the program HYPERQUAD.

Table 3

Table 3 shows the results of the ESAB and HYPERQUAD regression analysis of a selected part of the particular titration curve when the minimization process terminates. Both the common and the group parameters are refined and the best curve-fitting is proven by the results of a statistical analysis of the residuals. The reliability of the dissociation constant may be determined according to the goodness-of-fit test in with an increasing number of group parameters are refined a better fit is achieved and therefore a more reliable estimate of dissociation constants results. As further group parameters are refined the fit is improved. A quite sensitive criteria of the reliability of the dissociation constant is the mean of absolute values of residuals $E|\hat{e}|$. Comparing residuals with the instrumental noise, $s_{inst}(y)$, represented here by either $s_{inst}(y) = s(V) = 0.0001$ mL or $s_{inst}(y) = s(pH) = 0.01$, an excellent fit is confirmed because the mean $E|\hat{e}|$ and the residual standard deviation $s(\hat{e})$ are nearly the same and lower than the experimental noise $s_{inst}(y)$. Here, $E|\hat{e}| = 0.0001$ mL and $s(\hat{e}) = 0.0002$ mL are similar and both are lower than the microburette error $s(V) = 0.0001$ mL. As the bias $E(\hat{e})$ is equal to -2.6×10^{-6} in ESAB which may be taken as near to zero, no systematic error in curve fitting is expected. All residuals oscillate between lower -0.0002 mL and upper 0.0001 mL Hoaglin's inner bounds and therefore no outlying residuals lay outside these bounds. Residuals exhibit a normal distribution as confirmed by the Jarque-Berra normality test for combined sample skewness and kurtosis (*cf.* page 80 in ref. [57]), and also by the skewness $g_1(\hat{e})$ is near 0 (which is proving a symmetric distribution), and the kurtosis $g_2(\hat{e})$ is near 3 (which proves a symmetric normal distribution). Excellent fitness is indicated and the regression parameter estimates are considered sufficiently reliable. ESAB has reached constantly better fitness than HYPERQUAD and therefore it can be concluded that estimates of the dissociation constants estimated by ESAB are more reliable. The individual pair of one dissociation constant estimated with ESAB and HYPERQUAD, in which the same points of titration curve were used differ mostly on the second decimal place. The curve-fitting is significantly improved using the refinement of the group parameter L_0 , the concentration of the titrated drug Eltrombopag.

The ESAB program minimizing residuals $e_i = (V_{exp, i} - V_{calc, i})$ reaches 0.1 or 0.2 microliters and HYPERQUAD minimizing $e_i = (pH_{exp, i} - pH_{calc, i})$ reaches SIGMA value about 1 or less, thus proving an excellent fit. It may be concluded that the reliability of the dissociation constants of Eltrombopag was proven even when *group parameters* L_0 , H_T were ill-conditioned in a model. Their determination is uncertain and might lead to false estimate of *common parameters* pK_a and therefore make the computational strategy important. These group parameters can have great influence on a systematic error in the estimated pK_a and they should be refined together with common parameters pK_a . External calibration of pH of the glass electrode cell performed during titration is sufficiently accurate. Comparing two computational approaches, the ESAB and the HYPERQUAD programs, ESAB led to

better a fitness of potentiometric titration curve. The goodness-of-fit proved sufficient reliability of parameter estimates for four dissociation constants of drug Eltrombopag at 25°C.

Conclusion

Spectrophotometric and potentiometric pH-titration allowed the measurement of five dissociation constants of Eltrombopag, but worse solubility at pH above 9 at Eltrombopag concentration of micromoles also pH down 5 limits an estimation of the pK_a higher than 10 and in potentiometry lower than 5.

1) In the neutral pH the Eltrombopag occurs in water sparingly soluble form LH_3 , which is capable of protonation to form still soluble species LH_4 . The species LH_3 can be dissociated into water soluble species L . Acid-base titration of the triprotic molecule LH_3 with KOH leads to a mixture of six species H_3O^+ , OH^- , LH_3 , LH_2 , LH , L and the potassium species K^+ . The graph of molar absorption coefficients of variously protonated species according to wavelength shows that the spectrum of species LH_3 and LH_2 are of only a little different colour. The same is true for the proton chromophore at LH to LH_2 , while protonation of chromophore LH_2 to LH_3 has greater influence on chromophores in Eltrombopag and the results on the spectral change are therefore considerable.

2) We have proven that in the range of pH 2 to 10 five dissociation constants can be reliably estimated from the spectra when concentration of Eltrombopag is less than $10^{-4}M$. Although the change of pH somewhat less affected changes in the chromophore, five mixed dissociation constants at an ionic strength $I = 0,005$ can be reliably determined with REACTLAB and SQUAD84 reaching the similar values with both programs. From a dependence on ionic strength the thermodynamic dissociation constants were estimated at 25°C (Fig. 8): $pK_{a1}^T = 2.69$, $pK_{a2}^T = 6.97$, $pK_{a3}^T = 7.13$, $pK_{a4}^T = 7.65$, $pK_{a5}^T = 8.30$

Fig. 8

3) Four dissociation constants of Eltrombopag in concentration of 5 micromoles were determined by regression analysis of potentiometric titration curves without adjusting the ionic strength $I = 0.005$ and using ESAB and HYPERQUAD, reaching the similar values with both programs $pK_{a2} = 6.59(01)$, $pK_{a3} = 7.56(04)$, $pK_{a4} = 8.48(59)$, $pK_{a5} = 9.29(34)$ at 25°C (Table 3). The standard deviations in the last valid unit number are in the brackets.

4) Prediction of the dissociation constants of Eltrombopag was performed using MARVIN program to specify protonation locations to give the values in Table 1. Comparing two predictive with two experimental techniques it may be concluded that the prediction programs often vary considerably in an estimation of pK_a . It was proven that the most reliable regression estimate of the dissociation constants comes from the reliable experimental data. In case of close dissociation constants the higher degree of uncertainty in estimates should be expected and therefore usually two independent instrumental methods should be to applied and the results calculated using several independent programs to compare.

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Figures captions:

Fig. 1 Structural formula of Eltrombopag

Fig. 2 Structural formula with protonated ionization sites in Eltrombopag. The whole molecule of Eltrombopag was subdivided into four auxiliary fragments containing functional groups on which protonation occurred. These predicted pK_a values served to compare with predicted values throughout the structure of the Eltrombopag molecule

Fig. 3 The 3D-absorbance-response-surface concerning 71 measured absorption spectra of protonation equilibria for $9,5 \cdot 10^{-5}$ M Eltrombopag in dependence on pH at 25°C represents the input for the SQUAD84 and SPECFIT/32 programs (upper part). The Cattell's scree plot of the Wernimont-Kankare procedure for the determination of the rank of the absorbance matrix of Eltrombopag $k^* = 6$ leads to six light-absorbing species in the mixture, $n_c = 6$, with the use of Kankare's $s(A)$, RSD and RSM (lower part)

Fig. 4 In a spectra set the five analytical wavelengths *a* through *e* were selected at which the absorbance-pH curves were plotted. Six following figures from pH = 3.10 through pH = 10.02 show the consecutive deprotonation response in spectra, when each spectrum was deconvoluted on the spectrum of differently protonated species in mixture of Eltrombopag. At pH = 3.10 the species LH₅ accompanied species LH₄ predominates in the solution. At pH = 6.14 together with the species LH₃ two species LH₅, LH₄ exhibit absorption bands at the same wavelength of absorption maximum λ_{\max} . At pH = 7.61 the experimental spectrum is decomposed to three absorption bands concerning the species LH₃ which dissociate to species LH₂ and LH. At pH = 8.85 and 9.16 the species L occurs with species LH₂ and LH, and concentration of L in the solution increases up to pH = 10.02

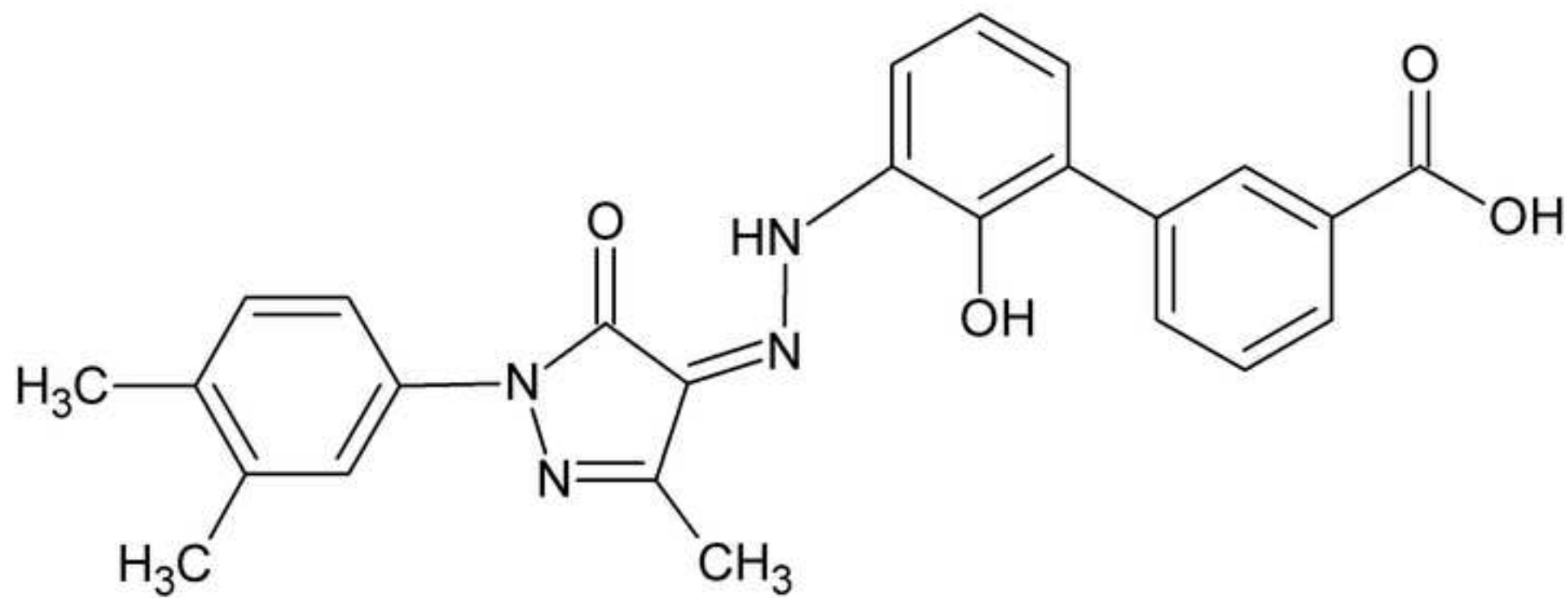
Fig. 5 Inputs of the absorbance data matrix show four regions of selected wavelengths of the 2D-absorbance-response spectra set for $9,5 \times 10^{-5}$ M Eltrombopag in dependence on pH at 25°C

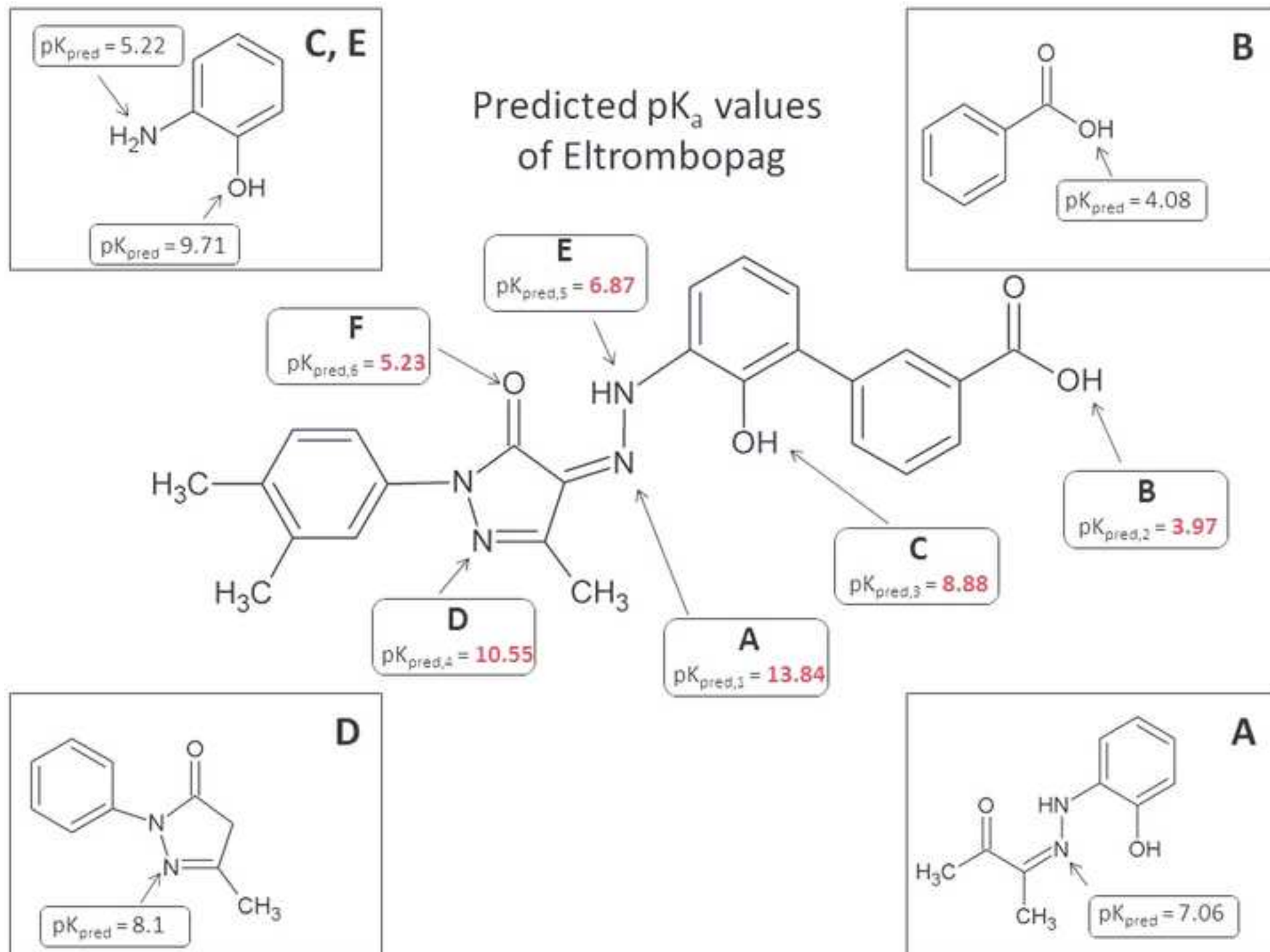
Fig. 6 The graph of molar absorption coefficients for six variously protonated species of Eltrombopag *versus* wavelength (upper part). Corresponding distribution diagram of the relative concentration of six variously protonated species for Eltrombopag (lower part), (SPECFIT, ORIGIN)

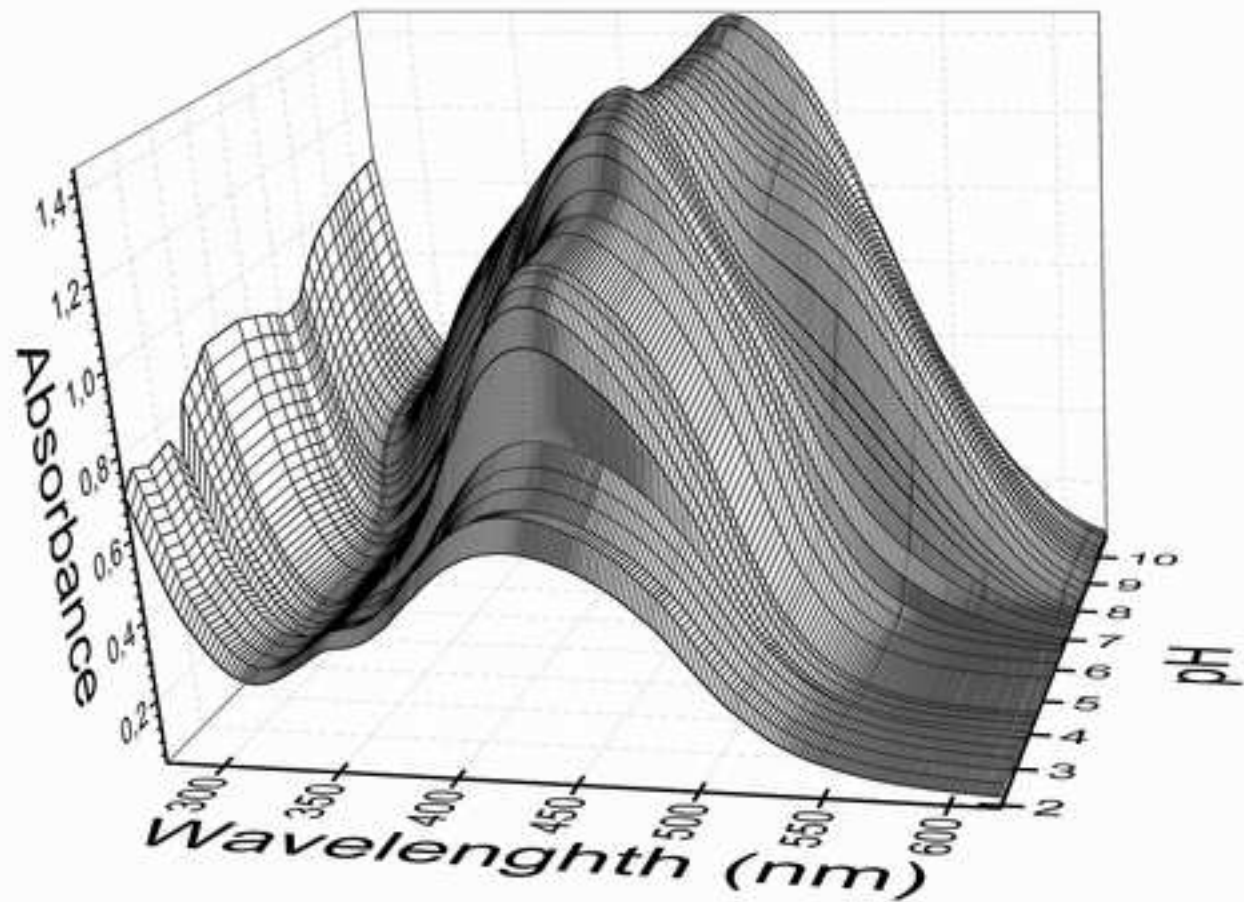
Fig. 7 Protonation equilibria of Eltrombopag analyzed with ESAB: the pH-potentiometric titration curve of acidified Eltrombopag plus HCl titrated with KOH is plotted with the Bjerrum protonation function indicating pK values (upper part). The distribution diagram of a relative presentation of variously protonated species L, LH, LH₂, LH₃ and LH₄ of Eltrombopag in dependence on pH at 25°C (lower part). Charges of ions were for a sake on simplicity omitted in the graph, (ESAB, HYPERQUAD, ORIGIN)

Fig. 8 Dependence of the mixed dissociation constants of Eltrombopag on the square root of the ionic strength for five dissociation constants at 25°C

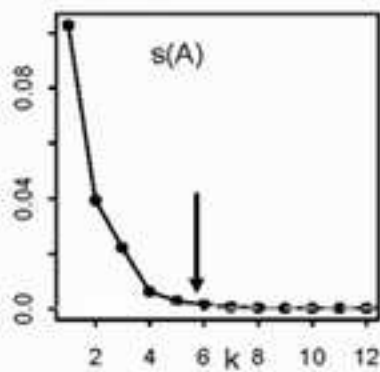
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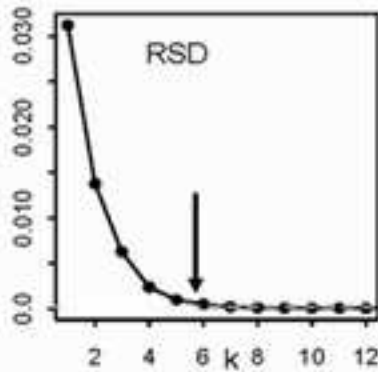




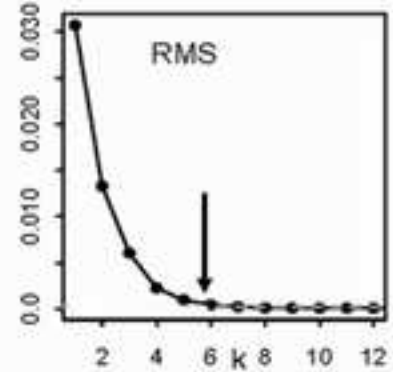
Kankare - residual standard deviation



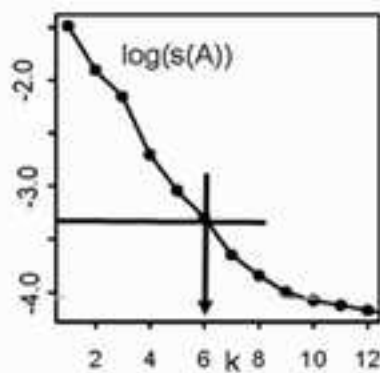
Residual standard deviation



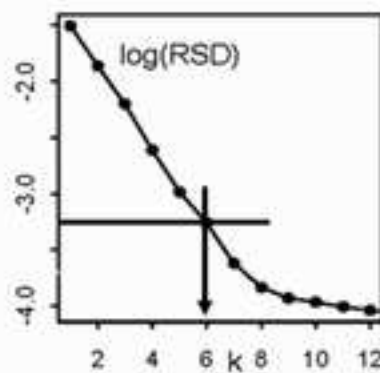
Root mean square error



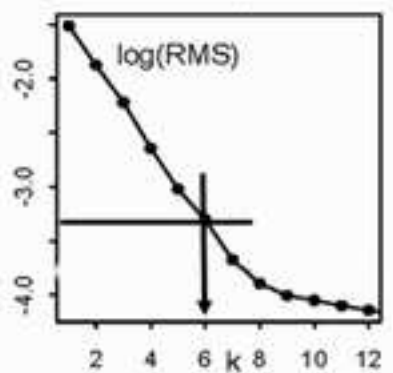
Kankare - residual standard deviation

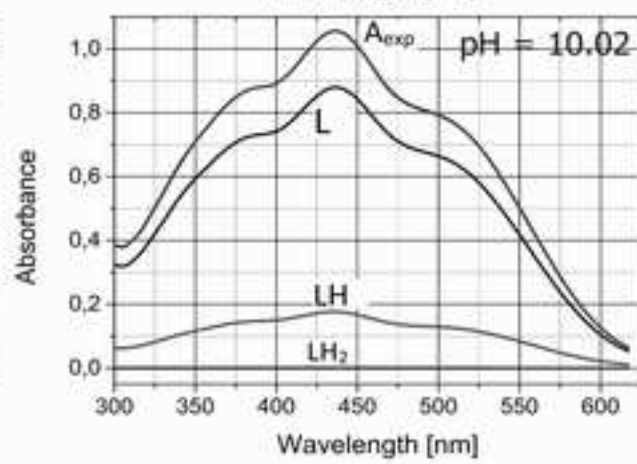
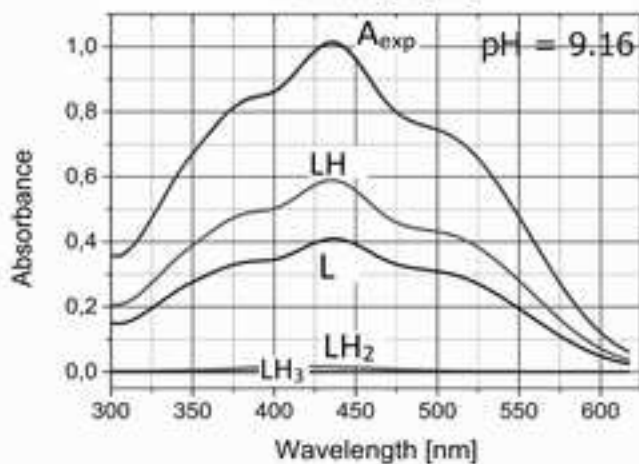
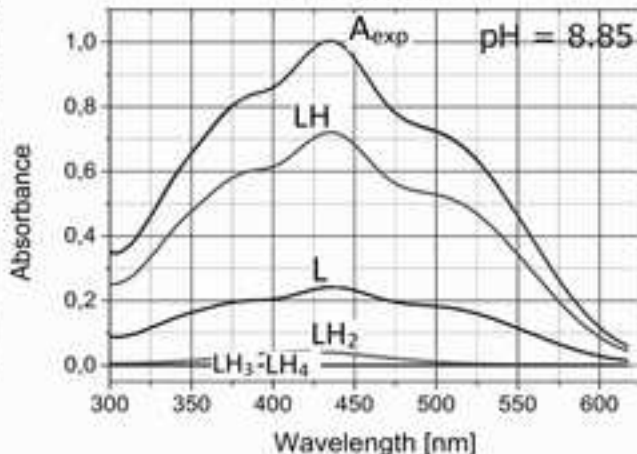
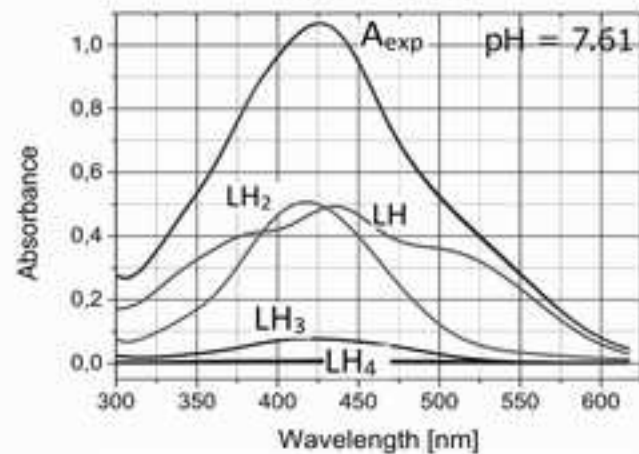
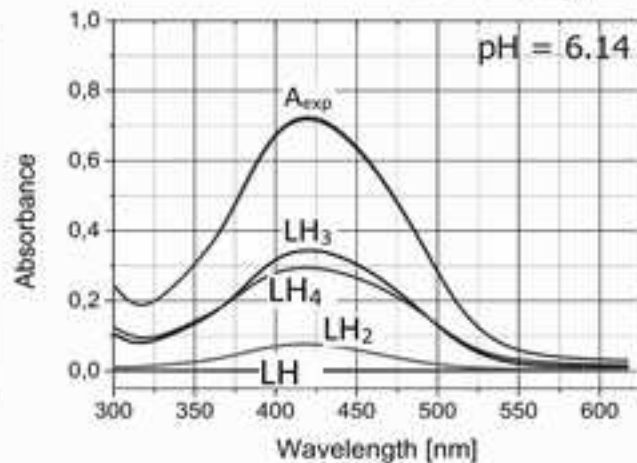
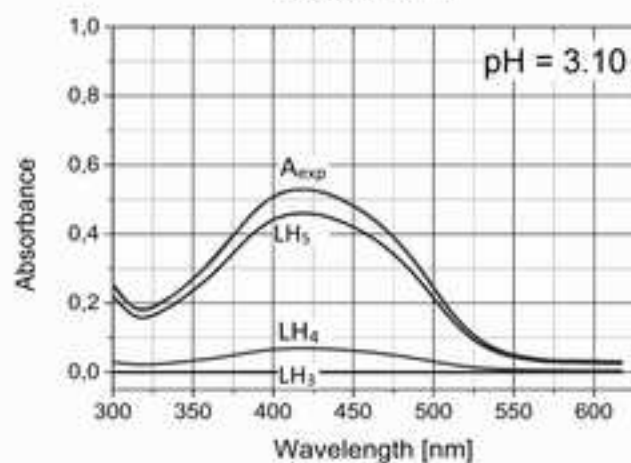
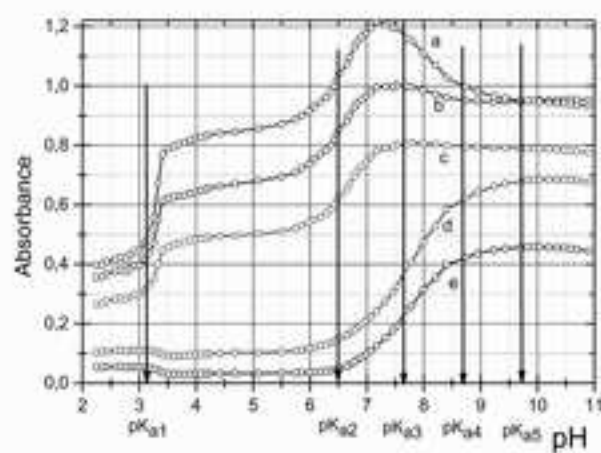
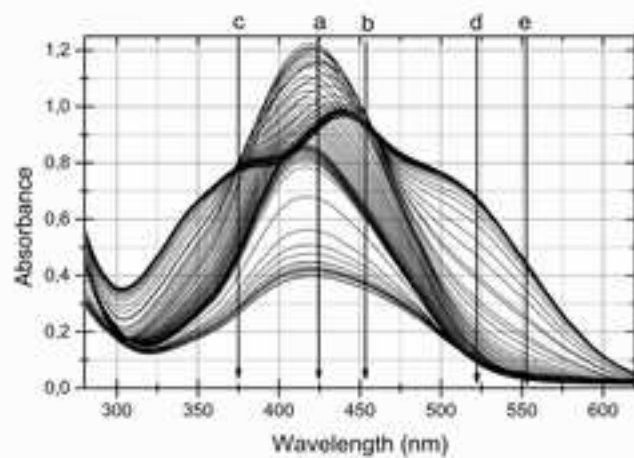


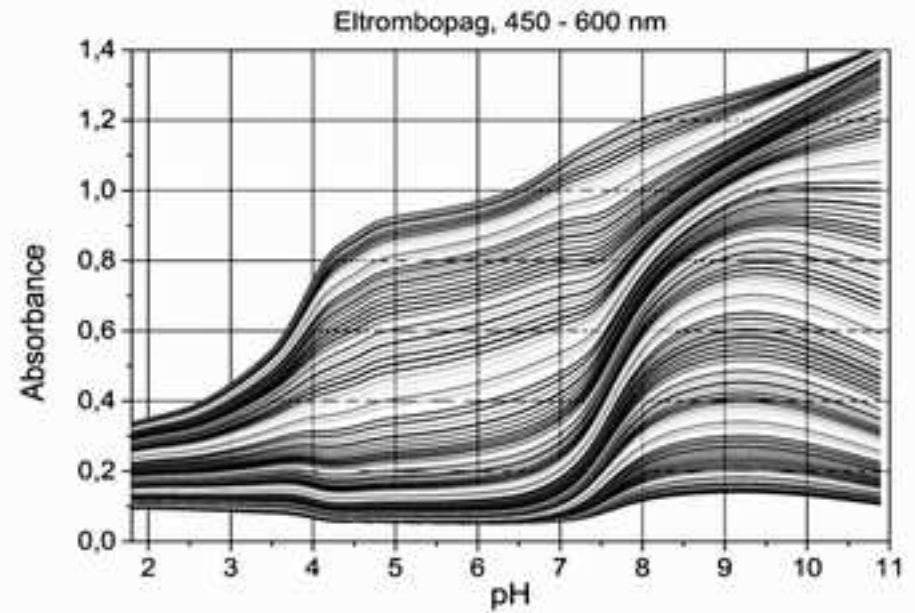
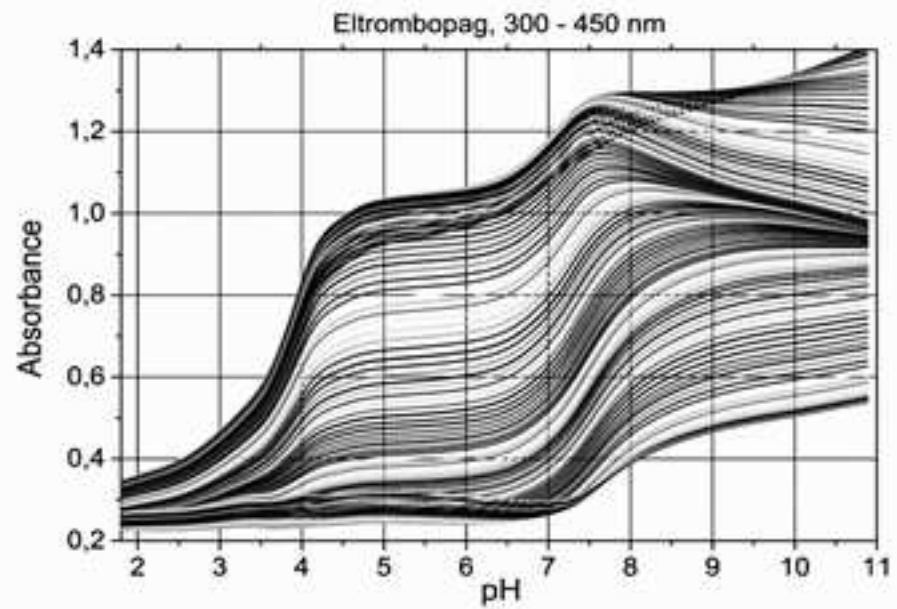
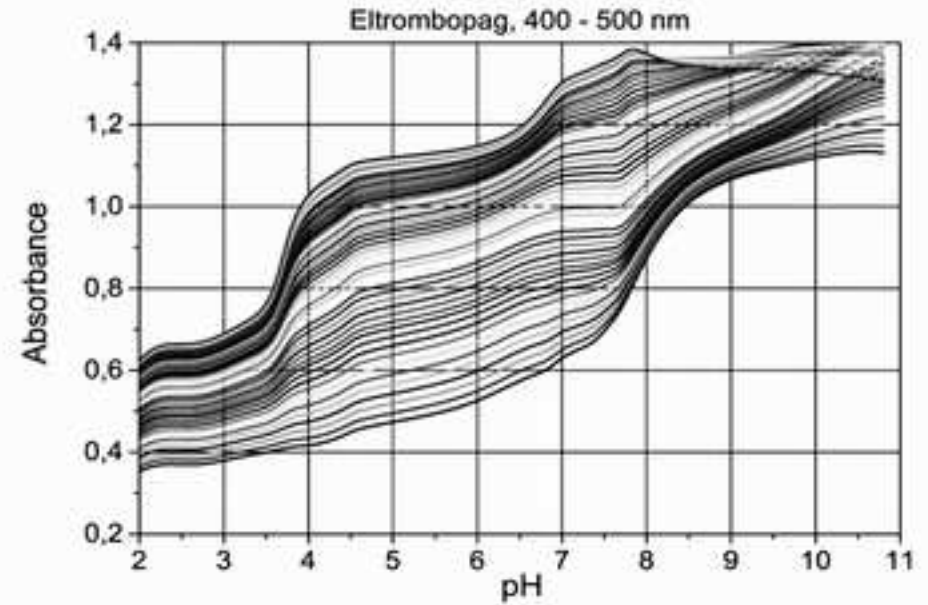
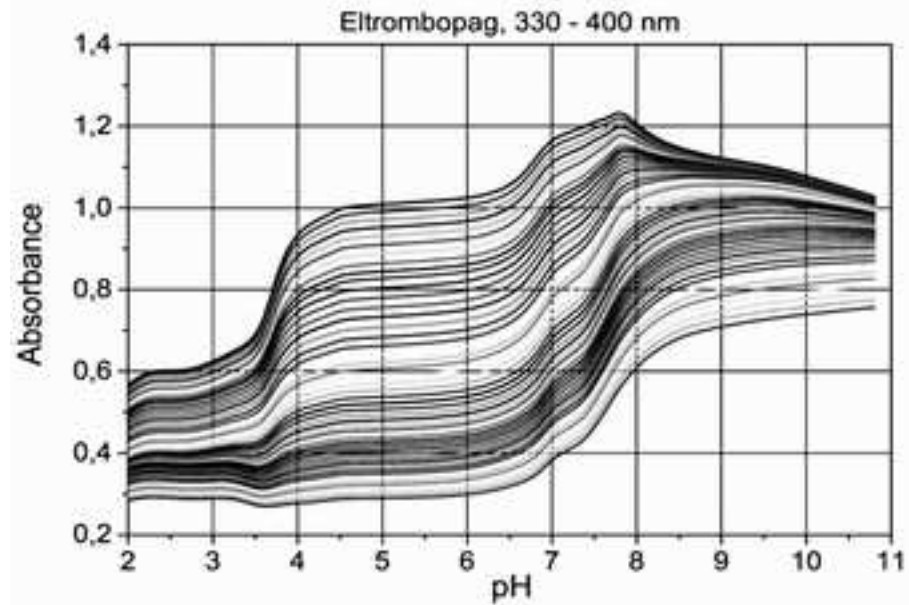
Residual standard deviation

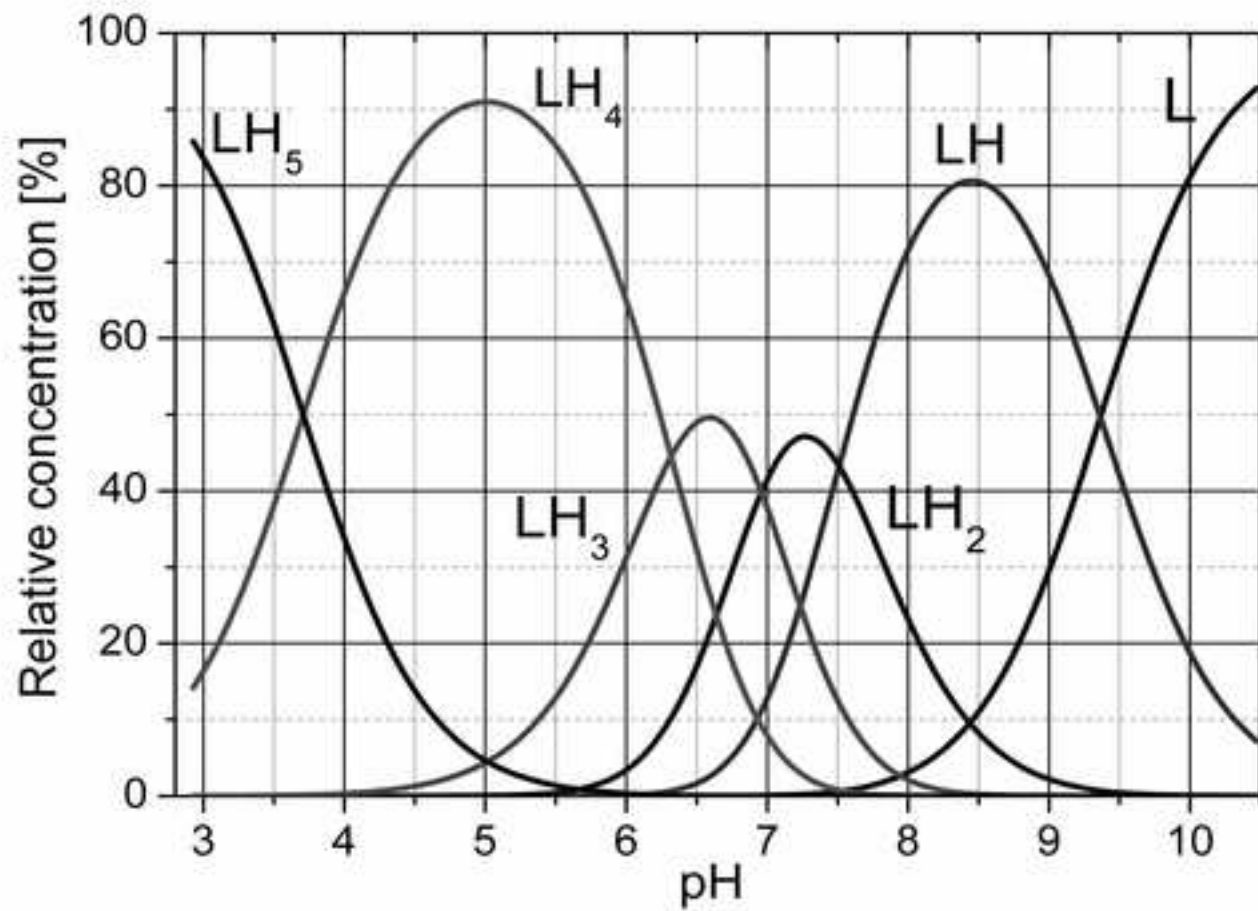
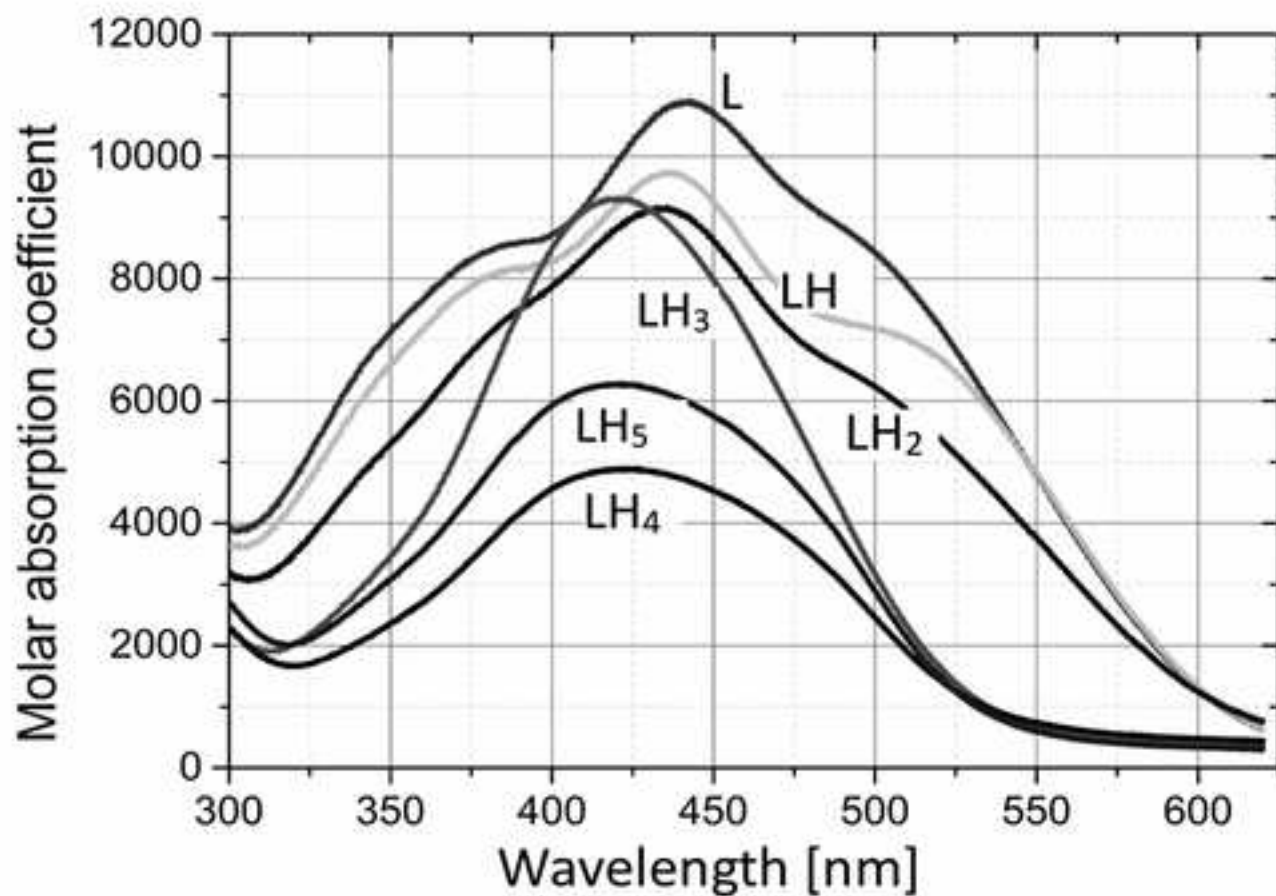


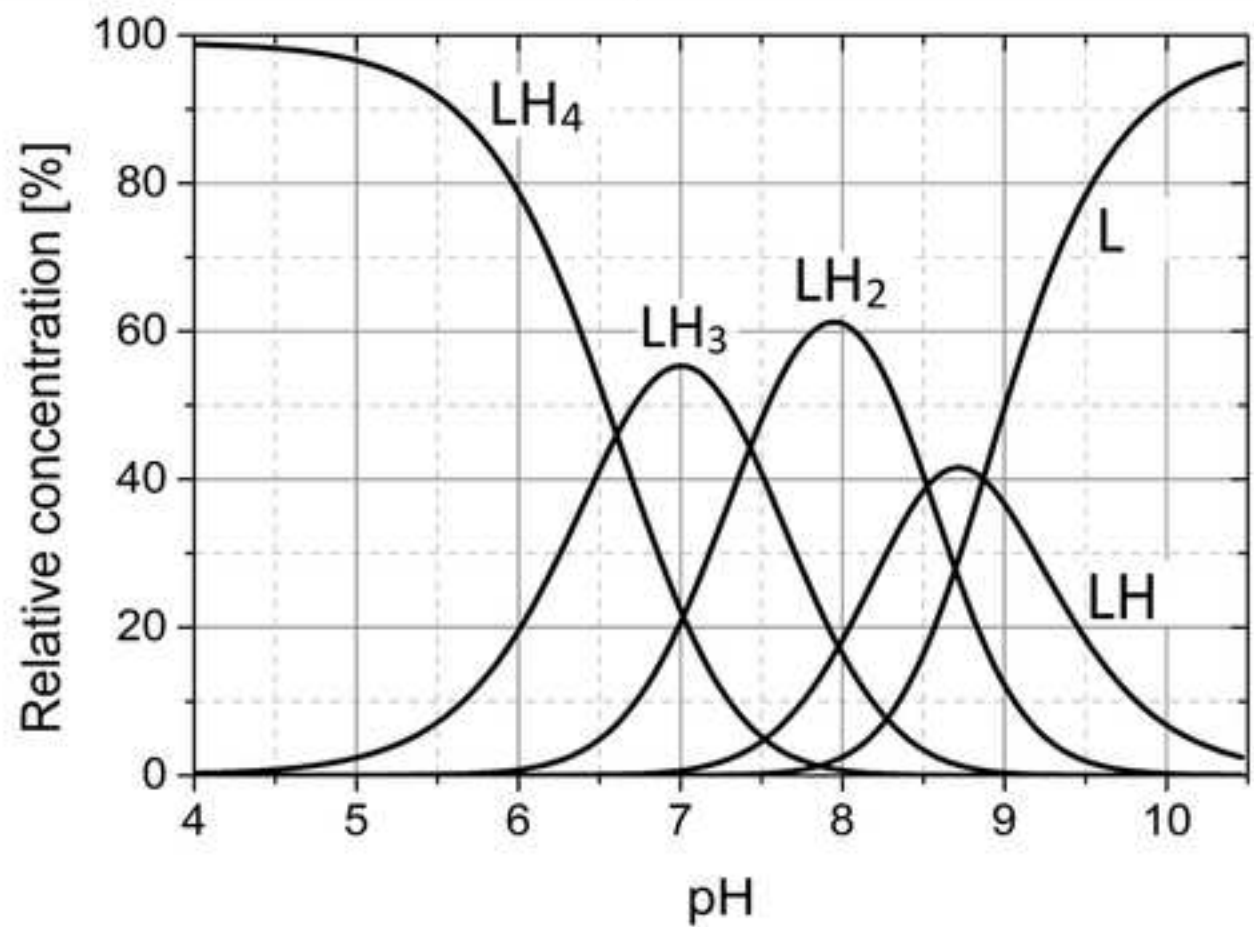
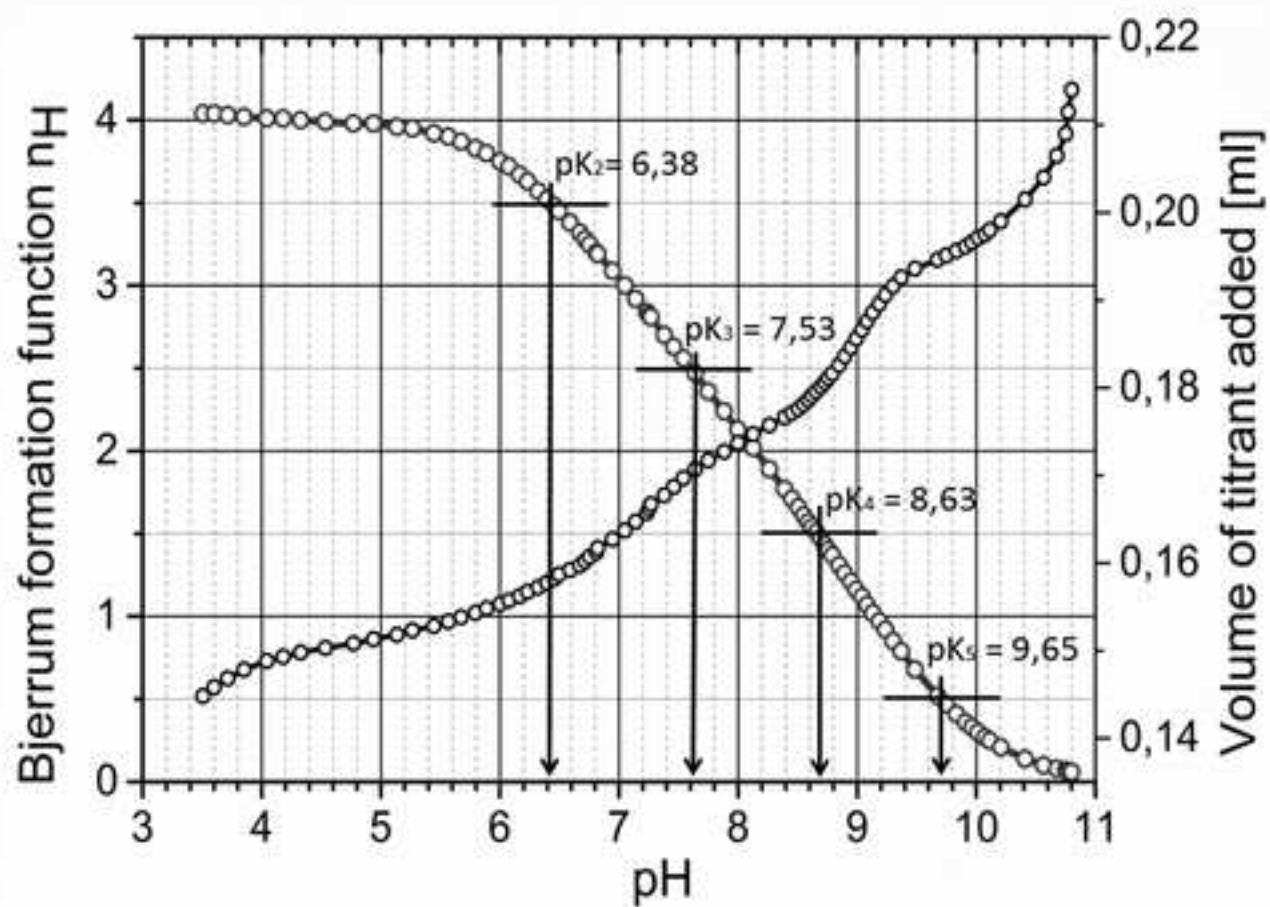
Root mean square error

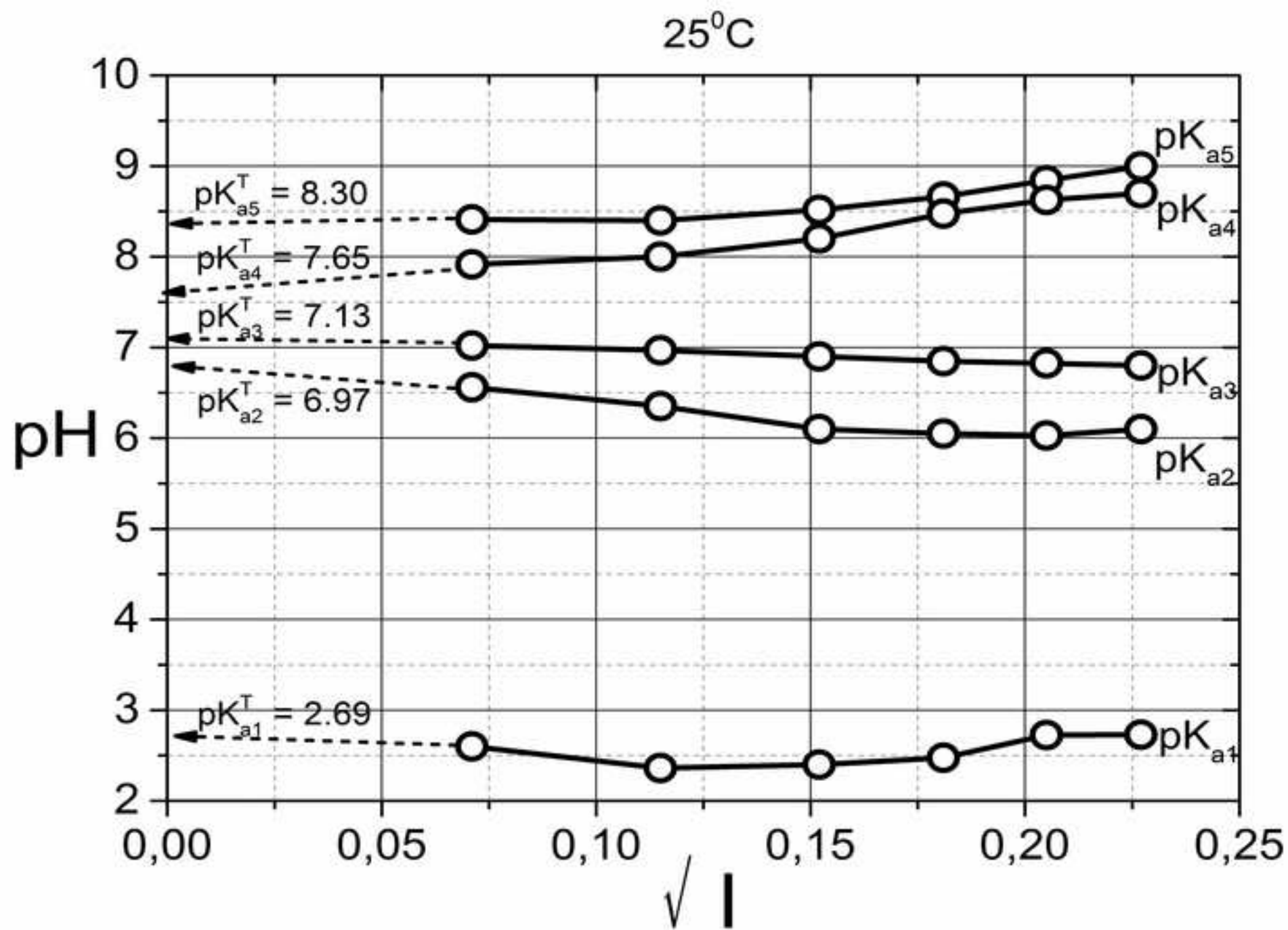












Eltrombopag

25C

pK1 = 2,69

pK2 = 6,97

pK3 = 7,13

pK4 = 7,65

pK5 = 8,30

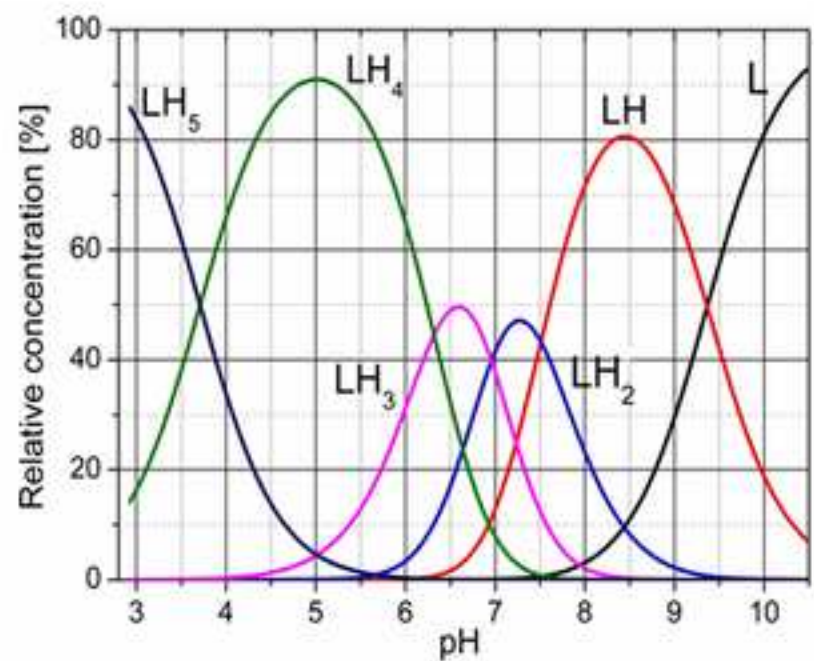
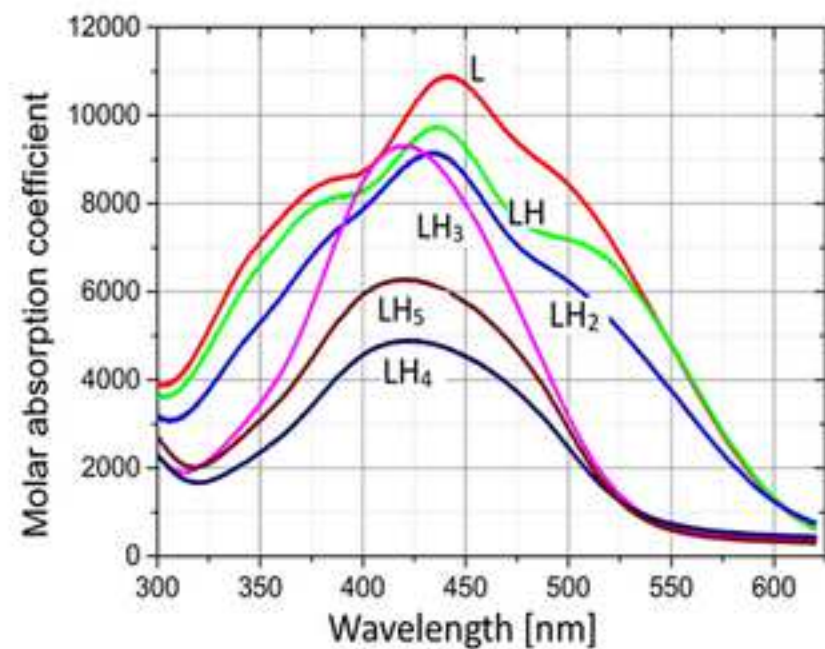
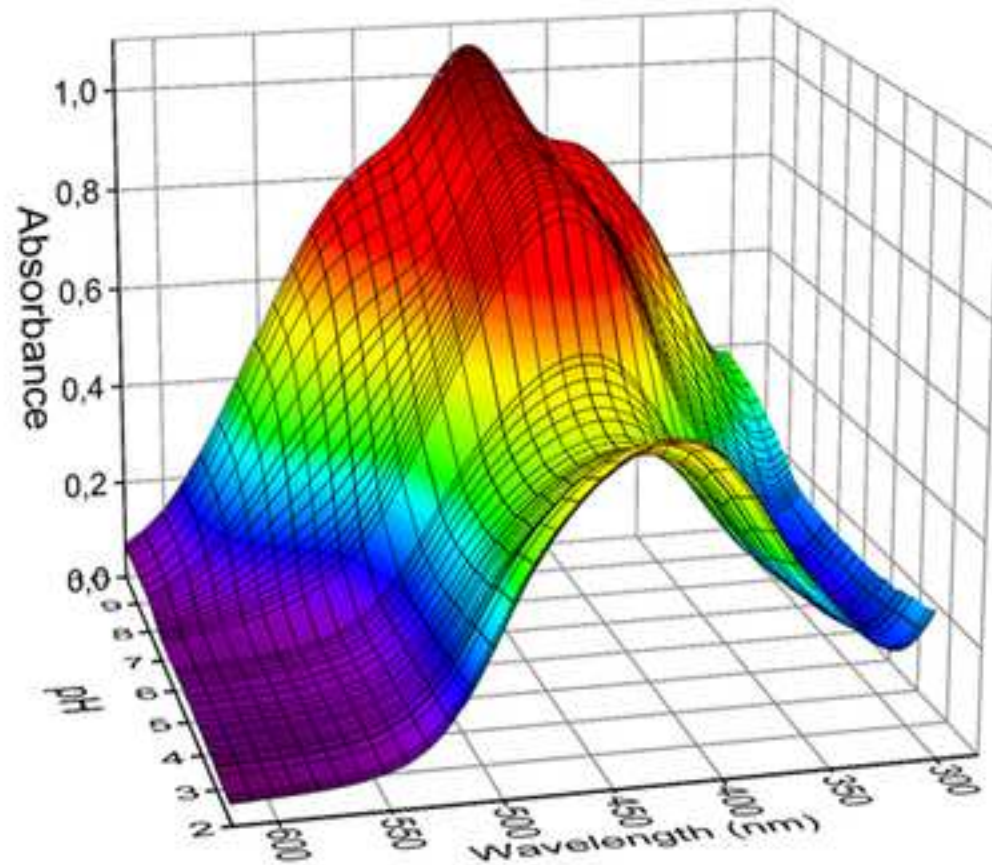
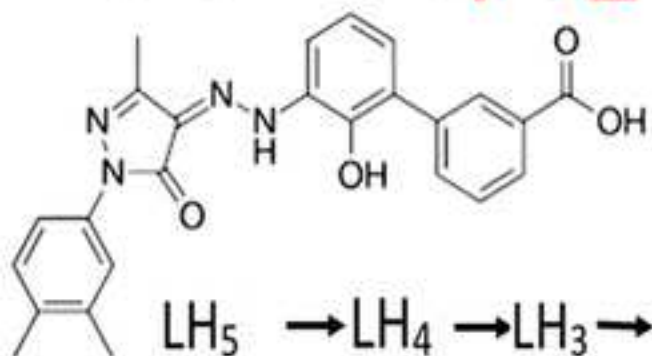


Table 1 Predicted dissociation constants at the suggested ionization sites **A** through **F** of the molecule Eltrombopag with the use of program MARVIN

Tautomer, Protonated ionization sites	p<i>K</i>_{a1}	p<i>K</i>_{a2}	p<i>K</i>_{a3}	p<i>K</i>_{a4}	p<i>K</i>_{a5}	p<i>K</i>_{a6}
1 A, B, C	0.13	3.97	9.36	-----	13.13	-----
2 A, B, C, D	4.70	3.61	9.40	-----	14.56	-----
3 B, C, D	0.51	3.97	7.20	6.22	-----	-----
4 B, C, F	-----	3.94	12.17	1.53	-----	5.23
5 B, C	-0.80	3.97	7.68	-----	-----	-----
6 B, C, D, F	1.05	3.96	7.78	10.15	-----	5.54
7 B, C, D	-----	4.00	7.68	2.89	-----	-----
Auxiliary fragments	For B is 4.08	For E is 5.22	For A is 7.06	For D is 8.1	For C is 9.71	Not found
Site in molecule of Eltrombopag	For B is 3.97	For E is 6.87	For A is 13.84	For D is 10.55	For C is 8.88	For F is 5.23

Table 3 Four dissociation constants pK_{a2} , pK_{a3} , pK_{a4} , pK_{a5} of Eltrombopag when their standard deviations in last valid digits are in parentheses. The reliability of parameter estimation is proven with a goodness-of-fit statistics: the sigma in pH units from HYPERQUAD, the bias or arithmetic mean of residuals $E(\hat{e})$ [mL], the residual mean $E|\bar{e}|$ [mL], the standard deviation of residuals $s(\hat{e})$ [mL], the residual skewness $g_1(\hat{e})$ and the residual kurtosis $g_2(\hat{e})$ proving a Gaussian distribution and Jarque-Berra normality test. ESAB and HYPERQUAD refinement of common and group parameters for a titration of Eltrombopag with HCl and KOH were performed.

* means that statistics is not available in the program's output.

Common parameters refined: pK_{a2} , pK_{a3} , pK_{a4} , pK_{a5} . **Group parameters refined:** L_0 . **Constants:** $H_T = -0.8138 \text{ mol. dm}^{-3}$, $t = 25.0 \text{ }^\circ\text{C}$, $pK_w = 13.9799$, $V_0 = 20.22 \text{ mL}$, $s(V) = s_{\text{inst}}(y) = 0.0001 \text{ mL}$, $s(pH) = s_{\text{inst}}(y) = 0.01$, $I_0 = 0.004$ (in vessel), $I_T = 0.8138$ (in burette KOH) or 1.0442 (in burette HCl).

	HYPERQUAD	ESAB
Number of points n	39	39
pK_{a2} (s_1), $H_4L = H^+ + H_3L$	6.60(13)	6.59(01)
pK_{a3} (s_2), $H_3L = H^+ + H_2L^-$	7.38(12)	7.56(04)
pK_{a4} (s_3), $H_2L = H^+ + HL$	8.46(09)	8.48(59)
pK_{a5} (s_4), $HL = H^+ + L$	8.77(07)	9.29(340)
L_0 concentration of drug [mol. dm^{-3}]	4.6E-04	1.7E-04
Goodness of fit test by the statistical analysis of residuals in pH (HYPERQUAD) or in V [mL] (ESAB)		
Sigma in pH units (HYPERQUAD)	0.615 pH units	*
Bias, arithmetic mean of residuals $E(\hat{e})$	-9.05E-03 pH units	6.9E-05 mL
Residual mean, $E \bar{e} $	0.0228 pH units	0.0001 mL
Standard deviation of residuals, $s(\hat{e})$	0.0335 pH units	0.0004 mL
Residual skewness, $g_1(\hat{e})$	1.08	0.14
Residual kurtosis, $g_2(\hat{e})$	8.42	3.39
Jarque-Berra normality test	Rejected	Accepted

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Editorial Office

25. 04. 2017

Dear Sir,

enclosed I send you the manuscript and figures of our paper

Overlapping pK_a of the multiprotic hemostyptic Eltrombopag using UV/VIS multiwavelength spectroscopy and potentiometry

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Novelty:

(a) Scientific motivation: The hemostyptic Eltrombopag treats low blood platelet counts in adults with chronic immune idiopathic thrombocytopenia ITP. It works by causing the cells in the bone marrow to produce more platelets. Medicine and pharmacology needs physical constants (spectra, pK_a 's, solubility, etc.) of new drugs.

(b) Relevance: To date, no spectra, no dissociation constants or no pH-distribution diagrams of the relative concentration of variously protonated ions of the new drug Eltrombopag have been published in literature.

(c) Significance of the obtained results: Five dissociation constants were estimated using a computer-assisted pH-absorbance spectra analysis when the reliability of the used regression analysis was proven. Due to the strongly overlapping protonation equilibria and a limited solubility behind the spectrophotometric, potentiometric data analysis was also used to evaluate mixed pK_a . By using a dependence on the ionic strength, thermodynamic dissociation constants at 25°C were estimated.

Yours sincerely

Prof. RNDr. Milan Meloun, DrSc.