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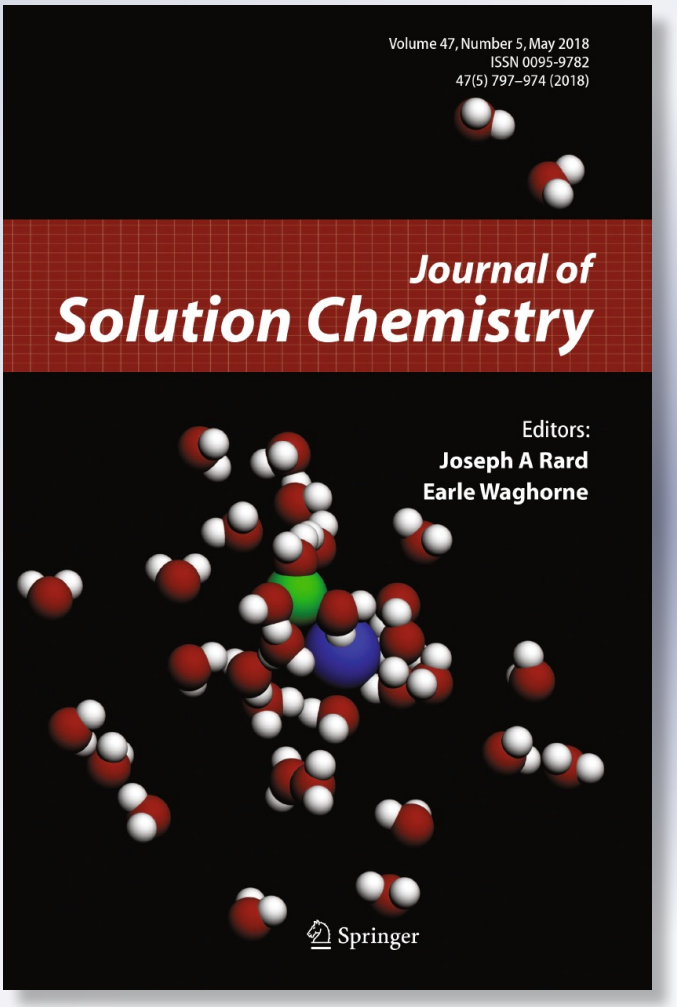

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The Overlapping Thermodynamic Dissociation Constants of the Antidepressant Vortioxetine Using UV–VIS Multiwavelength pH-Titration Data

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Abstract Potentiometric and spectrophotometric pH-titrations of the antidepressant drug Vortioxetine were compared for dissociation constants determinations. Vortioxetine is an atypical antidepressant, i.e., it is a serotonin modulator and stimulator. Depressive disorders are common mental health conditions that are thought to be caused by an imbalance in serotonin and norepinephrine in addition to multiple situational, cognitive, and medical factors. A chemometrics approach to the nonlinear regression of the pH-spectra (REACTLAB, SQUAD84) and pH-titration (ESAB) were used to determine the two overlapping dissociation constants. A sparingly soluble neutral base LH of Vortioxetine hydrobromide was protonated to form the two still-soluble cations LH_2^+ and LH_3^{2+} in pure water. In the range of pH (5–10), the two dissociation constants could be reliably estimated from small changes in the spectra of 9.2×10^{-5} mol·dm^{-3} Vortioxetine. Although the change of pH affected changes in the chromophore to a small extent, two thermodynamic dissociation constants were estimated: $pK_{a1}^T = 7.22$ and $pK_{a2}^T = 8.67$ at 25 °C and $pK_{a1}^T = 7.27$ and $pK_{a2}^T = 8.79$ at 37 °C. The graph of molar absorption coefficients of variously protonated species as a function of wavelength shows that the spectra of species LH_2^+ and LH vary in color, while protonation of the chromophore LH_2^+ to LH_3^{2+} has less influence on the chromophores of the Vortioxetine hydrobromide molecule. Two

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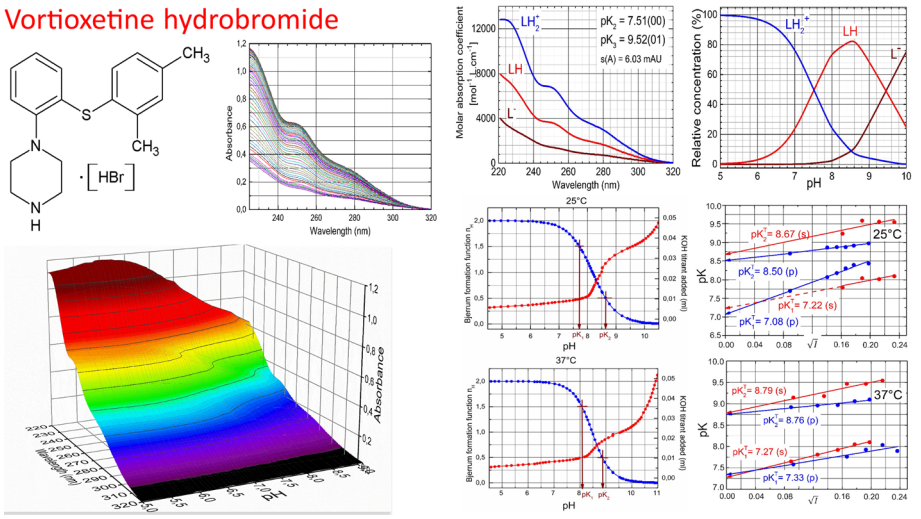
thermodynamic dissociation constants of 3×10^{-4} mol·dm^{-3} Vortioxetine were determined by regression analysis of the potentiometric titration curves, $pK_{a1}^{T} = 7.08$ and $pK_{a2}^{T} = 8.50$ at 25 °C and $pK_{a1}^{T} = 7.33$ and $pK_{a2}^{T} = 8.76$ at 37 °C. A prediction of the dissociation constants of Vortioxetine was carried out using the MARVIN and ACD/Percepta programs and only two dissociation constants were proposed theoretically.

Graphical Abstract



Keywords Dissociation constants · Vortioxetine · Spectrophotometric titration · REACTLAB · SQUAD84 · ESAB

1 Introduction

Vortioxetine hydrobromide (trade name Brintellix, Trintellix in Canada) with the IUPAC name 1-(2-((2,4-dimethyl-phenyl)thio)phenyl)piperazine hydrobromide, a molecular formula of $C_{18}H_{22}N_2S{\cdot}HBr$, and molecular weight of 379.36 g·mol^{-1}, is an atypical antidepressant (a serotonin modulator and stimulator) made by Lundbeck [1] and Takeda [2]. It relieves depression symptoms by increasing serotonin concentrations in the brain, by inhibiting its reuptake in the synapses and by modulating (activating certain receptors while blocking, or antagonizing, others) certain serotonin receptors. This puts it in the class of atypical antidepressants known as serotonin modulators and stimulators [3, 4]. On 30 September 2013 it was approved by the U.S. FDA for the treatment of major depressive disorders in adults. Vortioxetine was also examined as a treatment for generalized anxiety disorder but was not found to be superior to the placebo [5]. Depressive disorders, including major depressive disorders, are common mental health conditions thought to be caused by an imbalance in serotonin (5-HT) and norepinephrine, in addition to having multiple situational, cognitive, and medical factors [6]. The most common side effects reported with Vortioxetine are nausea, diarrhea, dry mouth, constipation, vomiting, flatulence, dizziness, and sexual dysfunction. Vortioxetine used alone in a high dose or in

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combination with other medications, such as other antidepressants, can produce a potentially life-threatening drug reaction known as serotonin syndrome [7].

One of the most important physico-chemical characteristics of the drug is its pK_a value, which can be used to determine the extent of drug absorption and is applied in pharmacokinetic and bioavailability studies [8–10]. The pK_a value allow prediction of the degree of ionization of the molecule at a particular pH by using the Henderson–Hasselbach equation [11] and is vital for understanding many properties essential to drug development. The extent of ionization of a compound plays a crucial role in the characterization of its absorption, distribution, metabolism and excretion (ADME) profile. In the case of poorly water-soluble drugs, the significance of pK_a increases intensely, particularly within the context of its ADME properties [12]. It is an essential parameter in drug discovery, particularly in physiological systems where the ionization state will affect the rate at which the compound is able to diffuse across membranes including the blood–brain barrier [13]. The level of general interest in such ionization phenomena is evident from the number of recent publications on the topic [14–16]. pK_a values can be either experimentally measured or theoretically predicted:

Many new drugs are poorly soluble in aqueous solutions and thus conventional potentiometric pH-metric determination of dissociation constants of these compounds can often be difficult. Spectrophotometry, and UV-titration (also called UV-metric [17]) in particular, is a convenient method to determine pK_a values in very dilute aqueous solutions since it requires relatively simple equipment and can work with sub-micromolar compound concentration (about 10^{-5}–10^{-6} mol·dm^{-3}). The compound should possess pH-dependent light absorption due to the presence of a chromophore in proximity to the ionization center, cf. Refs. [18–20]. In previous works [21–24] the authors have shown that the spectrophotometric method in combination with suitable chemometric tools can be used to determine dissociation constants pK_a even for sparingly soluble drugs. The most relevant algorithms are SQUAD84 [19] and REACTLAB [25]. This approach fails for the majority of active pharmaceutical ingredients (APIs) due to their low solubility. Improving the solubility of APIs may be achieved by a preparation of its salts. In general, salts have higher solubility than co-crystals, which are in turn more soluble than the pure APIs. Vortioxetine hydrobromide contains an acidic proton that is transferred to the secondary N atom on the piperazine ring of Vortioxetine, forming a charge-assisted hydrogen bond [26].

Nine commercially available or free programs for predicting ionization constants were compared [27]. Meloun et al. [28] used the REGDIA regression diagnostics algorithm written in S-Plus [29] to critically examine the accuracy of pK_a predictions with two programs (ACD/Percepta [1, 30], Marvin [30]) being considered the best. Balogh et al. [30] also found the most predictive and reliable predictors to be MARVIN and ACD/Percepta. Since knowledge of the acid dissociation constants is essential for the development of new compounds with biological activity, we have determined in this paper the overlapping pK_a values (i.e., $|pK_{a2} - pK_{a1}| < 3$) of the antidepressant drug Vortioxetine in water.

The aim of our study was to examine the spectrophotometric UV-metric analysis of the pH-absorbance matrix with small changes in spectra and to carry out a potentiometric pH-metric determination of the protonation model to find suitable conditions for a reliable regression determination of the overlapping dissociation constants. The experimental results were also evaluated by two different LFER-based pK_a predictions tools, the MARVIN and ACD/Percepta software.

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2 Computational Details

The general procedure for the spectrophotometric study of the protonation equilibria, called the UV-metric spectra analysis, was previously described [23, 24] by the following brief scheme:

(1) *Instrumental error of absorbance measurement, $s_{inst}(A)$, and a number of light-absorbing species* The INDICES program [31] estimates the minimum numbers of light-absorbing species n_c using the Wernimont–Kankare method of factor analysis [20, 31] and the instrumental error of absorbance measurement $s_{inst}(A)$. The second moment matrix is formed from the product of the absorbance matrix $\boldsymbol{A}$ and its transpose $\boldsymbol{A}^T$ giving a symmetric (square) matrix $\boldsymbol{M}$ of order n_s and rank n_c, $\text{rank}(\boldsymbol{M}) \leq n_c$. Applying the eigenvalues g_j of matrix $\boldsymbol{M}$, *the residual standard deviation of absorbance* $s_k(A)$ is estimated as $s_k(A) = \left\{ \left[\text{tr}(M) - \sum_{i=1}^{k} \alpha_i \right] / (n_w - k) \right\}^{1/2}$, where tr($\boldsymbol{M}$) is a trace of the matrix $\boldsymbol{M}$, and r is the estimated number of components in a mixture. The Cattell's plot of eigenvalues shows the values $s_k(A)$ for the different number of components k being plotted against an integer k, $s_k(A) = f(k)$, and the number of light-absorbing components is such an integer $n_c = k$ for which $s_k(A)$ is close to the instrumental standard deviation of absorbance, $s_{inst}(A)$. Let the precision of the absorbance measurement be given by the standard deviation of absorbance for the spectrophotometer used, $s_{inst}(A)$. We then determine that, if $s_k(A) < s_{inst}(A)$, it is possible to say that $n_c < k$, details may be found on page 104 in Ref. [32].

(2) *Theoretically predicted pK_a estimates* Two programs, MARVIN and ACD/Percepta, provide a set of powerful tools for theoretical predicting pK_a values on the basis of the structural formula of the compound.

(3) *Choice of computational strategy* Two programs for the numerical analysis of spectra were used, the hard modelling technique SQUAD84 [32] and the soft-modelling technique REACTLAB [25, 33, 34].

(4) *Diagnostics of the chemical model building and testing* A detailed procedure of the graphical and numerical analysis of residuals is described in [21, 23, 24] and is in brief stated as:

   (a) *The physical meaning of parametric estimates* The physical meaning of the dissociation constants and associated molar absorptivities is examined: $pK_{a,i}$ and ε_i should be neither too high nor too low, and ε_i should not be negative. The empirical rule that is often used is that a parameter is considered to be significant when the relation $s(\beta_j) \times F_\sigma < \beta_j$ is met and where F_σ is equal to 3 at a 99.9% statistical probability level.

   (b) *The physical meaning of the species concentrations* Physical constraints are generally applied to concentrations of species and their molar absorptivities as they must be positive numbers. The free concentrations of the basic components and the variously protonated species of the chemical model should show realistic molarities, i.e., greater than 10^{-8} mol·dm^{-3}.

   (c) *Goodness-of-fit test using the statistical analysis of residuals* To identify the "best" or true chemical model when several are possible or proposed, and so to establish whether the chemical model represents the data adequately, the residuals vector $\boldsymbol{e}$ should be carefully analyzed. One of the most important statistics calculated is *the standard deviation of the absorbance*, $s(A)$,

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estimated at the termination of the minimization process as $s(A) = \sqrt{U\min/df}$ where $U_{\min}$ stands for the residuals-square-sum function at its minimum and df is the degree of freedom, cf. page 101 in Ref. [32] and page 290 in Ref. [35].

(d) *The deconvolution of spectra* The resolution of each experimental spectrum into the spectra for the individual variously protonated species shows whether the experimental design, i.e., the proposed pH range, was sufficient. If for a particular pH value the spectrum consists of just a single component, further spectra for that pH or a similar value would be redundant even though they should improve the precision. In concentrations or pH ranges where more components contribute significantly to the spectrum, several spectra should be measured. A minor species has a relative concentration in a distribution diagram of less than 5% of the total concentration of the basic component c_L.

(e) *The signal-to-error ratio in the analysis of small spectra changes* [24] The absorbance change Δ_{ij} is the absorbance difference for the jth-wavelength at the ith-spectrum $\Delta_{ij} = A_{ij} - A_{i,\text{acid}}$, where $A_{i,\text{acid}}$ is the limiting spectrum of the acid form of the drug measured and this Δ_{ij} is then divided with the instrumental standard deviation $s_{\text{inst}}(A)$ and the resulting ratio $\Delta/s_{\text{inst}}(A)$ is called *the signal-to-error ratio SER* of the spectra studied. The *SER* ratio is examined for all absorbance matrix elements in the whole range of wavelength λ. It has been proven that when the ratio $\Delta/s_{\text{inst}}(A)$ is equal to or greater than 10, factor analysis is able to predict the correct number of components in the equilibrium mixture.

(f) *Determination of the thermodynamic dissociation constants* The limited form of the Debye–Hűckel equation for the data for aqueous solutions at 25 °C is applied so that the mixed dissociation constant pK_a is a dependent variable while the ionic strength, I, is the independent variable.

Potentiometric pH-metric determination using the ESAB program has been previously described [36, 37].

3 Materials and Methods

3.1 Materials

Vortioxetine, donated by the ZENTIVA GROUP, Ltd. (Prague) had a declared purity, checked by a HPLC method and alkalimetrically, that was always > 99%. This drug was weighed straight into a reaction vessel, resulting in a concentration of about 9.2×10^{-5} mol·dm^{-3}. Other chemicals have been previously described [22].

3.2 Apparatus

The apparatus used and both titration procedures were described in detail previously [23, 33, 34]. The experimental and computation scheme to determine the dissociation constants of the multi-component system was taken from Meloun et al. cf. page 226 in Ref. [32], and the five steps were described in detail [23]. The free hydrogen-ion concentration [H^+] was measured on the digital voltmeter (Hanna HI 3220) with a precision of ± 0.002 pH units,

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using a Theta HC 103-VFR combined glass electrode. The potentiometric titrations of drugs with potassium hydroxide were performed using the hydrogen activity scale. Standardization of the pH meter was performed using WTW standard buffers withvalues: 4.006 (4.024), 6.865 (6.841) and 9.180 (9.088) at 25 and 37 °C, respectively, in parentheses.

3.3 Software

Estimation of dissociation constants was performed by regression analysis of the UV-metric spectra analysis using the SQUAD84, REACTLAB programs and potentiometric pH-metric titration data using the ESAB program, and the spectra interpretation using the INDICES program [31]. Most graphs were plotted using ORIGIN 9 [5]. ACD/Percepta and MARVIN programs for predictions of pK_as are based on the structural formulae of the compounds.

4 Results and Discussion

The spectrophotometric UV-metric analysis of the pH-absorbance matrix and the potentiometric pH-metric determination of the protonation model found suitable conditions for a reliable regression determination of the dissociation constants.

4.1 UV-Metric Spectra Analysis

The strategy for an efficient experiment followed by spectral data treatment was according to the published tutorial [23]. A qualitative interpretation of the spectra aims to evaluate the quality of the data set, remove spurious data, and estimate the minimum number of factors that contribute aqueous species which are necessary to describe the experimental data.

4.1.1 Instrumental Error of Absorbance Measurement, $s_{inst}(A)$, and the Number of Light-Absorbing Species n_c

Vortioxetine has the molecular structure shown in Figs. 1 and 3 and several protonation equilibria were monitored spectrophotometrically to analyze the spectral data set (Fig. 2a, b). The spectral data set in the form of the absorbance matrix A, obtained at various pH values, was subjected to factor analysis to determine the number of independent light absorbing species, n_c, using the INDICES algorithm [31].

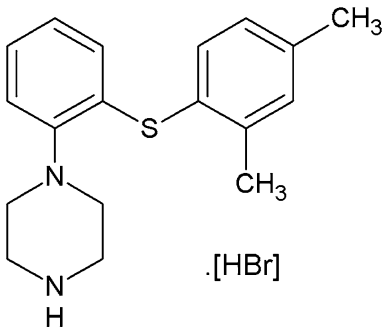

Fig. 1 Structural formula of Vortioxetine hydrobromide

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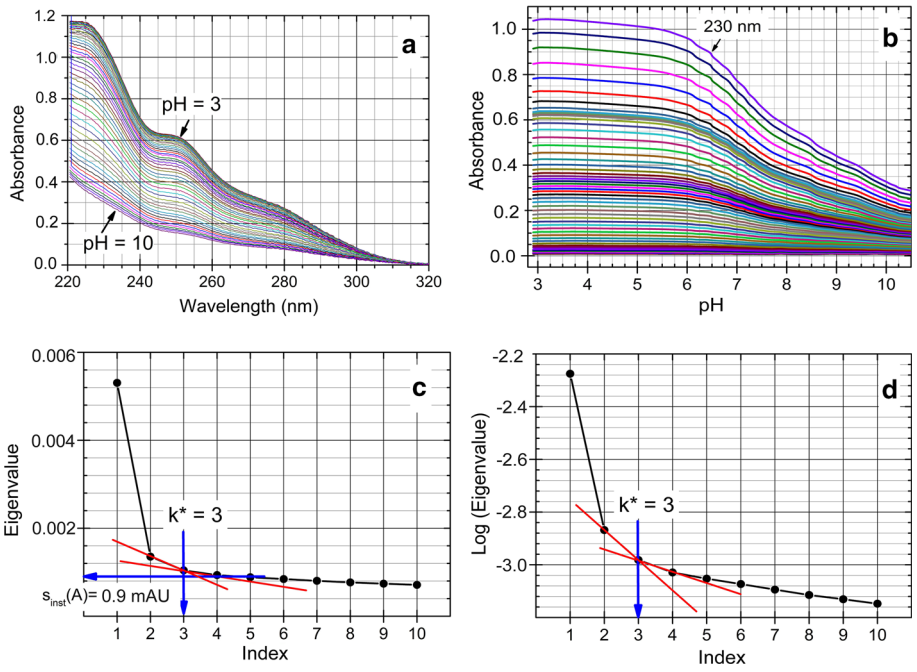

Fig. 2 **a** The 2D-absorbance-response-matrix and **b** the set of A–pH curves concerning 70 measured UV/VIS-absorption spectra of the protonation equilibria for 9.2×10^{-5} mol·dm^{-3} Vortioxetine at $I = 0.002$ mol·dm^{-3} in pure water as functions of pH at 25 °C, which forms the input for the SQUAD84 and REACTLAB programs. **c** Graphical determination of the rank of the absorbance matrix of Vortioxetine that permits determination of the minimum number of colored species, n_c, in a mixture using the Cattell's scree plot $s_k(A) = f(k)$ based on the Wernimont–Kankare criterion of singular value decomposition (SVD). The quantity $s_k(A)$ is the calculated standard deviation of absorbance as estimated by factor analysis of the absorbance matrix, and k is the rank of the matrix. Here $k^* = 3$ leads to four light-absorbing species in the mixture, $n_c = 3$, and the actual instrumental error of the spectrophotometer used is $s_{inst}(A) = 0.9$ mAU. **d** Cattell's scree plot $s_k(A) = f(k)$ in logarithmic scale (INDICES in S-PLUS)

The INDICES indicate the position of break points on the $s_k(A) = f(k)$ curve in the Cattell's scree plot $s_k(A) = f(k)$ using the most reliable approaches by Wernimont–Kankare's $s(A)$, cf. Refs. [20, 31]) and gives $k = 3$ with the corresponding co-ordinate $s_3(A) = 0.9$ mAU (Fig. 2c). This value also represents the actual instrumental error $s_{inst}(A) = 0.9$ mAU of the experimental equipment with the spectrophotometer CINTRA 5 (GBC, Australia). The number of light-absorbing species n_c helps to establish a protonation model. This means that the two dissociation constants will be preferred and three species LH_3^{2+}, LH_2^{+}, and LH are supposed to be present. For the large variations in the indicator values, these latter graphs are plotted on a logarithmic scale and the number of light-absorbing species n_c can be predicted from the index function by finding the point $n_c = k = 3$ where the slope of the index function $PC(k) = f(k)$ changes (Fig. 2d).

4.1.2 Theoretical Prediction of the pK_a

Marvin predicts two ionization sites (denoted as A and B in Fig. 3) that can be associated with dissociation constants; both ionization sites are associated with the nitrogen atom.

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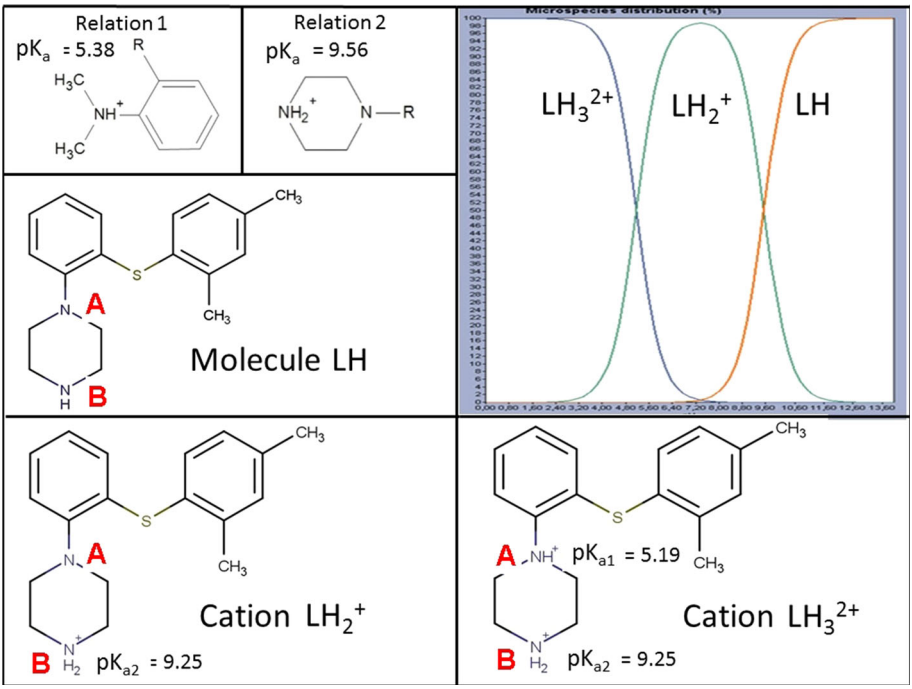

Fig. 3 Schematic representation of the protonated molecular structure of Vortioxetine with predicted dissociation constants and the distribution diagram of the relative concentrations of variously protonated ions using the MARVIN prediction. **a** relation 1 predicts the pK_a of protonated fragment which is similar with Vortioxetine; **b** relation 2 predict the pK_a of another protonated fragment

The macrodissociation constants of Vortioxetine were predicted according to the chemical structure analyzed by two reliable pK_a prediction tools. ACD/Percepta was run using the GALAS model. Marvin's pK_a predictions are based on the calculated partial charge of the atoms located in the analyzed structure, using the Hammett–Taft approach (Fig. 3). According to the Marvin-distribution diagram in Fig. 3, Vortioxetine is supposed to behave mostly as a neutral molecule LH in pH from 8 to 11. When the solution is acidified from pH 6–8, the cation LH_2^+ is formed as the ionization site B is protonated. In changing the pH from 6 to 3, the other cation LH_3^{2+} appeared and the ionization site A on nitrogen is protonated. We also performed an ionic distribution analysis using Marvin and the obtained data were similar to the ones obtained with ACD/Percepta. The structural prediction of dissociation constants of Vortioxetine was performed using the MARVIN program to specify protonation locations.

4.1.3 Choice of Computational Strategy

The hard modelling technique SQUAD84 and soft-modeling technique REACTLAB were used. In both programs the same computational strategy was applied, i.e., the *regression triplet* (criticism of data, model and method), cf. Refs. [21, 35].

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4.1.4 Diagnostics of the Chemical Model Building and Testing

The search for the best hypothesis of the chemical model containing either one or two dissociation constants is shown in Fig. 4: the spectra set of useful analytical wavelengths ranges was examined to indicate the best wavelength range in which the actual chromophore is active and reflects the dissociation in the molecule. The best regression model was sought by testing three working hypotheses of the protonation model: the first concerning one and the other with two dissociation constants. The criterion of reliability between the proposed hypotheses was the criterion $s(A)$ in the goodness-of-fit test. At the same time the estimates of the dissociation constants using two regression programs, i.e., SQUAD84 and REACTLAB were compared (Table 1). The mean residual $E|e|$ [mAU], the standard deviation of residuals $s(e)$ [mAU] and the Hamilton R-factor of relative fitness [%] in SQUAD84 generally showed that better fit of the calculated spectra was always achieved for the protonation model with three dissociation constants. REACTLAB seemed to offer the more reliable parameter estimates as it always reached a better curve fitting than the older program of SQUAD84.

Two dissociation constants pK_{a1} and pK_{a2}, and three molar absorptivities ε_{LH}, ε_{LH2}, and ε_{LH3}, were estimated using SQUAD84 and REACTLAB in the first run. The reliability of the parameter estimates may be tested using the following general diagnostics (Fig. 4) as was described in detail in Ref. [23]:

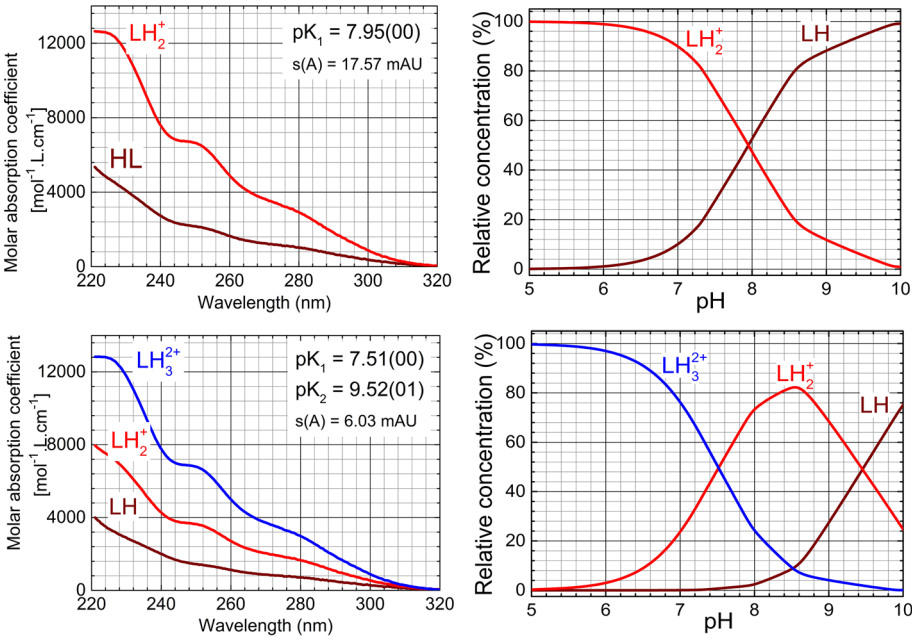

Fig. 4 Typical SQUAD84 working environment searching for the best protonation model of Vortioxetine in the pH range from 4 to 10 for one and two dissociation constants, pK_{a1} and pK_{a2}, using 9.2×10^{-5} mol·dm^{-3} Vortioxetine at $I = 0.002$ mol·dm^{-3} at 25 °C. Left panel the pure spectra profiles of molar absorptivities versus wavelength (nm) for all of the variously protonated species. Right panel the distribution diagram of the relative concentrations of all of the variously protonated species depending on pH (SQUAD84, ORIGIN)

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Table 1 The dependence of estimates of two dissociation constants for an ionic strength of Vortioxetin hydrobromide with SQUAD84 and REACTLAB at 25 °C

25 °C						
Ionic strength I_0 (mol·dm^{-3})	0.0262	0.0357	0.0451	0.0544		
Cattel's scree plot indicating the rank of the absorbance matrix (INDICES)						
Number of spectra measured, n_s	33	35	41	31		
Number of wavelengths, n_w	96	95	95	97		
Number of light-absorbing species, k^*	3	3	3	3		
Residual standard deviation, $s_k^*(A)$ (mAU)	0.98	1.02	1.22	1.11		
Estimates of dissociation constants in the searched protonation model						
pK_{a1} (s_1), $LH_4^{3+} \rightleftharpoons H^+ + LH_3^{2+}$						
SQUAD84	7.80 (04)	8.05 (04)	8.03 (11)	8.09 (06)		
REACTLAB	7.79 (07)	8.04 (01)	8.02 (01)	8.09 (01)		
pK_{a2} (s_2), $LH_3^{2+} \rightleftharpoons H^+ + LH_2^+$						
SQUAD84	9.27 (03)	9.20 (03)	9.60 (09)	9.55 (05)		
REACTLAB	9.23 (02)	9.58 (02)	9.55 (04)	9.54 (03)		
Goodness-of-fit test with the statistical analysis of residuals						
Mean residual $E\|\bar{e}\|$ (mAU)						
SQUAD84	11.50	15.35	14.5	13.32		
REACTLAB	11.47	15.21	14.43	13.16		
Standard deviation of residuals $s(\hat{e})$ (mAU)						
SQUAD84	17.79	29.05	30.50	26.90		
REACTLAB	16.96	27.79	29.33	25.21		
Sigma from ReactLab (mAU)						
REACTLAB	17.22	28.19	29.69	25.98		
Hamilton R-factor from SQUAD84 (%)						
SQUAD84	0.021	0.035	0.029	0.027		
37 °C						
Ionic strength I_0 (mol·dm^{-3})	0.0085	0.0181	0.0277	0.0372	0.0466	0.0558
Cattel's scree plot indicating the rank of the absorbance matrix (INDICES)						
Number of spectra measured, n_s	46	53	49	50	49	47
Number of wavelengths, n_w	234	117	117	117	117	117
Number of light-absorbing species, k^*	3	3	3	3	3	3
Residual standard deviation, $s_k^*(A)$ (mAU)	1.21	0.82	0.88	0.84	0.83	0.86
Estimates of dissociation constants in the searched protonation model						
pK_{a1} (s_1), $LH_4^{3+} \rightleftharpoons H^+ + LH_3^{2+}$						
SQUAD84	7.58 (02)	7.50 (02)	7.77 (02)	7.93 (04)	8.08 (06)	7.89 (02)
REACTLAB	7.58 (00)	7.50 (01)	7.76 (00)	7.93 (01)	8.03 (01)	7.89 (01)
pK_{a2} (s_2), $LH_3^{2+} \rightleftharpoons H^+ + LH_2^+$						
SQUAD84	9.15 (01)	9.19 (01)	9.49 (01)	9.48 (03)	9.66 (05)	9.38 (02)
REACTLAB	9.14 (01)	9.18 (01)	9.47 (01)	9.47 (02)	9.54 (03)	9.38 (01)
Goodness-of-fit test with the statistical analysis of residuals						
Mean residual $E\|\bar{e}\|$ (mAU)						
SQUAD84	68.50	4.56	6.11	10.9	15.61	8.01
REACTLAB	2.29	5.09	4.27	7.62	10.69	7.98
Standard deviation of residuals $s(\hat{e})$ (mAU)						
SQUAD84	16.30	9.65	11.80	24.70	35.25	19.70
REACTLAB	4.58	6.01	7.48	15.71	22.27	9.54

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Table 1 continued

Sigma from ReactLab (mAU)						
REACTLAB	19.90	9.47	11.63	24.23	34.45	19.25
Hamilton R-factor from SQUAD84 (%)						
SQUAD84	0.028	0.019	0.021	0.043	0.058	0.029

A solution of 9.2×10^{-5} mol·dm^{-3} Vortioxetin at $I = 0.002$ mol·dm^{-3} at 25 °C for n_s spectra measured at n_w wavelengths for $n_z = 2$ basic components L and H forms variously protonated species. The standard deviations of the parameter estimates are in the last valid digits in brackets. The resolution criterion and reliability of parameter estimates found are proven with goodness-of-fit statistics such as the residual standard deviation by factor analysis $s_k(A)$ (mAU), the mean residual $E|\bar{e}|$ (mAU), the standard deviation of absorbance after termination of the regression process $s(\hat{e})$ (mAU), the sigma $s(A)$ (mAU) from REACTLAB and the Hamilton R-factor of relative fitness (%) from SQUAD84

(a) *The physical meaning of parametric estimates* The first diagnostic value indicates whether all of the parametric estimates $pK_{a,i}$ and ε_i have physical meaning and reach realistic values. As the standard deviations $s(pK_{a,i})$ of parameters $pK_{a,i}$ and $s(\varepsilon_i)$ of parameters ε_i are significantly smaller than their corresponding parameter estimates, all the variously protonated species are statistically significant at a significance level of $\alpha = 0.05$. In the left hand part of Fig. 4 are shown the estimated molar absorptivities of all of the variously protonated species ε_{LH}, ε_{LH2}, and ε_{LH3} of Vortioxetine with as a function of wavelength.

(b) *The physical meaning of the species concentrations* The second diagnostic examines whether all of the calculated free concentrations of variously protonated species on the distribution diagram of the relative concentration expressed as a percentage have physical meaning, which proved to be the case (right hand panel of Fig. 4). The distribution diagram shows the protonation equilibria of LH_3^{2+}, LH_2^+ and LH. At neutral pH from 6 to 9 Vortioxetine prevails as the species LH_2^+ and LH_3^{2+}, and from pH 8–9 it does so in the form of the species LH_2^+ and the neutral molecule LH. Acidification of the species LH_2^+ creates the cation LH_3^{2+}. At concentrations of 10^{-4}–10^{-6} mol·dm^{-3} the Vortioxetine is sufficiently soluble that all its dissociation constants can be spectrophotometrically determined.

(c) *Goodness-of-fit test* Although the statistical analysis of residuals [23] gives the most rigorous test of the goodness-of-fit, realistic empirical limits must be used. The statistical measures of all residuals e prove that the minimum of the elliptic hyperparaboloid *RSS* has been reached (Table 1): the mean residual $E|\bar{e}|$(mAU) and the standard deviation of residuals $s(\hat{e})$ (mAU) always have sufficiently low values to indicate a good fit. This is also proven by the Hamilton *R-factor*. Dissociation constants estimated with SQUAD84 and REACTLAB estimated from all reproduced measurements are in good agreement. The SQUAD84 approach has a great advantage in a rigorous goodness-of-fit test made by the statistical analysis of residuals. Reproducibility of three experimental spectra sets with the use of two regression programs shows that two dissociation constants $pK_{a1} = 7.74$ and $pK_{a2} = 9.15$ are well-conditioned in the regression model, and therefore their numerical evaluation is quite reliable (Table 1).

(d) *The deconvolution of spectra* Figure 5 presents six figures from pH 5.25 through pH 10.07 to show the consecutive deprotonation response in spectra, when each experimental spectrum was decomposed into the spectra of differently protonated species in a mixture of Vortioxetine. At pH 5.35 the cation LH_3^{2+} predominates in

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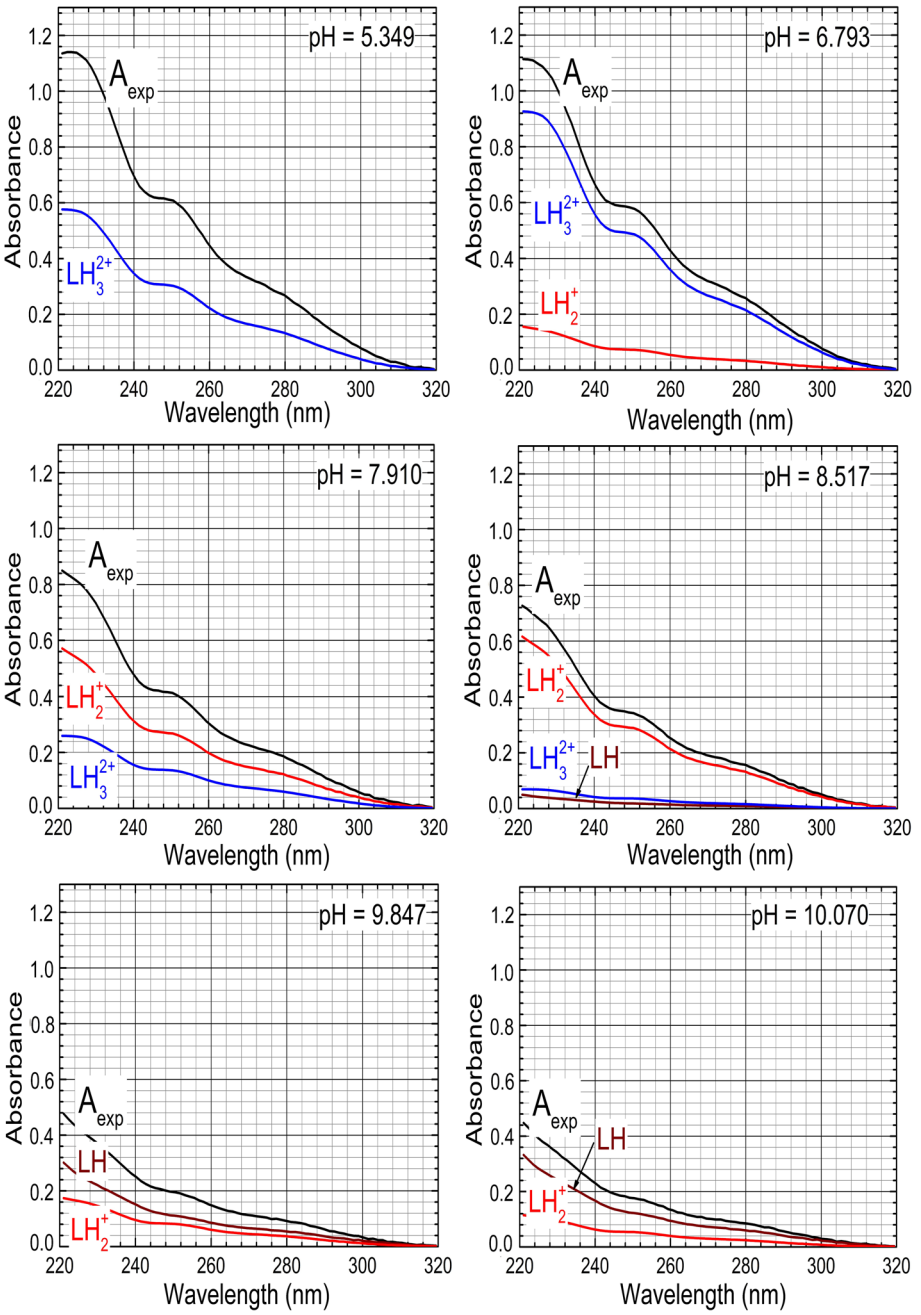

Fig. 5 Deconvolution of each experimental spectrum of 9.2×10^{-5} mol·dm^{-3} Vortioxetine at $I = 0.002$ mol·dm^{-3} at 25 °C into spectra for the individual variously protonated species in mixtures for pH values of 5.35, 6.79, 7.91, 8.52, 9.85, and 10.07 using SQUAD84

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solution. At pH 6.79, together with the cation LH_2^+, one dominant species LH_3^{2+} exhibits an absorption band at the same wavelength of the absorption maximum λ_{max}. At pH 7.91 and 8.52 the experimental spectrum is decomposed into two absorption bands involving the cations LH_3^{2+} and LH_2^+. At pH 9.85 the neutral molecule LH occurs with cation LH_2^+, and the concentration of LH in the solution increases up to pH 10.07.

(e) *Signal-to-error ratio in analysis of small spectra changes* A 3D-plot of the absorbance–response-matrix was analyzed (Fig. 6a). The resulting ratio of the normalized spectra changes, $SER = \Delta/s_{inst}(A)$, are plotted as a function of wavelength λ for all absorbance matrix elements (Fig. 6b). The *SER* ratio is then compared to the limiting *SER* value and used to test if the small absorbance changes are still significantly larger than the instrumental noise. When the *SER* value is greater than 10, a factor analysis is able to predict the correct number of light-absorbing components in the equilibrium mixture. To prove that the non-linear regression can analyze such spectral data, the residuals set was compared to the instrumental noise, $s_{inst}(A)$. Figure 6c shows a comparison of the ratio of the residuals of spectra normalized against instrumental noise, $e/s_{inst}(A)$, against wavelength for the measured Vortioxetine solution. It is clear that most of the residuals are of the same magnitude as the instrumental noise and the ratio $e/s_{inst}(A)$ is less than 2.

4.2 pH-Metric Data Analysis

The potentiometric titration of a mixture of HCl and Vortioxetine with potassium hydroxide concerning the pH-metric data analysis was carried out at 25 °C at the adjusted value of ionic strength (Fig. 7). The initial tentative value of the dissociation constant of the Vortioxetine studied, corresponding to the midpoint value in each plateau of the potentiometric titration curve, was refined by the ESAB program.

Since Vortioxetine has two dissociation constants, their numerical estimation was performed using a computer-assisted nonlinear regression. Regression analysis was employed by using the plateau of the middle part of the titration curve for alkalized Vortioxetine titrated with hydrochloric acid, followed by a subsequent retitration with potassium hydroxide. Also calculated on the assessed point titration curve was the Bjerrum formation protonation function, which is shown in the graph in Fig. 7. The estimates of two dissociation constants pK_{a1} and pK_{a2} are plotted on the Bjerrum formation curves. Since pHs above 10 and pH below 4 occur during the titrations (a very fine precipitate of Vortioxetine initially forms a slight opalescence), these parts of the titration curve with pH over 10 and pH below 4 did not undergo regression analysis for estimating pK_{a1} and pK_{a2}.

The ESAB residuals are defined as the difference between the experimental and calculated titrant volumes (Table 2). The goodness-of-fit test is performed with the statistical analysis of residuals. As further group parameters are refined, the fit is improved. A quite sensitive criterion of the reliability of the dissociation constants estimated is the mean of absolute values of residuals $E|\bar{e}|$ (mL). Comparing residuals with the instrumental noise, $s_{inst}(y)$, represented here by $s_{inst}(y) = s(V) = 0.0001$ mL, an excellent fit is confirmed since the mean $E|\bar{e}|$ (mL) and also the residual standard deviation $s(\hat{e})$ (mL) are nearly the same, and are lower than the experimental noise $s_{inst}(y)$. Here, $E|\bar{e}| = 0.0001$ mL and $s(\hat{e}) = 0.0002$ mL are similar and both are essentially the same as the microburette volume error $s(V) = 0.0001$ mL. All residuals oscillate between the lower − 0.0002 mL and upper

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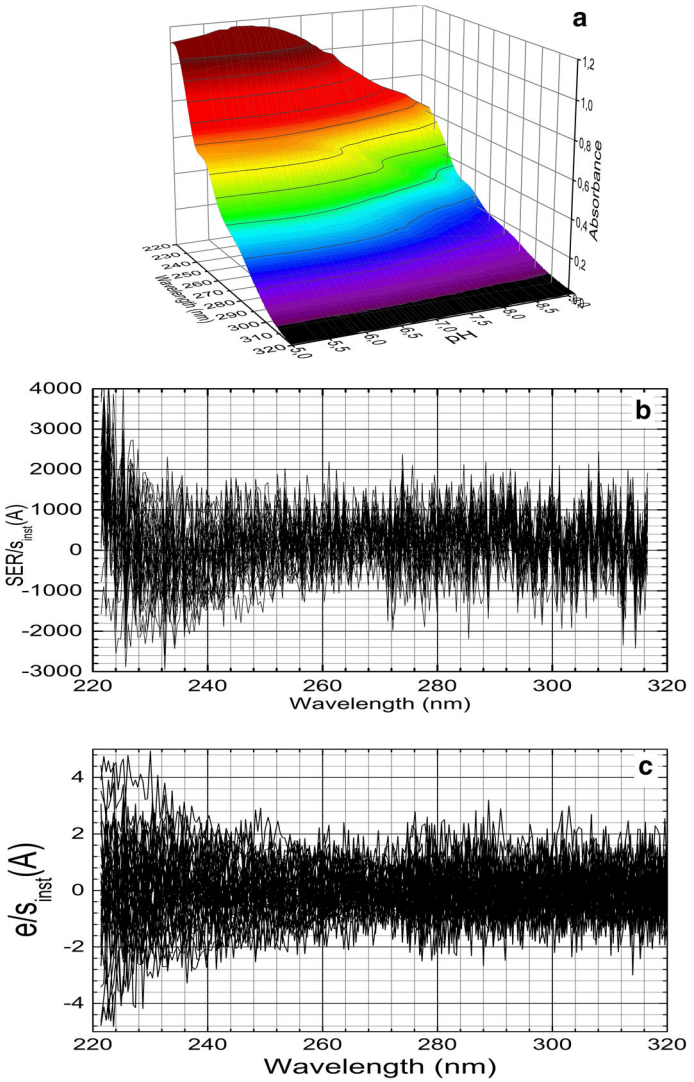

Fig. 6 Plot of small absorbance changes in the Vortioxetine spectrum within the pH-titrations. **a** The 3D plot of the absorbance–response matrix. **b** Plot of small absorbance shifts in the Vortioxetine spectrum within pH-titration when the value of the absorbance difference for the *j*th-wavelength of the *i*th-spectrum, $\mathrm{SER}_{ij} = A_{ij} - A_{i,\mathrm{acid}}$, is divided by the instrumental standard deviation, leading to $\mathrm{SER}/s_{\mathrm{inst}}(A)$. This ratio is plotted against the wavelength λ. Here $A_{i,\mathrm{acid}}$ is the limiting spectrum of the acid form of the drug. **c** The plot of the ratio $e/s_{\mathrm{inst}}(A)$, that is, the ratio of the residuals divided by the instrumental standard deviation $s_{\mathrm{inst}}(A)$ versus the wavelength λ for all the residual matrix elements for Vortioxetine tests, provided the residuals that are of the same magnitude as the instrumental noise (REACTLAB, ORIGIN 9)

limit 0.0002 mL of Hoaglin's inner bounds and therefore no outlying residuals were indicated outside these bounds (cf. page 80 in Ref. [38]). Thus the estimates of the dissociation constants estimated by ESAB are reliable. The curve-fitting is significantly improved by using the refinement of the group parameter L_0, the concentration of the titrated drug Vortioxetine.

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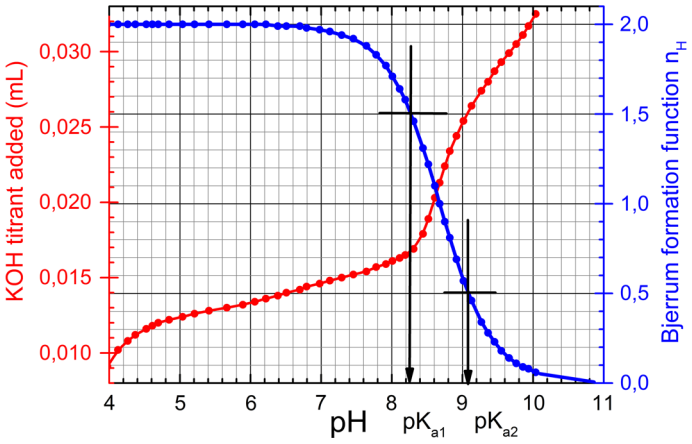

Fig. 7 The potentiometric titration curve of acidified Vortioxetine hydrobromide plus HCl titrated with KOH, plotted with the Bjerrum protonation function, indicating pK values, and analyzed with ESAB for a hypothesis of the protonation model with two dissociation constants at 25 and 37 °C (ESAB, ORIGIN)

Figure 8 brings the extrapolation of the mixed dissociation constants to the zero value of ionic strength according to the Debye–Hückel limiting law for the protonation model of two and three dissociation constants at temperatures 25 and 37 °C.

4.3 Discussion

Spectroscopic titration has been used as an alternative to determine the pK_a values of substances with large molar absorptivities because of its sensitivity to concentrations of substance as low as 10^{-5} mol·dm^{-3}. However, the examined compound must posses chromophore(s) in proximity to the ionization center(s) so that the protonated and deprotonated species exhibit sufficient spectral dissimilarity. In the UV titrations, the measured spectral data of Vortioxetine are a series of spectra acquired at different pH values. Acidifying the solution of the species LH_2^+ leading to cation LH_3^{2+} may be disturbed by Vortioxetine precipitation, which manifests itself especially at higher concentrations in potentiometric determinations. Both the REACTLAB and SQUAD84 programs for spectral analysis produce for a spectrophotometric concentration 9.2×10^{-5} mol·dm^{-3} Vortioxetine the same estimates of both dissociation constants, which exhibit identical goodness-of-fit tests. The influence of temperature at 25 and 37 °C does not seem to be very significant here.

The ESAB program minimizing residuals $e_i = (V_{\mathrm{exp},i} - V_{\mathrm{calc},i})$ reaches 0.1 or 0.2 microlitres, thus proving an excellent fit. It may be concluded that the reliability of the dissociation constants of Vortioxetine is proven even though the group parameters L_0, H_T were ill-conditioned in the model. The goodness-of-fit proved sufficient reliability of the parameter estimates for three dissociation constants of the Vortioxetine at 25 and 37 °C.

The determined dissociation constants are in agreement with the predicted values from the MARVIN program as stated in the results and conclusion sections. Any discrepancy might be caused by the unclear resonance structure of the heterocyclic molecule core, and, consequently, different electrones distribution, which can further lead to different predicted values according to the proposed structure. In such cases the prediction programs MARVIN and ACD may fail and an experimental laboratory determination is needed. As both

Table 2 ESAB refinement of common and group parameters for a pH-metric titration of Vortioxetine hydrobromide with HCl and KOH: the estimated dissociation constants pK_{a1}, pK_{a2} of Vortioxetine at 25 and 37 °C where their standard deviations in last valid digits are in parentheses

25 °C					
Ionic strength I_0 (mol·dm^{-3})	0.0080	0.0158	0.0237	0.0314	0.0391
Estimates of the group parameters H_0, H_T and L_0 in the searched protonation model					
Number of points n	52	57	53	52	56
H_0 × 1E+04 (mol·dm^{-3})	5.08 (02)	5.08 (01)	5.25 (02)	4.36 (05)	4.86 (06)
H_T (mol/L)	0.9729 (0396)	0.9668 (0208)	0.94081 (0306)	0.9304	0.9375 (0104)
L_0 × 1E+04 (mol·dm^{-3})	3.16 (02)	3.26 (01)	2.60 (01)	2.98 (05)	2.99 (06)
Estimates of the common parameters, i.e., dissociation constants in the searched protonation model					
pK_{a1}	8.39 (06)	8.48 (05)	8.82 (09)	8.70 (09)	8.82 (09)
pK_{a2}	8.99 (09)	8.96 (07)	8.79 (09)	8.65 (08)	8.54 (08)
Goodness-of-fit test with the statistical analysis of residuals					
Arithmetic mean of residuals $E(\hat{e})$, (mL)	− 1.77E−20	− 9.51E−21	− 1.89E−06	3.34E−20	− 3.04E−05
Hoaglin limit LL	− 0.0002	− 0.0002	− 0.0001	− 0.0002	− 0.0002
Hoaglin limit LU	0.0002	0.0002	0.0001	0.0002	0.0002
Mean of absolute value of residuals, $E\|\hat{e}\|$, (mL)	0.0005	0.0005	0.0004	0.0005	0.0006
Residual standard deviation, pH (ESAB)	0.1271	0.1175	0.0907	0.1296	0.1456
Residual standard deviation, $s(\hat{e})$, (mL)	0.0006	0.0006	0.0004	0.0006	0.0007
Residual skewness $g_1(\hat{e})$	0.11	0.16	− 0.18	− 0.02	0.06
Residual kurtosis $g_2(\hat{e})$	2.63	2.36	2.32	2.38	2.45
Jarque–Berra test of normality: p; normality is	0.894, Accepted	0.818, Accepted	0.780, Accepted	0.974, Accepted	0.952. Accepted
37 °C					
Ionic strength I_0 (mol·dm^{-3})	0.0080	0.0158	0.0237	0.0314	0.0391
Estimates of the group parameters H_0, H_T and L_0 in the searched protonation model					
Number of points n	57	57	58	54	48
H_0 × 1E+04 (mol·dm^{-3})	5.00 (05)	3.99 (06)	4.15 (06)	5.39 (05)	4.37 (05)
H_T (mol/L)	0.9304	0.9304	0.9304	0.9304	0.9304

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Table 2 continued

L_0 × 1E+04 (mol·dm^{-3})	3.74 (01)	4.26 (12)	4.14 (09)	3.55 (12)	3.68 (09)
Estimates of the common parameters, i.e., dissociation constants in the searched protonation model					
pK_{a1}	8.16 (04)	8.17 (04)	8.18 (04)	8.29 (04)	8.32 (04)
pK_{a2}	9.26 (08)	9.36 (08)	9.13 (07)	9.36 (10)	9.25 (08)
Goodness-of-fit test with the statistical analysis of residuals					
Arithmetic mean of residuals $E(\hat{e})$, (mL)	3.51E−06	1.75E−06	3.45E−06	1.85E−06	− 4.52E−21
Hoaglin limit LL	− 0.0002	− 0.0002	− 0.0002	− 0.0002	− 0.0002
Hoaglin limit LU	0.0002	0.0002	0.0002	0.0002	0.0002
Mean of absolute value of residuals, $E\|\hat{e}\|$, (mL)	0.0005	0.0006	0.0006	0.0005	0.0005
Residual standard deviation, pH (ESAB)	0.1214	0.146	0.1506	0.1254	0.1187
Residual standard deviation, $s(\hat{e})$, (mL)	0.0006	0.0007	0.0007	0.0006	0.0006
Residual skewness $g_1(\hat{e})$	0.00	− 0.18	− 0.15	− 0.22	− 0.10
Residual kurtosis $g_2(\hat{e})$	1.83	1.66	1.71	1.76	1.74
Jarque–Berra test of normality: p; normality is	0.977, accepted	0.771, accepted	0.827, accepted	0.712, accepted	0.911, Accepted

The reliability of parameter estimation is proven with thr goodness-of-fit statistics: the bias or arithmetic mean of residuals $E(\hat{e})$ (mL), the mean of absolute value of residuals, $E|\hat{e}|$ (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. *Common parameters refined:* pK_{a1}, pK_{a2}; *group parameters refined:* H_0, H_T, L_0; *constants*: t = 25.0 °C, pK_w = 13.9799, V_0 = 20.22 mL, $s(V) = s_{inst}(y)$ = 0.0001 mL, I_0 adjusted (in vessel), I_T = 0.9304 (in burette KOH) or 1.0442 (in burette HCl)

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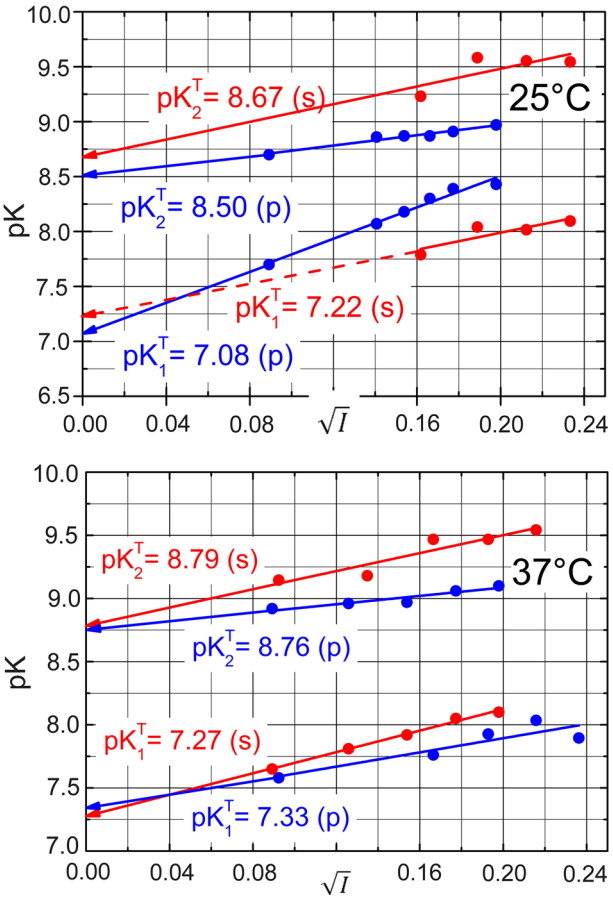

Fig. 8 Dependence of the mixed dissociation constant pK_a of Vortioxetine on the square-root of the ionic strength leading to the thermodynamic dissociation constant pK_a^T at 25 and 37 °C using UV-metric spectra analysis **(s)** and the same type of calculation using the pH-metric technique **(p)**

potentiometric and spectrophotometric results are similar, and regarding the goodness of fit tests, the conclusion can be drawn that the obtained experimental results are reliable and that they show the real dissociation of the investigated substance.

5 Conclusions

Spectrophotometric UV-metric and potentiometric pH-metric analysis allowed the measurement of the two dissociation constants of Vortioxetine. However, poorer solubility was observed at pHs above 10 and also below 4 for the Vortioxetine concentration in micromoles per liter, leading to the conclusion that an estimation of the pK_a higher than 10, or in potentiometry lower than 4, is not reliable.

(1) The sparingly soluble neutral molecule LH of Vortioxetine is capable of protonation to form the still soluble cations LH_2^+ and LH_3^{2+} in pure water. The graph of molar

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absorption coefficients of variously protonated species as a function of wavelength shows that the spectra of species LH_2^+ and LH vary slightly in color, while protonation of the chromophore LH_2^+ to LH_3^{2+} has a greater influence on chromophores in the Vortioxetine molecule.

(2) We have proven that in the range of pH 4–10 two dissociation constants can be reliably estimated from the spectra when the concentration of Vortioxetine is about 9.2×10^{-5} mol·dm^{-3}. Although the change of pH gave small changes in the chromophore, two overlapping thermodynamic dissociation constants were reliably determined with SQUAD84 and REACTLAB, yealding similar values with both programs: $pK_{a1}^T = 7.22$, $pK_{a2}^T = 8.67$ at 25 °C and $pK_{a1}^T = 7.27$, $pK_{a2}^T = 8.79$ at 37 °C.

(3) Two overlapping thermodynamic dissociation constants of Vortioxetine at a concentration of 3×10^{-4} mol·dm^{-3} were determined by the regression analysis of potentiometric titration curves using ESAB: $pK_{a1}^T = 7.08$, $pK_{a2}^T = 8.50$ at 25 °C and $pK_{a1}^T = 7.33$, and $pK_{a2}^T = 8.76$ at 37 °C.

(4) Prediction of the dissociation constants of Vortioxetine was performed using the MARVIN program to specify protonation locations and using the ACD/pK program. In comparing the two predictive with two experimental techniques, it may be concluded that the prediction programs often vary in their estimated pK_a values.

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