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Analytica Chimica Acta 584 (2007) 419–432
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

The thermodynamic dissociation constants of the anticancer drugs camptothecine, 7-ethyl-10-hydroxycamptothecine, 10-hydroxycamptothecine and 7-ethylcamptothecine by the least-squares nonlinear regression of multiwavelength spectrophotometric pH-titration data

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Received 14 June 2006; received in revised form 16 November 2006; accepted 17 November 2006
Available online 25 November 2006

Abstract

The mixed dissociation constants of four anticancer drugs – *camptothecine*, *7-ethyl-10-hydroxycamptothecine*, *10-hydroxycamptothecine* and *7-ethylcamptothecine*, including diprotic and triprotic molecules at various ionic strengths I of range 0.01 and 0.4, and at temperatures of 25 and 37 °C – were determined with the use of two different multiwavelength and multivariate treatments of spectral data, SPECFIT32 and SQUAD(84) nonlinear regression analyses and INDICES factor analysis. A proposed strategy for dissociation constants determination is presented on the acid–base equilibria of camptothecine. Indices of precise modifications of the factor analysis in the program INDICES predict the correct number of components, and even the presence of minor ones, when the data quality is high and the instrumental error is known. The thermodynamic dissociation constant pK_a^T was estimated by nonlinear regression of $\{pK_a, I\}$ data at 25 and 37 °C: for camptothecine $pK_{a,1}^T = 2.90(7)$ and 3.02(8), $pK_{a,2}^T = 10.18(30)$ and 10.23(8); for 7-ethyl-10-hydroxycamptothecine, $pK_{a,1}^T = 3.11(2)$ and 2.46(6), $pK_{a,2}^T = 8.91(4)$ and 8.74(3), $pK_{a,3}^T = 9.70(3)$ and 9.47(8); for 10-hydroxycamptothecine $pK_{a,1}^T = 2.93(4)$ and 2.84(5), $pK_{a,2}^T = 8.93(2)$ and 8.92(2), $pK_{a,3}^T = 9.45(10)$ and 9.98(4); and for 7-ethylcamptothecine $pK_{a,1}^T = 3.10(4)$ and 3.30(16), $pK_{a,2}^T = 9.94(9)$ and 10.98(18). Goodness-of-fit tests for various regression diagnostics enabled the reliability of the parameter estimates found to be proven. Pallas and Marvin predict pK_a being based on the structural formulae of drug compounds in agreement with the experimental value.

Keywords: Spectrophotometric titration; Dissociation constant; Protonation; Anticancer drug; Camptothecine; 7-Ethyl-10-hydroxycamptothecine; 10-Hydroxycamptothecine and 7-Ethylcamptothecine; SPECFIT; SQUAD; INDICES; PALLAS; MARVIN

1. Introduction

In the field of industrial pharmacy perhaps the most important physicochemical characteristics of drugs and excipients are their acidity or basicity expressed by their pK_a values, their hydrophobicity and it's dependence on pH. Before the drug can elicit an effect, for example if it is orally administered, it usually has to pass through a series of barriers, e.g. biological membranes either by passive diffusion and/or carrier-mediated uptake. Depending on the route of the administration of the drug and the location of the target site, the pH of the environments that the compound is exposed to may vary considerably. The affinity of the drug molecule for the target of interest and its ability to partition into a lipophilic environment at different pH values has to be quantified for a proper prediction of its ability to interact with the biological target and hence to be efficacious.

In previous work [1–9] the authors have shown that the spectrophotometric method in combination with suitable chemometric tools can be used for the determination of pro-

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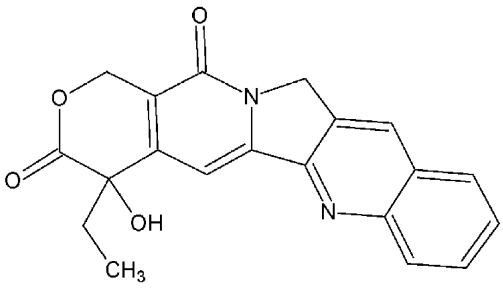
0003-2670/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2006.11.049

tonation constants β_{qr} or acid dissociation constants pK_a even for barely soluble drugs. Protonation constants or acid dissociation constants are very important both in the analysis of drugs and in the interpretation of their mechanisms of action as they are key parameters for predicting the extent of the ionisation of a molecule in solution at different pH. The acid–base properties of drugs affect the toxicity and pharmaceutical properties of organic acids and bases. Spectrophotometry is a convenient method for pK_a determination in very diluted aqueous solutions (about 10^{-5} to 10^{-6} M), provided that the compound possesses pH-dependent light absorption due to the presence of a chromophore in proximity to the ionisation centre *cf*. Refs. [10–25]: a series of 5–8 solutions of the sample with identical concentrations but with different pH can also be generated by titrating the sample solution alkalimetricaly, and the absorption spectra of the resulting solution of adjusted pH registered. When the components involved in the protonation equilibrium have distinct spectral responses their concentrations can be measured directly, and determination of the protonation constant is trivial. In many cases, however, the spectral responses of two and sometimes even more components overlap considerably, and analysis is no longer straightforward.

Problems arise because of strong overlapping chemical components involved in the equilibrium, and uncertainties arising from the mathematical algorithms used to solve such problems. In such cases, much more information can be extracted if multivariate spectrophotometric data are analyzed by means of an appropriate multivariate data analysis method. Hard modelling methods include traditional least-squares curve fitting approaches, based on a previous postulation of a chemical model, i.e. the postulation of a set of species defined by their stoichiometric coefficients and formation constants, which are then refined by least-squares minimization. These mathematical procedures require the fulfilment of the mass-balance equations and the mass-action law. The most relevant algorithms are SQUAD [14–19] and SPECFIT [22–24,31]. On the other hand, soft modelling techniques, such as multivariate curve resolution methods based on factor analysis, work without any assumption of a chemical model, and do not have the requirement of compliance with the mass-action law.

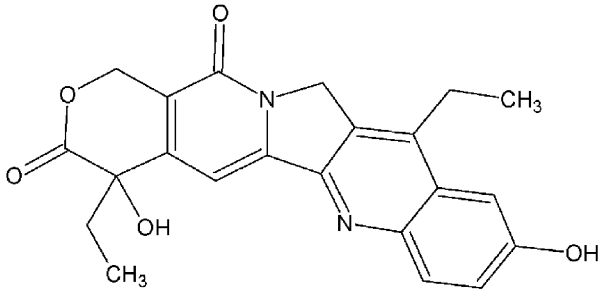
In this study, we have tried to complete the information on the protonation/dissociation constants for four anticancer drugs considered barely soluble or insoluble: the parent compound, *camptothecine*, and three related compounds *7-ethyl-10-hydroxycamptothecine*, *10-hydroxycamptothecine* and *7-ethylcamptothecine*. Concurrently, the experimental determination of protonation constants was combined with their computational prediction based on a knowledge of chemical structures.

Camptothecine (CPT) is a nearly water-insoluble monoterpene-derived indole alkaloid produced by the Chinese Camptotheca acuminatatree [26,27]. Camptothecine (chemically 4-ethyl-4-hydroxy-IH-pyrano(3′4′6′7) indolizino (1,2,-b) quinoline 3,14 (4H, 12H)-dione, CAS No. 7689-03-4, molecular formula $C_{20}H_{16}N_2O_4$, molecular weight 348.36) is of the structure

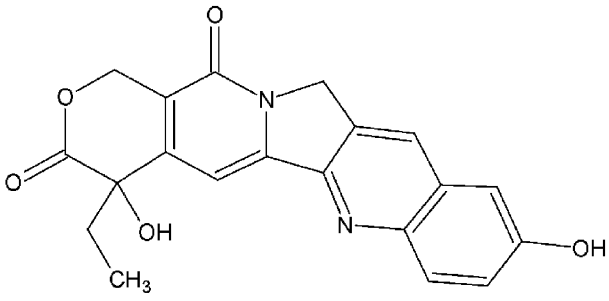


This pentacyclic alkaloid contains a quinoline ring system, a pyridone ring, and a terminal alpha-hydroxylactone ring. Above pH 7.4, solubility increases dramatically, with a slope of approximately 2 near pH 10, due to the ionization of the carboxylic group in the E-ring opened species, but the E-ring opened species of a camptothecine analog are therapeutically inactive, have a significantly shorter plasma half-life, and exhibit greater toxicity than the lactone. The active lactone form predominates only in acidic conditions [27]. Studies have also shown that the pH-dependent equilibrium shifts towards the inactive carboxylate form in plasma in a species-dependent manner. Equilibrium shift towards inactive carboxylate is favored in man, while equilibrium shift towards active lactone is favored in rodents.

7-Ethyl-10-hydroxycamptothecine is the pharmacologically active metabolite of the anticancer drug irinotecan (the prodrug) used globally in the first line treatment of advanced metastatic colorectal cancer. 7-Ethyl-10-hydroxycamptothecine (CAS No. 86639-52-3, molecular formula $C_{22}H_{20}N_2O_5$, molecular weight 392.40) is of the structure

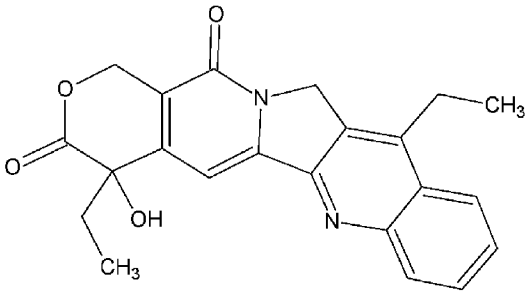


10-Hydroxycamptothecine is a minor alkaloid isolated from Camptotheca acuminata, or manufactured by semisynthesis from camptothecine. 10-Hydroxycamptothecine (CAS No. 19685-09-7, molecular formula $C_{20}H_{16}N_2O_5$, molecular weight 364.4) is of the structure



7-Ethylcamptothecine is one of the first semi-synthetic alkyl-derivatives of CPT [28,29]. It has been used as a model

compound, and as an intermediate for the preparation of other 7- and 10-substituted camptothecines. 7-ethylcamptothecine (syn.: ECPT, CAS No. 78287-27-1, molecular formula $C_{22}H_{20}N_2O_4$, molecular weight 376.44) is of the structure



2. Theoretical

2.1. Procedure for the determination of the chemical model and protonation 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

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

and in the case of a polyprotic species is protonated to yield a polyprotic acid H_JL:

$$L^{z-} + H^+ \rightleftarrows HL^{1-z}; K_{H1}$$

$$HL^{1-z} + H^+ \rightleftarrows H_2L^{2-z}; K_{H2}$$

The subscript to K_H indicates the ordinal number of the protonation step. The direct formation of each protonated species from the base L^{z-} can be expressed by the overall reaction $L^{z-1} + jH^+ \rightleftarrows H_jL^z$ and by the overall constant $\beta_{Hj} = K_{H1}K_{H2}\ldots K_{Hj}$, where j denotes the number of protons involved in the overall protonation. 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 the general formula L_qH_r in a particular chemical model and which are represented by p the number of species, $(q, r)_i$, $i = 1, \ldots, p$, where index i labels their particular stoichiometry; the overall protonation (stability) constant of the protonated species, β_{qr}, may then be expressed as

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

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

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

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

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

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 an element of the absorbance matrix $\boldsymbol{A}$ of size $(n \times m)$ being measured for n solutions with known total concentrations of two basic components, c_L and c_H, and at m wavelengths.

Throughout this paper, it is assumed that the $n \times m$ absorbance data matrix $\boldsymbol{A} = \varepsilon \boldsymbol{C}$ containing the n recorded spectra as rows can be written as the product of the $m \times p$ matrix of molar absorptivities ε and the $p \times n$ concentration matrix $\boldsymbol{C}$. Here p is the number of components that absorb in the chosen spectral range. The rank of the matrix $\boldsymbol{A}$ is obtained from the equation rank $(\boldsymbol{A}) = \min[\text{rank}\ (\varepsilon), \text{rank}\ (\boldsymbol{C})] \leq \min(m, p, n)$. Since the rank of $\boldsymbol{A}$ is equal to the rank of ε or $\boldsymbol{C}$, whichever is the smaller, and since rank $(\varepsilon) \leq p$ and rank $(\boldsymbol{C}) \leq p$, then provided that m and n are equal to or greater than p, it is only necessary to determine the rank of matrix $\boldsymbol{A}$, which is equivalent to the number of dominant light-absorbing components [1,11,20,36]. All spectra evaluation may be performed with the INDICES algorithm [1,36] in the S-Plus programming environment. Most index methods are functions of the number of principal components PC(k)'s into which the spectral data are usually plotted against an integer index k, PC(k) = $f(k)$, and when the PC(k) reaches the value of the instrumental error of the spectrophotometer used, $s_{inst}(A)$, the corresponding index k^* represents the number of light-absorbing components in a mixture, $p = k^*$. In a scree plot the value of PC(k) decreases steeply with increasing PCs as long as the PCs are significant. When k is exhausted the indices fall off, some even displaying a minimum. At this point $p = k^*$ for all indices. The index values at this point can be predicted from the properties of the noise, which may be used as a criterion to determine p [1,36].

The multi-component spectra analysing program SQUAD(84) [16] may adjust β_{qr} and ε_{qr} for a given absorption spectra set by minimising the residual-square sum function, U,

$$U = \sum_{i=1}^{n}\sum_{j=1}^{m} (A_{\text{exp},i,j} - A_{\text{calc},i,j})^2$$

$$= \sum_{i=1}^{n}\sum_{j=1}^{m} \left(A_{\text{exp},i,j} - \sum_{k=1}^{p} \varepsilon_{j,k} c_k \right)^2 = \text{minimum}$$

where $A_{i,j}$ represents the element of the experimental absorbance response-surface of size $n \times m$ and the independent variables c_k are the total concentrations of the basic components c_L and c_H being adjusted in n solutions. It means that the predicted absorbance-response surface is fitted to given spectral data, with one dimension representing the dependent variable (absorbance), and the other two dimensions representing the

independent variables, *viz*. the total component concentrations (or pH) of n solutions, at m wavelengths. The minimization may be done algorithmically or heuristically. The algorithmic process usually finds a global minimum whereas the heuristic process depends on human control. The user must decide whether a local or global minimum is required. In computational strategy, restrictions and initial guesses for the parameters and minimization steps for particular parameters should be supplied, and special care paid to parameters that are interdependent in the proposed regression model. Which computational strategy will prove optimal depends on the number of species, previous knowledge of some species in the chemical model, and the experimental design for changing the basic components in the equilibrium system, and therefore an *ad hoc* choice is necessary. Unknown parameters to be determined may be divided into two equal groups: (1) a hypothetical chemical model which is supplied by the user and should contain (a) an estimate of the number of light-absorbing species in solution, p, and (b) a list of variously protonated species of stoichiometry indices $(q, r)_i$, $i = 1,\ldots, p$; (2) the best estimates of the protonation constants, $\beta_{qr,i}$, $i = 1, \ldots, p$, which are adjusted by SQUAD(84) regression Gauss–Newton and Newton–Raphson algorithms. At the same time, a matrix of molar absorptivities $(\varepsilon_{qr,j}, j = 1, \ldots, m)_k$, $k = 1, \ldots, p$, 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 $[L_qH_r]_j$, $j = 1, \ldots, p$, forming for n solutions the matrix $\boldsymbol{C}$ are obtained. SQUAD(84) provides the user with two algorithms for solving the system of linear equations arising from Beer's law. The multiple regression algorithm is used during the initial data refinement. If negative molar absorptivities are detected the data should be first checked for data-entry and/or experimental errors. All plausible models are then tested to ascertain that the negative values are not due to fitting the wrong model. However, should all these strategies fail to remove the negative values, then the user would switch to the nonnegative least-squares algorithm NNLS. When the estimated β_{qr} and ε_{qr} values for the assumed chemical model have been refined, the agreement between the experimental and predicted data can be examined. If the agreement is not considered satisfactory, new chemical models are tried until a better fit with the experimental data is obtained. Various hypotheses of chemical models with refined parameters have been proposed and tested and the statistical characteristics describing the degree-of-fit of regression spectra through experimental points have been calculated. The residual are analyzed to test whether the refined parameters adequately represent the data, and should be randomly distributed about the predicted regression curve. To analyze the residuals, the following statistics are calculated: the residual mean $\bar{e}$, the standard deviation of the residuals $s(e)$, the skewness of the residuals set $\hat{g}_1(e)$, the kurtosis of the residuals set $\hat{g}_2(e)$ and the Hamilton *R-factor* for relative fit. The calculated standard deviation 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 $s(A) \approx s_{\text{inst}}(A)$ or $s(e) \approx s_{\text{inst}}(A)$ 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.

Another popular program is the SPECFIT/32 [31], based on singular value decomposition and nonlinear regression modeling using the Levenberg–Marquardt method for the determination of stability constants from spectrophotometric titration data. The method referred to as "model-free" does not require any assumption as to the chemistry of the system other than the number of active complexes present, not any assumptions as to the nature of absorbing complexes, their stoichiometry or a thermodynamic model. The solution is retrieved using constraints such as non-negativity for concentrations and absorptivities, closure (the sum of the concentrations of some species should be equal to a known quantity) and unimodality (only one maximum in the concentration profiles). The latest version of SPECFIT/32 [31] makes use of a multiwavelength and multivariate spectra treatment and enables a global analysis for equilibrium and kinetic systems with singular value decomposition and nonlinear least-squares regression modeling using the Levenberg–Marquardt method. The method has proved to be superior in discrimination between chemical models. Factor analysis is used as a powerful tool for the determination of independent components in a given data matrix is used.

2.2. Procedure for protonation model building and testing

An experimental and computational scheme for protonation model building of a multi-component and multiwavelength system was proposed by Meloun et al., *cf*. page 226 in Ref. [11] or Refs. [16,30] and is here revised with regard to SPECFIT/32:

(1) *Instrumental error of absorbance measurements,* $s_{\text{inst}}(A)$: The INDICES algorithm cf. Refs. [1,36] should be used to evaluate $s_{\text{inst}}(A)$. The Cartel's 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 and the instrumental error of the spectrophotometer used, $s_{\text{inst}}(A) = s_1^*(A)$ reaching a value of 0.25 mAU for the Cintra 40 (GBC, Australia) spectrophotometer employed.

(2) *Experimental design*: Simultaneous monitoring of absorbance and pH during titrations is used in a titration, when 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. 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*: A qualitative interpretation of the spectra aims to evaluate of 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. The INDICES [1,36] determine the number of dominant species present in the equilibrium mixture.

(4) *Choice of computational strategy*: The input data should specify whether β_{qr} or $\log \beta_{qr}$ values are to be refined whether 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 the protonation constants are to be refined or held constant, and whether molar absorptivities are to be refined.

(5) *Previously reported or theoretically predicted parameter β_{qr} estimates*: It is wise before starting a regression to analyze actual experimental data, to search for scientific library sources to obtain a good default for the number of ionizing groups, and numerical values for the initial guess as to relevant stability (protonation) constants and the probable spectral traces of all the expected components [37]. Two programs, PALLAS [38] and MARVIN [39] provide a collection of powerful tools for making *a prediction* of *the* pK_a values of any organic compound on the basis of base on the structural formulae of the compounds, using approximately 300 Hammett and Taft equations. Depending on the nature of the chemical structure and based on the hypothesis that the ionization state of a particular group is dependent upon its subenvironments constituted by its neighboring atoms and bonds, a hierarchical tree is constructed from the ionizing atom outward.

(6) *Diagnostic criteria indicating a correct chemical model*: When the minimization process of a regression spectra analysis terminates, some diagnostic criteria are examined to determine whether the results should be accepted. An incorrect hypothesis on the chemical model leads to divergency, cyclization, or the failure of the minimization. To attain a good chemical model, the following diagnostics should be considered:

1st diagnostic—the physical meaning of the parametric estimates: The physical meaning of the protonation constants, associated 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 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.

2nd diagnostic—the physical meaning of the species concentrations: There are some physical constraints which are generally applied to concentrations of species and their molar absorptivities: concentrations and molar absorptivities must be positive numbers. Moreover, the calculated distribution of the free concentration of the basic components and the variously protonated species of the chemical model should show realistic molarities, i.e. down to about 10^{-8} M.

3rd diagnostic—parametric correlation coefficients: Partial correlation coefficients, r_{ij}, indicate the interdependence of two parameters, i.e. stability constants β_i and β_j, when others are fixed in value.

4th diagnostic—goodness-of-fit test: 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 carefully analyzed. The goodness-of-fit achieved is easily seen by examination of the differences between the experimental and calculated values of absorbance, $e_i = A_{\mathrm{exp},i,j} - A_{\mathrm{calc},i,j}$. One of the most important statistics calculated is *the standard deviation of the absorbance*, $s(A)$, calculated from a set of refined parameters at the termination of the minimization process. This is usually compared with the standard deviation of absorbance calculated by the INDICES program [1,36] $s_k(A)$ and the instrumental error of the spectrophotometer used $s_{\mathrm{inst}}(A)$ and if it is valid that $s(A) \le s_k(A)$, or $s(A) \le s_{\mathrm{inst}}(A)$, then the fit is considered to be statistically acceptable. Some realistic empirical limits are employed: for example, when $s_{\mathrm{inst}}(A) \le s(A) \le 0.002$, the goodness-of-fit is still taken as acceptable, while $s(A) > 0.005$ indicated that a good fit has not been obtained. Alternatively, the statistical measures of residuals e can be calculated to examine the following criteria: the *residual mean* (known as the *residual bias*) $\bar{e}$ should be a value close to zero; the *mean residual* $|\bar{e}|$ and the *residual standard deviation* $s(e)$ being equal to the absorbance standard deviation $s(A)$ should be close to the *instrumental standard deviation* $s_{\mathrm{inst}}(A)$; the *residual skewness* $g_1(e)$ should be close to zero for a symmetric distribution of residuals; the *residual kurtosis* $g_2(e)$ should be close to 3 for a Gaussian distribution of residuals; a Hamilton *R-factor* of relative fit, expressed as a percentage, $(R \times 100\%)$, of <0.5% is taken as an excellent fit, but a value of >2% is taken to be 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).

5th diagnostic—deconvolution of spectra: Resolution of each experimental spectrum into spectra of the individual species proves whether the experimental design is efficient enough. If for a particular concentration range the spectrum consists of just a single component, further spectra for that range would be redundant. In ranges where many components contribute significantly to the spectrum, several spectra should be measured.

The details for the computer data treatment are collected in the *Supporting Information*.

2.3. Determination of the thermodynamic protonation/dissociation constants

The nonlinear estimation of the thermodynamic dissociation constant $K_a^T = a_{H^+} a_{L^-} / a_{HL}$, is simply a problem of optimization in the parameter space in which the pK_a and I are known and given values, while the parameters pK_a, $\mathring{a}$ and C of the Debye–Hückel equation are the unknown variables to be estimated [11,30].

2.4. Reliability of the estimated 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, and ε_{ij}, $j = 1, \ldots, m$, may be examined by the goodness-of-fit test, *cf.* page 101 in Ref. [32] or a previous paper [30].

3. Experimental

3.1. Chemicals and solutions

The camptothecine, 7-ethyl-10-hydroxycamptothecine, 10-hydroxycamptothecine and 7-ethylcamptothecine were purchased from Molcan Corporation, Canada, with a purity of 92.4, 98.2, 98.5 and 98.2%, respectively (HPLC). Two of their characteristics which may mostly affect the protonation behaviour, and thus, the HPLC-purity and residual amount of inorganic compounds, are summarized below:

Camptothecine: Batch No. 050611, Exp. date 2007-06-10, HPLC purity 94.3%, assay (on dried basis) 92.4%, residue on ignition 0.2%. *7-Ethyl-10-hydroxycamptothecine*: Batch No. 050709, Exp. date 2007-07-09, HPLC purity 98.5%, residue on ignition 0.2%. *10-Hydroxycamptothecine*: BatchNo.050818, Exp. date 2007-08-18, HPLC purity 98.2%, residue on ignition 0.5%. *7-ethylcamptothecine*: Batch No. 050819, Exp. date 2007-08-19, HPLC purity 98.2%, residue on ignition 0.6%.

Perchloric acid, 1 M, was prepared from conc. $HClO_4$ (p. a., Lachema Brno) using redestilled water and standardized against HgO and NaI with reproducibility of less than 0.20%. *Sodium hydroxide*, 1 M, was prepared from pellets (p. a., Aldrich Chemical Company) with carbondioxidefree redistilled water and standardized against a solution of potassium hydrogen-phthalate using the Gran Metod with a reproducibility of 0.1%. *Mercuric oxide*, *sodium iodide*, and *sodium perchlorate* (p. a., Lachema Brno) were not further purified. The preparation of other solutions from analytical reagent-grade chemicals have been described previously [30].

3.2. Apparatus and pH-spectrophotometric titration procedure

The apparatus used and the pH-spectrophotometric titration procedure have been described previously [30].

3.3. Software used

Computation relating to the determination of dissociation constants were performed by regression analysis of the UV/vis spectra using the SQUAD(84) [16] and SPECFIT/32 [31] programs. Most of graphs were plotted using ORIGIN 7.5 [33] and S-Plus [35]. The thermodynamic dissociation constant pK_a^T was estimated with the MINOPT nonlinear regression program in the ADSTAT statistical system (TriloByte Statistical Software, Ltd., Czech Republic), [34]. A qualitative interpretation of the spectra with the use of the INDICES program [36] aims to evaluate the quality of the dataset and remove spurious data, and to estimate the minimum number of *factors*, i.e. contributing aqueous species, which are necessary to describe the experimental data and determines the number of dominant species present in the equilibrium mixture.

3.4. Supporting information available

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

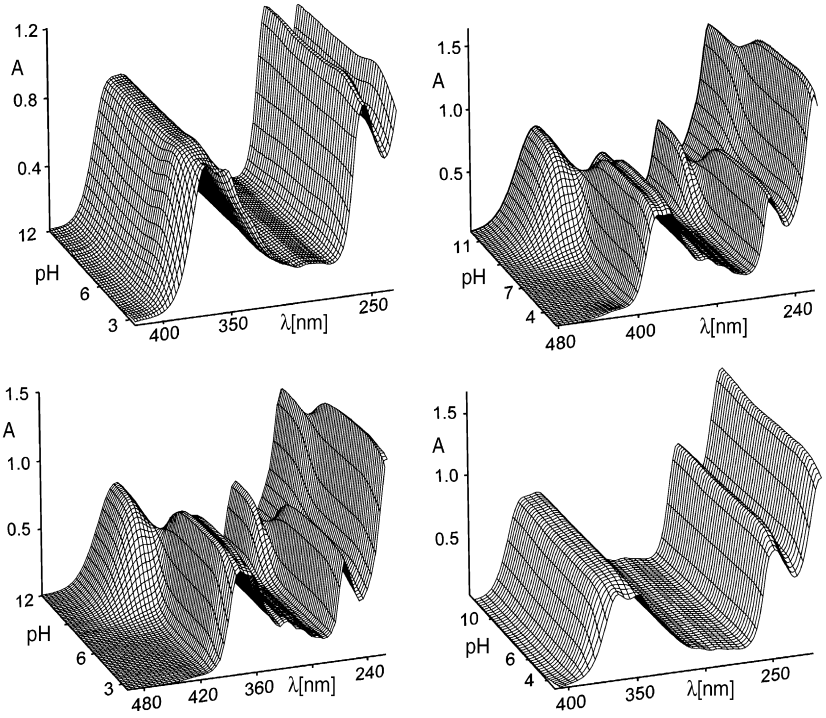


Fig. 1. The 3D-absorbance-response-surface representing the measured multiwavelength absorption spectra of (a) camptothecine, (b) 7-ethyl-10-hydroxycamptothecine, (c) 10-hydroxycamptothecine and (d) 7-ethylcamptothecine in dependence on pH at 25 °C (S-Plus).

4. Results and discussion

4.1. Camptothecine

The deprotonated camptothecine LH form exhibits two sharp isosbestic points in spectra, and these two points indicate one simple equilibrium. pH-spectrophotometric titration enables absorbance-response data (Fig. 1a) to be obtained for analysis by nonlinear regression, and the reliability of parameter estimates (pK's and ε's) can be evaluated on the basis of the goodness-of-fit test of residuals. The A–pH curves at 251, 373, 363, 352 and 392 nm show that the dissociation constant of camptothecine may be indicated. As the changes in spectra are quite small within deprotonation, however, both of the variously protonated species L and LH exhibit quite similar absorption bands. The shift of a band maximum to lower wavelengths in the spectra set may also be indicated (left and middle graph in the upper row of Fig. 2). The adjustment of pH value from 8.5 to 11.0 causes the absorbance to change by 0.022 of the A–pH curve only, so that the monitoring of both components L and LH of the protonation equilibrium is rather unsure. As the changes in spectra are very small, a very precise measurement of absorbance is necessary for a reliable detection of the deprotonation equilibrium studied.

In the first step of the regression spectra analysis, the number of light-absorbing species was estimated using the INDICES algorithm [36] (Fig. 2). The position of the break point on the $s_k(A) = f(k)$ curve in the factor analysis scree plot is calculated and gives $k^* = 3$ with the corresponding co-ordinate $s_k^*(A) = 0.52$ mAU, which may also be taken as the actual instrumental error $s_{\text{inst}}(A)$ of the spectrophotometer used. All six selected methods of modified factor analysis estimate the three light-absorbing components L, LH and LH_2 of the protonation equilibrium. The number of light-absorbing species p can be predicted from the index function values by finding the point

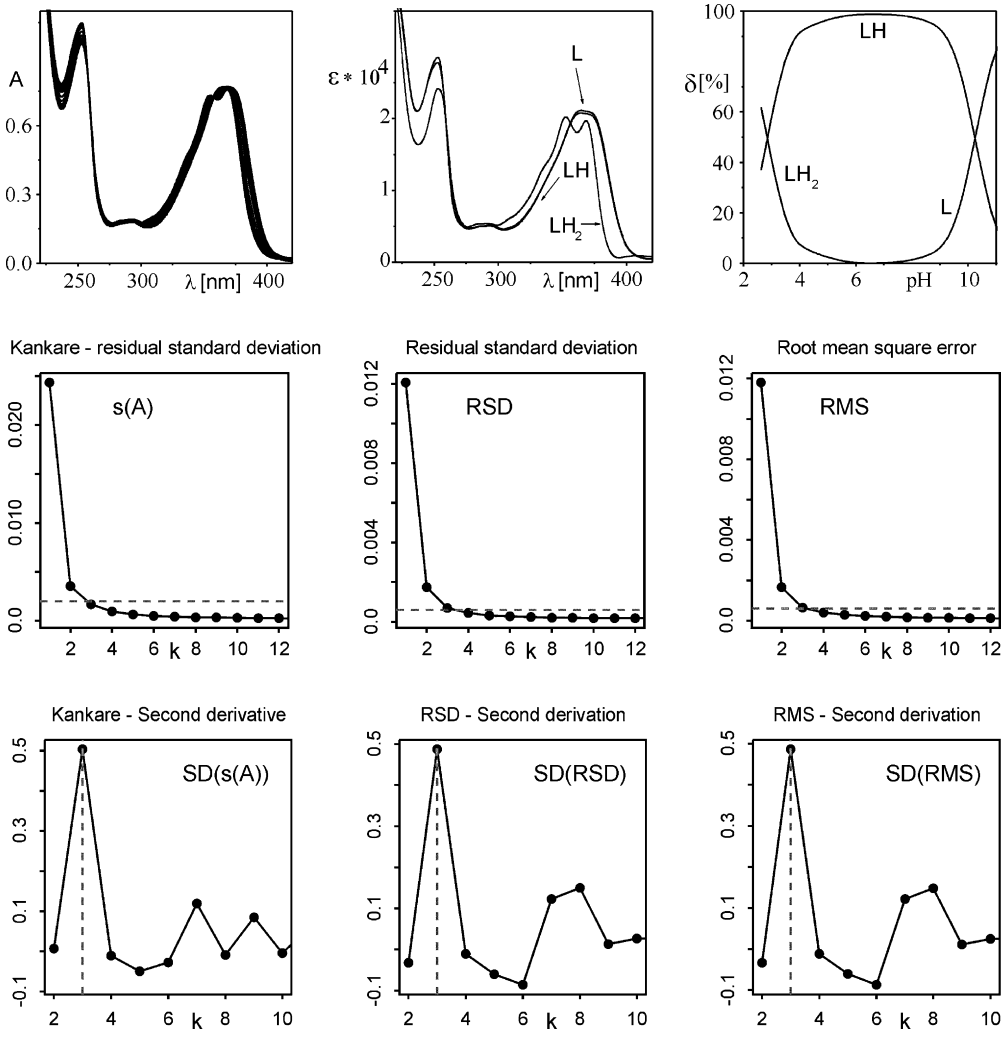


Fig. 2. Regression analysis of the protonation equilibria model of camptothecine in dependence on pH at 25 °C (SPECFIT, ORIGIN): *1st row*: The absorption spectra measured for various pH values (left), pure spectra profiles of molar absorptivities vs. wavelengths for variously protonated species L, LH, LH_2 (middle), distribution diagram of the relative concentrations of all of the variously protonated species L, LH, LH_2, of camptothecine in dependence on pH at 25 °C (right) (SPECFIT, ORIGIN). *2nd row*: Cartel's scree plot for determination of the number of light-absorbing species in mixture $k^* = 3$ leads to the actual instrumental error of the spectrophotometer used $s_3^*(A) = 0.52$ mAU and Kankare's residual standard deviation $s_k(A)$ (left), residual standard deviation R.S.D. (middle), root mean square error RMS (right). *3rd* row: The derivatives detection criteria of some indices functions SD($s_{(A)}$), SD(R.S.D.), SD(RMS) applied to the absorbance data indicate three light-absorbing species (INDICES in S-Plus).

$p = k$ where the slope of index function $PC(k) = f(k)$ changes, or by comparing $PC(k)$ values to the instrumental error $s_{inst}(A)$. This is the common criterion for determining p (the second and third rows in Fig. 2). Very low values of $s_{inst}(A)$ prove that a sufficiently precise spectrophotometer and efficient experimental technique were used. The two dissociation constants and three molar absorptivities of camptothecine calculated for 39 wavelengths constitute $2 + (3 \times 39) = 119$ unknown parameters, which are estimated and refined by SQUAD(84) or SPECFIT/32 in the first run. The reliability of the parameter estimates may be tested using the following diagnostics:

The *1st diagnostic* indicates whether all of the parametric estimates β_{qr} and ε_{qr} have physical meaning and reach realistic values. As the standard deviations $s(\log \beta_{qr})$ of parameters $\log \beta_{qr}$ and $s(\varepsilon_{qr})$ of parameters ε_{qr} are significantly smaller than their corresponding parameter estimates (Table 1), all the variously protonated species are statistically significant at a significance level $\alpha = 0.05$. The physical meaning of the protonation constant β_{qr}, molar absorptivities ε_{qr}, and stoichiometric indices q, r are examined in a search of the protonation equilibria model in Tables 2 and 3. The 2nd and 5th hypotheses of the protonation model are rejected, as the standard deviations of the parameter estimates are too large, and a poor fitness was achieved. The absolute values of $s(\beta_j)$, $s(\varepsilon_j)$ give information about the last U-contour of the hyperparaboloid in the neighbourhood 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 standard deviations of ε_{qr} for various wavelengths, $s(\varepsilon_{qr}) = f(\lambda)$, should have a Gaussian distribution; otherwise, erroneous estimates of ε_{qr} are obtained. The middle graph in the upper row of Fig. 2 shows that the estimated molar absorptivities of all of the variously protonated species ε_L, ε_{LH} and ε_{LH_2} of camptothecine in dependence on wavelength are realistic. Some spectra quite overlap and may cause some resolution difficulties in regression analysis. As the three protonation models in the model search of Tables 2 and 3 (1st model: L, LH, LH_2, 3rd model: L, LH, L_2H and 4th model:

Table 1
The best chemical model found for protonation equilibria of camptothecine using double checked nonlinear least squares regression analysis of multiwavelengths and multivariate pH-spectra with SQUAD(84) and SPECFIT/32 for $n_s = 18$ spectra measured at $n_w = 39$ wavelengths for $n_z = 2$ basic components L and H forming $n_c = 3$ variously protonated species

L_qH_r	Protonation constants estimated with SQUAD(84) and SPECFIT/32		Partial correlation coefficients	
	$\log \beta_{qr}$	$s(\log \beta_{qr})$	L_1H_1	L_1H_2
L_1H_1	10.77, 10.55	0.04, 0.051	1	–
L_1H_2	13.61, 13.39	0.04, 0.012	0.9576	1

Determination of the number of light-absorbing species by factor analysis

	SQUAD(84)	SPECFIT/32
Number of light-absorbing species k^*	3	3
Residual standard deviation $s_k^*(A)$	0.52	Not estimated

Goodness-of-fit test by the statistical analysis of residuals

Residual mean $\bar{e}$ [mAU]	-9.52×10^{-8}	1.21×10^{-8}
Mean residual $\lvert\bar{e}\rvert$ [mAU]	0.6	0.57
Standard deviation of residuals $s(e)$ [mAU]	0.83	0.62
Residual skewness $g_1(e)$	0.29	−0.27
Residual kurtosis $\hat{g}_2(e)$	2.8	3.61
Hamilton *R-factor* [%]	0.17	Not estimated
ε (all species) vs. λ are	Realistic	Realistic

The charges of the ions are omitted for the sake of simplicity and the standard deviations of the parameter estimates are in the last valid digits in brackets. The resolution criterion and reliability of parameter estimates found is proven with goodness-of-fit statistics such as the residual square sum *RSS*, the standard deviation of absorbance after termination of the regression process, $s(A)$ [mAU], the residual standard deviation by factor analysis $s_k(A)$ [mAU], the mean residual e, the residual standard deviation $s(e)$, the residual skewness $g_1(e)$ and the residual kurtosis $g_2(e)$ proving the Gaussian distribution; Hamilton *R*-factor [%] and nonnegative and realistic estimates of calculated molar absorptivities of all variously protonated species ε vs. λ.

Table 2
The search for a protonation equilibria model of camptothecine using nonlinear least-squares regression analysis of multiwavelength pH-spectra of Table 1

Estimated $\log \beta_{qr}$ using a hypothesis of					
q, r	1st model	2nd model	3rd model	4th model	5th model
1, 1	10.767(41)	6.771(146)	5.886(14)	–	–
1, 2	13.609(44)	–	–	9.953(34)	–
2, 1	–	–	15.115(69)	15.096(65)	8.500(3804)
2, 2	–	14.500(2745)	–	–	11.500(3642)
Degree-of-fit test by the statistical analysis of residuals as the resolution criterion, $s_k(A) = 0.52$ [mAU], $p = 3$					
$s(A)$ or $s(e)$ [mAU]	0.83	2.5	0.83	0.79	1.6
$\bar{e}$	0.6	1.43	0.59	0.56	1.11
$g_1(e)$	0.29	1.28	0.14	0.26	−0.17
$g_2(e)$	2.8	9.7	2.8	3.13	2.58
R-factor [%]	0.17	0.52	0.17	0.16	0.33
ε (all species) vs. λ are	Realistic	Realistic	Realistic	Realistic	Realistic
Model hypothesis is	Accepted	Rejected	Accepted	Accepted	Rejected

Table 3
Dependence of the mixed dissociation constants of camptothecine on ionic strength using regression analysis of pH-spectrophotometric data with SPECFIT and SQUAD, with the standard deviations of the parameter in the last valid digits in brackets

	Ionic strength								
	0.003	0.006	0.011	0.012	0.027	0.057	0.065	0.073	0.081
Estimated dissociation constants $pK_{a,1}$ and $pK_{a,2}$ at 25 °C									
SPECFIT									
$pK_{a,1}$	2.893(19)	2.840(12)	2.912(11)		2.605(23)		2.475(38)	2.613(28)	2.632(18)
$pK_{a,2}$		10.55(5)		9.450(58)	9.844(66)	9.550(49)	9.562(63)	9.720(66)	9.492(54)
$s(A)$ [mAU]	0.68	0.62	0.73	0.81	0.62	0.61	0.77	0.71	0.71
SQUAD									
$pK_{a,1}$	2.890(16)	2.842(44)			2.607(78)		2.465(73)	2.607(92)	2.624(79)
$pK_{a,2}$		10.77(41)		9.340(51)	9.792(73)	9.858(55)	9.702(61)	9.586(87)	9.578(77)
$s(A)$ [mAU]	0.87	0.83	0.99	0.98	0.79	0.83	0.94	0.92	0.88

	Ionic strength												
	0.002	0.004	0.026	0.034	0.041	0.042	0.048	0.050	0.056	0.071	0.078	0.08	0.096	0.119
Estimated dissociation constants $pK_{a,1}$ and $pK_{a,2}$ at 37 °C														
SPECFIT														
$pK_{a,1}$		3.009(13)		3.023(24)		2.881(11)							3.062(24)	2.981(24)
$pK_{a,2}$	10.16(2)		10.43(3)		10.46(3)	10.25(3)	10.44(4)	10.46(2)	10.27(3)	10.46(3)	10.45(3)	10.32(3)	10.63(1)	0.75
$s(A)$ [mAU]	0.46	0.46	0.39	0.56	0.33	0.59	0.41	0.85	0.35	0.44	0.47	0.46	0.66	
SQUAD														
$pK_{a,1}$		3.013(36)		3.018(19)		2.890(31)						2.821(35)	3.025(36)	2.978(26)
$pK_{a,2}$	10.33(18)	10.54(32)	10.48(28)		10.49(34)		10.39(31)	10.49(22)	10.55(23)	10.58(21)	10.57(23)		10.65(17)	
$s(A)$ [mAU]	0.60	0.63	0.63	0.78	0.64	0.77	0.65	0.99	0.52	0.53	0.62	0.52	0.91	0.88

L, LH_2, L_2H) are accepted, it may be concluded that regression spectra analysis cannot distinguish among these three models. All of these models also attain a very good spectra fitting.

The *2nd diagnostic* tests whether all of the calculated free concentrations of the three variously protonated species on the distribution diagram of the relative concentration expressed as a percentage have physical meaning, which proved to be the case (the right graph in Fig. 2). The calculated free concentration of the basic components and variously protonated species of the protonation equilibria model should show molarities down to about 10^{-8} M. Expressed in percentage terms, a species present at about 1% relative concentration or less in an equilibrium behaves as numerical noise in a 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 generally be in the range 10^3 to10^5 L mol^{-1} cm^{-1}, species present at less than *ca*. 0.1% relative concentration will affect the absorbance significantly only if their s is extremely high. The diagram shows the protonation equilibria of L, LH and LH_2.

The *3rd diagnostic* concerning the matrix of correlation coefficients in Table 1 proves that there is an interdependence of one pair of protonation constants of camptothecine r (β_{11} versus β_{12}).

The *4th diagnostic* concerns the goodness-of-fit and indicates nine outlying spectra. The goodness-of-fit achieved is easily seen by examination of the differences between the experimental and calculated values of absorbance, $e_i = A_{\mathrm{exp},i,j} - A_{\mathrm{calc},i,j}$. Examination of the spectra and of the graph of the predicted absorbance response-surface through all the experimental points should reveal whether the results calculated are consistent and whether any gross experimental errors have been made in the measurement of the spectra. One of the most important statistics calculated is the standard deviation of absorbance, $s(A)$, calculated from a set of refined parameters at the termination of the minimization process. This is usually compared to the standard deviation of absorbance calculated by the INDICES program [35], $s_k(A)$, and if $s(A) \leq s_k(A)$, or $s(A) \leq s_{\mathrm{inst}}(A)$, the instrumental error of the spectrophotometer used, the fit is considered to be statistically acceptable (Table 1). This proves that the $s_3(A)$ value is equal to 0.52 mAU and is close to the standard deviation of absorbance when the minimization process terminates, $s(e) = 0.83$ mAU (or 0.62 mAU SPECFIT). Although this statistical analysis of residuals gives the most rigorous test of the degree-of-fit, realistic empirical limits must be used. After removal of outlying spectra, the statistical measures of all residuals e prove that the minimum of the eliptic hyperparaboloid U is reached: the residual standard deviation $s(e)$ always has sufficiently low values, below than 1 mAU. The statistical measures of all the residuals prove that the minimum of the eliptic hyperparaboloid is reached: the residual mean $e = -9.52 \times 10^{-8}$ (or 1.21×10^{-8} SPECFIT) proves that there is no bias or systematic error in the spectra fitting. The mean residual $|\bar{e}| = 0.60$ mAU (or 0.57 mAU SPECFIT) and the residual standard deviation $s(e) = 0.83$ mAU (or 0.62 mAU SPECFIT) have sufficiently low values. The skewness $g_1(e) = 0.29$ (or -0.27 SPECFIT) is close to zero and proves a symmetric distribution of the residuals set, while the kurtosis $g_2(e) = 2.80$ (or 3.61 SPECFIT) is close to 3 proving a Gaussian distribution. The Hamilton *R-factor* of relative fitness is 0.17% calculated with SQUAD(84) only, proving so an excellent achieved fitness, and the parameter estimates may therefore be considered reliable. The criteria of resolution used for the hypotheses were: (1) a failure of the minimization process in a divergency or a cyclization; (2) an examination of the physical meaning of the estimated parameters to ensure that they were both realistic and positive; and (3) the residuals should be randomly distributed about the predicted regression spectrum, and systematic departures from randomness were taken to indicate that either the chemical model or the parameter estimates were unsatisfactory.

The *5th diagnostic*, the spectra deconvolution shows the deconvolution of the experimental spectrum into spectra of 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 an efficient experimentation strategy. Such a spectrum provides sufficient information for a regression analysis which monitors at least two species in equilibrium, where none is a minor species. 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. When, on the other hand, only one species prevails in solution, the spectrum yields quite poor information into the regression analysis, and the parameter estimate is somewhat uncertain, and definitely not reliable enough. To test the reliability of protonation constants at different ionic strengths, a goodness-of-fit test is applied with the use of a statistical analysis of the residuals, and the results are given in Tables 1–3. For the drug 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 than 0.2%, 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 1.0 mAU, and the proposal of a good protonation equilibria model and of reliable parameter estimates is proven.

4.2. Other derivatives of camptothecine

Using the experimental and evaluation strategy, the protonation equilibria of 7-ethyl-10-hydroxycamptothecine (Figs. 1b and 3), 10-hydroxycamptothecine (Figs. 1c and 4) and 7-ethylcamptothecine (Figs. 1d and 5) were also examined. To test the reliability of the protonation/dissociation constants at different ionic strengths, a goodness-of-fit test with the use of statistical analysis of the residuals was applied, and the results are given in Tables 2 and 3. For all four drugs studied the most efficient tool, such as the standard deviation of residuals, was applied. The standard deviation of absorbance $s(A)$ after termination of the minimization process is always better than 1 mAU, and the proposal of a good protonation equilibria model and reliable parameter estimates is thus proven. Pallas and Marvin [38,39] are both a collection of powerful tools for making predictions based on the structural formulae of drug compounds. Entering the compound topological

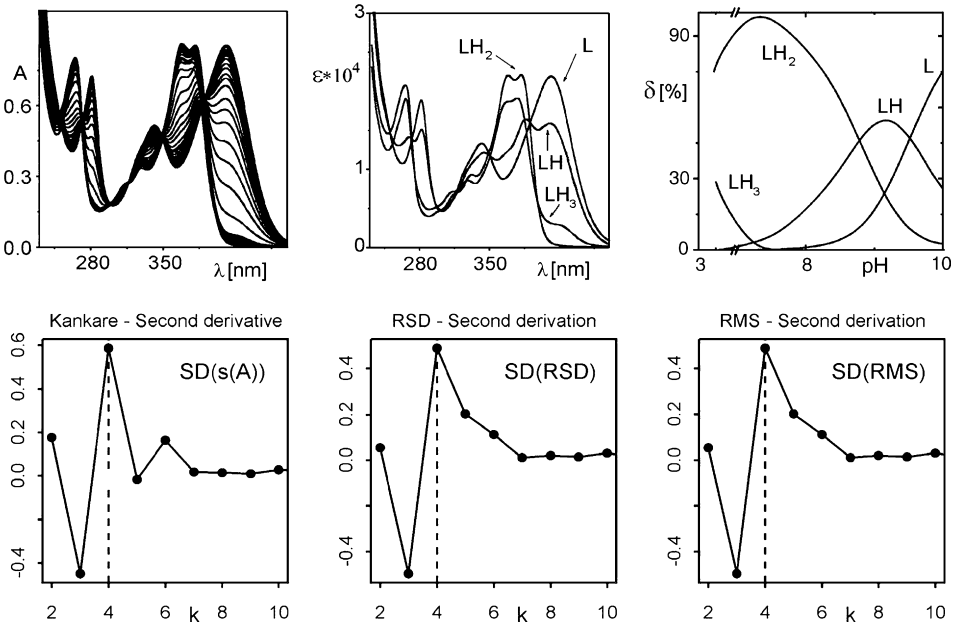


Fig. 3. *1st row*: The search for a chemical model of protonation equilibria in solution of 7-ethyl-10-hydroxycamptothecine: Absorption spectra measured for various pH values (left), pure spectra profiles of molar absorptivities vs. wavelengths for variously protonated species L, LH, LH_2, LH_3 (middle), distribution diagram of the relative concentrations of all of the variously protonated species L, LH, LH_2, LH_3 in dependence on pH at 25 °C (right) (SPECFIT, ORIGIN). *2nd row*: The derivatives detection criteria of some indices functions SD($s_k(A)$), SD(R.S.D.), SD(RMS) applied to the absorbance data indicate 4 light-absorbing species (INDICES in S-Plus).

structure descriptors graphically, pK_a values of organic compound are predicted using approximately hundreds Hammett and Taft equations and quantum chemistry calculus. The correlation between theory (the predicted value of pK_a) and experiment (the experimentally determined pK_a value) for the pK_a calculation is quite high. Fitting the points to the equation of a line $pK_{a,exp} = 1.33\ (s(\beta_0) = 0.48) + 1.01\ (s(\beta_1) = 0.07)\ pK_{a,predict}$ yields values of the slope $\beta_1 = 1.01$ with its standard deviation $s(\beta_1) = 0.07$, intercept $\beta_0 = 1.33$ with its standard deviation $s(\beta_1) = 0.48$, correlation coefficient $R = 0.9822$ and the determi-

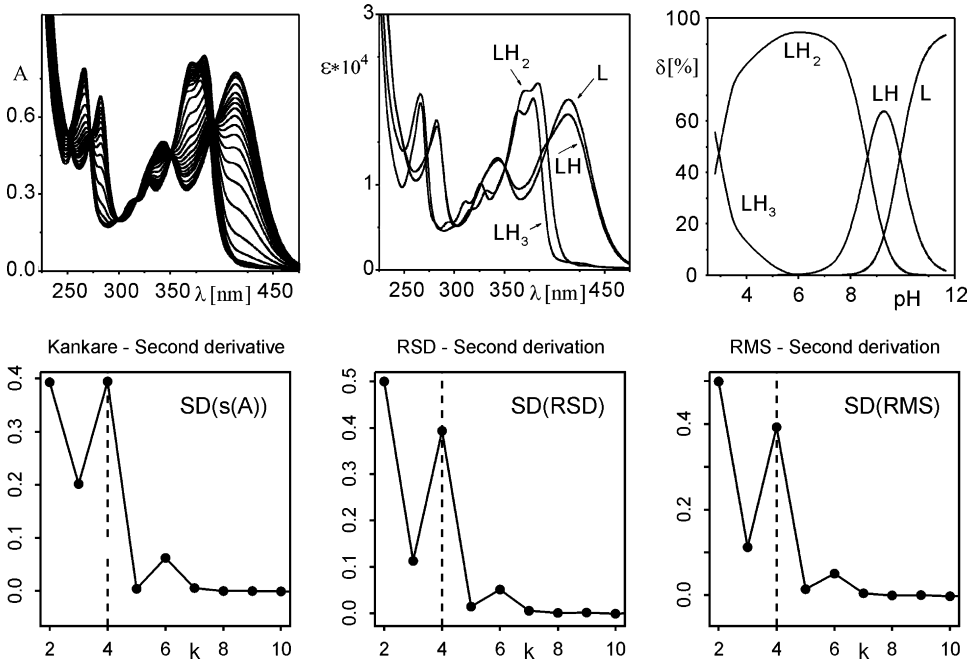


Fig. 4. *1st row*: The search for a chemical model of protonation equilibria in solution of 10-hydroxycamptothecine: absorption spectra measured for various pH values (left), pure spectra profiles of molar absorptivities vs. wavelengths for variously protonated species L, LH, LH_2, LH_3 (middle), distribution diagram of the relative concentrations of all of the variously protonated species L, LH, LH_2, LH_3 in dependence on pH at 25 °C (right) (SPECFIT, ORIGIN). *2nd row*: The derivatives detection criteria of some indices functions SD($s_k(A)$), SD(R.S.D.), SD(RMS) applied to the absorbance data indicate four light-absorbing species (INDICES in S-Plus).

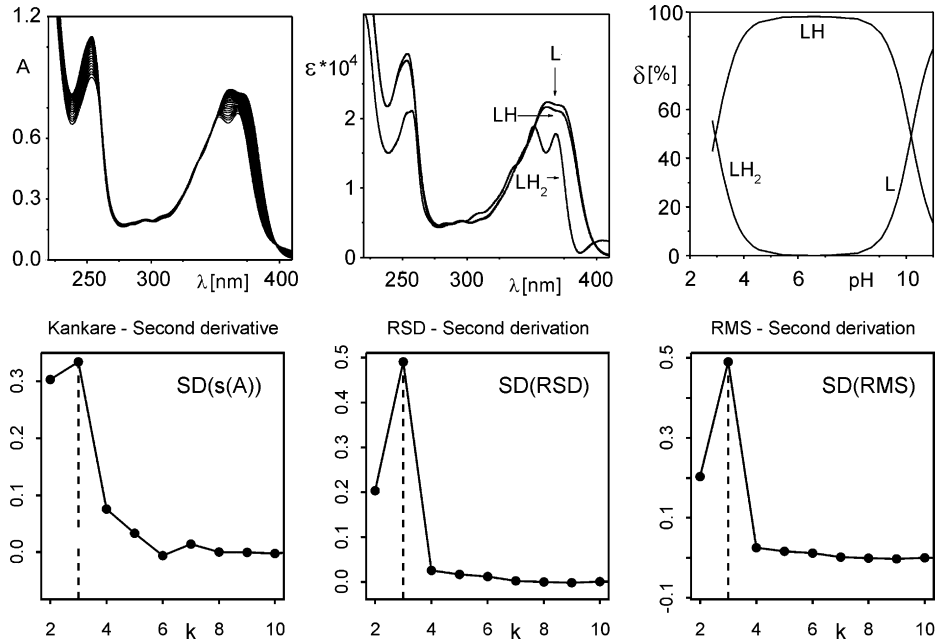


Fig. 5. *1st row*: The search for a chemical model of protonation equilibria in solution of 7-ethyl-camptothecine: absorption spectra measured for various pH values (left), pure spectra profiles of molar absorptivities vs. wavelengths for variously protonated species L, LH, LH_2 (middle), distribution diagram of the relative concentrations of all of the variously protonated species L, LH, LH_2, in dependence on pH at 25 °C (right) (SPECFIT, ORIGIN). *2nd row*: The derivatives detection criteria of some indices functions SD($s_k(A)$), SD(R.S.D.), SD(RMS) applied to the absorbance data indicate 3 light-absorbing species (INDICES in S-Plus).

nation coefficient R^2 100% = 96.47% and standard deviation of dependent variable $s(pK_a) = 0.55$. It is clear that both algorithms Pallas and Marvin [38,39] have an exceptionally close fit of experimental and predicted values. The high R and R^2 100% values indicate very good fit and good predictive capability for pK_a estimate.

4.3. Thermodynamic dissociation constants

The thermodynamic dissociation constants of the unknown parameter pK_a^T were estimated by applying a Debye–Hückel equation to the data in Tables 1–3, and Fig. 6 according to the regression criterion [33]; Table 4 shows point estimates of the

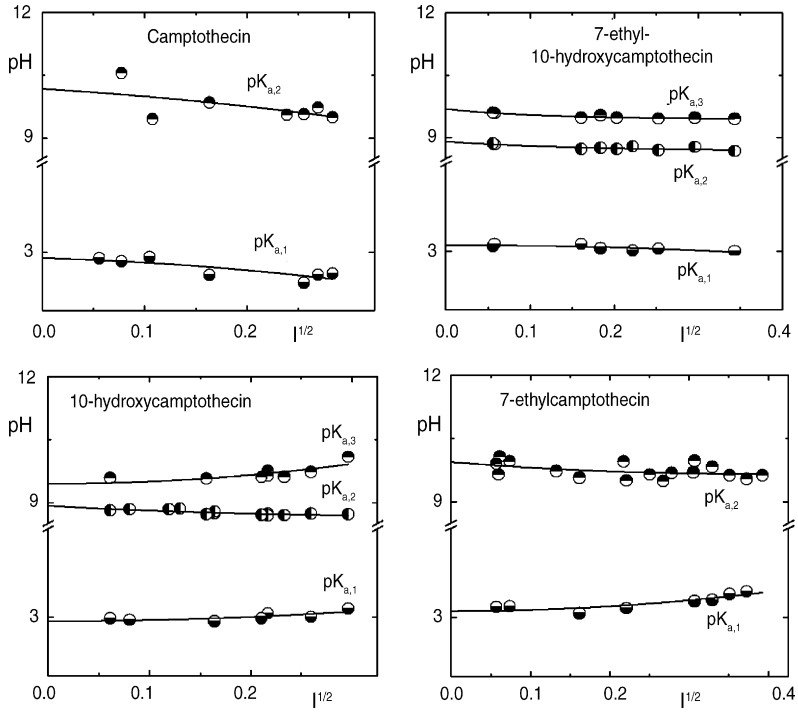


Fig. 6. Dependence of the mixed dissociation constant pK_a of four drugs of the campthothecine family on the square root of ionic strength, leading to parameter pK_a^T, at 25 °C.

Table 4
Thermodynamic dissociation constants for four anticancer drugs camptothecine, 7ethyl-10-hydroxycamptothecine, 10-hydroxycamptothecine and 7-ethylcamptothecine at two temperatures 25 and 37 °C

	SPECFIT		SQUAD		Predicted with MARVIN	Predicted with PALLAS
	Value at 25 °C	Value at 37 °C	Value at 25 °C	Value at 37 °C		
Camptothecine						
$pK^{T}_{a,1}$	2.90(7)	3.02(8)	2.83(9)	2.92(8)	3.07	4.17
$pK^{T}_{a,2}$	10.18(30)	10.23(8)	10.11(36)	10.43(3)	8.63	10.64
7-Ethyl-10-hydroxycamptothecine						
$pK^{T}_{a,1}$	3.11(2)	2.46(6)	3.04(5)	2.30(6)	3.92	5.66
$pK^{T}_{a,2}$	8.91(4)	8.74(3)	8.90(3)	8.84(3)	8.24	9.06
$pK^{T}_{a,3}$	9.70(3)	9.47(8)	9.71(5)	9.53(10)	9.12	10.65
10-Hydroxycamptothecine						
$pK^{T}_{a,1}$	2.93(4)	2.84(5)	2.92(4)	2.77(5)	3.17	4.56
$pK^{T}_{a,2}$	8.93(2)	8.92(2)	8.93(3)	8.90(2)	8.41	8.88
$pK^{T}_{a,3}$	9.45(10)	9.98(4)	9.46(9)	10.02(7)	9.14	10.64
7-Ethylcamptothecine						
$pK^{T}_{a,1}$	3.10(4)	3.30(16)	2.94(3)	3.26(22)	3.86	5.27
$pK^{T}_{a,2}$	9.94(9)	10.98(18)	9.73(9)	10.96(18)	8.44	10.65

The standard deviations in the last valid digits are in brackets.

thermodynamic dissociation constants of the four drugs at two temperatures. Because of the narrow range of ionic strengths, the ion-size parameter $å$ and the salting-out coefficient C could not be estimated.

5. Conclusions

When drugs are very poorly soluble then pH-spectrophotometric titration may be used with the non-linear regression of the absorbance-response-surface data instead of a potentiometric determination of dissociation constants. The reliability of the dissociation constants of the four drugs (i.e. camptothecine, 7-ethyl-10-hydroxycamptothecine, 10-hydroxycamptothecine and 7-ethylcamptothecine) may be proven with goodness-of-fit tests of the absorption spectra measured at various pH. Goodness-of-fit tests for various regression diagnostics enabled the reliability of the parameter estimates to be determined.

Acknowledgments

The financial support of the Grant Agency IGA (Grant No. NR9055-4/2006) and of the Czech Ministry of Education (Grant No. MSM253100002) is gratefully acknowledged.

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