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A novel computational strategy for the pK_a estimation of drugs by non-linear regression of multiwavelength spectrophotometric pH-titration data exhibiting small spectral changes

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ABSTRACT: A new computational procedure for the protonation model building of a multiwavelength and multivariate spectra treatment is proposed for the special case of small changes in spectra. The absorbance change Δ_i for the *i*th spectrum divided with the instrumental standard deviation $s_{inst}(A)$ represents *the signal-to-error ratio SER* of the spectra studied. The determination of the number of chemical components in a mixture is the first important step for further quantitative analysis in all forms of spectral data treatment. Most index-based methods of the factor analysis can always predict the correct number of components, and even the presence of a minor one, when the *SER* is higher than 10. The Wernimont–Kankare procedure in the program INDICES performs reliable determinations of the instrumental standard deviation of the spectrophotometer used $s_{inst}(A)$, correctly predicts the number of light-absorbing components present, and also solves ill-defined problems with severe collinearity in spectra or very small changes in spectra. The mixed dissociation constants of three drugs, haemanthamine, lisuride, and losartan, including diprotic molecules at ionic strengths of $I = 0.5$ and 0.01 and at 25°C were determined using two different multi-wavelength and multivariate treatments of the spectral data, SPECFIT32 and SQUAD(84) non-linear regression analyses and INDICES factor analysis, even in the case of small absorbance changes in spectra. The dissociation constant pK_a was estimated by non-linear regression of {pK_a, I} data at 25°C: for haemanthamine p$K_a = 7.28(1)$ at $I = 0.50$, for lisuride p$K_a = 7.86(1)$ and for losartan p$K_{a,1} = 3.60(1)$, p$K_{a,2} = 4.73(1)$ at $I = 0.01$. Goodness-of-fit tests for the 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 the drug compounds in agreement with the experimental value. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: spectrophotometric titration; dissociation constant; protonation; haemanthamine; lisuride; losartane; SPECFIT; SQUAD; INDICES; PALLAS; MARVIN

INTRODUCTION

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 ionization of a drug molecule in solution at different pHs. Spectrophotometry is a convenient method for pK_a determination in very diluted aqueous solutions (about 10^{-5}–10^{-6} M), provided that the compound possesses pH-dependent light absorption due to the presence of a chromophore in the proximity to the ionization center. In previous work, [1–9] the authors have shown that the spectrophotometric method can be used in combination with suitable chemometric tools for the determination of protonation constants β_{qr} or acid dissociation constants pK_a, even of barely soluble drugs. Problems arise because of strong overlapping chemical components involved in the equilibrium or small changes of spectral responses caused by a drug protonation, 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 cf. References [10–25].

Much work has been devoted to the development of methods for the resolution of multicomponent spectra, but less work has been carried out to reveal the limitations of

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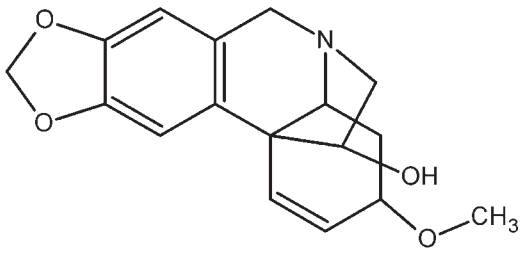
the methods and to estimate the minor components of the resolved spectra [1]. When no noise in spectra exists, the number of eigenvalues of the covariance matrix $\boldsymbol{A}^{\mathbf{T}}\boldsymbol{A}$ larger than zero is equivalent to the number of components, *r*, assuming that the spectra of the components in mixture are linearly independent. As all real data always contains experimental noise, the number of eigenvalues different from zero is usually larger than the number of components *r*. Experimental and/or random error can mask the identification of the true dimensionality of a data set. Chen *et al*. [26,27] have concluded that many multivariate statistical methods have been designed, and have solved certain problems encountered in spectra sets, when the spectra of components are similar and changes in absorbance are very small, and when there exist minor components or when the signal to noise ratio is low. In any study of this type, the level of 'experimental noise' used will be a critical factor. It is therefore necessary to have a consistent definition of the *signal-to-noise ratio SNR* so that the impact of this parameter can be critically assessed. Traditional approaches to *SNR* are typically based on the ratio of the maximum signal to maximum noise value. As an alternative, the concept of instrumental error was again employed and the *signal-to-error ratio SER* is defined, where for an error the instrumental standard deviation of absorbance, $s_{inst}(A)$, is used. Attention should be paid to a method's ability to detect a minor component in the presence of major ones. The detection limit is equivalent to the amount of 'detectable impurity' or the smallest relative concentration of the minor component. Approaching the detection limit, no method can accurately determine the minor component in the mixture. The detection limit depends on several factors, such as (i) the spectral similarity of the minor component to the other components, (ii) instrumental resolution, (iii) noise level and noise type, and (iv) the signal-to-noise ratio *SNR* with respect to the minor component.

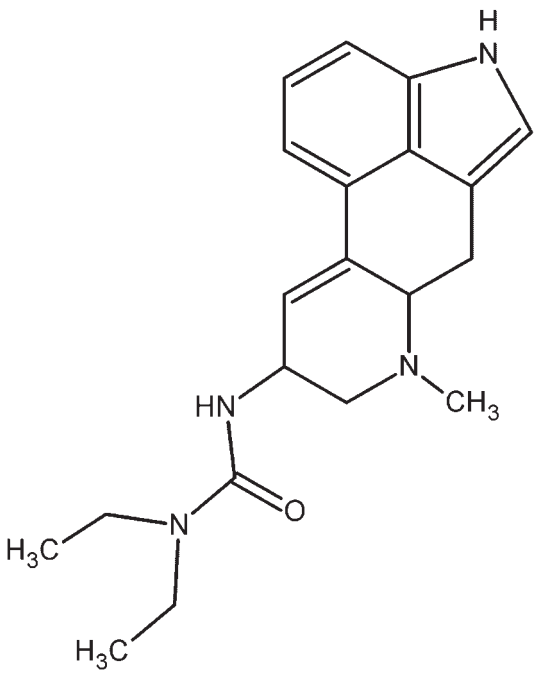
The regression methods include traditional least-squares curve fitting approaches, based on a previous postulation of a chemical model, that is, the postulation of a set of species defined by their stoichiometric coefficients and formation constants, which are then refined by least-squares minimization. These mathematical procedures require the fulfillment of the mass-balance equations and the mass-action law. The most relevant algorithms are SQUAD(84) [14–19] and SPECFIT32 [22–24,31].

Recently, haemanthamine, lisuride, and losartan were studied in our laboratory [7,8], and these three drugs were taken as examples of acid drugs which exhibit small changes in spectra.

Haemanthamine belongs to the class of 5,10B-ethanophenanthridines. It possesses relatively high antiretroviral properties, antiproliferative effects and also has potent antimalarial properties (against *Plasmodium falciparum*).[8] It is also found in the bulbs of Amaryllidaceae (Clivia species) and Liliaceae (Hippeastrum, Lycoris, Narcissus). CAS No.: 466-75-1, summary molecular formula: $C_{17}H_{19}NO_4$, molecular weight: 301.4, octanol/water partition coefficient as $\log P_{o/w}$: 1,47–1,56 (calculated); pK_a not known. Haemanthamine is of the structure



Lisuride: The systematic chemical name of lisuride is 3-(9,10-Didehydro-6-methyl-8α-ergolinyl)-1,1-diethylurea [8] and it is of the structure



Recommended INN name: lisuride, CAS Number: 18016-80-3, EINECS: 241-925-1, ACX. [7] Number: X1063856-3, description: almost white to light yellow or brownish crystalline powder, molecular formula: $C_{20}H_{26}N_4O$, molecular weight: 338.5, melting point**:** 169–172°C, solubility: slightly soluble in methanol, ethanol, dimethylformamide, dimethylsulfoxide, chloroform, and dichloromethane, sparingly soluble in ether and practically insoluble in water and hexane; pK_a is not known due to insolubility in water.

Losartan is a biphenylimidazole type drug, chemically 2-Butyl-4-chloro-1-[[2′-(1H-tetrazol-5-yl) [[1,1′-biphenyl]-4-yl]methyl]-1H-imidazole-5-methanol, CAS No. 0114798-26-4, molecular formula $C_{22}H_{23}ClN_6O$, molecular weight 422.93 [7] is of the structure

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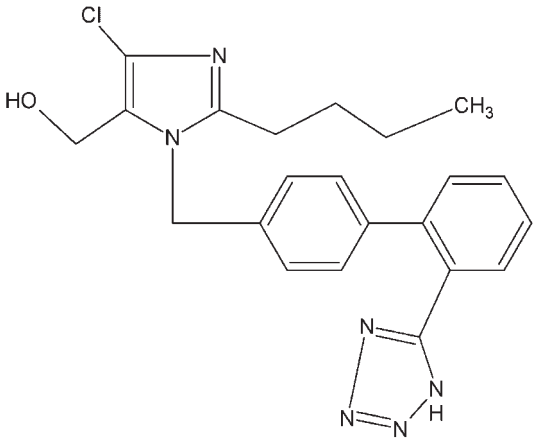

It is an AT1 antagonist. Angiotensin II binds to the AT1 receptor found in many tissues (e.g., in the vascular smooth muscle, adrenal gland, kidneys and heart), and elicits several important biological actions, including vasoconstriction and the release of aldosterone. These effects of angiotensin II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

THEORETICAL

Procedure for the determination of the protonation constants

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 n_c the number of species, $(q, r)_i$, $i = 1, \ldots, n_c$ where index i labels their particular stoicheiometry; 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 $K_{a,j} = [H_{j-1}L]a_{H^+}/[H_jL]$. 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 $A_{i,j} = \sum_{n=1}^{n_c} \varepsilon_{j,n}c_n = \sum_{n=1}^{n_c} (\varepsilon_{qr,j}\beta_{qr}l^q h^r)_n$ 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 $(n_s \times n_w)$ being measured for n_s solutions with known total concentrations of $n_z = 2$ basic components, c_L and c_H, at n_w wavelengths. The multicomponent spectra analyzing program SQUAD(84) [16] may adjust $\boldsymbol{\beta}_{qr}$ and $\boldsymbol{\varepsilon}_{\mathbf{qr}}$ for a given absorption spectra set by minimizing the residual-square sum function, U,

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

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

where $A_{i,\ j}$ represents the element of the experimental absorbance response-surface of size $n_s \times n_w$ (Fig. 1a–c) and the independent variables c_k are the total concentrations of the basic components c_L and c_H being adjusted in n_s solutions. This 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_s solutions, at n_w wavelengths. The best estimates of the protonation constants, $\boldsymbol{\beta}_{qr,\ i}$, $i = 1, \ldots, p$, are adjusted by SQUAD(84) Gauss–Newton and Newton–Raphson regression algorithms. At the same time, a matrix of the molar absorptivities $(\varepsilon_{qr,\ j}$, $j = 1, \ldots, n_w)_k$, $k = 1, \ldots, p$, as non-negative real is estimated, based on the current values of the protonation constants. For a set of current values of $\boldsymbol{\beta}_{qr,\ i}$, the free concentrations of ligand l (as h is known from pH measurement) is calculated for each solution, followed by the concentrations of all the species in equilibrium mixture $[L_qH_r]_j$, $j = 1, \ldots, p$, forming for n_s solutions of the matrix $\boldsymbol{C}$. When the estimated $\boldsymbol{\beta}_{\mathbf{qr}}$ and $\boldsymbol{\varepsilon}_{\mathbf{qr}}$ values for the assumed chemical model have been refined, the agreement between the experimental and predicted data can be examined. The residuals are analyzed to test whether the refined parameters adequately represent the data, and should be randomly distributed about the predicted regression curve. The following statistics are calculated: the residual mean $\overline{e}$ the standard deviation of the residuals $s(e)$, the skewness of the residuals set $\hat{g}_1(e)$, and the kurtosis of the residuals set $\hat{g}_2(e)$. If, after termination of the minimization process, the condition $s(A) \approx s_{\mathrm{inst}}(A)$ or $s(e) \approx s_{\mathrm{inst}}(A)$ is met, the hypothesis of the chemical model is taken as the most probable one and is accepted.Another popular program is SPECFIT/32,[31] based on singular value decomposition and non-linear 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 assumptions as to the chemistry of the system, other than the number of active complexes present, nor any assumptions as to the nature of absorbing complexes, their stoichiometry or a thermodynamic model. The latest version of SPECFIT/32 [31] makes use of a multiwavelength and multivariate spectra treatment, and enables a global analysis for

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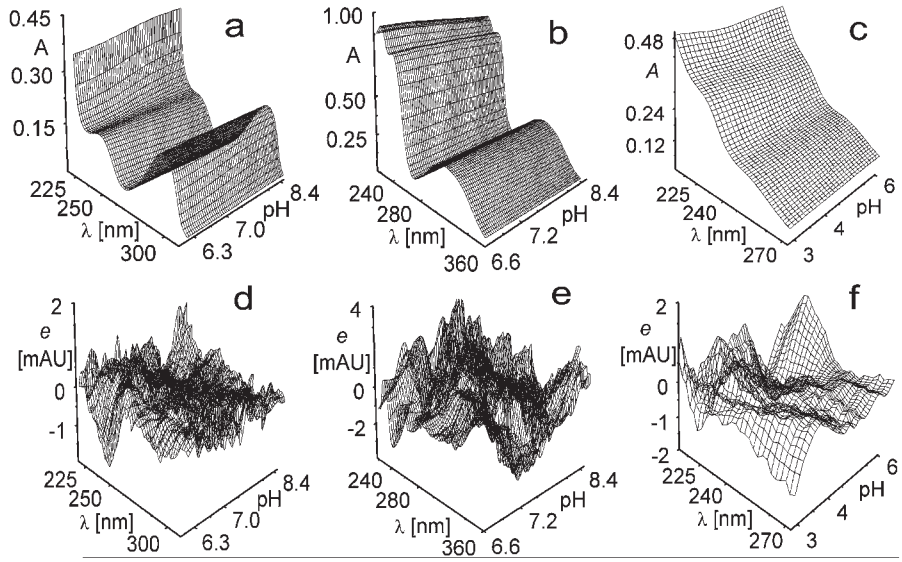

Figure 1. pH-dependence of the 3D-absorbance-surface representing the measured multiwavelength absorption spectra at 25°C for (a) haemanthamine, (b) lisuride, (c) losartan, and the 3D-residuals map after non-linear regression performed for (d) haemanthamine, which exhibits 912 residuals of the *residual bias* $\bar{e} = -3.36\text{E-}09$, close to zero, and the *residual standard deviation* $s(e) = 0.58$ mAU, close to the *instrumental standard deviation* $s_{inst}(A) = 0.45$ mAU; the *residual skewness* $g_1(e) = 2.16$ is not close to zero and indicates an asymmetric distribution of the residuals; the *residual kurtosis* $g_2(e) = 10.52$ is not close to 3 and indicates a non-Gaussian distribution of the residuals. The accuracy test of the bias proves that the bias is not significantly different from zero, and 34 outlying residuals were indicated, (e) lisuride exhibits 1440 residuals of $\bar{e} = 3.9\text{E-}08$ being close to zero, $s(e) = 0.76$ mAU is higher than $s_{inst}(A) = 0.28$ mAU; and both values $g_1(e) = -0.43$ and $g_2(e) = 3.82$ indicate an asymmetric distribution of the residuals. The accuracy test of the bias $\bar{e}$ proves significant bias, and 4 outlying residuals were detected, (f) losartan exhibits 780 residuals of $\bar{e} = 2.0\text{E-}08$, close to zero, $s(e) = 0.40$ mAU higher than $s_{inst}(A) = 0.13$ mAU; and $g_1(e) = 0.15$ proves symmetric distribution while $g_2(e) = 5.00$ indicates a non-Gaussian distribution of the residuals. The accuracy test of the bias proves insignificant bias and 18 outlying residuals; (S-Plus)

equilibrium and kinetic systems with singular value decomposition and non-linear least-squares regression modeling using the Levenberg–Marquardt method. This method has proven to be superior in discriminating between chemical models. The experimental and computational schemes for the determination of the protonation constants in a multicomponent system for data exhibiting small changes in spectra are taken from Meloun *et al.*,[11,30] and the details for the computer data treatment are collected in the *Supporting Information*.

Throughout this paper, it is assumed that the $n_s \times n_w$ absorbance data matrix $\boldsymbol{A} = \boldsymbol{\varepsilon}\ \boldsymbol{C}$ containing the n_s recorded spectra as rows can be written as the product of the $n_w \times p$ matrix of molar absorptivities $\boldsymbol{\varepsilon}$ and the $p \times n_s$ 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 $\text{rank}(\boldsymbol{A}) = \min[\text{rank}(\boldsymbol{\varepsilon}), \text{rank}(\boldsymbol{C})] \leq \min(n_w, p, n_s)$. Since the rank of $\boldsymbol{A}$ is equal to the rank of $\boldsymbol{\varepsilon}$ or $\boldsymbol{C}$, whichever is the smaller, and since $\text{rank}(\boldsymbol{\varepsilon}) \leq p$ and $\text{rank}(\boldsymbol{C}) \leq p$, then provided that n_w and n_s 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.[1,11,20,28] All spectra evaluations may be performed with the INDICES algorithm [1,28] in the S-Plus programming environment [29]. 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 the 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.[1,28]

Computational schema for protonation model building with SPECFIT32

An experimental and computational scheme for the protonation model building of a multi-component and multiwavelength system was proposed by Meloun *et al.* cf. page 226 in Reference[11] or References[16,30] and is here revised with regard to the SPECFIT/32 and INDICES applications for a case of data exhibiting small changes in spectra:

(1) *Instrumental error of absorbance measurements,* $s_{inst}(A)$: The INDICES algorithm cf. References[1,28] should be used to evaluate $s_{inst}(A)$. Cattel's scree plot of $s_k(A) = f(k)$ of the Wernimont–Kankare procedure

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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, $s_{inst}(A) = s_1^*(A)$ reaching a value of 0.25 mAU in range 225–360 nm for the Cintra 40 (GBC, Australia) spectrophotometer employed. This value can be used for a prediction of the *signal-to-error* ratio *SER* for experimental data. It was proven that the indices are able to accurately predict the correct number of components that contribute to a set of absorption spectra for data sets even exhibiting small changes in spectra but with an *SER* of equal to or higher than 10.

(2) *Experimental design:* Simultaneous monitoring of absorbance spectra and pH during titrations is used in a titration where 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 the quality of the dataset and remove spurious data, and to estimate the minimum number of *factors*, that is, contributing aqueous species, necessary to describe the experimental data. The INDICES[1,28] determine the number of dominant species present in the equilibrium mixture. It has been proven[1] that the Wernimont–Kankare procedure is a reliable method for determination of the instrumental standard deviation of the spectrophotometer used $s_{inst}(A)$, as it is stable in many situations and can correctly predict a minor component in a mixture even if its relative concentration is about 0.5–1% relative to the remaining components. This method can detect minor components and solve an ill-defined problem with severe collinearity in spectra, and predicts the correct number of components for data sets with a signal-to-error ratio *SER* of equal to or higher than 10. For the signal value *S* in a numerator of the ratio *S/E*, the absorbance difference for the *j*th-wavelength at the *i*th-spectrum $\Delta_{ij} = A_{ij} - A_{i,\ acid}$ can be used, where $A_{i,\ acid}$ is the limiting spectrum of acid form of drug measured. This absorbance change Δ_{ij} is then divided with the instrumental standard deviation $s_{inst}(A)$ and the resulting ratio $\Delta/s_{inst}(A)$ here represents the signal-to-error ratio *SER* of the spectra studied. This *SER* ratio is examined for all absorbance matrix elements in the whole range of wavelength λ and is compared with the limiting *SER* value. It has been proven that when the ratio $\Delta/s_{inst}(A)$ is equal to or higher than 10, the factor analysis is able to predict the correct number of components in the equilibrium mixture.

(4) *Choice of computational strategy of regression process:* The input data should specify whether β_{qr} or log β_{qr} values are to be refined with an application of two procedures of non-linear regression, and whether multiple regression (MR) or non-negative linear least-squares (NNLS) are desired. It should be indicated whether the protonation constants are to be refined or held constant, and whether molar absorptivities are to be refined.

(5) *The initial estimates of predicted parameter β_{qr} from the molecular structure*: It is wise before starting a regression to analyze the 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 the relevant protonation constants and the probable spectral traces of all the expected components. Some programs, PALLAS [32] and MARVIN [33], provide a collection of powerful tools for making *a prediction* of the pK_a values of any organic compound on the basis of the 3D-structural formulae of the compounds.

(6) *Diagnostic criteria indicating a 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[34]:

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 at a 99.9% statistical probability level.

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 concentrations of the basic components and the variously protonated species of the chemical model should show realistic molarities, that is, down to about 10^{-8} M.

3rd diagnostic – parametric correlation coefficients: Partial correlation coefficients, r_{ij}, indicate the interdependence of two parameters, that is, the 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 the chemical model represents the data adequately, the residuals $\boldsymbol{e}$ should be carefully analyzed. The goodness-of-fit achieved is easily seen by examination of the differences between the exper-

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imental and calculated values of absorbance, $e_i = A_{exp,\ i,\ j} - A_{calc,\ i,\ j}$. One of the most important statistics calculated is *the standard deviation of the absorbance*, $s(A)$, calculated at the termination of the minimization process as $s(A) = \sqrt{U_{min}}/df$ where U_{min} stands for the residuals-square-sum function in minimum and df is the degree of freedom. This is usually compared with the standard deviation of absorbance calculated by the INDICES program [1,28] $s_k(A)$ and the instrumental error of the spectrophotometer used $s_{inst}(A)$: if it is valid that $s(A) \leq s_k(A)$, or $s(A) \leq s_{inst}(A)$, then the fit is considered to be statistically acceptable. Some realistic empirical limits are employed: for example, when $s_{inst}(A) \leq s(A) \leq 0.002$, the goodness-of-fit is still taken as acceptable, while $s(A) > 0.005$ indicates that a good fit has not been obtained. Alternatively, the statistical measures of residuals $\boldsymbol{e}$ can be calculated to examine the following criteria: the *residual bias* $\bar{e}$ should be a value close to zero; the *residual standard deviation* $s(e)$ being equal to the absorbance standard deviation, $s(A)$ should be close to the *instrumental standard deviation* $s_{inst}(A)$; 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.

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

EXPERIMENTAL

Chemicals and solutions

The haemanthamine and lisuride were the kind gifts of IVAX Pharmaceuticals s.r.o., Czech Republic. The *haemanthamine* 100.0% (HPLC) was calculated as an area ratio with the use of the internal standard method. The *lisuride*, batch No. SC041200/4, was of assay 99.9% (HPLC) calculated as an area ratio using the internal standard method. *Losartan potassium* was purchased from SMS Pharmaceuticals s.r.o., India, with a purity of 99.7%. *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 hydrogenphthalate using the Gran Method with a reproducibility of 0.1%. The preparation of the other solutions from analytical reagent-grade chemicals has been described previously.[30]

Apparatus and pH-spectrophotometric titration procedure

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

Software used

Computations 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 the graphs were plotted using ORIGIN 7.5[36] and S-Plus.[29] A qualitative interpretation of the spectra with the use of the INDICES program[28] aims to evaluate the quality of the dataset and remove spurious data, and to estimate the minimum number of *factors*, that is, contributing aqueous species, necessary to describe the experimental data, as well as determining the number of dominant species present in the equilibrium mixture. pK_a are predicted using version of the PALLAS[32] and MARVIN[33] programs from 2004 and 2007.

Supporting information available

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

RESULTS AND DISCUSSION

Recently, haemanthamine, lisuride, and losartan were studied in our laboratory for a time[7,8], and these three drugs were therefore taken as examples of drug acids which exhibit quite small changes in spectra.

Haemanthamine: The deprotonation haemanthamine LH form exhibits two isosbestic points in the spectra, and these two points indicate one simple equilibrium. pH-spectrophotometric titration enables absorbance-response data (Fig. 1a and Fig. 2a) to be obtained for analysis by non-linear regression, and the reliability of parameter estimates (pK's and ε's) can be evaluated on the basis of a goodness-of-fit test of the residuals (Fig. 1d and Fig. 2d). The A-pH curves at 228, 252, and 294 nm (Fig. 2c) show that a dissociation constant 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 small shift of a band maximum to lower wavelengths in the spectra set is shown in Fig. 2a and Fig. 2e. The adjustment of pH value from 6.3 to 8.4 causes the absorbance to change by 22 mAU only,

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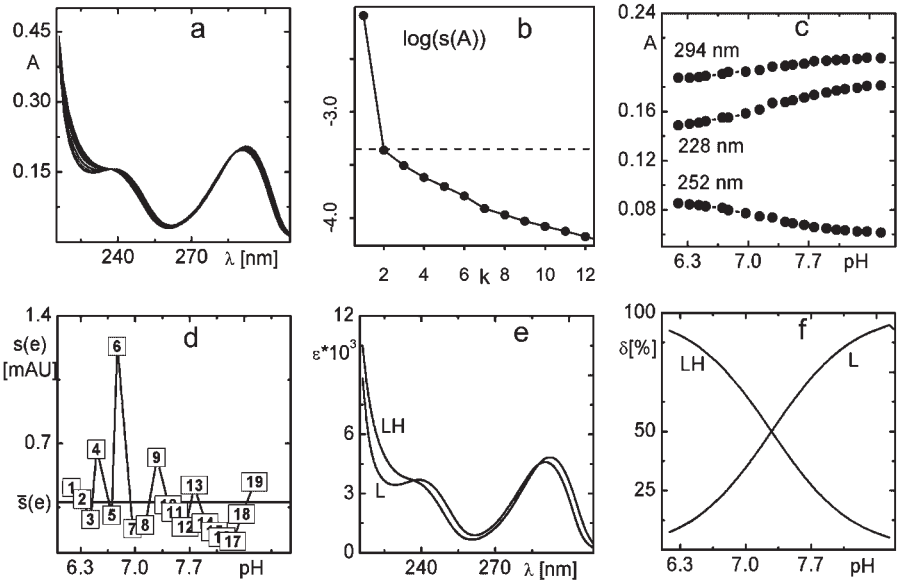

Figure 2. Non-linear regression analysis of the protonation equilibria model and factor analysis of haemanthamine: (a) pH-dependent absorption spectra at 25°C, (b) Cattel's scree plot of the Wernimont–Kankare procedure for the determination of the number of light-absorbing species in the mixture $k^* = 2$ leads to the actual instrumental error of the spectrophotometer used $s_{\text{inst}}(A) = 0.45$ mAU (INDICES in S-Plus), (c) the absorbance versus pH curves for 228, 252, and 294 nm at 25°C, (d) detecting influential outlying spectra with the use of the goodness-of-fit test and the plot of the residual standard deviation $s(e)$ versus pH for 19 spectra in dependence on pH at 25°C, (e) pure spectra profiles of molar absorptivities versus wavelengths for the variously protonated species L, LH, (f) distribution diagram of the relative concentrations of both variously protonated species L, LH, of haemanthamine in dependence on pH at 25°C, (SPECFIT, ORIGIN)

so that the monitoring of both components L and LH of the protonation equilibrium is rather uncertain. As the changes in spectra are very small, a very precise measurement of absorbance is required for the reliable estimation of the deprotonation equilibrium studied.

In the first step of the regression spectra analysis the number of light-absorbing species is estimated by the INDICES algorithm (Fig. 2b). The position of the break point on the $s_k(A) = f(k)$ curve in the factor analysis scree plot is calculated and gives $k^* = 2$ with corresponding co-ordinate $\log s_2^*(A) = -3.35$, that is, $s_2^*(A) = 0.45$ mAU, which also represents the actual instrumental error $s_{\text{inst}}(A)$ of the spectrophotometer used. Due to the large variations in the indicator values, these latter are plotted on a logarithmic scale. All other selected methods of the modified factor analysis in the INDICES algorithm estimate the two light-absorbing components L and LH of the protonation equilibrium. 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 to the instrumental error $s_{inst}(A) \approx 0.45$ mAU. This is the common criterion for determining p. Very low values of $s_{\text{inst}}(A)$ prove that a reliable spectrophotometer and experimental techniques were used.

The dissociation constant and the two molar absorptivities of haemanthamine calculated for 48 wavelengths of 19 spectra constitute $(2 \times 48) + 1 = 97$ unknown regression parameters, which are estimated and refined by SQUAD(84) or SPECFIT32 in the first run. The reliability of the parameter estimates may be tested with the use of the 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: for haemanthamine $pK_a = 7.28 (s = 0.007)$ at $I = 0.50$ and 25°C and PALLAS(2004) predicts $pK_a = 6.94$ while MARVIN(2007) $pK_a = 7.37$. 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, all the variously protonated species are statistically significant at a significance level $\alpha = 0.05$. The physical meaning of the dissociation constant, molar absorptivities, and stoichiometric indices is examined. The absolute values of $s(\beta_j)$, $s(\varepsilon_j)$ give information about the last U-contour of the hyperparaboloid in the neighborhood of the pit, $U_{\min}$. For well-conditioned parameters, the last U-contour is a regular ellipsoid, and the standard deviations are reasonably low. High s values are found with ill-conditioned parameters and a 'saucer'-shaped pit. The relationship $s(\beta_j) \times F_\sigma < \beta_j$ should be met where F_σ is equal to 3 for a 99.9% statistical probability level. 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. Figure 2e shows the estimated molar absorptivities of all of the variously protonated species ε_L, ε_{LH}, of haemanthamine in dependence on wavelength.

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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 a percentage have physical meaning, which proved to be the case (Fig. 2f). A distribution diagram makes it easier to quickly judge the contributions of the individual species to the total concentration. Since the molar absorptivities will generally be in the range 10^3–$10^5 1 \cdot mol^{-1} \cdot cm^{-1}$, 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 the protonation equilibria of LH and L.

The next diagnostic concerns the goodness-of-fit (Fig. 2d). The goodness-of-fit achieved is easily seen by examination of the differences between the experimental and calculated values of absorbance, $e_i = A_{exp,\ i,\ j} - A_{calc,\ i,\ j}$. 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. It is usually compared with the standard deviation of absorbance calculated by the INDICES program,[19] $s_k(A)$, 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. This proves that the $s_2(A)$ value is equal to 0.45 mAU and is quite close to the standard deviation of absorbance when the minimization process terminates, $s(A) = 0.58$ mAU. Although this statistical analysis of the residuals[20,35] 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 eliptic hyperparaboloid U is reached: the residual standard deviation $s(e)$ always has sufficiently low values, lower than 1 mAU. 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, systematic departures from randomness being taken to indicate that either the chemical model or the parameter estimates were unsatisfactory.

Lisuride: Lisuride also exhibits very small changes in spectra (Fig. 1b and Fig. 3a) within the protonation of anion L. The adjustment of pH from 6.1–8.8 causes an absorbance change of 80 mAU at 228 nm only, making monitoring of the L and LH components rather difficult (Fig. 3c). The best region of the spectrum seems to be 216–358 nm and p$K_a = 7.86$($s = 0.012$) at $I = 0.01$ and 25°C, and PALLAS(2007) predicts p$K_a = 6.65$ while MARVIN(2004) predicts p$K_a = 7.47$. The curves of the molar absorption coefficients for the forms L and LH cross at the wavelengths 250 and 280 nm, forming two isosbestic points. Most of the selected methods of factor analysis by the INDICES algorithm lead to two light-

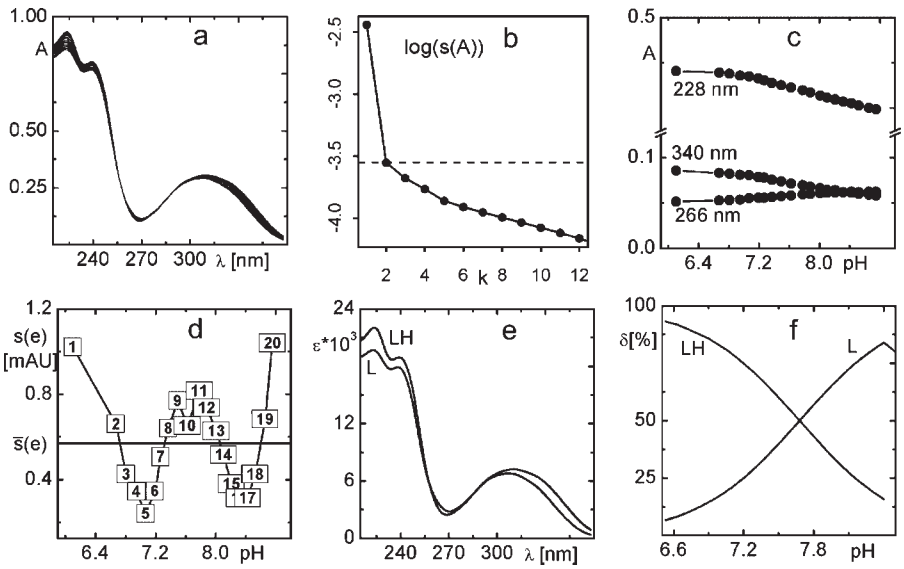

Figure 3. Non-linear regression analysis of the protonation equilibria model and factor analysis of lisuride: (a) pH-dependent absorption spectra at 25°C, (b) Cattel's scree plot of the Wernimont–Kankare procedure for determination of the number of light-absorbing species in mixture $k^* = 2$ leads to the actual instrumental error of the spectrophotometer used $s_{inst}(A) = 0.28$ mAU (INDICES in S-Plus); (c) the absorbance versus pH curves for 228, 266, and 340 nm in dependence on pH at 25°C, (d) detecting the influential outlying spectra with the use of the goodness-of-fit test and the plot of the residual standard deviation $s(e)$ versus pH for 20 spectra at 25°C, (e) pure spectra profiles of molar absorptivities versus wavelengths for the variously protonated species L, LH, (f) distribution diagram of the relative concentrations of all of the variously protonated species L, LH, of lisuride in dependence on pH at 25°C, (SPECFIT, ORIGIN)

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absorbing components in the equilibrium mixture (Fig. 3b). Even small changes in the spectra of the proposed chemical model of lisuride protonation led to small values of standard deviation of absorbance $s(A)$, these being mostly under 1 mAU. This goodness-of-fit (Fig. 3d) proves a sufficiently reliable estimates of the dissociation constant and molar absorption coefficient.

Losartan: A proposed strategy for efficient experimentation in dissociation constants determination followed by spectral data treatment in case of very small changes in spectra also is presented on the protonation equilibria of losartan (Fig. 1c and Fig. 4a). Losartan contains a complicated molecular structure, and two protonation equilibria can be monitored spectrophotometrically with close dissociation constants only. As all the variously protonated anions exhibit quite similar absorption bands, a part of the spectrum from 212 to 272 nm was selected as the most convenient for an estimation of the protonation constants. pH-spectrophotometric titration enables absorbance-response-surface data (Fig. 1c) to be obtained for analysis with non-linear regression, and the reliability of parameter estimates (pK's and ε's) can be evaluated on the basis of a goodness-of-fit test of the residuals. The A-pH curves at 228, 253, and 266 nm show that two protonation constants are indicated. The SPECFIT32 or SQUAD(84) program[7] analysis process starts with data smoothing followed by a factor analysis using the INDICES procedure[1,12]. The position of a break-point on the $s_k(A) = f(k)$ curve in the scree plot of the three most reliable approaches (Kankare's $s(A)$, *RSD* and *RSM* in References[1,12]) is calculated and gives $k^* = 3$ with the corresponding co-ordinate $\log s_3^*(A) = -3.90$, that is, $s_3^*(A) = 0.13$ mAU (Fig. 4b), which also represents the actual instrumental error $s_{\text{inst}}(A)$ of the spectrophotometer used. Due to the large variations in the indicator values, these latter are plotted on a logarithmic scale. 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 the index function $PC(k) = f(k)$ changes, or by comparing $PC(k)$ values with the instrumental error $s_{\text{inst}}(A)$. This is the common criterion for to determining p. Very low values of $s_{\text{inst}}(A)$ prove that a quite reliable spectrophotometer and experimental techniques were used. All index methods of the INDICES program 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 $(2 \times 39) + 1$ unknown regression parameters which are estimated and refined by 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, all the variously protonated species are statistically significant at a significance level $\alpha = 0.05$ and $\text{p}K_{\text{a},1} = 3.60 (s = 0.01)$, $\text{p}K_{\text{a},2} = 4.73 (s = 0.01)$ at

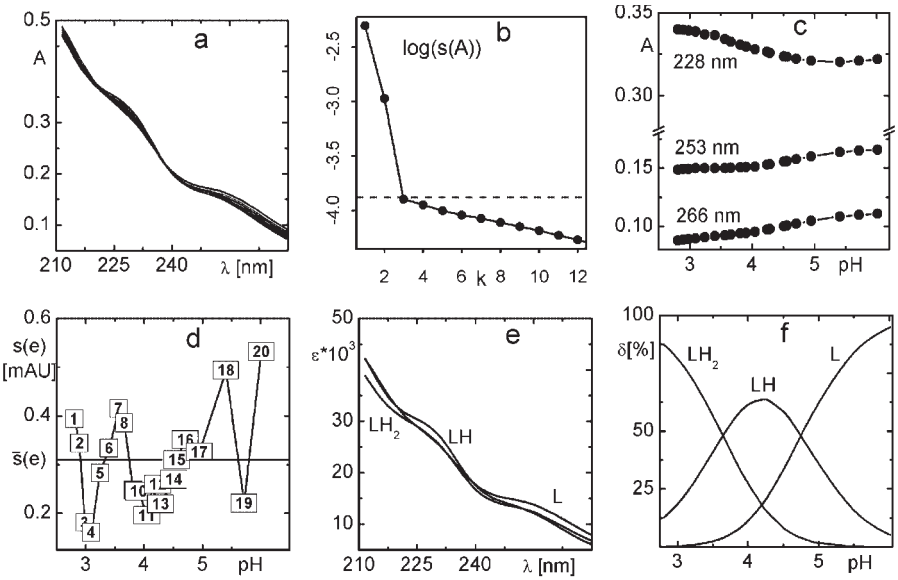

Figure 4. Non-linear regression analysis of the protonation equilibria model and factor analysis of losartan: (a) pH-dependent absorption spectra at 25°C, (b) Cattel's scree plot of the Wernimont–Kankare procedure for the determination of the number of light-absorbing species in the mixture $k^* = 3$ leads to the actual instrumental error of the spectrophotometer used $s_{\text{inst}}(A) = 0.13$ mAU (INDICES in S-Plus), (c) the absorbance versus pH curves for 228, 253, and 266 nm at 25°C, (d) detecting the influential outlying spectra with the use of the goodness-of-fit test and the plot of the residual standard deviation $s(e)$ versus pH for 20 spectra in dependence on pH at 25°C, (e) pure spectra profiles of molar absorptivities versus wavelengths for the variously protonated species L, LH, and LH_2, (f) distribution diagram of the relative concentrations of all three protonated species L, LH, and LH_2, of losartan in dependence on pH at 25°C, (SPECFIT, ORIGIN)

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$I = 0.01$ and 25°C and PALLAS(2004) predicts p$K_{a,\,1} = 4.25$ and p$K_{a,\,2} = 4.83$ while MARVIN(2004) p$K_{a,\,1} = 4.07$ and p$K_{a,\,2} = 5.04$. Figure 4e shows the estimated molar absorptivities of all of the variously protonated species ε_L, ε_{LH}, ε_{LH_2} 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 a percentage have physical meaning, which proved to be the case (Fig. 4f). A distribution diagram makes it easier to quickly judge the contributions of the individual species to the total concentration. The diagram shows that overlapping protonation equilibria of LH_2 with LH and L exist.

The next diagnostic concerns the goodness-of-fit (Fig. 4d). The goodness-of-fit achieved is easily seen by examination of the differences between the experimental and calculated values of absorbance, $e_i = A_{exp,\,i,\,j} - A_{calc,\,i,\,j}$. 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. This proves that the $s_3(A)$ value is equal to 0.14 mAU and is close to the standard deviation of absorbance when the minimization process terminates, $s(A) = 0.48$ mAU (SQUAD(84)) or 0.39 mAU (SPECFIT32). Although this statistical analysis of residuals[13] 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: the residual bias $\overline{e} = 2.00 \times 10^{-8}$ proves that there is no bias or systemic error in the spectra fitting. The residual standard deviation $s(e) = 0.48$ (SQUAD(84)) or 0.39 (SPECFIT32) mAU has a sufficiently low value. The skewness $g_1(e) = 0.15$ is quite close to zero and proves a symmetric distribution of the residuals set, while the kurtosis $g_2(e) = 5.00$ proves a non-Gaussian distribution.

To express small changes of absorbance in the spectral set, the absorbance differences for the jth wavelength of the ith spectrum $\Delta_i = A_{ij} - A_{i,\,acid}$ were calculated so that from the absorbance value of the spectrum measured at the actual pH the absorbance value of the acidic form was subtracted. The absorbance difference Δ_i was then divided by the actual instrumental standard deviation $s_{inst}(A)$ of the spectrophotometer used, and the resulting value represents the *signal-to-error* value *SER*. Figure 5 is a graph of the *SER* in dependence on wavelength in the measured range for all three drugs. When the *SER* is larger than 10, a factor analysis is able to predict the correct number of light-absorbing components in the equilibrium mixture.

To prove that non-linear regression can analyze such data the residuals set was compared with the instrumental noise $s_{inst}(A)$. If the ratio $e/s_{inst}(A)$ is of similar magnitude, that is, nearly equal to one, it means that sufficient curve fitting was achieved by the non-linear regression of the spectra set and that the minimization process found the minimum of the residual-square-sum function U_{min}. Figure 6 shows a comparison of the ratio $e/s_{inst}(A)$ in dependence on wavelength for all three drugs measured. From the figure it is obvious that most of the residuals are of the same magnitude as the instrumental noise.

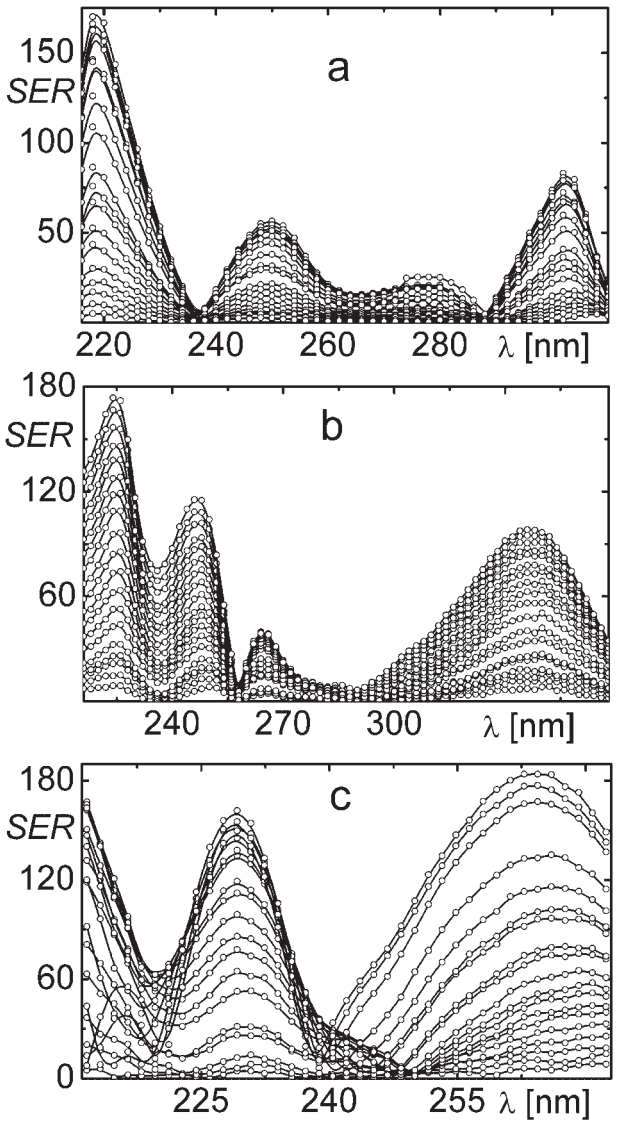

Figure 5. Plot of small absorbance changes in the spectrum. The value of the absorbance difference for the jth-wavelength of the ith-spectrum $\Delta_{ij} = A_{ij} - A_{i,acid}$ is divided by the instrumental standard deviation $s_{inst}(A)$, and the resulting ratios $SER = \Delta/s_{inst}(A)$ are plotted in dependence of wavelength λ for all absorbance matrix elements, where $A_{i,\,acid}$ is the limiting spectrum of the acid form of the drug. This ratio is compared with the limiting *SER* value for (a) haemanthamine, (b) lisuride, and (c) losartan to test if the absorbance changes are significantly larger than the instrumental noise.

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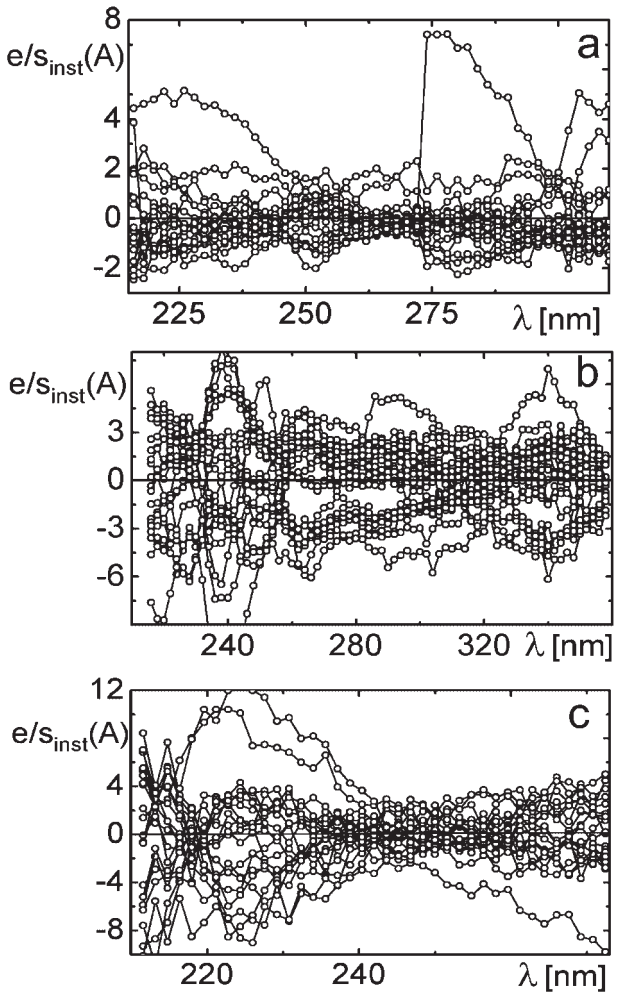

Figure 6. Plot of the ratio $e/s_{inst}(A)$, that is, the ratio of the residuals divided by the instrumental standard deviation $s_{inst}(A)$ versus the wavelength λ for all the residual matrix elements for (a) haemanthamine, (b) lisuride, and (c) losartan tests if the residuals are of the same magnitude as the instrumental noise

CONCLUSIONS

When drugs are very poorly soluble, pH-spectrophotometric titration may be used with the non-linear regression of the absorbance-response-surface data instead of performing a potentiometric determination of the dissociation constants. The reliability of the dissociation constants of the three drugs (haemanthamine, lisuride and losartan) may be proven with goodness-of-fit tests of the absorption spectra measured at various pH. The dissociation constant pK_a was estimated by non-linear regression of $\{pK_a, I\}$ data at 25°C: for haemanthamine $pK_a = 7.28(1)$ at $I = 0.50$, for lisuride $pK_a = 7.86(1)$ and for losartan $pK_{a,1} = 3.60(1)$, $pK_{a,2} = 4.73(1)$ at $I = 0.01$. Goodness-of-fit tests for the various regression diagnostics enabled the reliability of the parameter estimates to be determined. Most indices always predict the correct number of components and even the presence of a minor one when the *signal-to-error ratio SER* is higher than 10. The Wernimont–Kankare procedure in INDICES performs a reliable determination of the instrumental standard deviation of spectrophotometer used $s_{inst}(A)$, correctly predicts the number of light-absorbing components present and can also solve an ill-defined problem with severe collinearity in the spectra or very small changes in spectra.

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