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Cent. Eur. J. Chem. • 10(2) • 2012 • 338-353
DOI: 10.2478/s11532-011-0150-3

Central European Journal of **Chemistry**

Thermodynamic dissociation constants of risedronate using spectrophotometric and potentiometric pH-titration

Research Article

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Received 7 September 2011; Accepted 15 November 2011

Abstract: Risedronate inhibits bone resorption in diseases like osteoporosis, Paget´s disease, tumor bone diseases or the malfunction of phosphocalcium metabolism. The acid-base properties of risedronate in an aqueous solution have been studied in a pH range from 2 to 12 and can be described in terms of four dissociation steps: $pK_{a,2}$, $pK_{a,4}$, $pK_{a,5}$ (related to the dissociation of POH groups) and $pK_{a,3}$ related to the dissociation of protonated amino group NH_3^+. The mixed dissociation constants were determined at different ionic strengths I = 0.02 to 0.20 mol dm^{-3} KCl and of 25ºC and 37ºC using pH-spectrophotometric and pH-potentiometric titration methods. Determination of *group parameters* L_0, H_T might lead to false estimates of *common parameters* $\mathbf{pK_a}$;therefore, the computational strategy employed is important. A comparison between the two programs ESAB and HYPERQUAD demonstrated that the ESAB program provides a better fit of potentiometric titration curve. The thermodynamic dissociation constants pK^T_a were estimated by a nonlinear regression of (pK_a, I) data and a Debye-Hückel equation at 25ºC and 37ºC, $pK^T_{a,2}$ = 2.37(1) and 2.44(1), $pK^T_{a,3}$ = 6.29(3) and 6.26(1), $pK^T_{a,4}$ = 7.48(1) and 7.46(2) and $pK^T_{a,5}$ = 9.31(7) and 8.70(3) at 25ºC and 37ºC using pH-spectroscopic data and $pK^T_{a,2}$ = 2.48(3) and 2.43(1), $pK^T_{a,3}$ = 6.12(2) and 6.10(2), $pK^T_{a,4}$ = 7.25(2) and 7.23(1) and $pK^T_{a,5}$ = 12.04(5) and 11.81(2) at 25ºC and 37ºC. The ascertained estimates of three dissociation constants $pK_{a,3}$, $pK_{a,4}$, $pK_{a,5}$ are in agreement with the predicted values obtained using PALLAS.

Keywords: *Spectrophotometric titration • Potentiometric titration • Dissociation constant • Risedronate*

1. Introduction

Risedronate sodium, a chemically (1-hydroxy-2-(3-pyridinyl) ethylidene) bisphosphonic acid monosodium salt (Fig. 1), is a member of the drugs called nitrogen-containing bisphosphonates (N-BPs). These N-BPs have been developed over the past 30 years and are used in medicine [1-3]. A number of clinical studies were published demonstrating the importance of risedronate in osteoporosis therapy in the latter half of the 1990s [4-6]. The main effect of N-BPs is an inhibition of bone resorption. They may also play a role in supporting bone formation in some way [7-9]. N-BPs are synthetic substances which do not occur naturally in humans, and enzymes capable of decomposing a P-C-P binding are not known. Because of this, N-BP can be absorbed, stored and excreted, but not metabolized. Risedronate sodium belongs to the 3rd generation of N-BPs whose inhibition potential is ten times greater than the 2nd generation (of which a typical representative is alendronate sodium) [10], has a rapid and steady effect and demonstrably improves a bone's micro-architecture [11-13]. Risendronate rapidly reduces the risk of fractures [14-15], effects to all skeleton parts, including a femur cervix [16], and plays a role in preventing and treating corticosteroid-induced osteoporosis [17-19]. BPs are characterized by low bioavailability, typically 1% or less of the dose is absorbed. Risedronate has been determined in human urine using GC-MS following acylation and silylation to form volatile derivates [20] or using very

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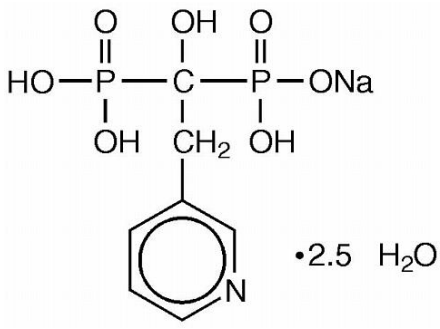

Figure 1. The structure of risedronate sodium.

sensitive enzyme-linked immunosorbent assay (ELISA) [21]. Vallano *et al.* [22] used column-switching ion-pair HPLC with UV detection to determine risedronate in human urine whereas ion-pair HPLC on BDS C_{18} analytical column is better to determine risedronate in bulk material due to its short analytical time [23].

Risedronate can dissociate: five H^+ donors (four POH groups, one gemine OH^- group) and one amino group as the H^+ acceptor. The algebraic description of a titration neglecting activities is completely specified by five ionization steps $K_{a,1}$, $K_{a,2}$, $K_{a,3}$, $K_{a,4}$, and $K_{a,5}$, and the equation for ionization of water K_w. Thus, the important dissociation constants in the physiological pH range are $pK_{a,3}$ and $pK_{a,4}$ and the dissociation constants associated with the loss of the third and fourth protons from the pentaprotic acid. These constants result from ionization, in unknown order, of the third P-OH and the N-H function explained by Hounslow *et al.* [24]. In order to determine dissociation constants for the polyprotic system in which the dissociation steps are well separated ($\Delta pK_a = |pK_{a,i+1} - pK_{a,i}| \geq 3$) the following is possible: if the dissociation steps are close and overlapping, the $pK_{a,i}$ values describe the stoichiometry but not the site of protonation as there are intermediate species that have the same number of protons but at different protonation sites.

In the present study, the potentiometric titration was also used due to the good water solubility of risedronate, and dissociation constants are compared with those by spectrophotometric titration.

2. Theoretical procedure

2.1. Determination of protonation model

An acid-base equilibrium of the studied drug is described in terms of the protonation of the Brönstedt base L^{z-1} according to the equation $L^{z-1} + H^+ \rightleftarrows HL^z$. 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, HL, H_2L, and H_3L ...*etc.*, which have the general formula H_rL in a particular chemical model and which are represented by the number of species, n_c, defined as r_i, $i = 1, ..., n_c$ where index i indicates their particular stoichiometry; the overall protonation (stability) constant of the protonated species, β_r, may then be expressed as

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

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

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

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

$$A_{i,j} = \sum_{n=1}^{n_c} \varepsilon_{j,n}\, c_n = \sum_{n=1}^{n_c} (\varepsilon_{r,j}\, b_r\, l\, h^r)_n \quad (3)$$

where $\varepsilon_{r,j}$ is the molar absorptivity of the H_rL species with the stoichiometric coefficient r measured at the jth wavelength. The absorbance $A_{i,j}$ is an element of the absorbance matrix $\boldsymbol{A}$ 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 [25,26].

2.2. Computation

In order to determine the dissociation constant of risedronate, several programs were used: SQUAD(84) [27,28] and SPECFIT/32 [29-31] for spectrophotometric pH titration data, ESAB2M and HYPERQUAD2008 for potentiometric titration data, and this procedure was previously published [32].

2.3. Signal-to-noise ratio SER in spectra

Direct results from experimental and instrumental operations in a laboratory are always approximate due mainly to the limited accuracy and precision of measuring instruments. The level of "experimental noise" should be used in the experiment as a critical factor. Therefore, it is necessary to have a consistent definition of the *signal-to-noise ratio SNR* so that the impact of this characteristic 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 the instrumental standard deviation of absorbance $s_{inst}(A)$ is used for an error. The plot of small absorbance changes in the spectrum with dissociation of the drug studied means that the value of the absorbance difference for the *j*th-wavelength of the *i*th-spectrum $\Delta_{ij} = A_{ij} - A_{i,acid}$ is divided by the instrumental standard deviation $s_{inst}(A)$, and the resulting ratios $SER = \Delta/s_{inst}(A)$ are plotted depending on wavelength λ for all absorbance matrix elements, where $A_{i,acid}$ is the initial spectrum of the acid form of the drug being measured for the starting pH value of the pH range studied. This *SER* ratio is then compared with the limiting *SER* value to test if the absorbance changes are significantly larger than the instrumental noise. The plot of the ratio $e/s_{inst}(A)$, *i.e.*, the ratio of the residuals divided by the instrumental standard deviation $s_{inst}(A)$ depending on wavelength λ for all the residual matrix elements, is for testing to ascertain if the residuals are of the same or similar magnitude as the instrumental noise to prove the best curve fitting achieved.

2.4. Determination of the thermodynamic protonation/dissociation constant

Let us consider the dependence of the mixed dissociation constant K_a on the ionic strength, when both ions HL^z and L^{z-1} have roughly the same ion-size parameter *å* in the dissociation equilibrium $HL^z \rightleftarrows L^{z-1} + H^+$ with the thermodynamic dissociation constant K_a^T and suppose that the overall salting-out coefficient is given by $C = C_{HL} - C_L$. This dependence is expressed by the Debye-Hückel equation

$$\mathrm{p}K_a = \mathrm{p}K_a^T - \frac{A\,(1-2\,z)\sqrt{I}}{1+B\,å\,\sqrt{I}} + C\,I \qquad (4)$$

where A = 0.5112 $mol^{-1/2}$ $L^{1/2}$ $K^{3/2}$ and B = 0.3291 $mol^{-1/2}$ m^{-1} $L^{1/2}$ $K^{1/2}$ 10^{10} for aqueous solutions at 25°C. The mixed dissociation constant pK_a represents a dependent variable while the ionic strength *I* stands for the independent variable. Three unknown parameters pK_a^T, *å*, *C* are to be estimated by minimising the sum of the squared residuals [33]

$$U(\boldsymbol{b}) = \sum_{i=1}^{n} w_i \left[\mathrm{p}K_{a,\exp,i} - \mathrm{p}K_{a,\mathrm{calc},i}\right]^2 = \\ = \sum_{i=1}^{n} w_i \left[\mathrm{p}K_{a,\exp,i} - f(I;\mathrm{p}K_a^T, å, C)\right]^2 = \mathrm{minimum} \qquad (5)$$

The nonlinear estimation problem 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^T, *å*, and *C* are unknown variables to be estimated. However, for small values of an ionic strength the pK_a^T can only be estimated.

3. Experimental procedure

3.1. Chemicals and solutions

Risedronate sodium (96%) was donated by ZENTIVA k.s. with declared purity confirmed by HPLC. *Hydrochloric acid,* 1 mol dm^{-3}, was prepared by diluting a concentrated HCl (p. a., Lachema Brno) with redistilled water and standardization against HgO and KI with a reproducibility of better than 0.2% according to the equation $HgO + 4\,KI + H_2O \leftrightarrow 2\,KOH + K_2[HgI_4]$ and $KOH + HCl \leftrightarrow KCl + H_2O$. *Potassium hydroxide,* 1 mol dm^{-3}, was prepared from the exact weight of pellets p. a., Aldrich Chemical Company with carbon-dioxide-free redistilled water kept for 50 minutes before that in an ultrasonic bath. The solution was stored for several days in a polyethylene bottle in an argon atmosphere. This solution was standardized against a solution of potassium hydrogen-phthalate using the derivative method with a reproducibility of 0.1%. *Mercury oxide, potassium iodide* and *potassium chloride,* p. a. Lachema Brno were not additionally purified. *Twice-redistilled water* kept for 50 minutes prior to that in an ultrasonic bath was used in the preparation of solutions.

3.2. pH-spectrophotometric titration

The apparatus used and the pH-spectrophotometric titration procedure have been described previously in detail [25,26,34]. The combined experimental and computational scheme used to determine the protonation constants of the multi-component system is taken from Meloun *et al.*, *cf.* page 226 in [35] and the five steps are described in detail elsewhere [25,26].

3.3. pH-potentiometric titration

The apparatus used and the pH-potentiometric titration procedure have been described previously in detail [32].

3.4. Computation and software

Computations relating to the determination of dissociation constants were performed by regression analysis of the UV/VIS spectra using the SQUAD(84) [27,28] and SPECFIT/32 [36] programs. Computations relating to the determination of the dissociation constants were performed by regression analysis of the titration curve using the ESAB program, version ESAB2M [37-39] and HYPERQUAD2008 program [39]. Most graphs were plotted using ORIGIN [40] and S-Plus [41]. The thermodynamic dissociation constant pK_a^T was estimated with the MINOPT nonlinear regression program in the ADSTAT statistical system (TriloByte Statistical Software, Ltd., Czech Republic), [42]. PALLAS

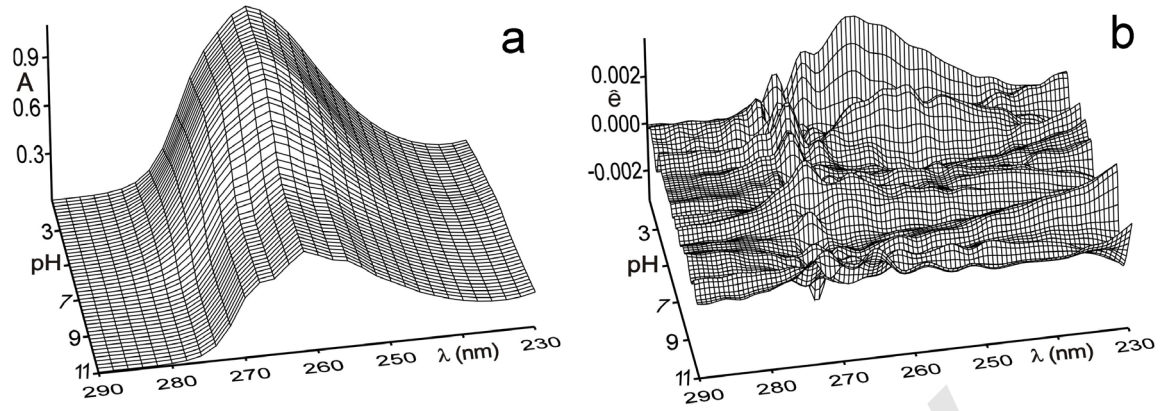

Figure 2. (a) The 3D-absorbance-response-surface representing the measured multiwavelength absorption spectra for risedronate depending on pH at 25°C, (b) the 3D-residuals map after a non-linear regression performed with SPECFIT/32 and SQUAD(84) programs, (S-Plus).

[43] is a program for making predictions based on the structural formulae of drug compounds. Entering the compound topological structure descriptors graphically, pK_a values of organic compounds are predicted using approximately hundreds of Hammett and Taft equations and quantum chemistry calculations.

3.5. 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), SPECFIT/32, ESAB2M and HYPERQUAD are available free of charge on line at http://meloun.upce.cz and in the menu DOWNLOAD and block DATA.

4. Results and discussion

4.1. The pH-spectrophotometric titration

The deprotonation of risedronate indicates five equilibria. The pH-spectrophotometric titration enables absorbance-response data (Figs. 2a and 3a) to be obtained for analysis by non-linear regression using the SPECFIT and SQUAD(84) programs. The reliability of estimated parameters (pK and ε´s) can be evaluated on the basis of the goodness-of-fit test of residuals (Fig. 2b).

Moreover, the A-pH curves at 238, 250, 264 and 274 nm (Fig. 3c) also show that the dissociation constants of risedronate may be indicated spectrophotometrically and that these wavelengths indicate individual protonation equilibria. Most wavelengths from 250 to 274 nm indicate dissociation equilibria of four ionic forms LH_4, LH_3, LH_2, and LH (the charges are here omitted for the sake of simplicity) containing a pyridinium ring between pH 4 and 9 as the pyridinium ring in risedronate has a larger molar absorptivity than pyridine ring at 262 nm. The changes in the spectra (Figs. 3a, 3d) are small within a deprotonation; in fact, both of the variously protonated species LH_2 and LH_3 exhibit similar absorption bands.

The adjustment of the pH value from 2 to 12 causes the absorbance to change by 0.6 of the A-pH curve so that the monitoring of five components L, LH, LH_2, LH_3 and LH_4 of the protonation equilibrium can be sometimes uncertain and a very precise measurement of absorbance is necessary for a reliable detection of the deprotonation equilibrium studied.

In the first step of the regression spectra analysis, the number of light-absorbing species was estimated using the INDICES algorithm [44] (Fig. 3b). The number of light-absorbing species k can be predicted from the index function values by finding the point $k^* = k$ where the slope of index function $PC(k) = f(k)$ changes, or by comparing $PC(k)$ values to the instrumental error $s_{inst}(A)$. This is the common criterion for determining the rank of absorbance matrix k^*. The very low value of $s_{inst}(A)$ on Fig. 3b proves that a sufficiently precise spectrophotometer and efficient experimental technique were used. The position of the break point on the $s_k(A) = f(k)$ curve in the Cattell´s scree plot is calculated and gives the rank value $k^* = 5$ with the corresponding co-ordinate $s_5(A) = 0.18$ mAU which may also be taken as the actual instrumental error $s_{inst}(A)$ of the spectrophotometer used.

The dissociation constants and the five molar absorptivities of risedronate ε_L, ε_{LH}, ε_{LH2}, ε_{LH3} and ε_{LH4} calculated for 39 wavelengths of 36 spectra (Figs. 2a, 3a) constitute (5×39) + 4 = 199 unknown regression parameters, which are estimated and refined with SQUAD(84) or SPECFIT32 programs in the first run. The reliability of the parameter estimates may be tested with the use of the following diagnostics:

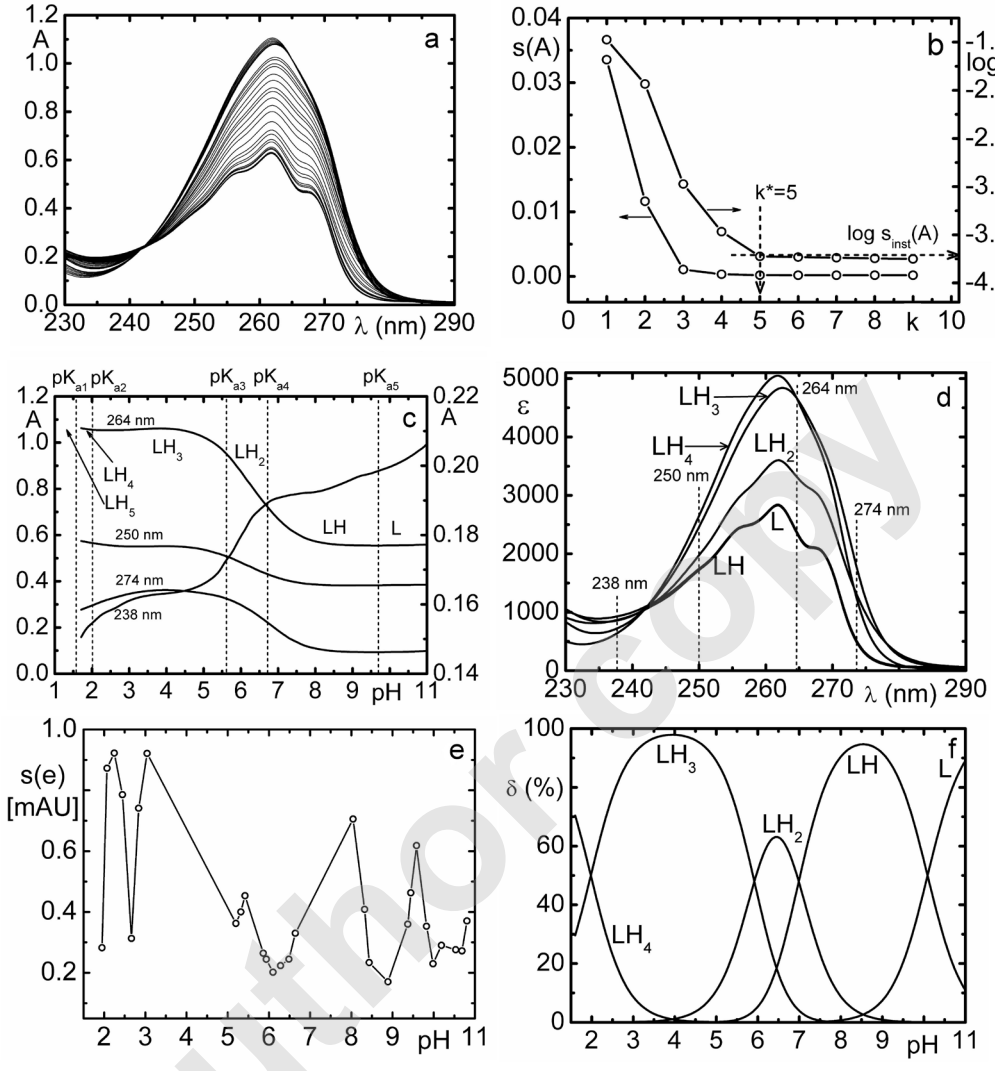

Figure 3. (a) Absorption spectra of 2×10^{-4} mol dm^{-3} of risedronate depending on pH at 25°C, (b) Cattell's scree plot of the Wernimont-Kankare procedure for the determination of the rank of the absorbance matrix $k^* = 5$ leads to the number of light-absorbing species in the mixture $n_c = 5$ and the actual instrumental error of the used spectrophotometer $s_{inst}(A) = 0.18$ mAU (INDICES in S-Plus), (c) The absorbance *vs.* pH curves for 238 nm, 274 nm, 250 nm and 264 nm in dependence on pH at 25ºC, (d) Pure spectra profiles of molar absorptivities vs. wavelengths for the variously protonated species L, HL, HL_2, LH_3 and LH_4 (e) Detecting influential outlying spectra with the use of the goodness-of-fit test and the plot of the residual standard deviation $s(\hat{e})$ *vs*. pH for 29 spectra depending on pH at 25ºC, (f) Distribution diagram of the relative concentrations of all variously protonated species L, HL, HL_2, LH_3 and LH_4 of risedronate depending on pH at 25ºC, (SPECFIT, SPLUS, ORIGIN). The charges of species are omitted for the sake of simplicity.

The *first diagnostic* value indicates whether all of the parametric estimates pK_{a2}, pK_{a3}, pK_{a4}, pK_{a5} and ε_L, ε_{LH}, ε_{LH2}, ε_{LH3} and ε_{LH4} have physical meaning and obtain realistic values: for risedronate $pK_{a,2}$ = 2.00(5), $pK_{a,3}$ = 5.99(1), $pK_{a,4}$ = 7.07(1) and $pK_{a,5}$ = 9.86(11) at 25˚C. The standard deviations of the parameters estimated are given in parentheses above, and pertain to the last valid digits of the parameters. As the standard deviations $s(pK_{a,i})$ of parameters $pK_{a,i}$ and $s(\varepsilon_i)$ of parameters ε_i are significantly smaller than their corresponding parameter estimates, all the variously protonated species are statistically significant at a significance level α = 0.05. The physical meaning of the dissociation constants $pK_{a,i}$, and molar absorptivities ε_i is so examined.

The regression spectra analysis begins with the determination of a chemical model (Table 1). The goodness-of-fit test proved that the L, LH, LH_2, LH_3 and LH_4 species participate in the dissociation. The 1st hypothesis of the protonation model L, LH, LH_2 is rejected, as a poor fit was achieved. The absolute values of $s(pK_{a,j})$, $s(\varepsilon_j)$ give information about the last *U*-contour of the hyperparaboloid in the neighbourhood of the pit, U_{min}. For well-conditioned parameters, the last *U*-contour is a regular ellipsoid, and the standard deviations are reasonably low. High *s* values are found

Table 1. The search of the best chemical model of dissociation of risedronate at I = 0.025 when five various hypotheses were tested. The charges were omitted for the sake of simplicity. Standard deviations of parameter estimated in last valid digits are in parentheses.

	Hypothesis of protonation model of L_qH_r with his dissociation constants pK_{qr}			
q, r	$pK_{q,r}$	$pK_{q,r}$	$pK_{q,r}$	$pK_{q,r}$
1, 0	-	-	-	-
1, 1	6.459(5)	5.819(26)	2.156(28)	2.117(21)
1, 2	-	6.916(11)	5.977(12)	5.990(18)
1, 3	-	-	7.077(6)	7.106(18)
1,4	-	-	-	9.573(18)
***s(A)* [mAU]**	10.37	4.86	1.74	0.70
$s_k(A)$ [mAU]	8.05,2	1.01,3	0.40,4	0.23,5
$\lvert\bar{e}\rvert$ [mAU]	6.83	2.52	0.98	0.46
$g_1(e)$	-0.06	1.06	0.07	0.22
$g_2(e)$	4.56	16.49	12.94	4.08
R-faktor [%]	2.43	1.12	0.39	0.16
Hypothesis is	Rejected	Rejected	Rejected	Accepted

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. As the four protonation models in the chemical model search summarized in Table 1 (1st model: L, LH, 2nd model: L, LH, LH_2, 3rd model: L, LH, LH_2, and LH_3, 4th model: L, LH, LH_2, LH_3 and LH_4) were tested, it may be concluded that regression spectra analysis can distinguish among these four models on the basis of a very close spectra fitting, and the model with ionic forms L, LH, LH_2, LH_3 and LH_4 was proven.

The *second diagnostic* tests determine whether all of the calculated free concentrations of the five variously protonated species on the distribution diagram of the relative concentration expressed as a percentage have physical meaning, which proved to be the case. The calculated free concentration of the basic components and variously protonated species of the protonation equilibria model should show molarities down to about 10^{-8} mol dm^{-3}. Expressed in percentage terms, a species present at about 1% relative concentration or less in the equilibrium behaves as numerical noise in a regression analysis. A distribution diagram of relative concentration shows the protonation equilibria of L, LH, LH_2, LH_3 and LH_4 depending on pH in Fig. 3f and 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 *third diagnostic* concerns the goodness-of-fit. The goodness-of-fit achieved is easily seen by examination of the differences between the experimental and calculated values of absorbance, $e_i = A_{exp,\ i,\ j} - A_{calc,\ i,\ j}$ in Fig. 3e. Examination of the spectra and of the graph of the predicted absorbance response-surface through all the experimental points (Fig. 2b) should reveal whether the results calculated are consistent and whether any gross experimental errors have been made in the measurement of the spectra. One of the most important statistics calculated is the standard deviation of absorbance, $s(A)$, calculated from a set of refined parameters at the termination of the minimization process. This is usually compared to the standard deviation of absorbance calculated by the INDICES program [44], $s_k(A)$, and if $s(A) \leq s_k(A)$, or $s(A) \leq s_{inst}(A)$ of the instrumental error of the spectrophotometer used, the fit is considered to be statistically acceptable (Table 1). Although this statistical analysis of residuals 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 with SQUAD(84): the residual standard deviation $s(e)$ = 0.70 mAU always has sufficiently low values. The graphical presentation of the residuals in Fig. 2b and

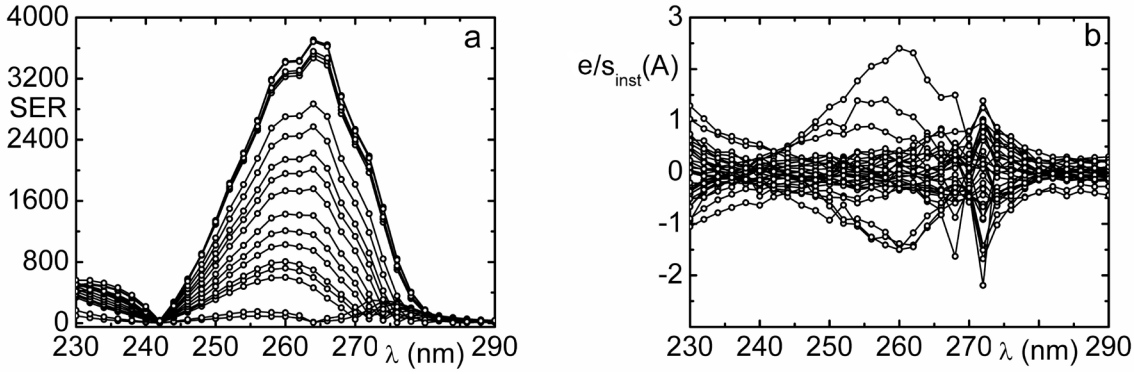

Figure 4. (a) The plot of small absorbance changes in the spectrum of risedronate means that the value of the absorbance difference for the *j*th-wavelength of the *i*th-spectrum $\Delta_{ij} = A_{ij} - A_{i,acid}$ is divided by the instrumental standard deviation $s_{inst}(A)$, and the resulting ratios $SER = \Delta/s_{inst}(A)$ are plotted depending on wavelength λ for all absorbance matrix elements, where $A_{i,acid}$ is the limiting spectrum of the acid form of the drug measured. This ratio is compared with the limiting *SER* value for the risedronate to test if the absorbance changes are significantly larger than the instrumental noise. (b) The plot of the ratio $e/s_{inst}(A)$, *i.e.*, the ratio of the residuals divided by the instrumental standard deviation $s_{inst}(A)$ depending on wavelength λ for all the residual matrix elements for risedronate, tests if the residuals are of the same magnitude as the instrumental noise, (SPECFIT/32, ORIGIN).

Fig. 3e assists the detection of an outlier spectrum point, a trend in the spectrum residuals, or an abrupt shift of level in the spectra. The statistical measures of all the residuals from Fig. 2b prove that the minimum of the elliptic hyperparaboloid is reached: the mean residual $|\overline{e}|$ = 0.46 mAU and the residual standard deviation $s(e)$ = 0.70 mAU have sufficiently low values. The skewness $g_1(e)$ = 0.22 is close to zero and proves a symmetric Gaussian distribution of the residuals set, while the kurtosis $g_2(e)$ = 4.08 is close to 3 proving a Gaussian distribution. The Hamilton *R-factor* of relative fit is 0.16% calculated with SQUAD(84) only, proving that an excellent fit is achieved, and the parameter estimates may therefore be considered reliable. If the Hamilton *R-factor* of relative fit, expressed as a percentage is < 0.5% the fit is excellent, but if it is > 2% the fit is poor. The criteria of resolution used for the hypotheses were: (1) a failure of the minimization process in a divergence or a cyclization; (2) an examination of the physical meaning of the estimated parameters to ensure that they were both realistic and positive; and (3) the residuals should be randomly distributed about the predicted regression spectrum, and systematic departures from randomness were taken to indicate that either the chemical model or the parameter estimates were unsatisfactory.

The fourth diagnostics: to express and analyze small changes of absorbance in the spectral set, the absorbance differences for the *j*-th wavelength of the *i*-th spectrum $\Delta_i = A_{ij} - A_{i,acid}$ were calculated so that the absorbance value of the acidic form was subtracted from the absorbance value of the spectrum measured at the actual pH. 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*. Fig. 4a shows a graph of the *SER* depending on wavelength in the measured range for the drug used. When the *SER* is larger than 10, it was proven [26] that a factor analysis is sufficiently able to predict the correct number of light-absorbing components in the equilibrium mixture. In order to prove that non-linear regression can also analyze such data of small absorbance changes, the residuals set was compared with the instrumental noise $s_{inst}(A)$. If the ratio $e/s_{inst}(A)$ is of a similar magnitude, *i.e.*, 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}. Fig. 4b shows a comparison of the ratio $e/s_{inst}(A)$ depending on the wavelength for risedronate measured. From the figure it is obvious that most of the residuals are of the same magnitude as the instrumental noise and thus indicates that changes of absorbance are 500 or 1,000 times larger and therefore sufficient reliability of the regression process is proven.

The fifth diagnostics: the spectra deconvolution in Fig. 5 shows the deconvolution of the experimental spectrum of the individual variously protonated species to examine whether the experimental design is efficient. If, for a particular pH range, the spectrum consists of just a single component, further spectra for that range would be redundant, although they could improve the precision. In pH ranges where more components contribute significantly to the spectrum, several spectra should be measured. Such a spectrum provides sufficient information for a regression analysis which monitors at least two species in equilibrium where none of them is a minor species. The minor species has a relative concentration in a distribution diagram of less than 5% of the total concentration of the basic component c_L.

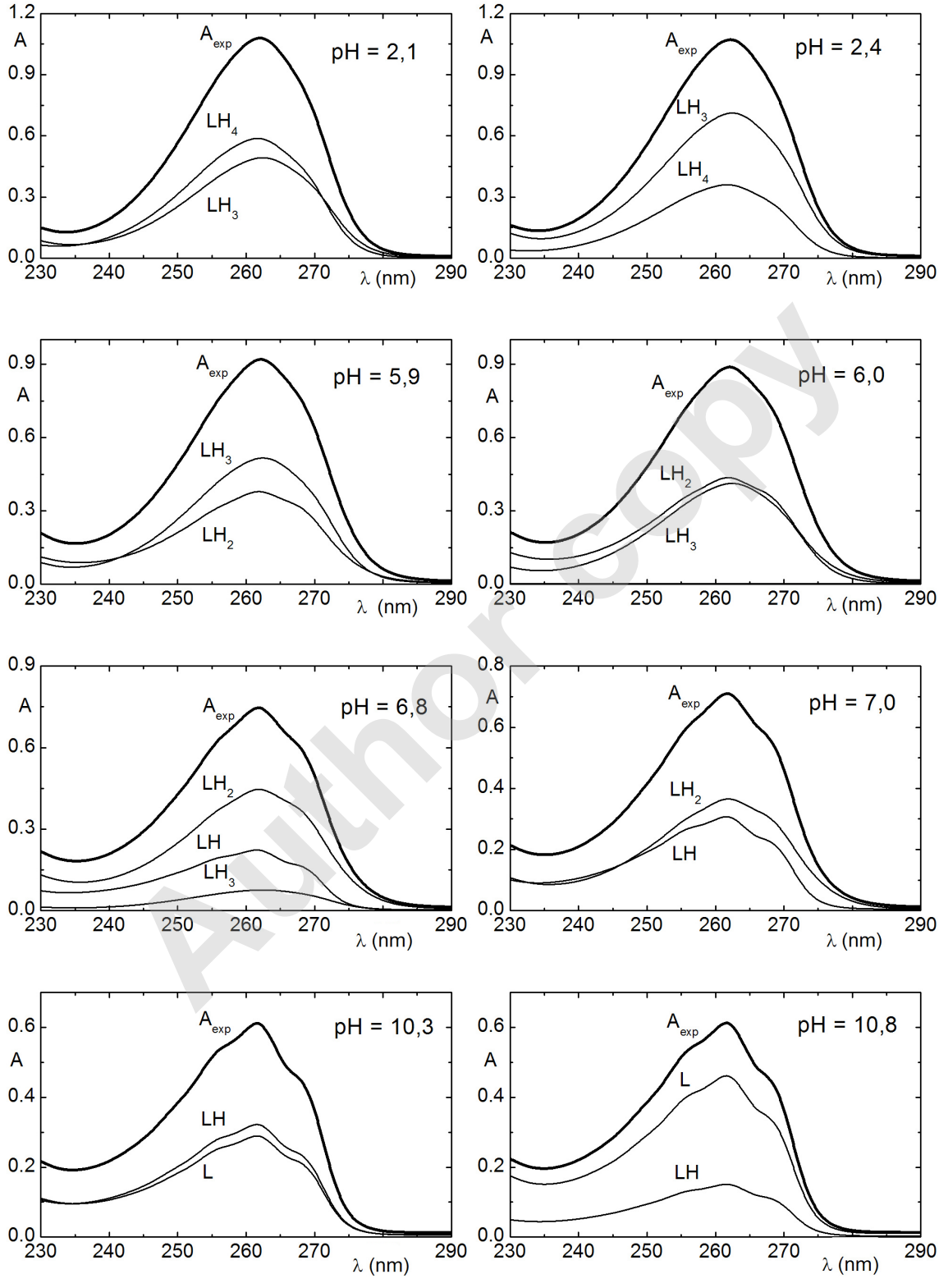

Figure 5. Deconvolution of the experimental absorption spectrum of risedronate for 39 wavelengths into spectra of the individual variously protonated species L, LH, LH_2, LH_3 and LH_4 in solution of each particular absorption spectrum for a selected value of pH equal 2.1, 2.4, 5.9, 6.0, 6.8, 7.0, 10.3, 10.8. The charges of species are omitted for the sake of simplicity (SQUAD, ORIGIN).

Table 2. Mixed dissociation constants $pK_{a,i}$ of risedronate at 25°C and various values of an ionic strength *I* estimated by nonlinear regression programs SPECFIT and SQUAD. Standard deviations of parameter estimates in last valid digits are in parentheses.

				25°C			
	Ionic strength	**0.058**	**0.068**	**0.075**	**0.089**	**0.099**	**0.110**
SPECFIT	$pK_{a,2}$	2.001(53)	1.966(54)	1.955(87)	1.927(72)	1.886(66)	1.859(86)
	$s(A)$[mAU]	0.56	0.97	0.66	0.85	0.77	0.68
	$pK_{a,3}$	5.996(13)	5.980(12)	5.979(14)	5.905(8)	5.922(11)	5.918(11)
	$pK_{a,4}$	7.073(12)	7.061(13)	7.018(14)	6.972(8)	6.970(12)	6.954(10)
	$s(A)$[mAU]	0.41	0.44	0.39	0.31	0.51	0.3
	$pK_{a,5}$	9.860(115)	9.720(94)	10.310(121)	10.560(103)	10.640(115)	10.810(41)
	$s(A)$[mAU]	0.30	0.22	0.41	0.37	0.27	0.19
SQUAD	$pK_{a,2}$	2.065(14.3)	2.016(11)	2.015(25)	1.992(18)	1.875(20)	1.897(17)
	$s(A)$[mAU]	0.73	0.94	0.88	1.01	1.08	0.64
	$pK_{a,3}$	6.001(7)	5.979(6)	5.98(8)	5.906(5)	5.917(7)	5.917(6)
	$pK_{a,4}$	7.078(4)	7.061(3)	7.017(4)	6.973(2)	6.963(4)	6.964(3)
	$s(A)$[mAU]	0.45	0.40	0.40	0.32	0.49	0.31
	$pK_{a,5}$	9.882(32)	9.719(35)	10.360(29)	10.870(40)	11.294(88)	11.099(55)
	$s(A)$[mAU]	0.46	0.45	0.53	0.57	0.57	0.29

When, on the other hand, only one species prevails in the solution, the spectrum yields quite poor information for a regression analysis and the parameter estimate is rather unsure and definitely not reliable enough. Spectrum deconvolution seems to be a quite useful tool in the proposal of an efficient experimentation strategy.

Applying a Debye-Hückel equation to the data in Table 2 and Table 3 according to the regression criterion, the unknown parameter pK_a^T has been estimated. Table 6 shows point estimates of the thermodynamic dissociation constants of risedronate studied at two temperatures. Because of the small range of ionic strength, the ion-size parameter *å* and the salting-out coefficient *C* could not be estimated here.

4.2. pH-potentiometric titration

Risedronate is soluble enough in water to use a potentiometry as a second independent instrumental method to determine the dissociation constants. For the adjusted value of an ionic strength the potentiometric titration of a mixture of HCl and risedronate with potassium hydroxide was carried out. The initial tentative value of the dissociation constants of the studied drug, corresponding to the midpoint value in each plateau of the potentiometric titration curve (Fig. 6a), was refined by the ESAB and/or the HYPERQUAD programs. Potentiometric titration of risedronate shows that two protons dissociate from this N-BP between pH 4 and pH 9, $^{+}LH_3^{2-} \rightarrow LH_2^{2-} \rightarrow LH^{3-}$ at $pK_{a,3}$ and $pK_{a,4}$. The overlapping pK_as were determined by deconvolution analysis with the ESAB or HYPERQUAD programs of the titration curves, and the values of $pK_{a,3}$ = 6.1 and $pK_{a,4}$ = 7.2 were found while Hounslow [24] found 5.5 and 6.8. The close proximity of $pK_{a,3}$ and $pK_{a,4}$ values indicated that it is not possible to measure directly the concentration of the ionic species LH_2 in Fig. 6.

Table 4 shows the results of the ESAB regression analysis of a part of a particular titration curve when the minimization process terminates. I addition to the original data {*V*, pa_{H+}}, residuals and the Bjerrum protonation function at each point are also given. Both the common and the group parameters are refined and the best curve-fitting is proven by the measures of a statistical analysis of the residuals. The strategy of an efficient computation in refinement of the group parameters was described earlier [32]. The reliability of the protonation constant may be determined according to the goodness-of-fit; when an increasing number of group parameters are refined, a better fit is achieved and therefore a more reliable estimate of protonation constants results. As further group parameters are refined, the fit is improved. A quite sensitive criterion of the reliability of the protonation constant is the mean of absolute values of residuals $E|\hat{e}|$ (Table 4). Comparing residuals with the instrumental noise, $s_{inst}(y)$, represented here by either $s(V)$ = 0.0010 cm³ or $s(E)$ = 0.2 mV, an excellent fit is confirmed because the mean $E|\hat{e}|$ and the residual standard deviation $s(\hat{e})$ are nearly the same and lower than the noise $s_{inst}(y)$. Here, $E|\hat{e}|$ = 0.00018 cm³ and $s(\hat{e})$ = 0.00021 cm³ are similar and both are lower than

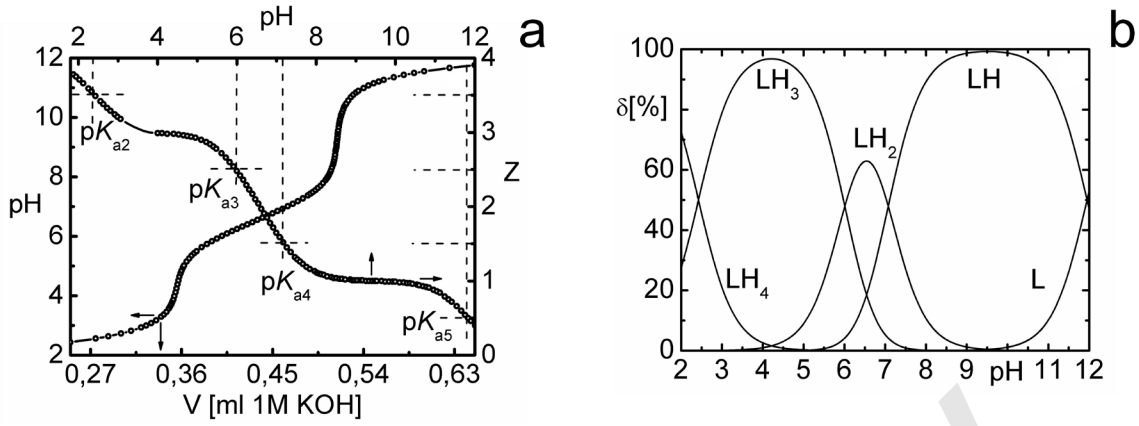

Figure 6. Protonation equilibria of risedronate analyzed with ESAB: (a) Potentiometric titration curve of risedronate with KOH and Bjerrum protonation *Z*-function; L_0 = 4.9035 ×10^{-3} mol dm^{-3}, H_T = 0.8961 mol dm^{-3}, V_0 = 15.3 cm^3, I = 0.025, t = 25°C; (b) Distribution diagram of relative presentation of all variously protonated species L, HL, HL_2, LH_3 and LH_4 of risedronate depending on pH at 25ºC, (ESAB, HYPERQUAD, ORIGIN).The charges of species are omitted for the sake of simplicity.

Table 3. Mixed dissociation constants p$K_{a,i}$ of risedronate at 37°C and various values of an ionic strength *I* estimated by nonlinear regression programs SPECFIT and SQUAD. Standard deviations of parameter estimates in last valid digits are in parentheses.

		37°C					
	Ionic strength	**0.058**	**0.068**	**0.075**	**0.089**	**0.099**	**0.110**
SPECFIT	p$K_{a,2}$	2.088(45)	1.972(55)	1.933(57)	1.880(62)	1.852(89)	1.816(65)
	s(*A*)[mAU]	0.44	0.80	0.71	1.17	0.87	0.76
	p$K_{a,3}$	5.958(15)	5.919(11)	5.904(8)	5.894(14)	5.858(21)	5.844(13)
	p$K_{a,4}$	7.068(19)	7.044(13)	6.994(12)	6.960(17)	6.895(23)	6.917(15)
	s(*A*)[mAU]	0.53	0.46	0.31	0.45	0.62	0.30
	p$K_{a,5}$	10.000(66)	9.806(127)	9.879(39)	10.150(62)	10.550(20)	10.840(126)
	s(*A*)[mAU]	0.30	0.68	0.28	0.34	0.58	0.29
SQUAD	p$K_{a,2}$	2.080(20)	1.972(18)	1.898(21)	1.911(13)	1.843(31)	1.814(20)
	p$K_{a,3}$	0.93	1.19	1.21	1.16	1.40	1.10
	s(*A*)[mAU]	5.956(10)	5.903(8)	5.904(5)	5.899(8)	5.860(13)	5.849(8)
	p$K_{a,4}$	7.071(6)	7.056(4)	6.994(3)	6.965(5)	6.894(7)	6.922(4)
	s(*A*)[mAU]	0.58	0.55	0.31	0.44	0.68	0.32
	p$K_{a,5}$	10.258(28)	9.800(28)	9.957(16)	10.335(20)	10.550(20)	11.024(47)
	s(*A*)[mAU]	0.56	0.66	0.55	0.49	0.58	0.48

the burette error *s*(*V*) = 0.0010 cm^3. As the bias $E(\hat{e})$ is equal to -5.4 ×10^{-6}, which may be taken as zero, no systematic error in curve fitting is expected. All residuals oscillate between lower -0.00007 cm^3 and upper 0.00006 cm^3 Hoaglin´s inner bounds and no outlying residuals lay outside these bounds. Residuals exhibit a normal distribution as confirmed by the Jarque-Berra normality test for combined sample skewness and kurtosis (*cf.* page 80 in [33]), and by the skewness $g_1(\hat{e})$ = -0.12 (which is not significantly different from zero and thus proves a symmetric distribution), and the kurtosis $g_2(\hat{e})$ = 1.96 (which proves a symmetric distribution between rectangular and Gaussian). An excellent fit is indicated and the regression parameter estimates are considered sufficiently reliable.

Fig. 6a shows a graphical presentation of the regression analysis results of the potentiometric titration curve of a mixture of HCl and risedronate at 25°C. The true model and the reliable parameter estimates are proven because the residuals exhibit a symmetric normal distribution with zero mean and also form a random pattern. No systematic departures from randomness indicate that the model proposed is true

Table 4a. ESAB refinement of common and group parameters for a titration of risedronate with KOH. *Common parameters refined:* $pK_{a,2}$ = 2.420(45), $pK_{a,3}$ = 5.983(2), $pK_{a,4}$ = 7.090(2), $pK_{a,5}$ = 11.914(3). *Group parameters refined:* L_0 = 4.903×10^{-3} mol dm^{-3}, H_T = -0.8961 mol dm^{-3}, *Constants:* H_0 = 2.149×10^{-2} mol dm^{-3}, t = 25.0°C, p_{Kw} = 13.9799, V_0 = 15.3 cm^3, $s(V)$ = 0.0001 cm^3, j_a = 0.0 mV, j_b = 0.0 mV, I_0 = 0.0 (in vessel), I_T = 0.8961 (in burette).

I	Titrant V [cm^3]	Residual $\hat{e}$ [cm^3]	pa_H	Protonation function
1	0.2250	0.0040	2.336	3.55
2	0.2500	-0.0004	2.433	3.49
3	0.2750	-0.0035	2.558	3.42
4	0.4000	0.0000	6.000	2.43
5	0.4050	0.0001	6.082	2.37
6	0.4100	0.0001	6.161	2.30
7	0.4150	0.0001	6.239	2.24
8	0.4300	0.0001	6.467	2.06
9	0.4350	0.0001	6.541	2.00
10	0.4400	-0.0001	6.614	1.94
11	0.4450	-0.0002	6.688	1.87
12	0.4500	-0.0002	6.764	1.81
13	0.4550	-0.0002	6.840	1.75
14	0.4600	-0.0003	6.916	1.69
15	0.4650	-0.0002	6.997	1.63
16	0.4700	-0.0001	7.080	1.56
17	0.4750	0.0000	7.167	1.50
18	0.4800	0.0001	7.260	1.44
19	0.4850	0.0002	7.358	1.38
20	0.4900	0.0003	7.469	1.31
21	0.4925	0.0003	7.528	1.28
22	0.4950	0.0003	7.594	1.25
23	0.5135	0.0003	8.734	1.02
24	0.5140	0.0003	8.858	1.01
25	0.5142	0.0002	8.913	1.01
26	0.5145	0.0002	8.978	1.01
27	0.5148	0.0001	9.041	1.01
28	0.5150	0.0001	9.120	1.01
29	0.5152	-0.0001	9.170	1.01
30	0.5155	-0.0001	9.260	1.00
31	0.5158	-0.0002	9.338	1.00
32	0.5160	-0.0002	9.414	1.00

Continued **Table 4a.** ESAB refinement of common and group parameters for a titration of risedronate with KOH. *Common parameters refined:* $pK_{a,2}$ = 2.420(45), $pK_{a,3}$ = 5.983(2), $pK_{a,4}$ = 7.090(2), $pK_{a,5}$ = 11.914(3). *Group parameters refined:* $L_0 = 4.903\times10^{-3}$ mol dm^{-3}, H_T = -0.8961 mol dm^{-3}, *Constants:* $H_0 = 2.149\times10^{-2}$ mol dm^{-3}, t = 25.0°C, p_{Kw} = 13.9799, V_0 = 15.3 cm^3, $s(V)$ = 0.0001 cm^3, j_a = 0.0 mV, j_b = 0.0 mV, I_0 = 0.0 (in vessel), I_T = 0.8961 (in burette).

I	Titrant *V* [cm³]	Residual *ê* [cm³]	pa_H	Protonation function
33	0.5162	-0.0003	9.482	1.00
34	0.5165	-0.0004	9.528	1.00
35	0.5250	-0.0001	10.542	0.96
36	0.5400	-0.0003	10.958	0.90
37	0.5450	0,0000	11.047	0.88
38	0.5500	0.0003	11.121	0.86
39	0.6750	0.0000	11.834	0.55

Table 4b. Reliability of estimated parameters proved by a statistical analysis of residuals.

Bias, *E (ê)*	0.0000054cm³
Lower and upper Hoaglin's limits	-0.00007 cm³ and 0.00006 cm³, no outliers
Mean of absolute values of residuals, *E* |*ê*|	0.00018 cm³
Standard deviation, *s (ê)*	0.00021 cm³
Skewness, g_1 *(ê)*	-0.12 (not differing from 0)
Kurtosis, g_2 *(ê)*	1.96 (not differing from 3)
Jarque-Berra normality test of a residuals	Normality accepted

and the estimates of the parameters are reliable. The distribution diagram in Fig. 6b of the relative abundance of all variously protonated species seems to be more interesting than a numerical value of just the protonation constant. The intersection of both curves gives a value of the protonation constant on the *pH*-axis.

Table 5 shows results for the protonation constant of risedronate acid determined at various values of ionic strength and 25°C as a result of regression analyses performed with two different mathematical approaches. The program ESAB minimising residuals $e_i = (V_{exp,\,i} - V_{calc,\,i})$ reaches 0.1 to 0.3 microliters and HYPERQUAD minimising $e_i = (pa_{H+,exp,\,i} - pa_{H+,calc,\,i})$ reaches a SIGMA value of about 2 or less thus proving an excellent fit.

Table 6 shows results for the protonation constant of risedronate determined at various values of ionic strength and 37°C also proving an excellent fit. Applying the extended Debye-Hückel Eq. 4 to data from Tables 5 and 6 according to a regression criterion *U*, the unknown parameter pK^T_a has been estimated. Table 6 shows the point estimates and calculated standard deviations when the minimization process terminates.

Applying the extended Debye-Hückel equation to data from Table 5 and Fig. 7 according to the regression criterion *U*, the unknown parameter $pK^T_{a,i}$ has been estimated. Table 6 shows the point estimates and calculated standard deviations when the minimization process terminates.

5. Literature comparison

Risedronate has six functional groups that could be ionized: five H^+ donors (four POH groups, geminate OH group) and one amino group as H^+ acceptor (Fig. 1). The mechanism of gradual dissociation of N-BPs was introduced by Hounslow *et al.* [24]: Risendronate N-BP is pentaprotic acid but after dissolution it may be treated as a tetraprotic or triprotic acid. Titration of risedronate with a strong base (*e.g.* KOH) involves nine solution species: H_3O^+, OH^-, $^+LH_5$, $^+LH_4^-$, $^+LH_3^{2-}$, LH_2^{2-}, LH^{3-}, L^{4-}, and the metal cation K^+. Dissociation constants $pK_{a,1}$ and $pK_{a,2}$ are in the region 1.0 and 2.0, respectively, and $pK_{a,5}$ is above 10.5, corresponding to the first, second, and fourth ionizations of the four P-OH groups. Thus, the

Table 5. Mixed dissociation constants $pK_{a,i}$ of risedronate at 25°C and various values of an ionic strength *I* estimated by nonlinear regression programs ESAB and HYPERQUAD. Standard deviations of parameter estimates in last valid digits are in parentheses.

I	pK_a	ESAB pK_a	ESAB |ê| [μL]	HYPERQUAD pK_a	HYPERQUAD SIGMA
0.025	$pK_{a,2}$	2.420(46)	0.3	2.391(9)	1.59
	$pK_{a,3}$	5.983(2)	0.1	6.011(9)	
	$pK_{a,4}$	7.090(2)		7.077(9)	
	$pK_{a,5}$	11.914(3)		11.951(8)	
0.062	$pK_{a,2}$	2.419(44)	0.3	2.417(9)	1.76
	$pK_{a,3}$	5.991(6)	0.2	5.962(11)	
	$pK_{a,4}$	7.031(4)		7.029(11)	
	$pK_{a,5}$	11.943(6)		11.983(10)	
0.099	$pK_{a,2}$	2.498(59)	0.3	2.431(9)	1.71
	$pK_{a,3}$	5.922(7)	0.2	5.959(18)	
	$pK_{a,4}$	6.991(3)		6.998(18)	
	$pK_{a,5}$	12.077(6)		12.121(18)	
0.134	$pK_{a,2}$	2.419(49)	0.3	2.427(10)	1.38
	$pK_{a,3}$	5.898(6)	0.1	5.904(11)	
	$pK_{a,4}$	6.919(2)		6.940(12)	
	$pK_{a,5}$	11.804(6)		11.886(12)	
0.169	$pK_{a,2}$	2.498(61)	0.3	2.482(11)	1.49
	$pK_{a,3}$	5.965(10)	0.2	5.908(8)	
	$pK_{a,4}$	6.935(4)		6.937(8)	
	$pK_{a,5}$	11.822(5)		11.875(7)	
0.203	$pK_{a,2}$	2.399(45)	0.3	2.504(17)	1.54
	$pK_{a,3}$	5.787(5)	0.2	5.849(9)	
	$pK_{a,4}$	6.849(3)		6.869(9)	
	$pK_{a,5}$	11.753(5)		11.786(8)	

important dissociation constants in the physiological pH range are $pK_{a,3}$ and $pK_{a,4}$ and the dissociation constants associated with loss of the third and fourth protons from the pentaprotic acid.

The zwitterions are the most probable structures of the electroneutral forms of risedronate. The $pK_{a,1}$ values of risedronate that correspond to the dissociation of the –POH group in cationic acid H_5L^+ with the formation of zwitterions were obtained by using NMR-controlled titrations [24]. It is evident that the application of the standard titrimetric procedure for $pK_{a,1}$ determination does not allow for the calculation of pK_{a1} due to the minor presence of H_5L^+ form in the equilibrium state of the risedronate solution. Thus, the proteolytic properties of risedronate in the pH range from 2 to 12 can be described in terms of four dissociation steps: $pK_{a,2}$, $pK_{a,4}$, $pK_{a,5}$ (related to the dissociation of POH groups) and $pK_{a,3}$ related to the dissociation of NH^+_3. The protonation of the sodium salt of risedronate acid has been studied at various temperatures and ionic strengths [24,45,46]. In only a few cases has the dependence of the dissociation constants on ionic strength been systematically investigated, and the methods reviewed [46]. The dissociation constants of three nitrogen-containing bisphosphonic acids have been obtained [47] and are widely cited by other authors and referral databases. However, it is interesting that other authors [48,49] have determined different dissociation constants and have not resolved this discrepancy (Table 6). Risedronate N-BP exists as an ionic species in which the nitrogen is either uncharged (N-species) or is protonated and positively charged (N^+-species). Provided that

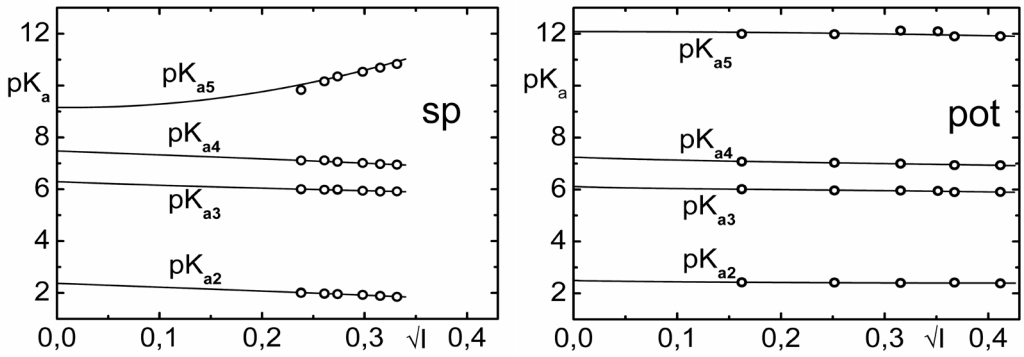

Figure 7. Plots of the dependence of the mixed dissociation constants $pK_{a,i}$ of risedronate estimated spectrophotometrically (sp) and potentiometrically (pot) on the square root of ionic strength, which lead to the parameter estimates of $pK^T_{a,2}$ = 2.37(1) and 2.44(1), $pK^T_{a,3}$ = 6.29(3) and 6.26(1), $pK^T_{a,4}$ = 7.48(1) and 7.46(2) and $pK^T_{a,5}$ = 9.31(7) and 8.70(3) at 25ºC and 37ºC.

Table 6. Dissociation constants of risedronate found in the literature and estimated in this work with program HYPERQUAD. *pot = potentiometric titration data, sp = spectrophotometric titration data. Standard deviations of parameter estimates in last valid digits are in parentheses.

Ionic strength *I*, tºC	Reference	$pK_{a,1}$	$pK_{a,2}$	$pK_{a,3}$	$pK_{a,4}$	$pK_{a,5}$
	Prediction with Pallas	1.75	4.68	6.25	6.85	11.56
	26	-	-	5.66	6.74	-
	25	-	2.77	5.25	6.79	10.45
H_2O	24	1.6	2.2	5.9	7.1	11.7
I = 0, 25ºC, pK^T_a	This work pot*	-	2.48(3)	6.12(2)	7.25(2)	12.04(5)
I = 0, 37ºC, pK^T_a	This work pot*	-	2.43(1)	6.10(2)	7.23(1)	11.81(2)
I = 0, 25ºC, pK^T_a	This work sp*	-	2.37(1)	6.29(3)	7.48(1)	9.31(7)
I = 0, 37ºC, pK^T_a	This work sp*	-	2.44(1)	6.26(1)	7.46(2)	8.70(3)

there is a wavelength at which the N-species has a significantly different molar absorptivity from that of the N^+-species, spectrophotometry may be used to determine the proportion of the risedronate existing as either of the two species at any pH. Hounslow *et al.* [24] used NMR-pH titration to determine dissociation constants of risedronate 5.66 and 6.74. Takami *et al.* [45] determined the dissociation constants of risedronate in water and found five pK_a values 1.6, 2.2, 5.9, 7.1 and 11.7. Nancollas *et al.* [46] determined only four dissociation constants 2.77, 5.25, 6.79 and 10.45.

6. Conclusions

The proteolytic properties of risedronate in the pH range from 2 to 12 can be described in terms of four dissociation steps: $pK_{a,2}$, $pK_{a,4}$, $pK_{a,5}$ (related to the dissociation of POH groups) and $pK_{a,3}$ related to the dissociation of protonated amino group NH_3^+. The reliability of the dissociation constants of risedronate was proven even when two *group parameters* L_0, H_T were ill-conditioned in a model. Their determination is uncertain and might lead to false estimates of *common parameters* pK_a,so the computational strategy employed is important. A comparison of two computational approaches, the ESAB and the HYPERQUAD programs, showed that ESAB can achieve a better fit of potentiometric titration curve. The thermodynamic dissociation constants pK^T_a were estimated by a nonlinear regression of (pK_a, *I*) data and a Debye-Hückel equation at 25ºC and 37ºC, $pK^T_{a,2}$ = 2.37(1) and 2.44(1), $pK^T_{a,3}$ = 6.29(3) and 6.26(1), $pK^T_{a,4}$ = 7.48(1) and 7.46(2) and $pK^T_{a,5}$ = 9.31(7) and 8.70(3) at 25ºC and 37ºC using pH-spectroscopic data and $pK^T_{a,2}$ = 2.48(3) and 2.43(1), $pK^T_{a,3}$ = 6.12(2) and 6.10)2), $pK^T_{a,4}$ = 7.25(2) and 7.23(1) and $pK^T_{a,5}$ = 12.04(5) and 11.81(2) at 25ºC and 37ºC. The ascertained estimates of three dissociation constants $pK_{a,3}$, $pK_{a,4}$, $pK_{a,5}$ are in agreement with predicted values obtained using PALLAS.

Acknowledgments

The financial support of the Grant Agency IGA MZ ČR (Grant No NS9831-4/2008) and of the Czech Ministry of Education (Grant No MSM0021627502) is gratefully acknowledged.

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