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Thermodynamic protonation constants of vardenafil by the nonlinear regression of multiwavelength pH-spectrophotometric titration data

Research Article

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Abstract: pH-spectrophotometric titration data were used to determine protonation constants of vardenafil at different ionic strengths *I* and temperatures of 25°C and 37°C. The use of two different multiwavelength and the multivariate treatment of spectral data, SPECFIT32 and SQUAD(84) nonlinear regression analyses and INDICES factor analysis is presented. The reliability of the protonation constants of the drug was proven with goodness-of-fit tests of the pH-spectra. The thermodynamic protonation constants log K^T_i were estimated by a nonlinear regression of (log *K, I*) data using the Debye-Hückel equation, yielding log $K_4^T = 3.59(1)$ and 3.26(1), log $K_3^T = 5.64(1)$ and 5.81(1), log $K_2^T = 9.41(1)$ and 8.59(2), log $K_1^T = 10.92(2)$ and 10.05(1) at 25ºC and 37ºC, where the figure in brackets is the standard deviation in last significant digit. Concurrently, the experimental determination of four thermodynamic protonation constants was combined with the computational prediction of the MARVIN program based on knowledge of the chemical structures of the drug and was in good agreement with its experimental value. The factor analysis of spectra in the INDICES program predicts the correct number of light-absorbing components when the instrument error is known and when the *signal-to-error ratio SER* is higher than 10.

Keywords: *Spectrophotometric titration • Protonation constant • Vardenafil • SPECFIT • SQUAD*

1. Introduction

Vardenafil is widely used as a selective inhibitor of cyclic guanosine monophosphate (*cGMP*)-specific phosphodiesterase type 5 (PDE5), in the treatment of erectile dysfunction (ED). It can also be effective as a therapy for a range of cardiovascular diseases, such as pulmonary arterial hypertension (PAH) [1-4]. It is sold under the trade names ***Levitra*** (Bayer AG, GSK, and SP) and ***Staxyn***. Vardenafil hydrochloride is designated chemically as 4-[2-Ethoxy-5-(4-ethylpiperazin-1-yl) sulfonyl-phenyl]-9-methyl-7-propyl-3,5,6,8-tetrazabicyclo [4.3.0]nona-3,7,9-trien-2-one and has the structure shown in Fig. 1. Dissociation/protonation constants can be predicted with the use of the quantum-chemistry programs MARVIN [5], SPARC [6], PALLAS [7] and ACD/Labs [8].

Vardenafil hydrochloride is a nearly colorless, solid substance with a molecular weight of 579.1 g mol^{-1} and a solubility of 0.11 mg mL^{-1} in water [9]. Vardenafil is an oral drug that is used to treat impotence, the inability to attain or maintain a penile erection. It has a mechanism of action that is similar to sildenafil (Viagra) and tadalafil (Cialis). Sexual stimulation that leads to engorgement and erection causes the production and release of nitric oxide in the penis [10,11]. Nitric oxide then activates the enzyme, guanylate cyclase to produce cyclic guanosine monophosphate (cGMP). The cGMP is primarily responsible for increasing and decreasing the size of the blood vessels carrying blood to and from the penis, respectively [12].

Protonation/dissociation constants are very important both in the analysis of drugs and in the interpretation

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of their mechanisms of action. It was shown that the spectrophotometric method can be used in combination with suitable chemometric tools for the determination of protonation constants even for sparingly soluble drugs [9].

In this study, pH-spectrophotometric titration was used due to the poor water solubility of vardenafil which does not enable an application of potentiometric titration with higher concentrations. Concurrently, the experimental determination of protonation constants was combined with their computational prediction based on knowledge of the chemical structures.

2. Theoretical details

2.1. Determination of protonation model

The protonation equilibria $L^{z-1} + H^+ \leftrightarrow HL^z$ between the anion L^- of a drug and a proton H^+ are considered to form a set of variously protonated species L, HL, H_2L, H_3L, *etc*. (the charges are omitted for the sake of generality). These have the general formula H_rL in a particular chemical model and are represented by r_i, i = 1, ..., n_c where the index i indicates their particular stoichiometry and n_c indicates the number of species. The overall protonation (stability, association) constant of the protonated species, β_r, may then be expressed as

$$\beta_r = [H_rL] / ([L]\ [H]^r) \quad (1)$$

where the [L], [H] and $[H_rL]$ represent the free concentrations of the species. For protonation reactions realized at constant ionic strength, the "protonation constants" are defined as

$$K_{a,j} = \frac{[H_jL]}{[H_{j-1}L]\,a_{H^+}} \quad (2)$$

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

$$A_{i,j} = \sum_{n=1}^{n_c} \varepsilon_{j,n}\ [H_rL] = \sum_{n=1}^{n_c} (\varepsilon_{r,j}\ \beta_r\ [L]\ [H]^r)_n \quad (3)$$

where $\varepsilon_{r,j}$ is the molar absorptivity of the H_rL species with the stoichiometric coefficient r measured at the jth wavelength. The absorbance $A_{i,j}$ is an element of the absorbance matrix ***A*** of size ($n_s \times n_w$), and is measured

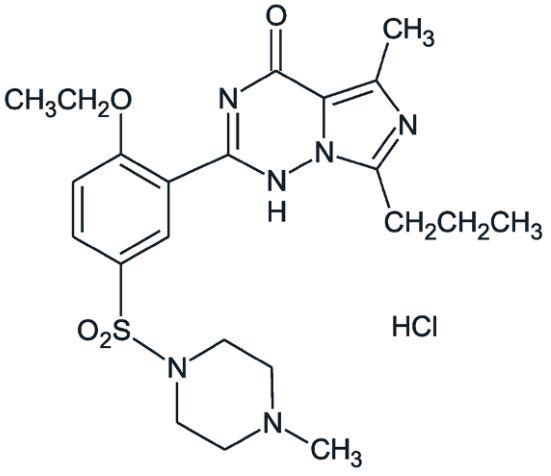

Figure 1. Chemical structure of vardenafil.

for n_s solutions with known total concentrations of n_z = 2 basic components, c_L and c_H, at n_w wavelengths [13,14]. To determine the protonation constant of vardenafil, the programs SQUAD(84) [15,16] and SPECFIT/32 [17-19] were used and the procedure was previously published [20].

2.2. Determination of the number of light-absorbing species

For the study of protonation equilibria, it is important to know the number of light-absorbing species. If we know this number, we can suggest the correct chemical model from which the non-linear regression model estimates the protonation constants. The factor analysis in the INDICES algorithm [21,22] is an efficient instrument for determining the number of light-absorbing species. The source matrix is the absorbance matrix **A**, whose columns represent the measured pH and the lines represent wavelengths. The source matrix **A** with dimensions $n \times m$ can be expressed as **A = L F + E** where **L** with dimensions $n \times k$ represents the factor score matrix, **F** with dimensions $k \times m$ is the matrix of factor weight and E with dimensions $n \times m$ expresses the matrix of error factors. The various indicator function $PC(k)$ techniques in the INDICES algorithm developed to deduce the exact size of the true component space can be classified into two general categories. They have been previously described in details [22]: (a) precise methods based upon knowledge of experimental error of the absorbance data $s_{inst}(A)$, and (b) approximate methods requiring no knowledge of the experimental error. In general, most methods are based on the procedure of finding the point where the slope of the indicator function $PC(k) = f(k)$ changes.

2.3. Determination of the thermodynamic protonation/protonation constant

The dependence of the protonation constant K_a = [HL^z]/(a_{H+} [L^{z-1}]) on ionic strength is expressed by the extended Debye-Hückel equation [9-12,22]. This assumes that both the HL^z and L^{z-1} ions have roughly the same ion-size parameter *å* in the protonation equilibrium L^{z-1} + H$^+$ ↔ HL^z with the thermodynamic protonation constant $K_a^T = a_{HL}/(a_{H+}\, a_L)$, and that the overall salting-out coefficients are given by $C = C_{HL} - C_L$.

2.4. Signal-to-noise ratio SNR

The level of "experimental noise" should be used in the experiment as a critical factor. Therefore, it is necessary to have a consistent definition of the *signal-to-noise ratio SNR* so that the impact of this parameter can be critically assessed.

The plot of the ratio $e/s_{inst}(A)$, *i.e.*, the ratio of the residuals divided by the instrument standard deviation $s_{inst}(A)$ depending on wavelength λ for all the residual matrix elements for tests if the residuals are of the same or similar magnitude as the instrument noise to prove the best curve fitting achieved. Analysis of the ratio e/sinst(A) was described previously [30].

3. Experimental procedure

3.1. Chemicals and solutions

Vardenafil donated by ZENTIVA Group k.s. with a declared purity of 100% by HPLC was used without further purification. Mercury oxide, potassium iodide and potassium chloride, p. a. Lachema Brno were not additionally purified. Twice-redistilled water previously kept for 50 minutes in a sonic bath was used in the preparation of solutions.

3.2. pH-spectrophotometric titration procedure

The spectral measurements were carried out using a model GBC Cintra 404 spectrophotometer with 1 cm optical path length [20]. The free hydrogen-ion concentration [H$^+$] was measured on a Hanna HI 3220 digital voltmeter with a precision of ±0.002pH with the use of a Theta HC 103-VFR combined glass electrode with a standard calomel electrode. Titrations were performed in a water-jacketed double-walled glass vessel of 100 mL.

The experimental and computational scheme to determine the protonation constants of the multicomponent system is taken from Meloun *et al.*, *cf.* page 226 in [24] and the detailed steps are described elsewhere [13,14,22,25,26].

3.2.1. Procedure

To determine the mixed association constants of the protonation equilibria of the drug acids, the following procedure was applied:

Step 1. *Calibration of glass electrode cell*: The hydrogen activity scale pa_H+ was used after standardization with 3 WTW standard buffers of values 4.006 (4.024), 6.865 (6.841) and 9.180 (9.088) at 25°C (37°C).

Step 2. *pH-spectrophotometric titration:* A mixture of 20.00 mL containing $L_0 = 4.2\times10^{-5}$ M drug, H_0 = 0.007 M hydrochloric acid and indifferent solutions of KCl for adjustment of ionic strength was titrated with standard H_T = 0.896 M KOH at 25°C and about 50 spectra were recorded. Since preparation of a large number of separate solutions is tedious, simultaneous monitoring of absorbance and pH during titrations is valuable. In a titration, the total concentration of one of the components changes incrementally over a relatively wide range, but the total concentrations of the other components change only by dilution, or not at all if they are present at the same concentration in the titrant and titrand. However, the absorbance cannot be varied over a large range without decreasing the precision of its measurement, and is effectively confined to a range of about one order of magnitude, *e.g.* 0.1 < *A* < 1.2, although the range of concentrations measured can be increased by use of different path-lengths, *e.g.* 5, 1 and 0.1 cm. The protonation equilibria of drugs were studied in the UV/visible region, 250 - 343 nm. The wavelength range selected is such that every species made a significant contribution to the absorbance [31]. If only a small number of wavelengths were used, those corresponding to maxima or shoulders in the spectra should be chosen, because small errors in setting the wavelength are then less important. It is best to use wavelengths at which the molar absorptivities of the species differ greatly, or a large number of wavelengths spaced at equal intervals [22].

Step 3. *Protonation equilibria of drug,* log $\beta_{pq\,j}$, j = 1, ..., J: The protonation constant log β_j, j = 1, ..., J was determined from analysis of a set of pH-spectra of a mixture of drug acid, HCl and KOH using the SPECFIT and SQUAD programs.

Step 4. *Reliability of protonation constant* log $\beta_{pq\,j}$, j = 1, ..., J: The reliability of the protonation constant log β_j, j = 1, ..., J and a chemical model determination was considered on the basis of goodness-of-fit tests performed by the statistical analysis of residuals.

3.3. Computation and software

Computations of the protonation constants were performed by regression analysis of the UV/VIS spectra using the SQUAD(84) [15,16] and SPECFIT/32 [27] programs. A qualitative interpretation of the spectra with the use of the INDICES program [22] in S-Plus [21] was used 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. Most graphs were plotted using ORIGIN 8 [28] and S-Plus [21]. The thermodynamic protonation constant was estimated with the MINOPT nonlinear regression program in the ADSTAT statistical system (TriloByte Statistical Software, Ltd., Czech Republic) [26,29]. MARVIN [5] is a program for predictions based on the structural formulae of drug compounds. Entering the compound topological structure descriptors graphically, log *K* values of organic compound are predicted using hundreds of Hammett and Taft equations and quantum chemistry computations [32].

3.4. Supporting information available

Complete experimental and computational procedures, input data specimens and corresponding output in numerical and graphical form for the programs SQUAD(84), SPECFIT/32 are available free of charge on line at *http://meloun.upce.cz* in the DOWNLOAD menu and DATA block.

4. Results and discussion

The deprotonation of vardenafil indicates four protonation equilibria. The pH-spectrophotometric titration enables absorbance-response 3D-data (Fig. 2a) used for the non-linear regression with the SPECFIT and SQUAD(84) programs. The reliability of the estimated parameters (log *K*´s and e´s) can be evaluated on the basis of the goodness-of-fit test of residuals (Fig. 2b). Other instrument methods for determining protonation constants do not seem to be suitable for very limited solubility in water.

As the changes in spectra are quite small in response to deprotonation (Figs. 2a and 4a), all of the variously protonated species exhibit similar absorption bands and a very precise measurement of absorbance is required for the reliable estimation of each deprotonation equilibrium. The spectrophotometric analysis of the protonation equilibria of vardenafil involves several steps: The first step concerns a determination of the number of light-absorbing species. The hard-modelling method (*e.g.* SQUAD(84)) requires knowing the value of the number of light-absorbing species (Fig. 3), their stoichiometry and an initial approximation of the protonation constants. The method of Kankare´s residual standard deviation $s(A)$ [25], identically lead to a significant break point on the curve at $k^* = 5$ with corresponding co-ordinates $\log s_k^*(A) = 3.76$, *i.e.*, $1000 s_k^*(A) = 0.17$ milli absorbance units. This is equivalent to the actual instrument error $s_{inst}(A)$ of the spectrophotometer used [22].

The next step of the spectra analysis is proving the reliability of the chemical model. The goodness-of-fit achieved is easily seen by examining the differences between the experimental and calculated values of absorbance, $e_i = A_{exp,i,j} - A_{calc,i,j}$. The reliability of the proposed chemical model is considered based on several criteria of the goodness-of-fit test:

(a) the estimation of the standard deviation of absorbance $s(A)$ after regression analysis should have a value close to residual standard deviation of absorbance $s_k(A)$ from the factor analysis of the INDICES method. It is usually compared to the standard deviation of absorbance calculated, and if $s(A) \le s_k(A)$, or $s(A) \le s_{inst}(A)$ (the instrument error of the spectrophotometer) used, the fit is considered to be statistically acceptable.

(b) the chemical model found should be equivalent to the hypothesis with a sufficiently lower estimation of the arithmetic mean of residuals $|\hat{e}|$ (Fig. 2b);

(c) the chemical model found should have the lowest value of the Hamilton *R*-factor of all tested hypotheses [22];

(d) the residual skewness $\hat{g}_1(\hat{e})$ should have a value close to zero and the residual kurtosis $\hat{g}_2(\hat{e})$ should have a value close to 3.

The hypothesis of the chemical model that complies with these criteria can be described as the best regression model showing the best goodness-of-fit of the calculated absorbance-response surface of measured spectra. When testing various chemical hypotheses for vardenafil at $I = 0.0291$ and 25°C, four proposed hypotheses of the protonation model were analysed and the regression spectra analysis can distinguish among these models. On the basis of very good spectra fitting, the protonation model of five species (L, HL, H_2L, H_3L, and H_4L) was proven. The statistical measures of all residuals from Fig. 2b prove that the minimum of the hyperparaboloid is reached: the mean residual $1000|\hat{e}| = 0.68$ absorbance units and the residual standard deviation $1000s(e) = 1.05$ absorbance units have sufficiently low values. The skewness $g_1(e) = 0.19$ is close to zero and proves a nearly symmetric distribution of the residuals, while the kurtosis $g_2(e) = 5.74$ is close to 6 proving a symmetric Laplace distribution. The Hamilton *R*-factor of relative fitness is 0.64% calculated with SQUAD(84)

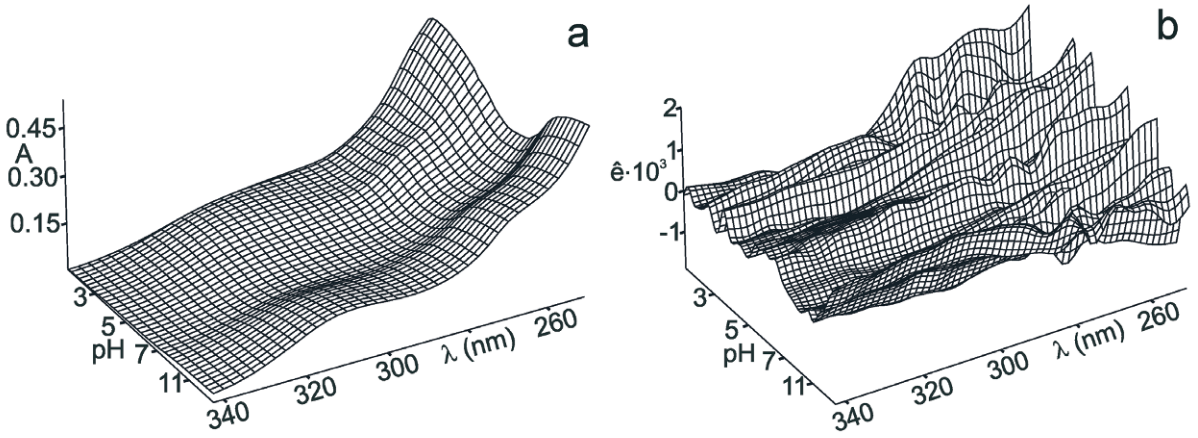

Figure 2. (a) The 3D-absorbance-response-surface representing the measured multiwavelength absorption spectra (n = 46, m = 19) for 4.2×10^{-5} mol dm^{-3} vardenafil depending on pH at 25°C, (b) the 3D-residuals map after a non-linear regression performed with SPECFIT/32 program, (S-Plus).

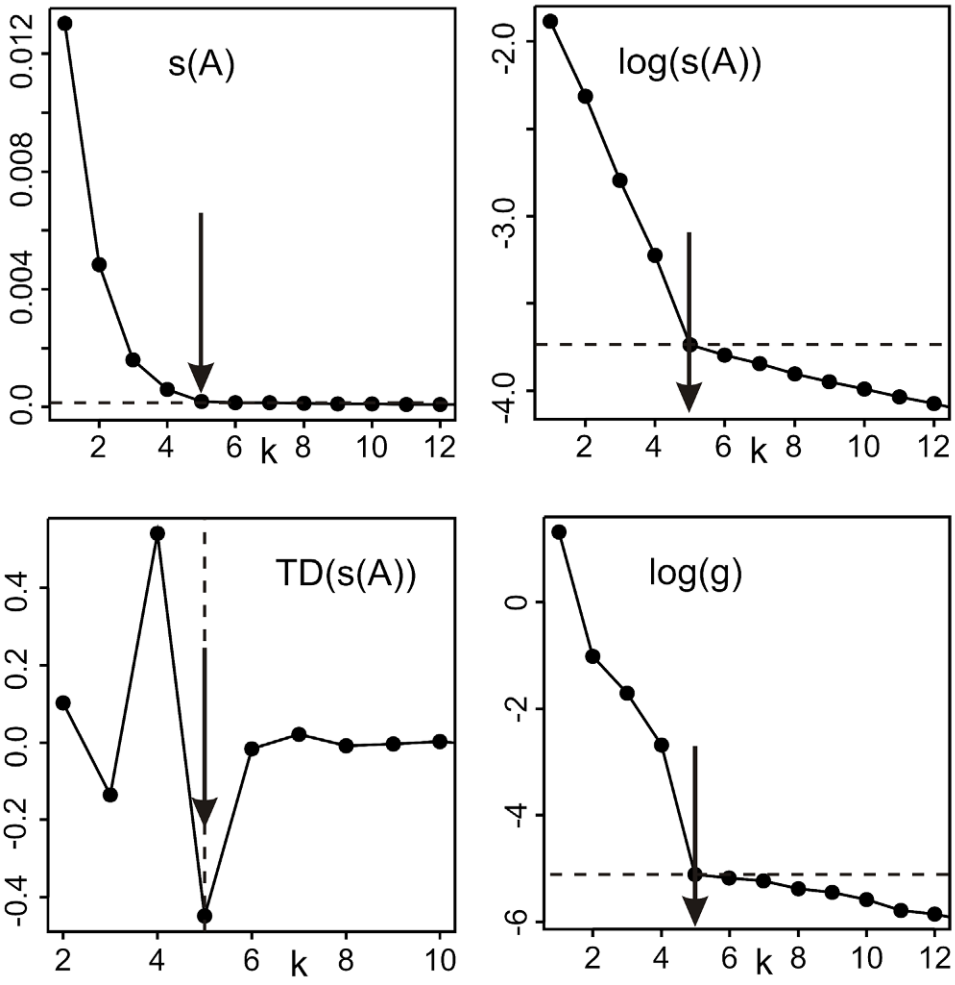

Figure 3. The dependence of the Cattel´s index plot of eigenvalues in form of four graphical methods as a function of the number of principal components k for the pH-absorbance matrix: *Kankare's residual standard deviation s(A), logarithm of Kankare's residual standard deviation log(s(A)), the third derivation of residual standard deviation TD(s(A)), logarithm of eigenvalues log(g)*. The arrows indicate that all methods lead to 5 light-absorbing species in pH-equilibrium mixture (S-Plus).

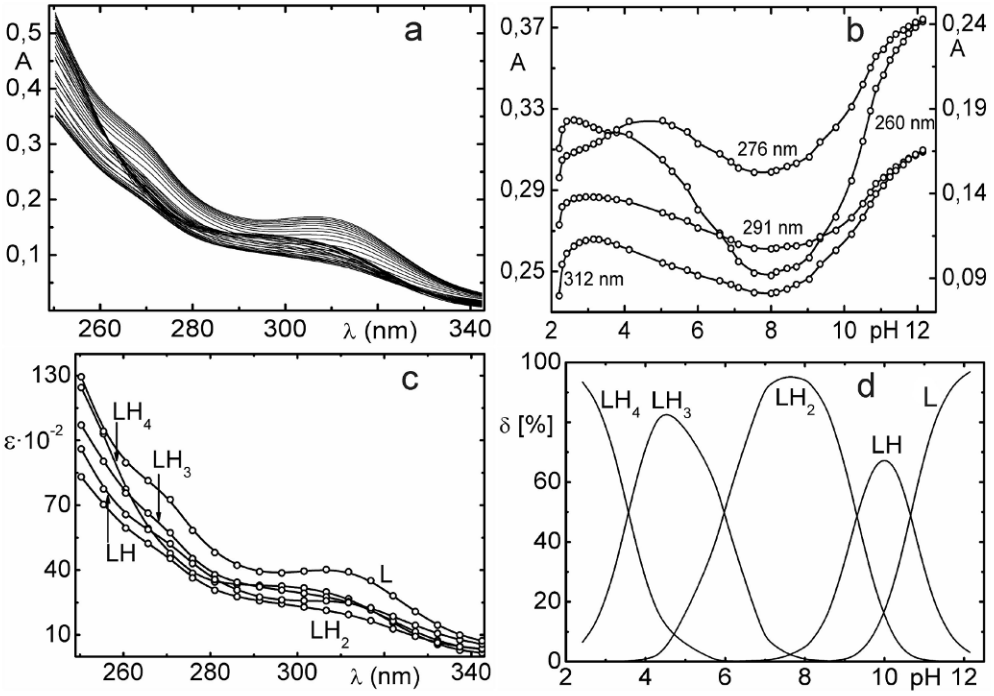

Figure 4. (a) Absorption spectra of $4.2 \cdot 10^{-5}$ mol·dm^{-3} vardenafil in dependence on pH at 25ºC, (b) The absorbance vs. pH curves for 260 nm, 276 nm, 291 nm and 312 nm at 25ºC,(c) Pure spectra profiles of molar absorptivity vs. wavelengths for the variously protonated species L, HL, H_2L, H_3L, and H_4L, (d) Distribution diagram of the relative concentrations of all variously protonated species L, HL, H_2L, H_3L, and H_4L of vardenafil depending on pH at 25ºC, (SPECFIT, SPLUS, ORIGIN). The charges of species are omitted for the sake of generality.

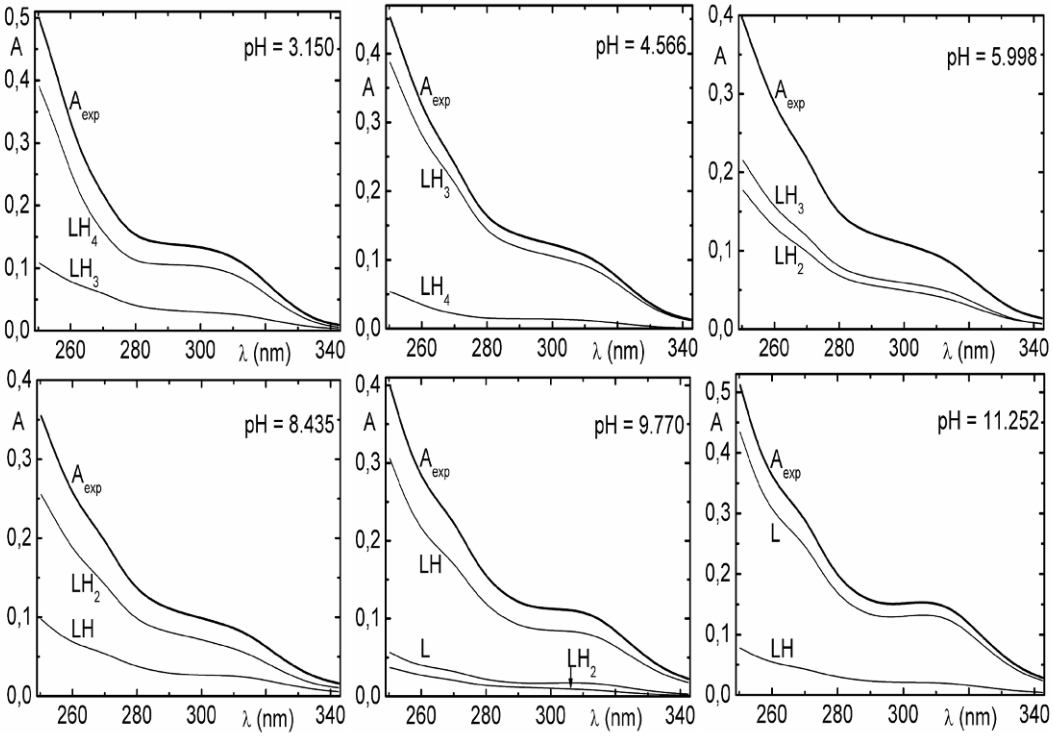

Figure 5. Decomposition of the experimental absorption spectrum of vardenafil for (n = 46, m = 19) into the spectra of the individual variously protonated species L, HL, H_2L, H_3L, and H_4L in solution of each particular absorption spectrum for a selected value of pH equal 3.150, 4.566, 5.998, 8.435, 9.770 and 11.252. The charges of species are omitted for the sake of generality (SQUAD, ORIGIN).

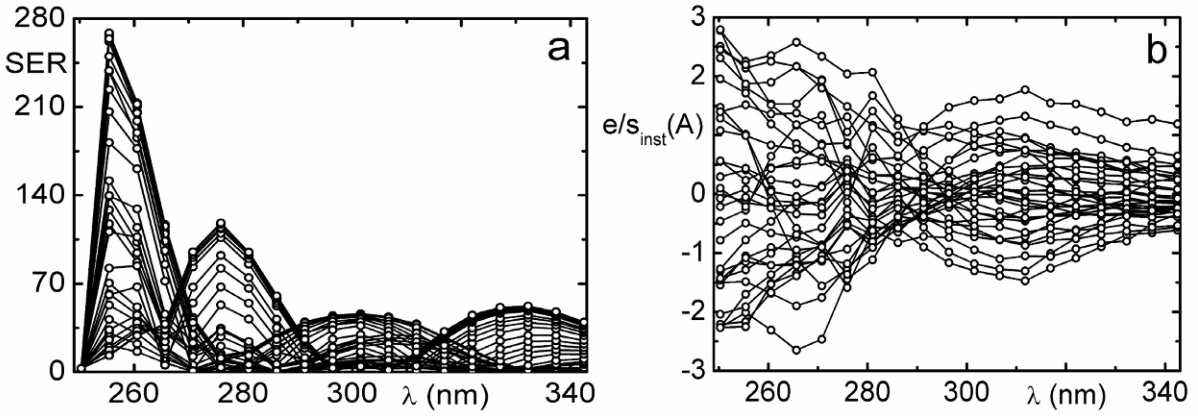

Figure 6. (a) The plot of small absorbance changes in the spectrum of vardenafil means that the value of the absorbance difference for the *j*th-wavelength of the *i*th-spectrum $\Delta_{ij} = A_{ij} - A_{i,acid}$ is divided by the instrument standard deviation $s_{inst}(A)$, and the resulting ratios $SER = \Delta/s_{inst}(A)$ are plotted in dependence of wavelength λ for all absorbance matrix elements, where $A_{i,acid}$ is the limiting spectrum of the acid form of the drug measured. This ratio is compared with the limiting *SER* value for the vardenafil to test if the absorbance changes are significantly larger than the instrument noise. (b) The plot of the ratio $e/s_{inst}(A)$, *i.e.*, the ratio of the residuals divided by the instrument standard deviation $s_{inst}(A)$ in dependence on wavelength λ for all the residual matrix elements for vardenafil tests if the residuals are of the same magnitude as the instrument noise.

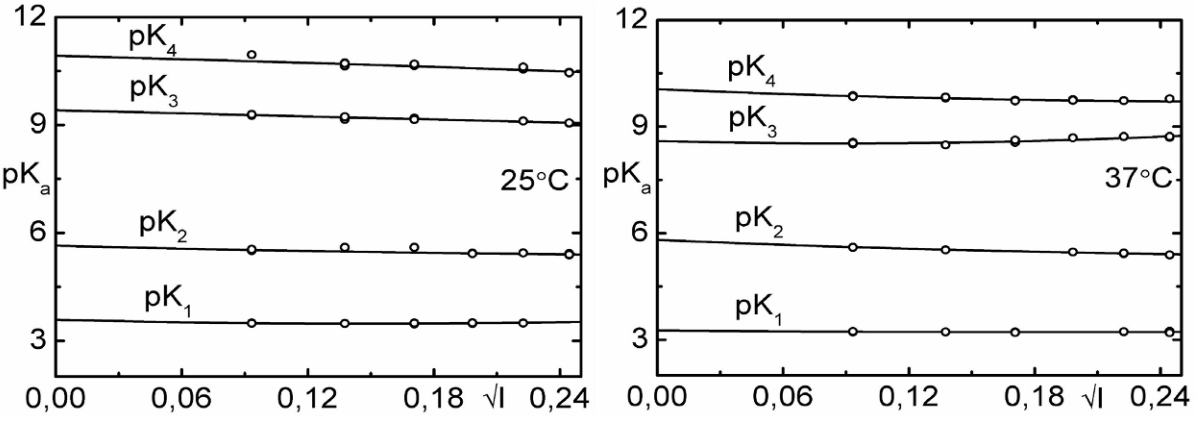

Figure 7. Dependence of the mixed dissociation constants pK_a of vardenafil on the square root of an ionic strength at 25°C and 37°C.

only, proving an excellent fit, and the parameter estimates may therefore be considered sufficiently reliable.

Four protonation constants and the five molar absorptivities of vardenafil, *i. e.*, e_L, e_{HL}, e_{H2L}, e_{H3L}, and e_{H4L} calculated for 19 wavelengths from 46 spectra (Fig. 2a) constitute (5×46) + 4 = 234 unknown regression parameters, which are estimated and refined with the SQUAD(84) or SPECFIT32 programs in the first run. Factor analysis of absorption spectra (Fig. 2a) leads to five light-absorbing species. The graph of molar absorptivity (Fig. 4c) for variously protonated species L, HL, H_2L, H_3L, and H_4L indicates that the selected chemical model is reliable. An important result of regression analysis is the distribution diagram (Fig. 4d) of the relative concentration of all variously protonated species with dependence on pH.

Fig. 5 shows the decomposition of the experimental spectrum of the individual variously protonated species to examine whether the experimental design is efficient.

As elucidated in [30] and [33] the vardenafil shows small changes in absorbance (Fig. 4a). Therefore we calculated *the signal-to-error* value *SER* (Fig. 6a) as the difference of each spectrum absorbance A_{ij} and absorbance of the most acid component $A_{i,acid}$ divided by the actual instrumental standard deviation $s_{inst}(A)$ of the spectrophotometer used. We can say that factor analysis was able to predict the correct number of variously protonated species in the mixture because the *SER* value is larger than 10. If we compare the residual set (Fig. 2b) with the instrumental noise $s_{inst}(A)$ we obtain $e/s_{inst}(A)$ ratio (Fig. 6b). As we can see the ratio is nearby one which prove that nonlinear regression is able to analyse this data and the minimization process

Table 1. Protonation constants of vardenafil at 25°C and 37°C and various values of an ionic strength estimated by non-linear regression using programs SPECFIT. Standard deviations of protonation constants in the last valid digits are in parentheses, *s(A)* is the calculated standard deviation of absorbance in milli absorbance units when regression process terminates.

		25°C					
Ionic strength		**0.0087**	**0.0189**	**0.0291**	**0.0393**	**0.0495**	**0.0597**
SPECFIT	log K_{a4}	3.48(3)	3.48(8)	3.47(23)	3.48(4)	3.49(6)	3.32(4)
	log K_{a3}	5.80(2)	5.80(6)	5.91(4)	5.75(3)	5.15(1)	5.40(2)
	log K_{a2}	9.51(11)	9.16(7)	9.15(5)	8.94(9)	9.21(1)	8.84(2)
	log K_{a1}	11.16(10)	10.64(2)	10.35(2)	10.25(4)	10.61(2)	10.46(3)
	1000 s(A) [mAU]	1.14	0.72	0.70	0.65	0.49	0.92
		37°C					
Ionic strength		**0.0087**	**0.0189**	**0.0291**	**0.0393**	**0.0495**	**0.0597**
SPECFIT	log K_{a4}	3.23(2)	3.22(3)	3.21(2)	3.47(2)	3.23(2)	3.20(4)
	log K_{a3}	5.60(3)	5.53(2)	5.63(1)	5.47(1)	5.42(2)	5.39(2)
	log K_{a2}	8.54(3)	8.48(5)	8.62(4)	8.68(2)	8.72(6)	8.72(5)
	log K_{a1}	9.85(2)	9.81(2)	9.73(2)	9.74(4)	9.73(4)	9.88(3)
	1000 s(A)	1.04	0.93	0.95	0.75	1.45	1.48

Table 2. The thermodynamic protonation constants of vardenafil at 25°C and 37°C. Standard deviations of protonation constants in the last valid digits are in parentheses.

	25°C	**37°C**	**MARVIN prediction**
log K^T_{a4}	3.59(1)	3.26(1)	2.41
log K^T_{a3}	5.64(1)	5.81(1)	6.21
log K^T_{a2}	9.41(1)	8.59(2)	8.10
log K^T_{a1}	10.92(2)	10.05(1)	11.46

can reach the global minimum U_{min}. It is obvious from the figure that most of the residuals are of the same magnitude as the instrument noise and thus proves a sufficient reliability of the regression process.

Applying a Debye-Hückel equation to the data in Table 1 according to the regression criterion, the unknown parameter log K^T has been estimated. Table 2 yields point estimates of the thermodynamic protonation constants of vardenafil studied at two temperatures (Fig. 7). Because of the small range of ionic strength, the ion-size parameter *å* and the salting-out coefficient *C* could not be estimated.

5. Conclusions

When drugs are poorly soluble, pH-spectrophotometric titration may be used along with non-linear regression of the absorbance-response-surface data. The reliability of the protonation constants of the drug vardenafil may be proven with goodness-of-fit tests of the absorption spectra measured at various pH. The thermodynamic protonation constants log K^T_i were estimated by a nonlinear regression of (log *K, I*) data using the Debye-Hückel equation: log K^T_4 = 3.59(1) and 3.26(1), log K^T_3= 5.64(1) and 5.81(1), log K^T_2 = 9.41(1) and 8.59(2), log K^T_1= 10.92(2) and 10.05(1) at 25ºC and 37ºC, where the figure in brackets is the standard deviation in last significant digits. Concurrently, the experimental determination of four thermodynamic protonation constants was combined with their computational prediction from the MARVIN program based on knowledge of the chemical structures of the drug, and good agreement was found between computational and experimental values. Most indices always predict the correct number of components when the *signal-to-error ratio SER* is still much higher than 10.

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