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The thermodynamic dissociation constants of losartan, paracetamol, phenylephrine and quinine by the regression analysis of spectrophotometric data

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Abstract

The mixed dissociation constants of four drug acids – losartan, paracetamol, phenylephrine and quinine – at various ionic strengths I of range 0.01 and 1.0 and at temperatures of 25 and 37 °C were determined using SPECFIT32 and SQUAD(84) regression analysis of the pH–spectrophotometric titration data. A proposed strategy of efficient experimentation in a dissociation constants determination, followed by a computational strategy for the chemical model with a dissociation constants determination, is presented on the protonation equilibria of losartan. Indices of precise methods 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. Improved identification of the number of species uses the second or third derivative function for some indices, namely when the number of species in the mixture is higher than 3 and when, due to large variations in the indicator values even at logarithmic scale, the indicator curve does not reach an obvious point where the slope changes. The thermodynamic dissociation constant pK_a^T was estimated by nonlinear regression of $\{pK_a, I\}$ data at 25 and 37 °C: for losartan $pK_{a,1}^T = 3.63(1)$ and 3.57(3), $pK_{a,2}^T = 4.84(1)$ and 4.80(3), for paracetamol $pK_{a,1}^T = 9.78(1)$ and 9.65(1), for phenylephrine $pK_{a,1}^T = 9.17(1)$ and 8.95(1), $pK_{a,2}^T = 10.45(1)$ and 10.22(1), for quinine $pK_{a,1}^T = 4.25(1)$ and 4.12(1), $pK_{a,2}^T = 8.72(1)$ and 8.46(2). Goodness-of-fit tests for various regression diagnostics enabled the reliability of the parameter estimates to be found.

Keywords: Spectrophotometric titration; Dissociation constant; Protonation; Losartan; Paracetamol; Phenylephrine; Quinine

1. Introduction

Dissociation constants/protonation constants are very important both in the analysis of drugs and in the interpretation of their mechanisms of action. Of the several physicochemical methods for studying the protonation equilibria in solution, UV–vis spectrophotometry under broad experimental conditions is in general highly sensitive, and with subsequent computer treatment of the data can be a very powerful method [1–17]. When the components involved in protonation equilibrium have distinct spectral responses, their concentrations can be measured directly, and the determination of the dissociation constant is trivial. In many cases, however, the spectral responses of two or sometimes even more components overlap considerably, and analysis is no longer straightforward. Much more information can be extracted if multivariate spectroscopic data are analyzed by means of an appropriate multivariate data analysis [12,14]. On the other hand, protonation equilibria sometimes involve minor changes in the electronic spectra, which make it difficult to employ spectrophotometry for determination of dissociation constants.

In this study, we try to complete the information on protonation equilibria and dissociation constants of four pharmaceutically active ingredients of different origin (losartan,

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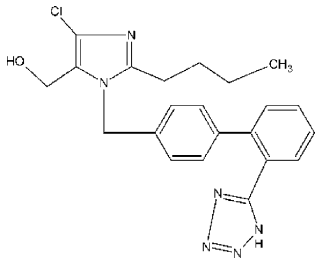
paracetamol, phenylephrine and quinine) which are formulated into a variety of different dosage forms, and whose exact pK_a knowledge is of special importance. Whereas fat-soluble (lipophilic) drugs diffuse through the membrane depending on their liposolubility, drugs of electrolyte character (acids or bases) diffuse according to the liposolubility of their *non-dissociated* part of molecules only. Ionized (dissociated) parts of molecules are lipophobic and generally can only hardly, with exemptions, diffuse through membranes. More precisely, the partition of a hydrophobic molecule across a membrane depends on its *membrane* partition coefficient, i.e. the proportion that will be present in the membrane phase compared to the aqueous phase. Most often, this coefficient is derived from measurements made between two isotropic phases [18–20]. Basic lipophilic compounds often exhibit higher affinities for biological membranes than predicted by their membrane partition coefficient value [21–23]. In particular, the protonated form of lipophilic bases has a high affinity as a result of electrostatic interactions with zwitterionic or anionic lipids [24]. In some cases, specific interactions between a drug and a particular lipid species have been observed [25]. The need of achieving the systemic effect and precise dose–control are crucial for cardiovascular drugs, which makes their nasal administration less practical. Even though interesting attempts were made also with nasal administration of some cardiovascular drugs as, for example, verapamil [26], it concerned mostly other than cardiovascular indications. Regularly taken cardiovascular drugs are more and more formulated into controlled-release solid dosage forms, where the knowledge of dissociation constant is of great importance. An example of a drug being typically formulated into solid dosage forms is losartan, a cardiovascular drug, an oral, specific angiotensin-II receptor (type AT1) antagonist [27].

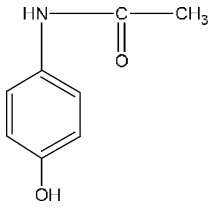
Losartan (biphenylimidazole-type drug, chemically 2-butyl-4-chloro-1-[[2′-(1*H*-tetrazol-5-yl)[[1,1′-biphenyl]-4-yl]methyl]-1*H*-imidazole-5-methanol, CAS No. 0114798-26-4, molecular formula $C_{22}H_{23}ClN_6O$, molecular weight 422.93) is of structure



Angiotensin II binds to the AT1 receptor found in many tissues (e.g. vascular smooth muscle, adrenal gland, kidneys, and the heart) and elicits several important biological actions, including vasoconstriction and the release of aldosterone. These effects of angiotensin II lead to elevation of blood pressure.

A representative of drugs available in different liquid and solid dosage forms as tablets, liquid suspensions, chewable tablets, coated caplets, soft and hard gelatin capsules, geltabs and suppositories, is paracetamol.

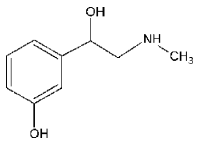
Paracetamol (syn.: acetaminophen, CAS No. 103-90-2; molecular formula $CH_3CONHC_6H_4OH$; molecular weight 151.16) is a long-established antipyretic/analgesic drug of structure



The painkilling properties of paracetamol were discovered by accident when a similar molecule (acetanilide) was added to a patients' prescription approximately 100 years ago. On the contrary to this, the precise mode of action of paracetamol is still unclear. It was thought that, like salicylates, it reduces the prostaglandin formation either by inhibiting the cyclooxygenase responsible for the biosynthesis of prostaglandins, or by enforcement of biotransformation of prostaglandin G_2 to prostaglandin H_2. However, in contrast to aspirin, paracetamol is observed to act almost exclusively on the central nervous system, with little peripheral effect. It is only slightly soluble in cold water, moderately soluble in water at room temperature (1 g dissolves in approx. 70 ml) but considerably more soluble in hot water and soluble in organic solvents such as methanol, ethanol, dimethylformamide, ethylene dichloride, acetone, and ethyl acetate [28,29]. Published literature on the analgesic and antipyretic effects of paracetamol revealed a therapeutically effective plasma concentration range of 10–30 μg/ml [30]. The relatively high dissociation constant of acetaminophen (pK_a = 9.78) compared to the pH of human milk (pH ∼ 6.2), high-affinity constant of association in milk ($K = 1.47 \times 10^4$ L/mol) at low concentration of binding sites B_0 ([concentration of binding sites] $\sim 9.01 \times 10^4$ L/mol) support the findings that the drug is excreted in milk and ingested by infants who are breast-fed [31] but that the amounts excreted are not clinically significant.

In the nasal and eye liquid dosage forms, sympathomimethic drugs such as phenylephrine have long been used.

Phenylephrine (chemically (α*R*)-3-hydroxy-α-[(methylamino)methyl]benzenemethanol, CAS No. 59-42-7, molecular formula $C_9H_{13}NO_2$, molecular weight 167.21) of structure

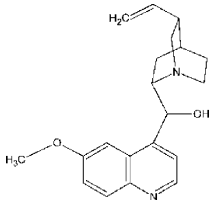


is a sympathomimetic agent with mainly direct effects on adrenergic receptors. It has predominantly alpha adrenergic activity and is without stimulating effects on the central nervous system. The sympathomimetic effect of phenylephrine produces vasoconstriction which in turn relieves nasal congestion. However, similarly as other sympathomimetics of

this group, phenylephrine is poorly absorbed across the nasal mucosa for systemic effect [32]. The transnasal administration requires the drug to be formulated mostly in aqueous solution, and both the stability of the drug and the permeability through the nasal mucosa is greatly affected by the drug dissociation constant. The extent of nasal absorption was found to be dependent on the pH of the solution of the drug. Generally, a greater nasal absorption is achieved at a pH lower than the pK_a at which the penetrant molecule exists as non-ionic species. With increase in pH over the pK_a, the rate of absorption decreases owing to the ionization of the penetrant molecule. On the other hand, non-physiological values of pH of the drug formulation must be avoided, and formulations having much lower pH than that of the nasal and/or eye liquid are unfavourable as they cause physiologically unpleasant perception during and after application. The pH at the site of absorption is affected not only by the pH of the drug formulation, but also by the local pH of the absorbing tissue. In nasal mucosa fluid, better absorption can be expected with those drugs having pK_a higher than the pH of nasal mucosa (slightly acidic, pH 5.5–5.6). In eyes, the pH of the conjunctival fluid (tears) is slightly basic (pH 7.4).

Dissociation constant plays a key role directly in the mode of action of another well-known drug, quinine.

Quinine (syn. Chinina; Chininum; Quinina; (8*S*,9*R*)-6′-methoxycinchonan-9-ol trihydrate; (*R*)-(6-methoxy-4-quinolyl)-(2*S*,4*S*,5*R*)-(5-vinylquinuclidin-2-yl)methanol trihydrate; (9*R*)-6′-methoxy-cinchonan-9-ol, CAS No. 130-95-0, molecular formula $C_{20}H_{24}N_2O_2$, molecular weight 324.42) of structure



is an alkaloid derived from cinchona bark. It is used mainly as antimalarial drug, but also for nocturnal leg cramps, and also in patients with myotonia. Quinine has been found to reverse a multidrug-resistance modulation MDR in a variety of tumor cell lines and has been safely used in combination with chemotherapy with leukemias, myelomas and lymphomas [33–35]. Eytan et al. [36] showed that the MDR modulators, among others quinine as well, had faster permeation kinetics through a model membrane than the substrates. Its mechanism of antimalarial action is cytotoxicity to the parasite.

This paper investigates the dissociation constants of the four drugs losartan, paracetamol, phenylephrine and quinine 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. The pK_a^T may be used for the prediction of the actual dissociation constant pK_a at the given value of an ionic strength. Critical comparison of nine selected index methods applied to the protonation equilibria of the four drugs will also be provided, namely when derivatives to improve the reliability of the identification of the number of components are used.

2. Theoretical

Computations related to the determination of protonation constants [1–4] may be performed by the regression analysis of spectra using versions of the SQUAD program family [2,5–10] and SPECFIT32 [37]. When considering a protonation of anion L^{z-1}

$$L^{z-1} + H^+ \rightleftarrows HL^z \tag{1a}$$

characterized by the protonation constant

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

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

$$L^{z-} + H^+ \rightleftarrows HL^{1-z};\ K_{H_1} \tag{2a}$$

$$HL^{1-z} + H^+ \rightleftarrows H_2L^{2-z};\ K_{H_2} \tag{2b}$$

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

$$L^{z-1} + jH^+ \rightleftarrows H_jL^z \tag{3a}$$

and by the overall protonation constant, where *j* denotes the number of protons involved in the overall protonation. For dissociation reactions realized at constant ionic strength, the so-called "mixed dissociation constants" are defined as

$$K_{a,j} = \frac{[H_{j-1}L]a_{H^+}}{[H_jL]} \tag{3b}$$

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.

If the protonation equilibria between the anion, L (the charges are now 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 are represented by *p* the number of species, $(q, r)_i$, $i = 1, \ldots, p$ where index *i* labels their particular stoichiometry, then the overall protonation constant of the protonated species, β_{qr}, may be expressed as

$$\beta_{qr} = [L_qH_r]/[L]^q[H]^r = c/I^q h^r \tag{4}$$

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

$$A_{i,j} = \sum_{n=1}^{p} \varepsilon_{j,n} c_n = \sum_{n=1}^{p} (\varepsilon_{qr,j} b_{qr} I^q h^r)_n \tag{5}$$

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 $(n \times m)$ being measured for n solutions with known total concentrations of two basic components, c_L and c_H, at m wavelengths. Throughout this paper, it is assumed that the $n \times m$ absorbance data matrix $\boldsymbol{A} = \boldsymbol{\varepsilon C}$ 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 $\text{rank}(\boldsymbol{A}) = \min[\text{rank}(\varepsilon), \text{rank}(\boldsymbol{C})] \leq \min(m, p, n)$. Since the rank of $\boldsymbol{A}$ is equal to the rank of ε or $\boldsymbol{C}$, whichever is the smaller, and since $\text{rank}(\varepsilon) \leq p$ and $\text{rank}(\boldsymbol{C}) \leq p$, 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 [2,11,12]. All spectra evaluation may be performed with the INDICES algorithm [12] 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 screen 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 [12].

The multi-component spectra analyzing program SQUAD(84) [7] may adjust β_{qr} and ε_{qr} for absorption spectra by minimizing the residual-square sum function, U,

$$U = \sum_{i=1}^{n}\sum_{j=1}^{n}(A_{\text{exp},i,j} - A_{\text{calc},i,j})^2 = \sum_{i=1}^{n}\sum_{j=1}^{m}\left(A_{\text{exp},i,j} - \sum_{k=1}^{p}\varepsilon_{j,k}c_k\right)^2 = \text{minimum} \quad (6)$$

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. The calculated standard deviation of absorbance $s(A)$ is used as the most important criterion for a fitness test. If, after termination of the minimization process the condition $s(A) \approx s_{inst}(A)$ is met and also the R-factor is less than 1%, the hypothesis of the chemical model is taken as the most probable one and is accepted. SPECFIT32 is the latest version of a global analysis program for equilibrium and kinetic systems with singular value decomposition and nonlinear regression modeling using the Levenberg–Marquardt method [37].

3. Experimental

3.1. Chemicals and solutions

All the drugs were used in the form of hydrochloride or nitrate.

Losartan potassium was purchased from SMS Pharmaceuticals, India, with a purity of 99.7%. Paracetamol was purchased from Aldrich Chemical Company with a purity of 98.0%. Phenylephrine hydrochloride was purchased from Aldrich Chemical Company with a purity 99.0%.

Quinine base was purchased from Aldrich Chemical Company with p. a. purity.

Perchloric acid, 1 M, was prepared from conc. $HClO_4$ (p. a., Lachema Brno) using redistilled water and standardized against HgO and NaI with a reproducibility of less than 0.20%. Sodium hydroxide, 1 M, was prepared from pellets (p. a., Aldrich Chemical Company) with carbondioxide-free redistilled water and standardized against a solution of potassium hydrogen-phthalate using the Gran method in the MAGEC program [2] 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 has been described previously [3–4]. Twice-redistilled water was used in the preparation of solutions.

3.2. Apparatus and pH–spectrophotometric titration procedure

The apparatus used and the pH–spectrophotometric titration procedure have been described previously [13].

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

The experimental and computational schemes for the determination of the protonation constants of the multicomponent system are taken from Meloun et al. [2] and are described in a previous paper [13]. When a minimization process terminates, some diagnostics are examined to determine whether the results should be accepted: the physical meaning of the parametric estimates, the physical meaning of the species concentrations, the goodness-of-fit test and the deconvolution of the spectra.

3.4. 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, å, and C are unknown variables to be estimated [2,13].

3.5. Reliability of estimated protonation/dissociation constants

The adequacy of a proposed regression chemical 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. Ref. [2, p. 101] or may be found in a previous paper [13] and also are explained in Ref. [39].

3.6. Software used

Computations were performed by regression analysis of UV–vis spectra using the SQUAD(84) program [7] and SPECFIT32 [37]. The thermodynamic dissociation constant pK_a^T was estimated with the MINOPT nonlinear regression program in the ADSTAT statistical system (TriloByte Statistical Software, Ltd., Pardubice) [38,39].

4. Results and discussion

4.1. Estimation of the protonation/dissociation constants of five drugs

4.1.1. Losartan

A proposed strategy for efficient experimentation in dissociation constants determination followed by spectral data treatment is presented on the protonation equilibria of losartan. Losartan contains a complicated molecular structure and two protonation equilibria can be monitored spectrophotometrically with close dissociation constants

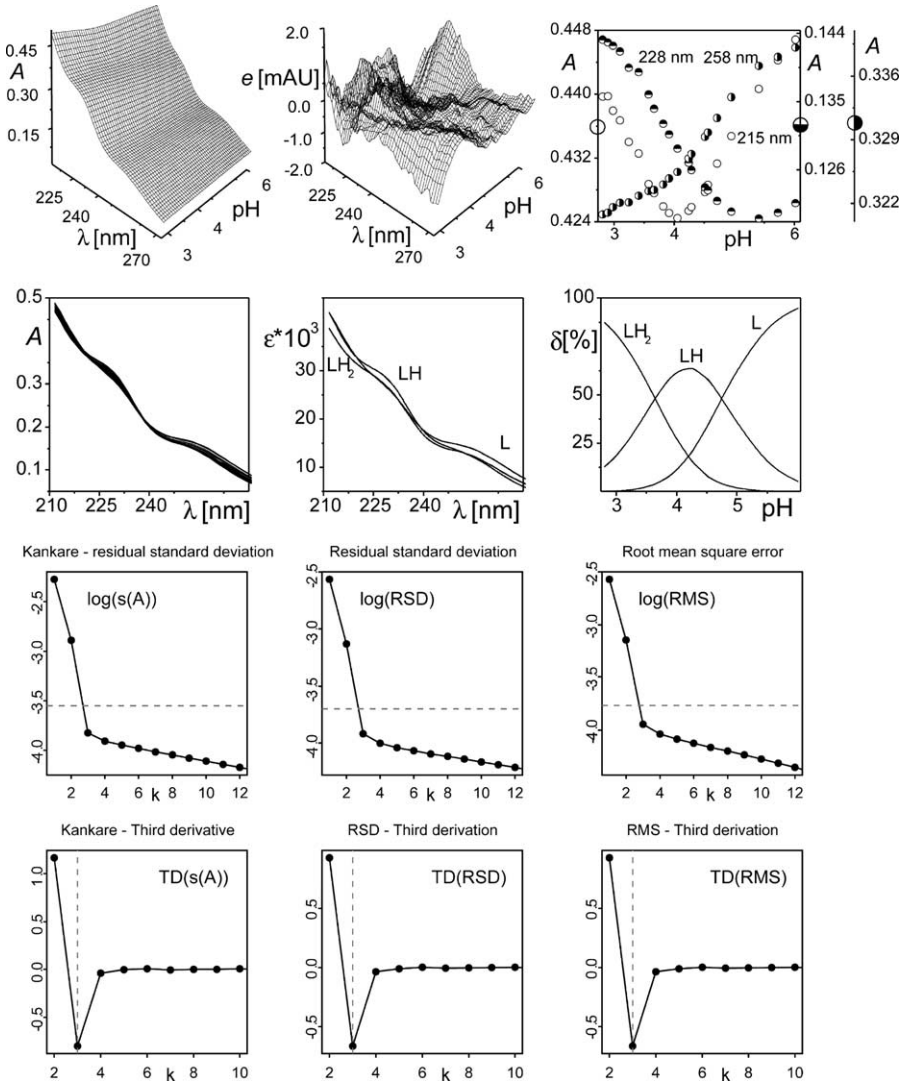


Fig. 1. Absorption spectra of the protonation equilibria of losartan in dependence on pH at 25 °C. First row: 3D absorbance-response-surface represents input for SQUAD(84) and SPECFIT32 programs, 3D overall diagram of residuals indicates the quality of a goodness-of-fit, *A*–pH curves at three selected wavelengths. Second row: absorption spectra measured, spectra of molar absorptivities vs. wavelengths for all of the variously protonated species, distribution diagram of the relative concentrations of all of the variously protonated species. Third row: Kankare's residual standard deviation $s_k(A)$, residual standard deviation RSD, root mean square error RMS. Fourth row: the third derivative of three index functions TD($s_k(A)$), TD(RSD), TD(RMS).

Table 1
Determination of the dissociation constants and molar absorptivities of variously protonated species of losartan by regression analysis of the UV–vis absorption spectra with SQUAD(84) and SPECFIT32 for $n = 20$ spectra measured at $m = 39$ wavelengths for two basic components L and H forming $p = 3$ variously protonated species

L_qH_r	SQUAD(84)		SPECFIT32	
	pK_a	s(pK_a)	pK_a	s(pK_a)
L_1H_1	3.642	0.041	3.642	0.038
L_2H_1	4.741	0.014	4.751	0.015
Determination of the number of light-absorbing species by factor analysis				
Number of light-absorbing species p		3		3
Residual standard deviation, $s_3(A)$ (mAU)		0.14		0.14
Goodness-of-fit test by the statistical analysis of residuals				
Residual-square-sum RSS $\times 10^3$		0.12		0.12
Hamilton R-factor		0.0019		Not calculated
Residual mean, $\bar{e}$ (mAU)		-4.01×10^{-9}		Not calculated
Mean residual, $\lvert\bar{e}\rvert$ (mAU)		0.34		Not calculated
Standard deviation of residuals, $s(e)$ (mAU)		0.56		0.49
Residual skewness, $g_1(e)$		−0.30		Not calculated
Residual kurtosis, $g_2(e)$		4.76		Not calculated

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

only. As all variously protonated anions exhibit quite similar absorption bands, a part of spectrum from 210 to 270 nm was selected as the most convenient for an estimation of protonation constants. pH–spectrophotometric titration enables absorbance-response-surface data (Fig. 1) to be obtained for analysis with 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 215, 228 and 258 nm show that two protonation constants can be indicated. The SPECFIT32 or SQUAD(84) program [7] analysis process starts with data smoothing followed by a factor analysis using the INDICES procedure [12]. The position of a break point on the $s_k(A) = f(k)$ curve in the screen plot of the three most reliable approaches (Kankare's $s(A)$, RSD and RSM) is calculated and gives $k^* = 3$ with the corresponding co-ordinate $s_3^*(A) = 0.14$ mAU (Table 1), which also represents the actual instrumental error $s_{inst}(A)$ of the spectrophotometer used (Fig. 1). Due to the large variations in the indicator values, these latter are plotted on a logarithmic scale (Figs. 1–4). The number of light-absorbing species p can be predicted from the index function values by finding the point $p = k$ where the slope of index function PC(k) = $f(k)$ changes, or by comparing PC(k) values with the instrumental error $s_{inst}(A)$. This is the common criterion for to determining p. Very low values of $s_{inst}(A)$ prove that quite reliable spectrophotometer and experimental techniques were used. When there are more than three components, derivative methods can be used: when the curve PC(k) = $f(k)$ does not exhibit a clear break point, the second derivative localizes this break more reliably. The second derivative SD(k) = log[PC($k+1$)] − 2 log[PC(k)] + log[PC($k-1$)] and p–k should be at the first maximum of the SD(k) function. The third derivative TD(k) = log[PC($k+2$)] − 3 log[PC($k+1$)] + 3 log[PC(k)] − log[PC($k-1$)] value crosses zero and reaches a negative minimum which can be used as a criterion. The TD(k) and p should be equal to the k-value where TD(k) has its first minimum. All three index methods predict the three variously protonated light-absorbing species of losartan in equilibrium. The two dissociation constants and three molar absorptivities of losartan calculated for 39 wavelengths of 20 spectra constitute 236 unknown parameters which are refined by the SQUAD(84) or SPECFIT32 in the first run. The reliability of the parameter estimates may be tested with the use of following diagnostics.

The first diagnostic value indicates whether all of the parametric estimates β_{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 dissociation 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 $s(\beta_j)$ and $s(\varepsilon_j)$ 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_{pqr} for various wavelengths, $s(\varepsilon_{qr}) = f(\lambda)$, should have a Gaussian distribution; otherwise, erroneous estimates of ε_{qr} are obtained. Fig. 1 shows the estimated molar absorptivities of all of the variously protonated species ε_L, ε_{LH}, ε_{L_2H} of losartan in dependence on wavelength. Some spectra overlap and such cases may cause some resolution difficulties.

The second diagnostic tests whether all of the calculated free concentrations of variously protonated species on the distribution diagram of the relative concentration expressed as

Table 2
The search for a chemical equilibrium model of losartan 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

q:*r*	pK_a				
	Model 1	Model 2	Model 3	Model 4	Model 5
1: 1	4.422 (48)	–	3.602 (13)	4.803 (13)	–
1: 2	–	8.945 (49)	4.733 (11)	–	9.529 (16)
1: 3	–	–	–	7.249 (18)	3.533 (23)
Goodness-of-fit test by the statistical analysis of residuals					
RSS × 10^3	6.19	6.42	0.12	0.24	0.33
$s(A)$ (mAU)	2.82	2.87	0.39	0.55	0.65
$s(e)$ (mAU)	2.82	2.87	0.39	0.55	0.65
$g_1(e)$	0.42	0.31	0.16	0.05	−0.10
$g_2(e)$	5.88	4.57	5.23	4.39	5.09
Conclusion of the chemical model search					
Model is	Rejected	Rejected	Accepted	Rejected	Rejected

The reliability of the parameter estimates found is proven with goodness-of-fit statistics, the residual standard deviation $s_k(A)$ (mAU) and the standard deviation of absorbance after termination of the regression process, $s(A)$ (mAU).

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

The chemical model attained contains L, LH, LH_2 at 25 °C ionic strength											
	0.007	0.02	0.034	0.047	0.066	0.086	0.106	0.145	0.165	0.185	0.204
p$K_{a,1}$	3.602 (13)	3.535 (18)	3.520 (30)	3.626 (24)	3.536 (18)	3.556 (33)	3.524 (15)	3.577 (16)	3.537 (47)	3.476 (15)	3.542 (37)
p$K_{a,2}$	4.733 (11)	4.723 (16)	4.697 (26)	4.672 (20)	4.649 (11)	4.612 (26)	4.631 (15)	4.677 (17)	4.688 (33)	4.642 (14)	4.591 (25)
Goodness-of-fit test											
RSS × 10^3	0.12	0.24	0.74	0.30	0.21	0.69	0.19	0.17	1.40	0.21	0.89
$s(A)$ (mAU)	0.39	0.56	0.97	0.66	0.52	0.94	0.49	0.47	1.34	0.52	1.07

The chemical model attained contains L, LH, LH_2 at 37 °C ionic strength								
	0.017	0.024	0.037	0.070	0.097	0.137	0.163	0.203
p$K_{a,1}$	3.642 (38)	3.509 (35)	3.440 (27)	3.447 (37)	3.415 (26)	3.498 (41)	3.442 (19)	3.447 (29)
p$K_{a,2}$	4.751 (15)	4.597 (15)	4.636 (18)	4.515 (15)	4.474 (14)	4.437 (22)	4.540 (11)	4.574 (26)
Goodness-of-fit test								
RSS × 10^3	0.12	0.27	0.40	0.23	0.22	0.41	0.13	0.72
$s(A)$ (mAU)	0.49	0.60	0.72	0.56	0.54	0.73	0.40	0.96

The reliability of the parameter estimates found is proven with goodness-of-fit statistics such as the residual square sum function RSS and the standard deviation of absorbance after termination of the regression process, $s(A)$ (mAU) at 25 °C (upper part) and 37 ° (lower part).

Table 4
Dependence of the mixed dissociation constants of paracetamol on ionic strength using regression analysis of pH–spectrophotometric data with SPECFIT, with the standard deviations of the parameter estimates in the last valid digits in brackets

The chemical model attained contains L, LH at 25 °C ionic strength								
	0.021	0.028	0.053	0.066	0.129	0.19	0.219	0.305
p$K_{a,1}$	9.728 (2)	9.739 (3)	9.723 (4)	9.711 (2)	9.711 (1)	9.700 (4)	9.709 (2)	9.720 (4)
Goodness-of-fit test								
RSS × 10^3	0.32	0.43	0.66	0.35	0.28	0.78	0.18	0.37
$s(A)$ (mAU)	0.73	0.82	1.04	0.73	0.62	1.17	0.58	0.90

The chemical model attained contains L, LH at 37 °C ionic strength										
	0.066	0.104	0.129	0.159	0.178	0.190	0.190	0.202	0.219	0.225
p$K_{a,1}$	9.551 (3)	9.551 (5)	9.535 (4)	9.541 (4)	9.534 (5)	9.529 (5)	9.538 (4)	9.525 (3)	9.518 (9)	9.540 (4)
Goodness-of-fit test										
RSS × 10^3	0.76	0.56	0.48	0.67	0.53	1.09	0.99	0.20	0.42	0.23
$s(A)$ (mAU)	1.15	0.93	0.89	0.94	0.91	1.34	1.24	0.55	0.92	0.59

The reliability of the parameter estimates found is proven with goodness-of-fit statistics such as the residual square sum function RSS and the standard deviation of absorbance after termination of the regression process, $s(A)$ (mAU) at 25 °C (upper part) and 37 °C (lower part).

a percentage have physical meaning, which proved to be the case (Fig. 1). The calculated free concentration of the basic components and variously protonated species of the chemical 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 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 generally be 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. The diagram shows that overlapping protonation equilibria of L_2H with LH and L exist.

The next diagnostic concerns the goodness-of-fit (Fig. 1). 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

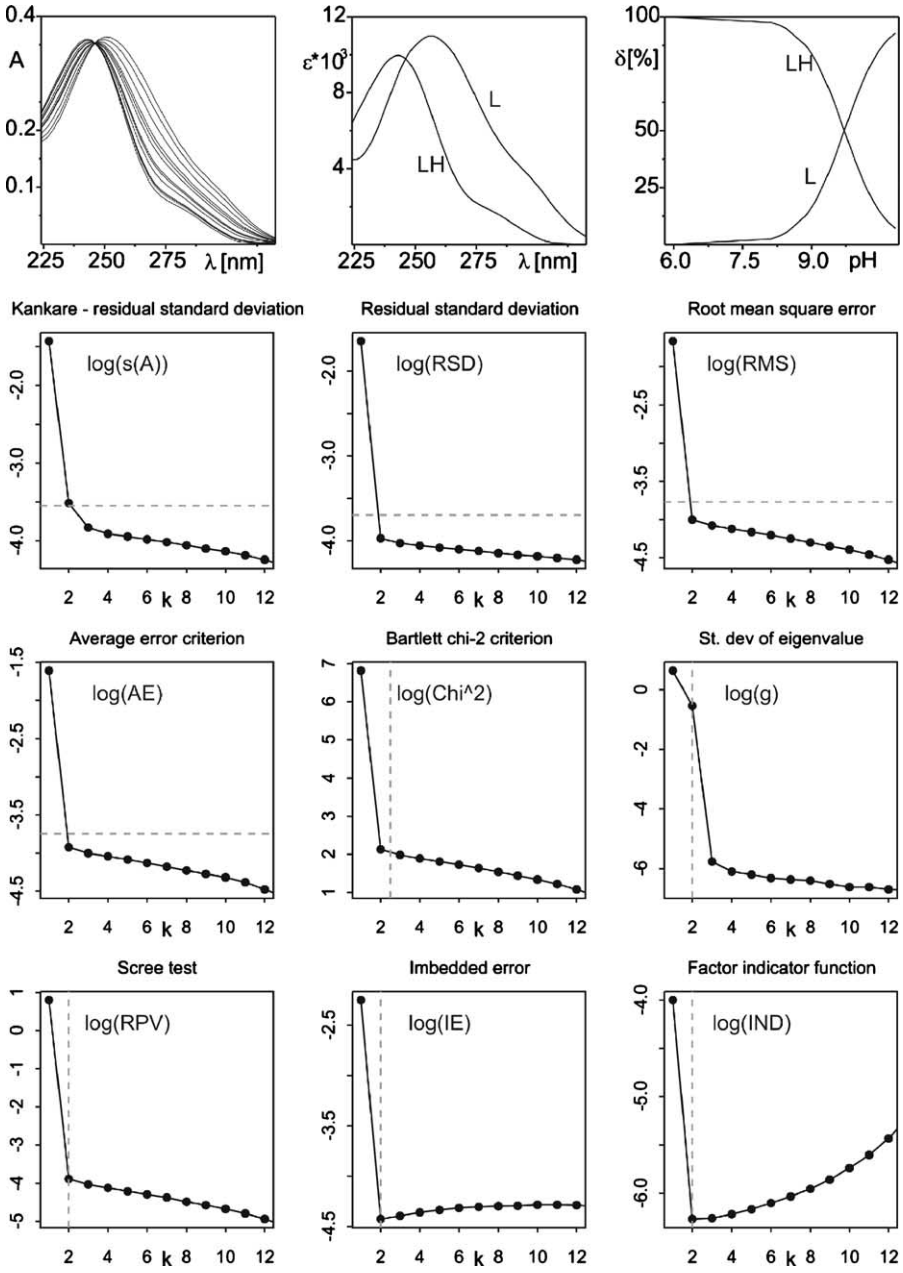


Fig. 2. Absorption spectra of the protonation equilibria of paracetamol in dependence on pH at 25 °C. First row: absorption spectra measured, spectra of molar absorptivities vs. wavelengths for all of the variously protonated species, distribution diagram of the relative concentrations of all of the variously protonated species. Second row: Kankare's residual standard deviation $s_k(A)$, residual standard deviation RSD, root mean square error RMS. Third row: average error criterion AE, Bartlett χ^2 criterion, standard deviation of eigenvalues $s(g)$. Fourth row: scree test RPV, imbedded error function IE, factor indicator function IND.

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. It is usually compared with the standard deviation of absorbance calculated by the INDICES program [12], $s_k(A)$, 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 (Table 1). This proves that the $s_3(A)$ value is equal to 0.14 mAU and is quite close to the standard deviation of absorbance when the minimization process terminates, $s(A) = 0.56$ mAU (SQUAD(84)) or 0.49 mAU (SPECFIT32). Although this statistical analysis of residuals [13,39] gives the most rigorous test of the degree-of-fit, realistic empirical limits must be used. The statistical measures of all residuals e prove that the minimum of the elliptic hyperparaboloid U is reached (Table 1):

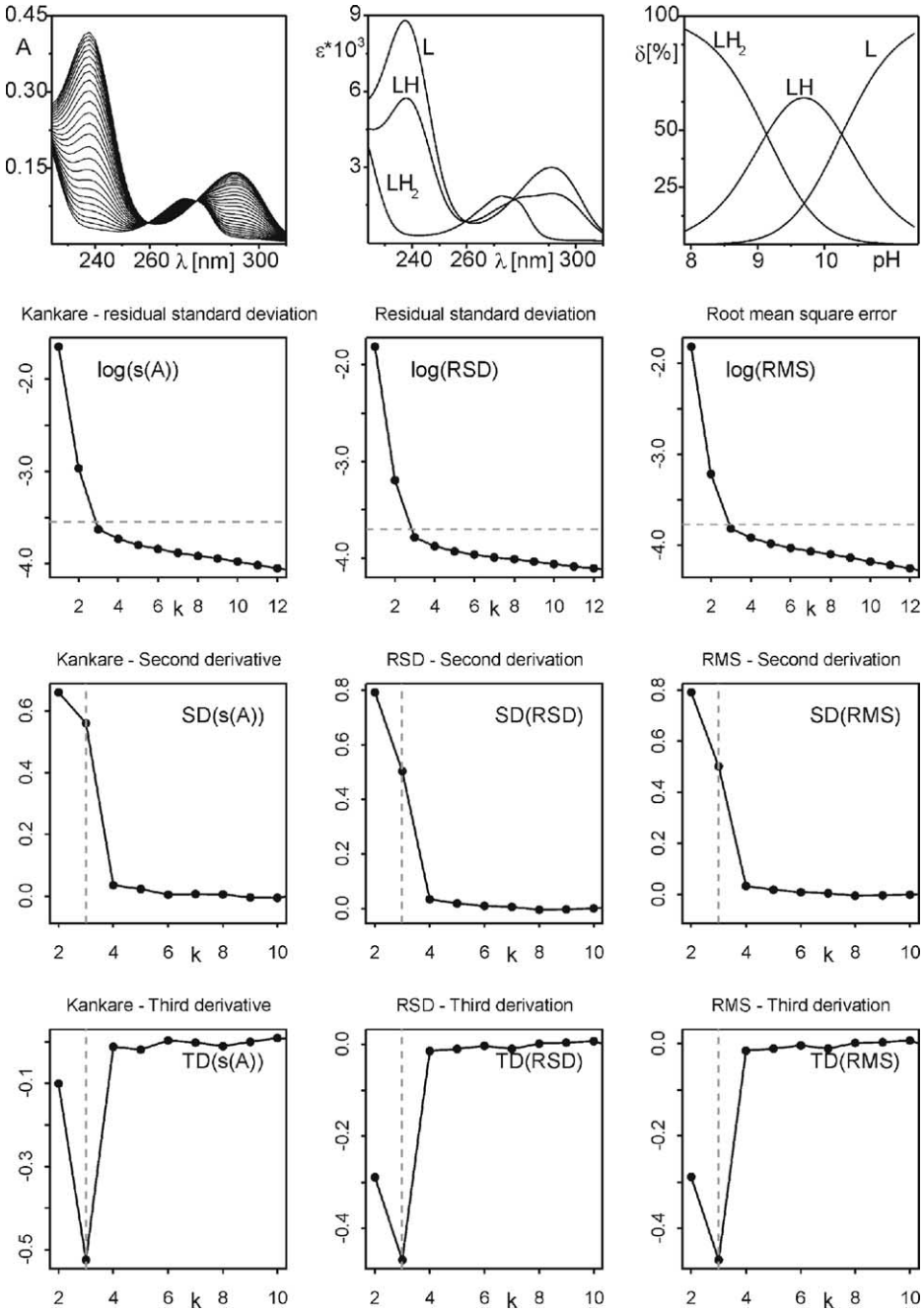


Fig. 3. Absorption spectra of the protonation equilibria of phenylephrine in dependence on pH at 25 °C. First row: absorption spectra measured, spectra of molar absorptivities vs. wavelengths for all of the variously protonated species, distribution diagram of the relative concentrations of all of the variously protonated species. Second row: Kankare's residual standard deviation $s_k(A)$, residual standard deviation RSD, root mean square error RMS. Third row: the second derivative of index functions SD($s_k(A)$), SD(RSD), SD(RMS). Fourth row: the third derivative of index functions TD($s_k(A)$), TD(RSD), TD(RMS).

the residual mean $\overline{e} = -4.01 \times 10^{-9}$ proves that there is no bias or systematic error in spectra fitting. The mean residual $|\overline{e}| = 0.34$ mAU and the residual standard deviation $s(e) = 0.56$ mAU (SQUAD(84)) or 0.49 mAU (SPECFIT32) have sufficiently low values. The skewness $g_1(e) = -0.30$ is quite close to zero and proves a symmetric distribution of the residuals set, while the kurtosis $g_2(e) = 4.76$ is close to 3, proving a Gaussian distribution.

In searching for the best chemical model of protonation equilibria, five various hypotheses of the stoichiometric indices q and r of L_qH_r acid were tested in order to find that which best represented the data (Table 2). 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 if they were both realistic and positive; and (3)

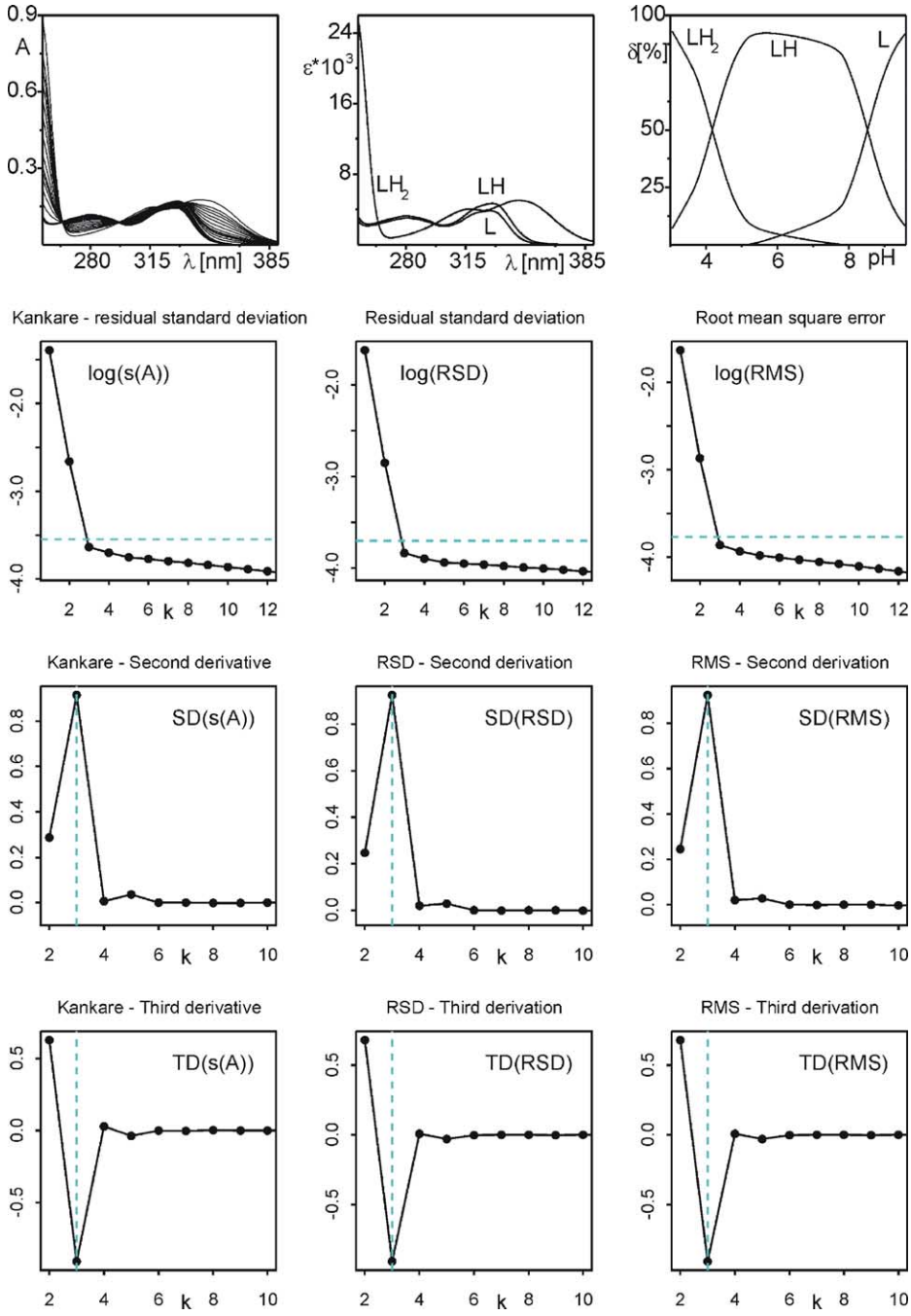


Fig. 4. Absorption spectra of the protonation equilibria of quinine in dependence on pH at 25 °C. First row: absorption spectra measured, spectra of molar absorptivities vs. wavelengths for all variously protonated species, distribution diagram of the relative concentrations of all of the variously protonated species. Second row: Kankare's residual standard deviation $s_k(A)$, residual standard deviation RSD, root mean square error RMS. Third row: the second derivative of index functions SD($s_k(A)$), SD(RSD), SD(RMS). Fourth row: the third derivative of index functions TD($s_k(A)$), TD(RSD), TD(RMS).

Table 5
Dependence of the mixed dissociation constants of phenylephrine on ionic strength using regression analysis of pH-spectrophotometric data with SPECFIT, with the standard deviations of the parameter estimates in the last valid digits in brackets

The chemical model attained contains L, LH, LH_2 at 25 °C Ionic strength												
	0.018	0.030	0.043	0.057	0.070	0.110	0.136	0.163	0.229	0.322	0.414	0.507
$pK_{a,1}$	9.164 (4)	9.103 (2)	9.132 (2)	9.104 (3)	9.119 (5)	9.113 (2)	9.134 (4)	9.098 (5)	9.155 (3)	9.150 (4)	9.178 (2)	9.176 (3)
$pK_{a,2}$	10.374 (7)	10.251 (4)	10.255 (4)	10.230 (6)	10.220 (8)	10.207 (4)	10.209 (7)	10.190 (8)	10.213 (6)	10.182 (7)	10.220 (4)	10.202 (6)
Goodness-of-fit test												
RSS $\times 10^3$	0.28	0.15	0.08	0.30	0.39	0.13	0.25	0.49	0.20	0.28	0.06	0.13
$s(A)$ (mAU)	0.52	0.35	0.27	0.49	0.61	0.33	0.47	0.65	0.40	0.47	0.23	0.33

The chemical model attained contains L, LH, LH_2 at 37 °C Ionic strength										
	0.017	0.017	0.030	0.043	0.057	0.070	0.093	0.096	0.110	0.136
$pK_{a,1}$	8.914 (3)	8.969 (6)	8.897 (5)	8.917 (5)	8.988 (7)	8.923 (5)	8.956 (6)	8.969 (8)	8.971 (6)	8.894 (5)
$pK_{a,2}$	10.064 (4)	10.106 (7)	10.029 (5)	10.016 6)	10.100 (8)	10.024 (6)	10.020 (8)	10.051 (10)	10.046 (8)	9.931 (5)
Goodness-of-fit test										
RSS $\times 10^3$	0.50	1.07	0.92	0.89	1.36	0.91	1.17	1.97	1.05	0.65
$s(A)$ (mAU)	0.65	0.99	0.86	0.87	1.11	0.87	0.99	1.31	0.98	0.74

The chemical model attained contains L, LH, LH_2 at 37 °C Ionic strength										
	0.189	0.216	0.242	0.282	0.322	0.375	0.414	0.467	0.534	0.560
$pK_{a,1}$	8.907 (8)	9.022 (6)	8.983 (5)	8.936 (6)	9.019 (5)	9.034 (6)	8.976 (5)	9.077 (7)	9.036 (5)	9.073 (6)
$pK_{a,2}$	9.898 (9)	10.057 (9)	10.013 (7)	9.929 (7)	10.022 (8)	10.045 (10)	9.978 (8)	10.089 (12)	10.046 (9)	10.093 (12)
Goodness-of-fit test										
RSS $\times 10^3$	1.40	1.01	0.86	0.45	0.73	1.11	0.95	1.31	1.05	1.36
$s(A)$ (mAU)	1.11	0.94	0.85	0.66	0.78	0.97	0.89	1.07	0.92	1.07

The reliability of the parameter estimates found is proven with goodness-of-fit statistics such as the residual square sum function RSS and the standard deviation of absorbance after termination of the regression process, $s(A)$ (mAU) at 25 °C (upper part) and 37 °C (lower part).

Table 6
Dependence of the mixed dissociation constants of quinine on ionic strength using regression analysis of pH–spectrophotometric data with SPECFIT, with the standard deviations of the parameter estimates in the last valid digits in brackets

The chemical model attained contains L, LH, LH_2 at 25 °C ionic strength									
	0.027	0.04	0.053	0.133	0.167	0.2	0.4	0.467	0.6
$pK_{a,1}$	4.175 (1)	4.192 (1)	4.208 (1)	4.341 (1)	4.343 (1)	4.372 (1)	4.454 (1)	4.568 (1)	4.670 (1)
$pK_{a,2}$	8.525 (6)	8.488 (7)	8.636 (16)	8.597 (13)	8.816 (11)	8.616 (12)	8.620 (18)	8.785 (12)	8.717 (12)
Goodness-of-fit test									
RSS × 10^3	0.28	0.34	1.3	0.61	0.88	0.57	1.39	1.08	0.75
$s(A)$ (mAU)	0.39	0.42	0.85	0.62	0.68	0.56	0.87	0.77	0.63
The chemical model attained contains L, LH, LH_2 at 37 °C ionic strength									
	0.018	0.054	0.072	0.09	0.108	0.126	0.18	0.234	0.27
$pK_{a,1}$	3.997 (3)	4.021 (4)	4.132 (2)	4.150 (2)	4.182 (1)	4.202 (2)	4.205 (2)	4.260 (2)	4.283 (3)
$pK_{a,2}$	8.418 (19)	8.353 (26)	8.452 (16)	8.412 (17)	8.437 (17)	8.420 (20)	8.485 (22)	8.462 (22)	8.472 (22)
Goodness-of-fit test									
RSS × 10^3	0.61	1.02	0.37	0.33	0.35	0.43	0.5	0.5	0.57
$s(A)$ (mAU)	0.57	0.73	0.44	0.43	0.43	0.48	0.53	0.51	0.55

The reliability of parameter the estimates found is proven with goodness-of-fit statistics such as the residual square sum function RSS and the standard deviation of absorbance after termination of the regression process, $s(A)$ (mAU) at 25 °C (upper part) and 37 °C (lower part).

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.

Using the experimental and evaluation strategy, the protonation equilibria of losartan (Tables 1–3 and Fig. 1), paracetamol (Table 4 and Fig. 2), phenylephrine (Table 5 and Fig. 3) and quinine (Table 6 and Fig. 4) were also examined. To test the reliability of the protonation/dissociation constants at different ionic strengths, the goodness-of-fit test with the use of statistical analysis of the residuals was applied, and the results are given in Tables 3–6. For all four drugs studied the most efficient tools, such as the mean residual and the standard deviation of residuals, were applied. The standard deviation of absorbance $s(A)$ after termination of the minimization process is always better than 1.3 mAU, and the proposal of a good chemical model and reliable parameter estimates is proven.

4.1.2. Paracetamol

For the conjugation of the hydroxyl group with the π-electron system of a benzene ring, the dissociation of paracetamol can easily be identified in spectra of the range 224–320 nm. One sharp isosbestic point proves one protonation equilibrium. All nine index methods in Fig. 2 (cf. Refs. [12,13]) lead to the same conclusion that there are two light-absorbing species in the equilibrium mixture. For only two species in the mixture can SD(k) and TD(k) not be used. The graph of the molar absorption coefficients of variously protonated species shows that the curve of HL significantly differs from the curve of L.

4.1.3. Phenylephrine

The molecule contains two hydroxyl groups and one amino-nitrogen which dissociate a proton and affect chromophor. The two dissociation constants $pK_{a1} = 3.63$ and $pK_{a2} = 4.84$ concerning both hydroxyls are close, as it is valid that $|pK_{a1} - pK_{a2}| < 3$. Spectra for various values of pH in the range 227–307 nm exhibit two sharp isosbestic points and prove the assumed protonation equilibria. The graph of molar absorption coefficients in dependence on wavelength (Fig. 3) shows that the dissociation of the two hydroxyl groups strongly affects the π-electron system in the molecule. The third derivative TD(k) obviously indicates three light-absorbing species in the equilibrium mixture.

4.1.4. Quinine

The molecule contains several groups which can be deprotonated. Two distant dissociation constants were indicated, $pK_{a1} = 4.26$ and $pK_{a2} = 8.74$, as it is valid that

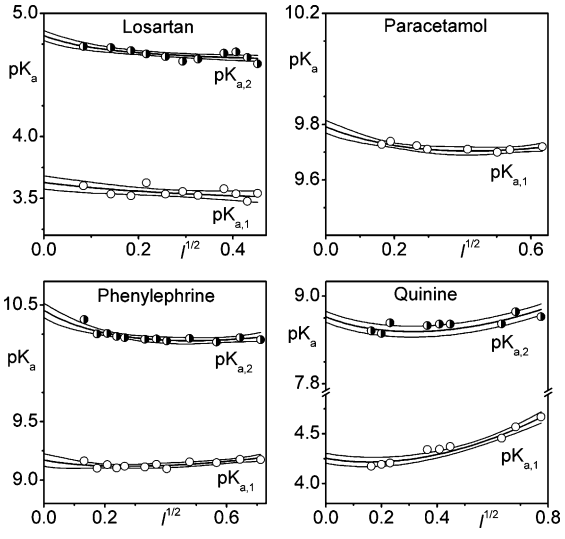


Fig. 5. Dependence of the mixed dissociation constant pK_a of four drugs on the square root of ionic strength, which lead to parameter estimate of pK_a^T at 25 °C.

Table 7
Thermodynamic dissociation constants for losartan, paracetamol, phenylephrine and quinine at two selected temperatures with the standard deviations in the last valid digits in brackets

		Value at 25 °C	Value at 37 °C
Losartan	$pK^{T}_{a,1}$	3.63 (1)	3.57 (3)
	$pK^{T}_{a,2}$	4.84 (1)	4.80 (3)
Paracetamol	$pK^{T}_{a,1}$	9.78 (1)	9.65 (1)
Phenylephrine	$pK^{T}_{a,1}$	9.17 (1)	8.95 (1)
	$pK^{T}_{a,2}$	10.45 (1)	10.22 (1)
Quinine	$pK^{T}_{a,1}$	4.25 (1)	4.12 (1)
	$pK^{T}_{a,2}$	8.72 (1)	8.46 (2)

$|pK_{a1} - pK_{a2}| > 3$. The second SD(k) and third TD(k) derivative prove that there are three light-absorbing species in the protonation equilibria mixture. The spectra show that the protonation of the quinine molecule affects the π-electron system of the molecule.

The unknown parameter pK^{T}_{a} was estimated by applying a Debye–Hückel equation to the data in Tables 3–6 and Fig. 5 according to the regression criterion; Table 7 shows point estimates of the 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 poorly soluble then pH–spectrophotometric titration may be used with the nonlinear regression of the absorbance-response-surface data instead of a potentiometric determination of dissociation constants. The reliability of the dissociation constants of the four drug acids (i.e. losartan, paracetamol, phenylephrine and quinine) 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

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