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Thermodynamic dissociation constants of silychristin, silybin, silydianin and mycophenolate by the regression analysis of spectrophotometric data

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Abstract

Mixed dissociation constants of four drug acids, i.e. silychristin, silybinin, silydianin and mycophenolate at various ionic strengths I of range 0.01 and 0.30 and at temperatures of 25 and 37 °C were determined using the SQUAD(84) regression analysis program applied to pH-spectrophotometric titration data. The proposed strategy of an efficient experimentation in a protonation constants determination, followed by a computational strategy for the chemical model with a protonation constants determination, is presented on the protonation equilibria of silychristin. The thermodynamic dissociation constant pK_a^T was estimated by non-linear regression of $\{pK_a, I\}$ data at 25 and 37 °C: for silychristin $pK_{a,1}^T = 6.52(16)$ and 6.62(1), $pK_{a,2}^T = 7.22(13)$ and 7.41(5), $pK_{a,3}^T = 8.96(9)$ and 8.94(9), $pK_{a,4}^T = 10.17(7)$ and 10.03(8), $pK_{a,5}^T = 11.89(4)$ and 11.63(7); for silybin $pK_{a,1}^T = 7.00(4)$ and 6.86(5), $pK_{a,2}^T = 8.77(11)$ and 8.77(3), $pK_{a,3}^T = 9.57(8)$ and 9.62(1), $pK_{a,4}^T = 11.66(3)$ and 11.38(1); for silydianin $pK_{a,1}^T = 6.64(7)$ and 7.10(6), $pK_{a,2}^T = 7.78(5)$ and 8.93(1), $pK_{a,3}^T = 9.66(9)$ and 10.06(11), $pK_{a,4}^T = 10.71(7)$ and 10.77(7), $pK_{a,5}^T = 12.26(5)$ and 12.14(5); for mycophenolate $pK_a^T = 8.32(1)$ and 8.14(1). Goodness-of-fit tests for various regression diagnostics enabled the reliability of parameter estimates to be found.

Keywords: Spectrophotometric titration; Dissociation constant; Protonation; Debye–Hückel equation; Silychristin; Silybinin; Silydianin; Mycophenolate

1. Introduction

In the 1990s, the pharmaceutical industry and regulation adopted a new system for the classification of drugs, the Biopharmaceutics Classification System (BCS) [1,2]. The BCS classifies every pharmaceutical active ingredient into one of four groups based on two basic characteristics: solubility and permeability. The system reflects contemporary experience in the evaluation of the most important features of drugs which affect the formulation of medicine preparation and regulatory consequences. When a poorly soluble drug is to be formulated, attention

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is paid mainly to an improvement of its solubility, and thus mostly to the selection of the appropriate pharmaceutical excipient(s). Less attention is paid to its behavior in water systems compared to more soluble drugs. We decided to complete such an information and to study the protonation equilibria of three pharmaceutical active ingredients of natural origin, silybin, silychristin and silydianin, all of which possess low water solubility. As we paid special attention to the methodology, we also studied the protonation equilibria of two other compounds, mycophenolate mofetil and mycophenolate sodium salt, in order to verify the validity of the results.

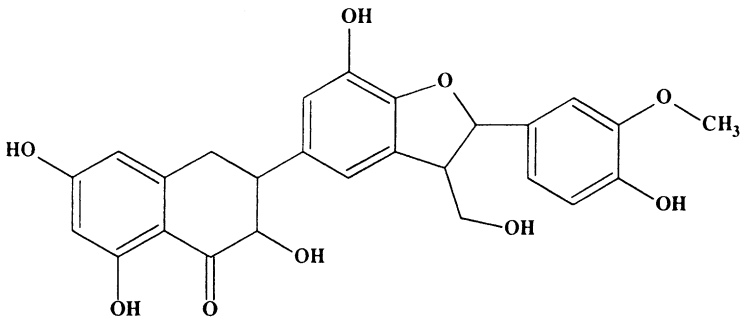
Silybin, silychristin and silydianin are flavanolignans belonging to a group of biologically active substances found in milk (or Saint-Mary) thistle, *Silybum marianum* (L.) *Gaertner*. They are almost certainly produced in the plant by a radical coupling of flavonoid and coniferyl alcohol [3]. In 1974, Wagner et al. [4] proposed giving the name silymarin to the whole group of active flavanolignan-like substances and the names silybin, silydianin and silychristin to its main constituents. Currently, silymarin is available as standardized mixture of four flavanolignan isomers: silybin (synonymous with silibinin), isosilybin (synonymous with isosilibinin), silydianin and silychristin (synonymous with silycristin). The major component of silymarin is silybin, which constitutes 60–70% of the drug [5].

The antioxidative activity of silymarin compounds consists in scavenging free radicals and inhibiting lipid peroxidation in cell membranes, chelating iron ions and inhibiting oxidative enzymes [6–10]. Hepatoprotective effects result from the promotion of mitotic activity in regenerating liver tissue ([11]), from the activation of hepatocyte RNA polymerase I and the restoration of their ATPase activity and glutathione content, and from strong inhibition of the production of pro-inflammatory cytokines (interleukin 1 and tumor necrosis factor-alpha) and pro-inflammatory agents-leukotrienes and prostaglandines [12].

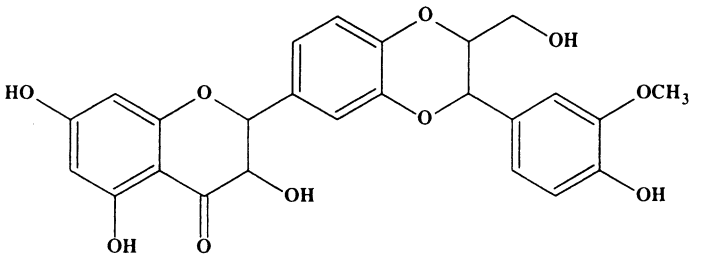
The hepatoprotective effect has been proved against a variety of hepatotoxic compounds [13–15]. Positive effects are also described in cases of acute liver injury after intoxication with mushroom toxins such as phalloidin or amanitin from *Amanita phalloides* [16–19] and cases of chronic hepatitis of either viral or alcoholic ethiology [20–23]. After a phospholipid complex of silybin was prepared, its bioavailability increased [24,25]. Even though the phospholipid moiety facilitates not only the solubility but also the permeation of the drug, there is still little known about the protonation behavior of silymarin compounds as such. In this work, we studied the protonation equilibria of silybin, silichristin and silydianin to complete their pre-formulation characteristics.

Mycophenolate mofetil (MPM), is a morpholinoethyl ester of mycophenolic acid (MPA), which itself is a fermentation product of several Penicillium species. MPA is a potent, non-competitive, and reversible inhibitor of inosine monophosphate dehydrogenases in eukaryotic cells. This enzyme is a branch point of purine biosynthesis.

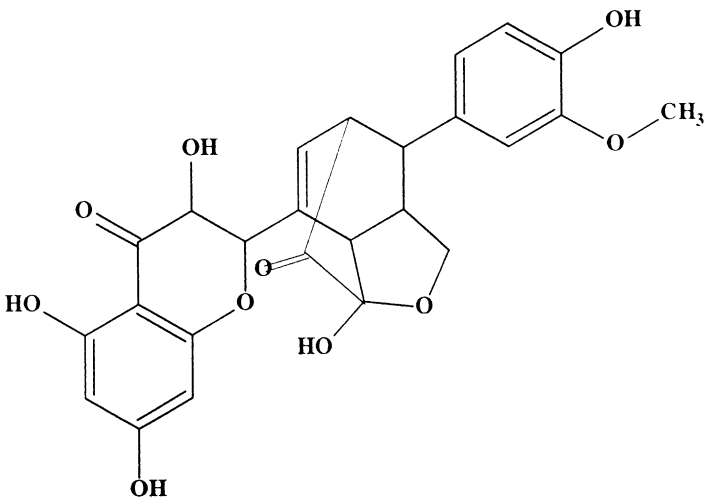
The original compound, MPA, is almost insoluble in water. However, neither of the pharmaceutical formulations using the more soluble sodium salt of MPA were succesful from the point of view of bioavailability. The reason for this is that sodium mycophenolate is not permeable as it is either dissociated or precipitates in the gastrointestinal tract. The first and theoretically expectable dissociation constant for the sodium salt is not published, and the second dissociation constant for the hydroxyl group on the mycophenolate part of the molecule should be close to that of the morpholinoester, i.e. is 8.5 (see Roche US Pharmaceuticals, 20. 2. 2002, available from http://www.roche.com). Thus, it behaves like a weak base, and in theory, can be absorbed in the small intestine if passes the stomach. Moreover, the solubility of sodium mycophenolate increases in alkalic media. The morpholinoester MPM is less soluble at neutral pH (43 μg/ml at pH 7.4 and 4.3 mg/ml at pH 3.6, see Roche US Pharmaceuticals, 20. 2. 2002, available from http://www.roche.com) than sodium mycophenolate but the solubility increases in acidic medium (4.27 mg/ml at pH 3.6), Therefore, MPM is more readily absorbed in stomach, as the first dissociation constant for the nitrogen-site on morpholino part of the molecule is 5.6 and it determines it behaves like a weak acid. The second dissociation site is on the phenolic group of the mycophenolate and its dissociation constant is 8.5.



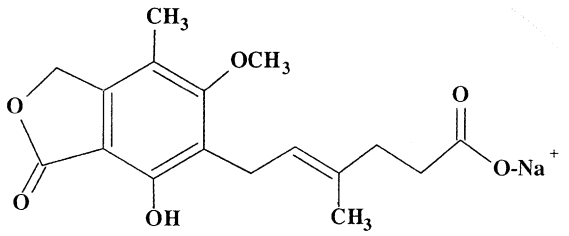
Silychristin, CAS: 33889-69-9



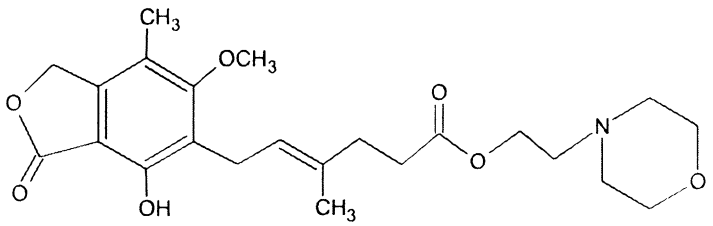
Silybin, CAS: 22888-70-6



Silydianin, CAS: 29782-68-1



Sodium mycophenolate, (Sodium 1,3- dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-4-hexenoate), $C_{17}H_{19}O_6Na$



Mycophenolate mofetil

The protonation constants of silybinin, silychristin, silydianin and mycophenolate at various ionic strengths have been studied at various temperatures. However, in only few cases has the dependence of the protonation constants on ionic strength been systematically investigated and the thermodynamic dissociation constant estimated. The dissociation constants of acid(s) can be estimated by analysis of acid–base titrations and these methods have been reviewed [26,27,30,31]. If the influence of various systematic errors is taken into account a computer-based method is necessary. The reliability of dissociation constants obtained by the regression analysis of potentiometric or pH-spectrophotometric data is dependent upon (i) the calibration of the glass electrode cell, (ii) the algorithm used, (iii) the instrumental method used and the parameters selected for refinement, and (iv) the strategy of an efficient experimentation.

In this paper we decided to investigate the dissociation constants of the four drugs a silychristin, silybinin, silydianin and mycophenolate at various ionic strengths and at 25 and 37 °C, to prove their reliability and also to estimate the thermodynamic dissociation constant pK_a^T at these two temperatures.

2. Theoretical

2.1. Determination of protonation/dissociation constants

An acid–base equilibrium of the drug studied is described in terms of the protonation of the Brönstedt base L^{z-1} according to the equation $L^{z-1} + H^+ \rightleftarrows Hl^z$ characterized by the protonation constant:

$$K_H = \frac{a_{HL^z}}{a_L^{z-1}} = \frac{[HL^z]}{[L^{z-1}][H^+]} \frac{y_{HL^z}}{y_{L^{z-1}} y_{H^+}} \quad (1)$$

For dissociation reactions realized at constant ionic strength so called “mixed dissociation constants” are defined as:

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

These constants are found in experiments where pH values are measured with glass and reference electrodes, standardized with the practical $pH(S) = pa_{H^+}$ activity scale recommended internationally [26,27]. The $pH(S) = p(a_{H^+})_c + \log \rho_s$ where index c means molar (and if relevant molal concentrations m) and ρ_s is the density of the solvent. For aqueous solutions

and temperatures up to 35 °C, this correction is less than 0.003 pH unit. The value of $[H_{j-1}L]/[H_jL]$ may be determined either by (a) a potentiometric titration when a determination of "the stoichiometric protonation/dissociation constant" pK_c is possible, [28,29] or by (b) a spectrophotometric-pH titration when a determination of the mixed protonation/dissociation constant pK_a is performed, [27,30–32]. If the protonation is studied at several ionic strengths or at a low value of ionic strength, the thermodynamic dissociation constant pK_a^T can be obtained by extrapolating to zero ionic strength ($I = 0$), the reference state for the activity coefficient being an infinitely diluted solution.

Computations related to the determination of protonation constants may be performed by the regression analysis of spectra using versions of the SQUAD family program [27,32–35]. If the protonation equilibria between the anion, L (the charges are omitted for the sake of simplicity) of a drug and a proton, H, are considered to form a set of variously protonated species L, LH, LH_2, LH_3, ... etc. which have a general formula L_qH_r in a particular chemical model and are represented by n_c the number of species, $(q, r)_i$, $i = 1, \ldots, n_c$ where index i labels their particular stoicheiometry, then the overall protonation (stability) constant of the protonated species, β_{qr} may be expressed as:

$$\beta_{qr} = \frac{[L_qH_r]}{[L]^q[H]^r} = \frac{c}{(l^q h^r)} \tag{3}$$

where the free concentration $[L] = l$, $[H] = h$ and $[L_qH_r] = c$. For the ith solution measured at the jth wavelength, the absorbance, $A_{i,j}$, is defined as:

$$A_{i,j} = \sum_{n=1}^{n_c} \varepsilon_{j,n} c_n = \sum_{n=1}^{n_c} (\varepsilon_{qr,j} \beta_{qr} l^q h^r)_n \tag{4}$$

where $\varepsilon_{qr,j}$ is the molar absorptivity of the L_qH_r species with the stoichiometric coefficients q, r measured at the jth wavelength. The absorbance $A_{i,j}$ is the element of the absorbance matrix $\boldsymbol{A}$ of size $(n_s \times n_w)$ being measured for n_s solutions with known total concentrations of $n_z = 2$ basic components, c_L and c_H, at n_w wavelengths. The rank of the matrix $\boldsymbol{A}$ is obtained from the equation $\text{rank}(\boldsymbol{A}) = \min [\text{rank}(\boldsymbol{E}), \text{rank}(\boldsymbol{C})] \leq \min (n_w, n_c, n_s)$. Since the rank of $\boldsymbol{A}$ is equal to the rank of $\boldsymbol{E}$ or $\boldsymbol{C}$, whichever is the smaller, and since $\text{rank}(\boldsymbol{E}) \leq n_c$ and $\text{rank}(\boldsymbol{C}) \leq n_c$, then provided n_w and n_s are equal to or greater than n_c, it will only be necessary to determine the rank of matrix $\boldsymbol{A}$ which is equivalent to the number of dominant light-absorbing components [27,36,37].

The multi-component spectra analysing program SQUAD(84) [32] may adjust β_{qr} and ε_{qr} for absorption spectra by minimising the residual-square sum function, U,

$$\begin{aligned} U &= \sum_{i=1}^{n_s}\sum_{j=1}^{n_w} (A_{\text{exp},i,j} - A_{\text{calc},i,j})^2 \\ &= \sum_{i=1}^{n_s}\sum_{j=1}^{n_w} \left(A_{\text{exp},i,j} - \sum_{k=1}^{n_c} \varepsilon_{j,k} c_k \right)^2 \\ &= \text{minimum} \end{aligned} \tag{5}$$

where $A_{i,j}$ represents the element of the experimental absorbance response-surface of size $n_s \times n_w$ and the independent variables c_k are the total concentrations of the basic components c_L and c_H being adjusted in n_s solutions. Unknown parameters are the best estimates of the protonation constants, $\beta_{qr,i}$ $i = 1, \ldots, n_c$, which are adjusted by SQUAD(84) regression algorithm. At the same time, a matrix of molar absorptivities $(\varepsilon_{qr,j}, j = 1, ..., n_w)_k$, $k = 1, \ldots, n_c$, as non-negative reals is estimated, based on the current values of protonation constants. For a set of current values of $\beta_{qr,i}$, the free concentrations of ligand l, as h is known from pH measurement, for each solution is calculated, followed by the concentrations of all the species in equilibrium mixture $[L_qH_r]_j$, $j = 1, \ldots, n_c$, forming for n_s solutions the matrix $\boldsymbol{C}$ are obtained. The calculated S.D. of absorbance $s(A)$ and the Hamilton *R-factor* are used as the most important criteria for a fitness test. If, after termination of the minimization process the condition $s(A) \approx s_{\text{inst}}(A)$ is met and the *R-factor* is less than 1%, the hypothesis of the chemical model is taken as the most probable one and is accepted.

2.2. Determination of the thermodynamic protonation/dissociation constant

Let us consider a dependence of the mixed dissociation constant $K_a = a_{H^+}[L^{z-1}]/[HL^z]$ on an ionic strength, when both ions HL^z and L^{z-1} have roughly the same ion-size parameter $\mathring{a}$ in the dissociation

equilibrium $HL^z \rightleftarrows L^{z-1} + H^+$ with the thermodynamic dissociation constant $K_a^T = a_{H^+}a_{L^-}/a_{HL}$, and suppose that the overall salting-out coefficients is given by $C = C_{HL} - C_L$. This dependence is expressed by the extended Debye–Hückel equation.

$$pK_a = pK_a^T - \frac{A(1-2z)\sqrt{I}}{(1+B\text{å}\sqrt{I})} + CI \quad (6)$$

where $A = 0.5112\,\text{mol}^{-1/2}\,\text{l}^{1/2}\,\text{K}^{3/2}$ and $B = 0.3291\,\text{mol}^{-1/2}\,\text{m}^{-1}\,\text{l}^{1/2}\,\text{K}^{1/2}\,10^{10}$ for aqueous solutions at 25 °C or 37 °C. The mixed dissociation constant pK_a represents a dependent variable while the ionic strength I stands for the independent variable. Three unknown parameters $b = \{pK_a, \text{å}, C\}$ are to be estimated by a minimization of the sum of the squared residuals [27]:

$$\begin{aligned} U(b) &= \sum_{i=1}^{n} w_i[pK_{a,\text{exp},i} - pK_{a,\text{calc},i}]^2 \\ &= \sum_{i=1}^{n} w_i[pK_{a,\text{exp},i} - f(I;\ pK_a^T, \text{å}, C)]^2 \\ &= \text{minimum} \end{aligned} \quad (7)$$

2.3. Reliability of estimated protonation/dissociation constants

The adequacy of a proposed regression model with experimental data and the reliability of parameter estimates $pK_{a,i}$ found (being denoted for the sake of simplicity as b_j, $j = 1, \ldots, m$,) and ε_{ij}, $j = 1, \ldots, n_w$, may be examined by the goodness-of-fit test, p. 101 [27]:

(1) *The quality of parameter estimates* b_j $j = 1, \ldots, m$, found is considered according to their variances $D(b_j)$. Often an empirical rule is used: parameter b_j significantly differs from zero when its estimate is greater than 3 S.D., $3\sqrt{D(b_j)} < |b_j|$, $j = 1, \ldots, m$.

(2) *The quality of the experimental data*, is examined by the identification of influential points with the use of regression diagnostics, p. 62 [38].

(3) *The quality of curve fit achieved*, the adequacy of a proposed model and m parameter estimates found with n values of experimental data is examined by a goodness-of-fit test based on the statistical analysis of classical residuals. If the proposed model adequately represents the data, the residuals should form a random pattern with a normal distribution $N(0, s^2)$, with the residual mean equal to zero, $\bar{e} = 0$, and the S.D. of residuals $s(e)$ being near to noise i.e. the experimental error ϵ of absorbance measured. Systematic departures from randomness indicate that the model and parameter estimates are not satisfactory. Examination of residual plots may be assisted by graphical analysis of residuals, p. 289, 290 [38].

3. Experimental

3.1. Chemicals and solutions

Drugs: Silybin, silydianin, silychristin, mycophenolate mofetil and sodium mycophenolate were generously donated by IVAX-CR, Czech Republic. A silymarin extract of pharmacopoeial quality (DAB IX) was prepared from *Silybum marianum*, var. Silyb (L.) Gaertn. (Asteraceae). Individual components were isolated and purified by ethylacetate extraction, crystallization and chromatography. The final purities achieved were: silybin: IVAX-CR company standard AB023, Batch No. 190194, 97.5% (HPLC). Silydianin IVAX-CR company standard RD, Batch No. 090680, 99.9% (HPLC). Silychristin: IVAX-CR company standard RD 0731, Batch No. SC 010498/10 90.3% (HPLC), expiry date May 2003. Mycophenolate mofetil was prepared by the direct esterification of mycophenolic acid with 2-morfolinoethanol. Sodium mycophenolate was prepared by neutralization reaction with sodium methanolate. The final purities achieved were: mycophenolate mofetil: IVAX-CR, Batch No. KB151200, 99.3% (HPLC) Sodium mycophenolate: IVAX-CR, Batch No. KB/060999/1.

Perchloric acid: 1 M, was prepared by dilution of concentrated $HClO_4$ (p. a. Lachema Brno) with redistilled water and standardization against HgO and NaI with a reproducibility better than 0.2% according to the equation $HgO + 4\ NaI + H_2O \rightleftarrows 2\ NaOH + Na_2[HgI_4]$ and $NaOH + HClO_4 \rightleftarrows NaClO_4 + H_2O$.

Sodium hydroxide: 1 M, was prepared from an exact weight of pellets (p. a. Aldrich) with a carbon-dioxide free redistilled water. The solution was stored for several days in a polyethylene bottle. This solution

was standardized against a solution of potassium hydrogen–phthalate using the Gran method in the MAGEC program [27,30] with a reproducibility of 0.1%.

Mercury oxide, *sodium iodide*, and *sodium perchlorate*: (p. a. Lachema Brno) were not further purified. The preparation of buffers and other solutions from analytical-reagent grade chemicals has also been described previously [28,29].

Twice-redistilled water: was used in the preparation of solutions.

3.2. Apparatus and pH-spectrophotometric titration procedure

The free hydrogen-ion concentration h was measured via *emf* on a digital voltmeter OP-208/1 (Radelkis, Budapest) with a precision of ±0.1 mV using of a glass electrode G202B (Radiometer, Copenhagen) and a commercial SCE reference electrode OP-8303P (Radelkis, Budapest). The spectrophotometric multiple-wavelength pH-titration was carried out as follows: an aqueous solution 20.00 cm^3 containing 10^{-5} mol dm^{-3} drug, 0.100 mol dm^{-3} hydrochloric acid and 10 cm^3 indifferent solution KCl for adjustment of ionic strength was titrated with standard 1.0 mol dm^{-3} KOH at 298 K and 20 absorption spectra were recorded. 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 °C±0.1° and 37.0 °C±0.1°. When the drugs were titrated, a stream of argon gas was bubbled through the solution both for stirring and for maintaining an inert atmosphere. The argon was passed through aqueous ionic medium by prior passage through one or two vessels also containing the titrand medium before entering the corresponding titrand solution. The burettes used were syringe micro-burettes of 1250 μl capacity (META, Brno) with a 2.50 cm micrometer screw, [39]. The polyethylene capillary tip of the micro-burette was immersed into the solution when adding reagent but pulled out after each addition in order to avoid leakage of the reagent during the pH read out. The micro-burette was calibrated by 10 replicate determinations of the total volume of delivered water by weighing on a Sartorius 1712 MP8 balance with results evaluated statistically, leading to a precision of ±0.015% in added volume over the whole volume range. The solution was pumped into the cuvette and spectrophotometric measurement was performed with the use of a Cintra 40 (GBC, Australia) spectrophotometer.

3.3. Procedure for determination of the chemical model and protonation constants

The experimental and computation scheme for the determination of the protonation constants of the multi-component system is taken from Meloun et al., p. 226 [27] or [32]:

(1) *Instrumental error of absorbance measurements*, $s_{inst}(A)$: The INDICES algorithm should be used with solutions of potassium dichromate to evaluate $s_{inst}(A)$, [37]. The scree plot of $s_k(A) = f(k)$ consists of two straight lines intersecting at $\{s_k^*(A); k^*\}$ where k^* is the matrix rank for the system. Since $k^* = 1$ for $K_2Cr_2O_7$, the value of $s_k(A)$ for $k^* = 1$ is a good estimate of the instrumental error of the spectrophotometer used, $s_{inst}(A) = s_*^1(A)$ reaching a value of 0.25 mAU for the Cintra 40 (GBC, Australia) spectrophotometer.

(2) *Experimental design*: Since preparation of a large number of separate solutions is tedious, simultaneous monitoring of absorbance and pH during titrations is valuable [32]. In a titration, the total concentration of one of the components changes incrementaly 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. $0.1 < A < 1.2$, though 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 are studied in the visible region, 190–760 nm. The wavelength range selected is such that every species made a significant contribution to the absorbance. Little information is obtained in regions

of great spectral overlap or where the molar absorptivities of two or more species are linearly interdependent as the change of absorbance following changes in c_L and c_H becomes rather small. If only a small number of wavelengths is used, those of maxima or shoulders 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.

(3) *Number of light-absorbing species:* When no outliers (grossly erroneous points) are present in the spectra examined, $s_k^*(A) \leq s_{inst}(A)$ is valid. The INDICES [37] determine the number of dominant species to be present in equilibrium mixture.

(4) *Choice of computational strategy:* The input data should specify whether β_{qr} or $\log \beta_{qr}$ values are to be refined, multiple regression (MR) or non-negative linear least-squares (NNLS) are desired, whether baseline correction has to be performed, etc. In description of the model, it should be indicated whether protonation constants are to be refined or held constant, and whether molar absorptivities are to be refined.

(5) *Diagnostics indicating a correct chemical model:* When a minimization process terminates, some diagnostics are examined to determine whether the results should be accepted. To attain a good chemical model, the following diagnostics should be considered [41]:

(5a) *First diagnostic—the physical meaning of parametric estimates*: The physical meaning of the protonation constants, molar absorptivities, and stoichiometric INDICES is examined: β_{qr} and ε_{qr} should be neither too high nor too low, and ε_{qr} should not be negative. The absolute values of $s(\beta_j)$, $s(\varepsilon_j)$ gives information about the last U-contour of the hyperparaboloid in neighborhood of the pit, U_{min}. For well-conditioned parameters, the last U-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 S.D. of ε_{pqr} for various wavelengths, $s(\varepsilon_{qr}) = f(\lambda)$, should have a Gaussian distribution; otherwise, erroneous estimates of ε_{qr} are obtained.

(5b) *Second diagnostic—the physical meaning of the species concentrations:* The calculated distribution of the free concentration of the basic components and variously protonated species of the chemical model should show molarities down to about 10^{-8} M. Since a species present at about 1% relative concentration or less in an equilibrium behaves as a numerical noise in regression analysis, a distribution diagram makes it easier to judge the contributions of individual species to the total concentration quickly. Since the molar absorptivities will be generally in the range 10^3–10^5 l mol^{-1} cm^{-1}, species present at less than ca. 0.1% relative concentration will affect the absorbance significantly only if their ε is extremely high.

(5c) *Third diagnostic—parametric correlation coefficients:* Partial correlation coefficients, r_{ij}, indicate the interdependence of two parameters β_i and β_j when the others are fixed in value. All these correlation coefficients may have values between -1 and $+1$. Zero means complete independence, and $+1$ or -1 means complete correlation. Two completely correlated species cannot be included in a chemical model, because the relevant protonation constants are strongly correlated.

(5d) *Fourth diagnostic—goodness-of-fit test:* This diagnostic contains the criteria for testing the correctness of a hypothetical chemical model. To identify the "best" or true chemical model when several are possible or proposed, and to establish whether or not the chemical model represents the data adequately, the residuals e should be analyzed. The goodness-of-fit achieved is easily seen by examination of the differences between the experimental and calculated values of absorbance, $e_i = A_{exp,i,j} - A_{calc,i,j}$. One of the most important statistics calculated is the S.D. of the absorbance, $s(A)$, calculated from a set of refined parameters at the termination of minimization process. It is usually compared with the standard deviation of absorbance calculated by the INDICES

program [37], $s_k(A)$, and if $s(A) \leq s_k(A)$, or $s(A) \leq s_{inst}(A)$, the instrumental error of the spectrophotometer used, the fit is considered to be statistically acceptable. Although this statistical analysis of residuals [38] gives the most rigorous test of the degree-of-fit, realistic empirical limits must be used. For example, when $s_{inst}(A) \leq s(A) \leq 0.003$, the goodness-of-fit is still taken as acceptable, whereas $s(A) > 0.010$ indicated that a good fit has not been obtained. Alternatively, the statistical measures of residuals e can be calculated: the residual mean (known as the bias) $\bar{e}$ should be a value close to zero; the mean residual and the residual S.D. $s(e)$ should be close to the absorbance S.D. $s_{inst}(A)$; the skewness $g_1(e)$ should be close to zero for a symmetric distribution; the kurtosis $g_2(e)$ should be close to 3 for the Gaussian distribution; a Hamilton *R-factor* of relative fit, expressed as a percentage, ($R \times 100\%$), of <0.5% is taken as an excellent fit, but of >2% is a poor one. The *R-factor* gives a rigorous test of the null hypothesis H_0 (giving R_0) against the alternative H_1 (giving R_1). H_1 could be rejected at the α significance level if $R_1/R_0 > R_{(m,n-m,\alpha)}$, where n is the number of experimental points, m is the number of unknown parameters, and $(n - m)$ is the number of degrees of freedom. The value of $R_{(m,n-m,\alpha)}$ can be found in statistical tables.

(5e) *Fifth diagnostic—deconvolution of spectra:* Resolution of each experimental spectrum into spectra of the individual species proves whether the experimental design is efficient.

3.4. Software used

Computation relating to the determination of dissociation constants was performed by regression analysis of UV/Vis spectra using the SQUAD(84) program [32]. The thermodynamic dissociation constant pK^T was estimated with the non-linear regression program MINOPT in the ADSTAT statistical system (TriloByte Statistical Software Ltd. Pardubice), [40].

4. Results and discussion

4.1. Estimation of protonation/disociation constants of four drugs

The proposed strategy for an efficient experimentation in protonation constants determination followed by spectral data treatment is presented on the protonation equilibria of silychristin. pH-spectrophotometric titration enables absorbance–response–surface data (Fig. 1a) to be obtained for analysis with the non-linear regression. The reliability of parameter estimates (pK's and ε's) may be evaluated on the basis of the goodnes-of-fit test of residuals (Fig. 1b). The SQUAD(84) program [32] analysis process starts with data smoothing followed by a factor analysis on the Kankare method using INDICES procedure [37]. The position of a break-point on the $s_k(A) = f(k)$ curve in the scree plot is calculated and gives $k^* = 6$ with the corresponding co-ordinate $s_6^*(A) = 0.25$ mAU which also represents the instrumental error $s_{inst}(A)$ of the spectrophotometer used (Fig. 2a). Five protonation constants and six molar absorptivities of silychristin for 39 wavelengths constitute 239 unknown parameters which are refined by the MR algorithm in the first run of the SQUAD program. In the second run, the NNLS algorithm makes the final refinement of all previously found parameter estimates with all molar absorptivities kept non-negative. The reliability of the parameter estimates may be tested with the use of SQUAD(84) diagnostics:

The first diagnostic indicates whether all parametric estimates β_{qr} and ε_{qr} have physical meaning and reach realistic values. As the S.D. $s(\log \beta_{qr})$ of parameters $\log \beta_{qr}$ and $s(\varepsilon_{qr})$ of parameters ε_{qr} are significantly smaller than their corresponding parameter estimates (Table 1), all variously protonated species are statisticaly significant. Fig. 2c show estimated molar absorptivities of all the variously protonated species ε_L, ε_{LH}, ε_{LH2}, ε_{LH3}, ε_{LH4} and ε_{LH5} of silychristin in dependence on wavelength. Some spectra quite overlap and such cases may cause some resolution difficulties in a non-linear regression approach.

The second diagnostic tests whether all of the calculated free concentrations of variously protonated species on the distribution diagram have physical meaning, which proved to be the case (Fig. 2d).

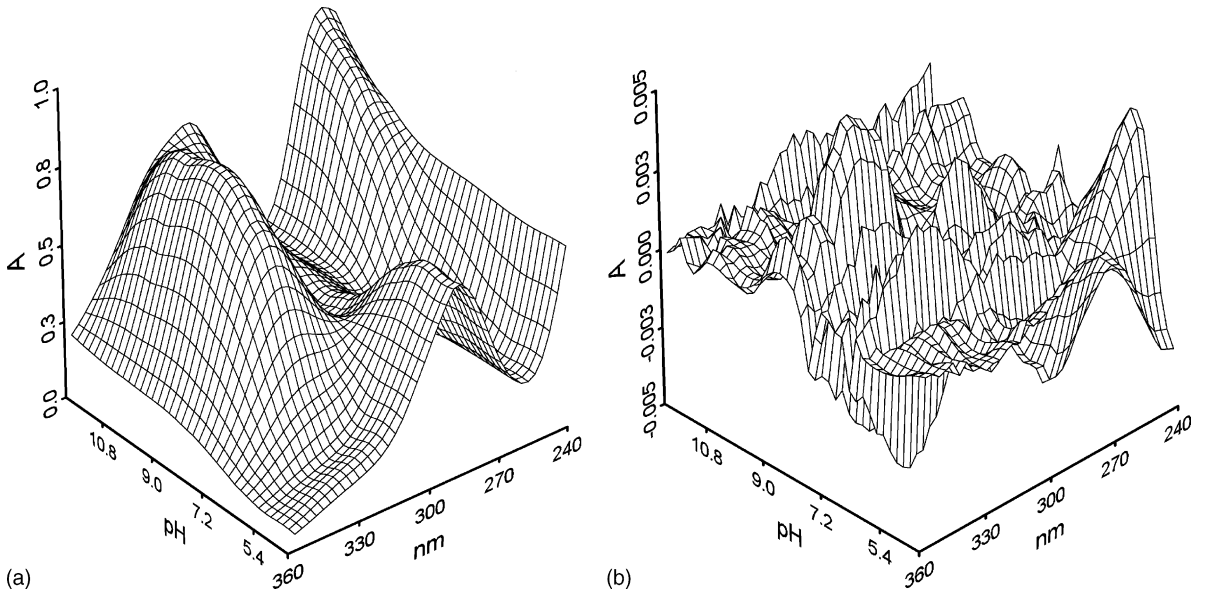


Fig. 1. Absorption spectra of protonation equilibria of silychristin in dependence on pH at 25 °C: (a) 3D-absorbance–response–surface representing SQUAD(84) input data, (b) the 3D-overall diagram of residuals represents a response surface indicating a quality of goodness-of-fit.

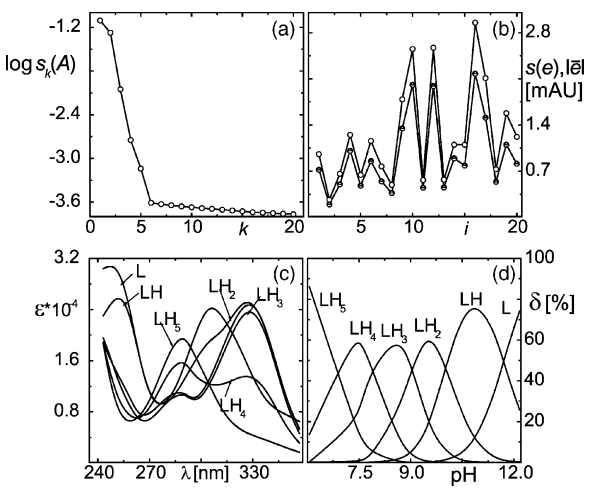


Fig. 2. Estimation of protonation constants and molar absorptivities of silychristin at 25 °C and $I = 0.013$: (a) the scree plot for determination of the number of light-absorbing species in mixture $k^* = 6$ and the instrumental error of the spectrophotometer used $s_6^*(A) = 0.25$ mAU, (b) the goodness-of-fit scatter plot: $s(e)$ and $|\bar{e}|$ for each spectrum, (c) the spectra of molar absorptivities vs. wavelengths for all variously protonated species, (d) distribution diagram of the relative concentrations of all of the variously protonated species.

The diagram shows that the overlapping protonation equilibria exist.

The third diagnostic concerning the matrix of correlation coefficients in Table 1 proves that there is an absence of an interdependence of any pair of protonation constants of silychristin except of species LH_3 versus LH_4 and LH_4 versus LH_5. Significant correlation of these two pairs may be explained by too close protonation constants, which concerns overlapping equilibria.

The fourth diagnostic concerning the goodness-of-fit (Fig. 1b) proves that the $s_6(A)$ value is equal to 0.25 mAU and is quite close to the standard deviation of absorbance when the minimization process terminates, $s(A) = 1.64$ mAU. The statistical measures of all residuals e from Fig. 1b proves that the minimum of the eliptic hyperparaboloid U is reached: the residual mean $\bar{e} = -1.01 \times 10^{-17}$ proves that there is no bias or systematic error in spectra fitting. The mean residual $|\bar{e}| = 0.94$ mAU and the residual S.D. $s(e) = 1.64$ mAU have sufficiently low values. The skewness $g_1(e) = -0.28$ is quite close to zero and proves a symmetric distribution of the residuals set while the kurtosis $g_2(e) = 3.94$ is close to 3 proving a Gaussian distribution. The Hamilton *R-factor* of relative fitness is 0.28% proving an excellent achieved

Table 1
Determination of protonation constants and molar absorptivities of variously protonated species of silychristin by regression analysis of the UV/Vis absorption spectra with SQUAD(84) for $n_s = 20$ spectra measured at $n_w = 39$ wavelengths for $n_z = 2$ basic components L and H forming $n_c = 6$ variously protonated species

Protonation constants			Partial correlation coefficients				
L_qH_r	$\log \beta_{qr}$	s ($\log \beta_{qr}$)	L_1H_1	L_1H_2	L_1H_3	L_1H_4	L_1H_5
L_1H_1	11.802	0.008	1	–	–	–	–
L_1H_2	21.546	0.015	0.6908	1	–	–	–
L_1H_3	30.095	0.075	0.2339	0.5885	1	–	–
L_1H_4	37.239	0.075	0.1811	0.4860	0.9119	1	–
L_1H_5	43.628	0.089	0.1173	0.3201	0.6719	0.9153	1

Determination of the number of light-absorbing species by factor analysis	
Number of light-absorbing species k^*	6
Residual S.D. $s_k^*(A)$	0.25 mAU
Goodnes-of-fit test by the statistical analysis of residuals	
Hamilton *R-factor*	0.28%
Residual mean $\bar{e}$	-1.01×10^{-17}
Mean residual $\lvert\bar{e}\rvert$	0.94 mAU
S.D. of residuals $s(e)$	1.64 mAU
Residual skewness $\hat{g}_1(e)$	−0.28
Residual kurtosis $\hat{g}_2(e)$	3.94

The charges of the ions are omitted for the sake of simplicity.

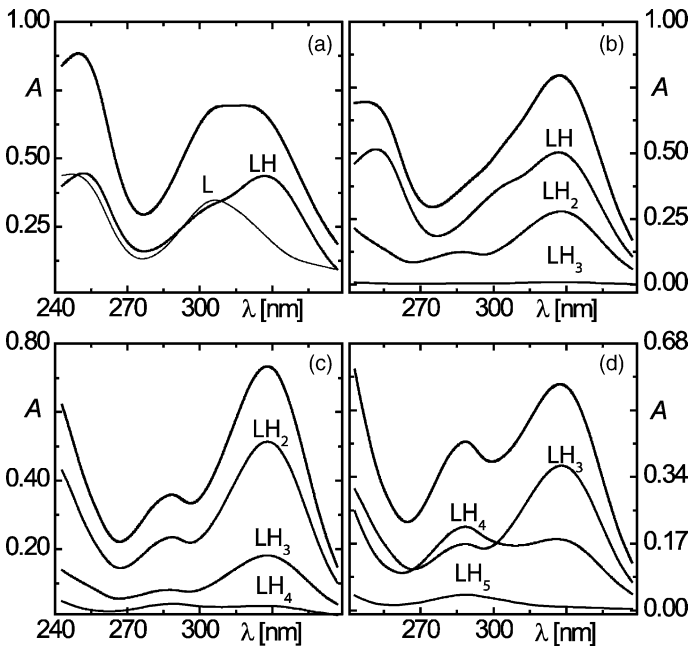


Fig. 3. Deconvolution of the experimental spectrum of silychristin into spectra for the individual variously protonated species in solution for pH equal to: (a) 11.7, (b) 9.9, (c) 8.1 and (d) 7.2.

fitness, and therefore the parameter estimates may be considered suitably as reliable.

The fifth diagnostic, the spectra deconvolution on Fig. 3, shows the deconvolution of the experimental spectrum into spectra for the individual variously protonated species to examine whether the experimental design is efficient. Spectrum deconvolution seems to be quite an useful tool in the proposal of a strategy of an efficient experimentation. Such a spectrum provides a sufficient information for a regression analysis which monitors at least two species in equilibrium where none of them is a minor species. The minor species has a relative concentration in a distribution diagram of less than 5% of the total concentration of the basic component c_L. When, on the other hand, only one species prevails in solution, the spectrum yields quite poor information into a regression analysis and the parameter estimate is rather unsure and definitely not reliable enough.

Using the experimental and evaluation strategy, the protonation equilibria of silybin (Table 2 and Fig. 4), silydianin (Fig. 5) and mycophenolate (Table 3 and Fig. 6) are also examined. To test the reliability of protonation/dissociation constants at different ionic strength the goodness-of-fit test with the use of statistical analysis of the residuals is applied and results appear in Tables 2 and 3. For all four drugs studied the most efficient tools, such as the Hamilton *R-factor*, the mean residual and the standard deviation of residuals are applied: as the *R-factor* in all cases reaches a value of less then 0.5% an excellent fitness and reliable parameter estimates are indicated. The standard deviation of absorbance $s(A)$ after termination of the minimization process is always better than 2 mAU, the proposal of a good chemical model and reliable parameter estimates are proven.

The first problem in the evaluation of the protonation equilibria of the first three drugs (silychristin,

Table 2
Dependence of the mixed dissociation constants of silybin on ionic strength using regression analysis of pH-spectrophotometric data with SQUAD(84) with the standard deviations of the parameter estimates in the last valid digits in brackets

The chemical model attained contains L, LH, LH_2, LH_3, LH_4 at 25 °C					
	Ionic strength				
	0.011	0.032	0.089	0.128	0.177
$pK_{a,4}$	11.644(9)	11.501(8)	11.666(15)	11.622(14)	11.621(12)
$pK_{a,3}$	9.721(18)	9.611(10)	9.579(19)	9.556(19)	9.735(24)
$pK_{a,2}$	8.938(43)	8.666(21)	8.549(38)	8.574(41)	8.645(30)
$pK_{a,1}$	6.871(44)	6.898(22)	6.858(40)	6.841(43)	6.727(35)
	Goodness-of-fit test				
R-factor (%)	0.29	0.2	0.35	0.26	0.27
$s_k(A)$ (mAU)	0.25	0.3	0.29	0.26	0.31
$s(A)$ (mAU)	1.6	1.01	1.61	1.84	1.12
The chemical model attained contains L, LH, LH_2, LH_3, LH_4 at 37 °C					
	Ionic strength				
	0.011	0.029	0.085	0.131	0.198
$pK_{a,4}$	11.340(22)	11.295(15)	11.263(16)	11.248(14)	11.224(17)
$pK_{a,3}$	9.504(26)	9.411(17)	9.340(23)	9.308(17)	9.280(19)
$pK_{a,2}$	8.543(46)	8.412(27)	8.205(32)	8.095(32)	8.090(30)
$pK_{a,1}$	6.795(49)	6.778(29)	6.773(37)	6.754(34)	6.659(35)
	Goodness-of-fit test				
R-factor (%)	0.32	0.26	0.27	0.28	0.27
$s_k(A)$ (mAU)	0.38	0.45	0.49	0.35	0.37
$s(A)$ (mAU)	2.09	1.43	1.66	1.42	1.36

The reliability of parameter estimates found is proven with goodness-of-fit statistics such as the Hamilton *R-factor* (%), the residual S.D. $s_k(A)$ (mAU) and the standard deviation of absorbance after termination of the regression process, $s(A)$ (mAU) at 25 (upper part) and 37 °C (lower part).

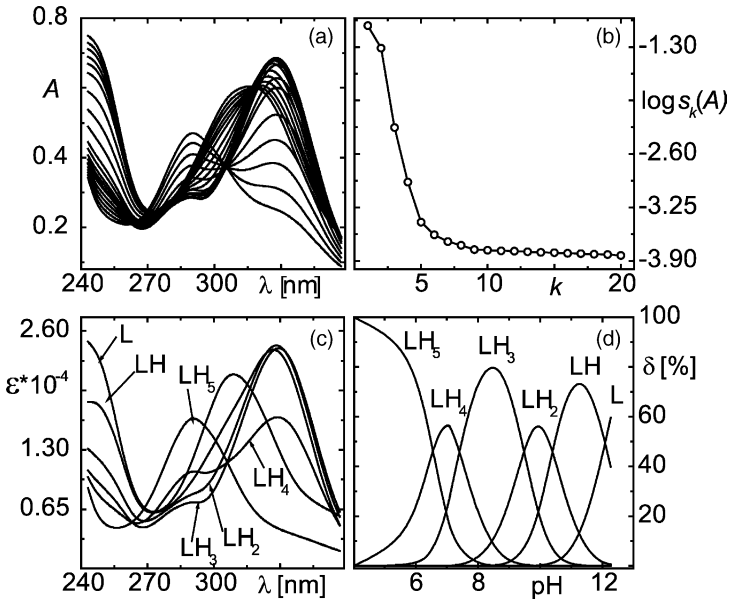


Fig. 4. Estimation of protonation constants and molar absorptivities of silybin at 25 °C and ionic strength $I = 0.015$: (a) experimental spectra, (b) the scree plot for determination of the number of light-absorbing species in mixture $k^* = 5$ and $s_5^*(A) = 0.32$ mAU, (c) the spectra of molar absorptivities vs. wavelengths for all of the variously protonated species, (d) distribution diagram of the relative concentrations of all variously protonated species.

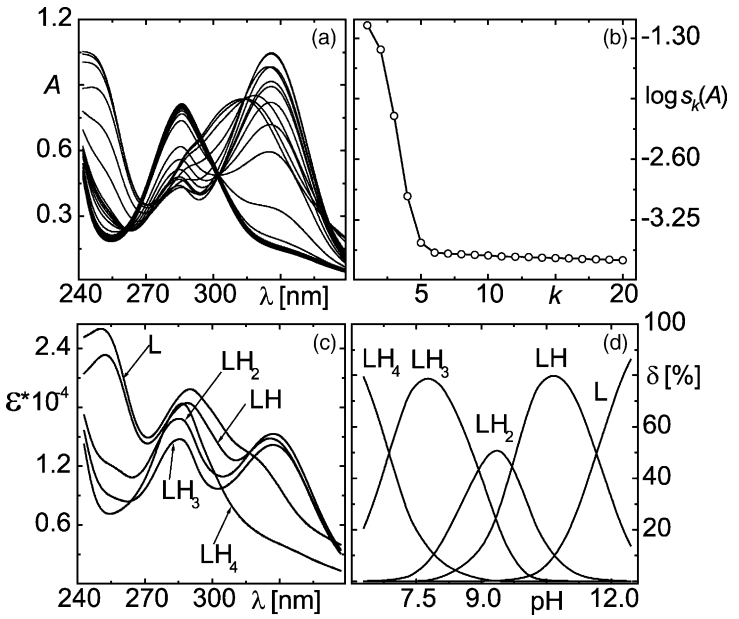


Fig. 5. Estimation of the protonation constants and molar absorptivities of silydianin at 25 °C and ionic strength $I = 0.009$: (a) experimental spectra, (b) the scree plot for determination of the number of light-absorbing species in mixture $k^* = 6$ and $s_6^*(A) = 0.23$ mAU, (c) the spectra of molar absorptivities vs. wavelengths for all of the variously protonated species, (d) distribution diagram of the relative concentrations of all of the variously protonated species.

Table 3
Dependence of the mixed dissociation constants of mycophenolate on ionic strength using regression analysis of pH-spectrophotometric data with SQUAD(84) with the standard deviations of parameter estimates in the last valid digits in brackets

The chemical model attained contains L, LH at 25 °C								
	Ionic strength							
	0.005	0.008	0.027	0.038	0.049	0.059	0.07	0.08
$pK_{a,1}$	8.271(2)	8.228(2)	8.156(2)	8.125(1)	8.102(1)	8.105(1)	8.082(2)	8.059(1)
	Goodness-of-fit test							
R-factor (%)	0.48	0.45	0.46	0.42	0.41	0.37	0.49	0.37
$s_k(A)$ (mAU)	0.29	0.14	0.12	0.14	0.16	0.17	0.32	0.14
$s(A)$ (mAU)	1.64	1.41	1.41	1.28	1.28	1.09	1.46	1.11

The chemical model attained contains L, LH at 37 °C										
	Ionic strength									
	0.073	0.073	0.083	0.095	0.104	0.107	0.113	0.124	0.144	0.16
$pK_{a,1}$	8.066(1)	8.070(1)	8.058(1)	8.047(1)	8.040(1)	8.039(1)	8.025(1)	8.026(2)	8.014(1)	8.001(3)
	Goodness-of-fit test									
R-factor (%)	0.23	0.23	0.22	0.12	0.21	0.21	0.23	0.34	0.16	0.5
$s_k(A)$ (mAU)	0.22	0.24	0.13	0.09	0.18	0.15	0.26	0.35	0.14	0.33
$s(A)$ (mAU)	0.6	0.64	0.51	0.27	0.51	0.46	0.57	0.82	0.37	1.26

The reliability of parameter estimates found is proven with goodness-of-fit statistics such as the Hamilton *R-factor* (%), the residual S.D. $s_k(A)$ (mAU) and the standard deviation of absorbance after termination of the regression process, $s(A)$ (mAU) at 25 (upper part) and 37 °C (lower part).

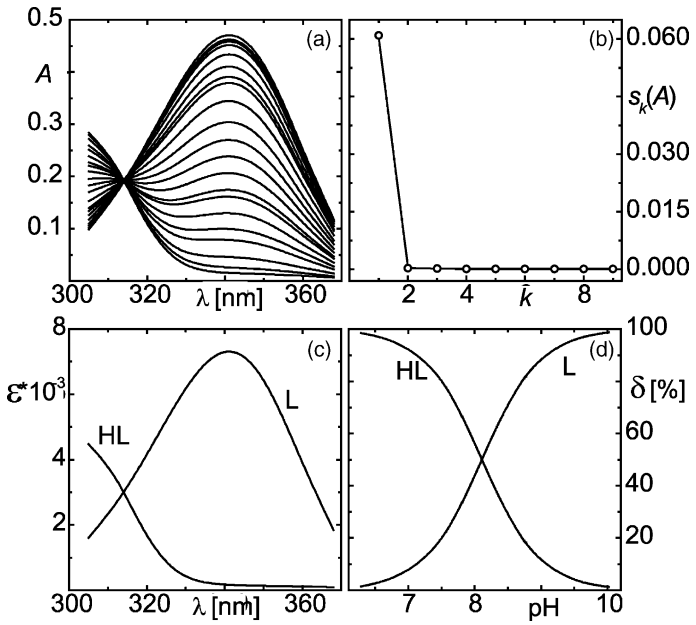


Fig. 6. Estimation of the protonation constants and molar absorptivities of sodium mycophenolate at 25 °C and $I = 0.008$: (a) experimental spectra, (b) the scree plot for determination of the number of light-absorbing species in mixture $k^* = 2$ and $s_2^*(A) = 0.14$ mAU, (c) the spectra of molar absorptivities vs. wavelengths for all of the variously protonated species, (d) the distribution diagram of the relative concentrations of all of the variously protonated species.

silybin and silydianin) concerns the strongly overlapping equilibria because the difference of two consecutive dissociation constants is less than 3 (here about 1.2). Such close equilibria are always difficult to evaluate and therefore the user should carefuly prove the reliability of each dissociation constant estimation. A distribution diagram of the relative concentrations of all of the variously protonated species demonstrates the overlapping protonation equilibria for close consecutive dissociation constants.

The second problem concerns small differences of molar absorptivities in variously protonated species within a spectrum (Figs. 2c, 4c and 5c). It may happen that non-linear regression fails when small differences of absorbance are of the same magnitude as instrumental noise, $s_{inst}(A)$.

4.2. Estimation of the thermodynamic protonation/dissociation constant

Applying a Debye–Hückel Eq. (6) to the data according to the regression criterion (7), the unknown parameter pK_a^T has been estimated. Table 4 brings point estimates of the thermodynamic dissociation constants of four drugs at two temperatures. Because of the narrow range of ionic strength the ion-size parameter $å$ and the salting-out coefficient C could not be estimated.

Table 4
Thermodynamic dissociation constants for silychristin, silybin, silydianin and mycophenolate at two selected temperatures

		Value at 25 °C	Value at 37 °C
Silychristin	$pK_{a,1}^T$	6.52(16)	6.62(1)
	$pK_{a,2}^T$	7.22(13)	7.41(5)
	$pK_{a,3}^T$	8.96(9)	8.94(9)
	$pK_{a,4}^T$	10.17(7)	10.03(8)
	$pK_{a,5}^T$	11.89(4)	11.63(7)
Silybin	$pK_{a,1}^T$	7.00(4)	6.86(5)
	$pK_{a,2}^T$	8.77(11)	8.77(3)
	$pK_{a,3}^T$	9.57(8)	9.62(1)
	$pK_{a,4}^T$	11.66(3)	11.38(1)
Silydianin	$pK_{a,1}^T$	6.64(7)	7.10(6)
	$pK_{a,2}^T$	7.78(5)	8.93(1)
	$pK_{a,3}^T$	9.66(9)	10.06(11)
	$pK_{a,4}^T$	10.71(7)	10.77(7)
	$pK_{a,5}^T$	12.26(5)	12.14(5)
Mycophenolate	pK_a^T	8.32(1)	8.14(1)

5. Conclusions

When drugs are poorly soluble then, instead of a potentiometric determination of dissociation constants, pH-spectrophotometric titration may be used with the non-linear regression of the absorbance–response–surface data. The reliability of the dissociation constants of four drug acids (i.e. silychristin, silybin, silydianin and mycophenolate) may be proven with goodness-of-fit tests of the absorption spectra measured at various pH. The thermodynamic dissociation constants pK_a^T of the four drugs are estimated by the non-linear regression of $\{pK_a, I\}$ data at 25 and 37 °C: for silychristin $pK_{a,1}^T = 6.52(16)$ and 6.62(1), $pK_{a,2}^T = 7.22(13)$ and 7.41(5), $pK_{a,3}^T = 8.96(9)$ and 8.94(9), $pK_{a,4}^T = 10.17(7)$ and 10.03(8), $pK_{a,5}^T = 11.89(4)$ and 11.63(7); for silybin $pK_{a,1}^T = 7.00(4)$ and 6.86(5), $pK_{a,2}^T = 8.77(11)$ and 8.77(3), $pK_{a,3}^T = 9.57(8)$ and 9.62(1), $pK_{a,4}^T = 11.66(3)$ and 11.38(1); for silydianin $pK_{a,1}^T = 6.64(7)$ and 7.10(6), $pK_{a,2}^T = 7.78(5)$ and 8.93(1), $pK_{a,3}^T = 9.66(9)$ and 10.06(11), $pK_{a,4}^T = 10.71(7)$ and 10.77(7), $pK_{a,5}^T = 12.26(5)$ and 12.14(5); for mycophenolate $pK_a^T = 8.32(1)$ and 8.14(1).

Acknowledgements

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