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Reliability of protonation constants of vildagliptin dimers by the regression analysis of pH-titration data

Research Article

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Abstract: Protonation constants of protonated monomers and dimers of the vildagliptin are determined potentiometrically. For the low concentration c_L = 3.3 mmol dm^{-3} the monomers L and LH dominate, while for a higher concentration c_L = 6.3 mmol dm^{-3} the dimers L_2H_2, L_2H_3, L_2H_4 and L_2H are mainly present. The algorithm used has little influence on the precision of the formation constants in comparison with the reproducibility of the titration. The mixed protonation constants of vildagliptin dimers L_qH_r at various temperatures are determined using FBSTAC4 and HYPERQUAD regression analysis of the potentiometric titration data. The accuracy of the protonation constants $\log_{10} \beta_{qr}$ depends on the accuracy of the group parameters. As two *group parameters* L_0, H_T are ill conditioned in a model, their determination is therefore uncertain; both can significantly cause a systematic error in the estimated *common parameters* $\log_{10} \beta_{qr}$. Using various regression diagnostics the goodness-of-fit proves the reliability of all parameter estimates. A rough estimation of thermodynamic enthalpies ΔH^0 (kJ mol^{-1}) and entropies ΔS^0 (J K^{-1} mol^{-1}) is determined from the temperature variation of protonation constants. The enthalpy shows the protonation process is exothermic, and the entropy indicates that it is spontaneous.

Keywords: *Regression analysis • pH-titration • Dimer • Vildagliptin • Protonation constant*

1. Introduction

Protonation equilibria of various drugs have been systematically studied in our laboratory. The protonation constants of vildagliptin L_qH_r may be determined by the regression analysis of potentiometric pH-titration curves. While at a low concentration of about 10^{-6} mol dm^{-3} only monomers are formed, above 0.001 mol dm^{-3} some oligomers can be present. Protonation constants of dimers estimated by the regression analysis of potentiometric titration curves can be affected by (1) the used instrumental technique, (2) temperature, (3) concentration of drug in solution, and (4) used regression algorithm.

Vildagliptin, chemical formula $C_{17}H_{25}N_3O_2$ (Fig. 1), IUPAC name (2S)-1-{2-[(3-hydroxy-adamantan-1-yl) amino]acetyl}pyrrolidine-2-carbonitrile has the CAS number 274901-16-5. Molecular weight is 303.3993. It is stable under ordinary conditions. Vildagliptin (previously identified as LAF237, trade names Zomelis, Galvus) is an oral anti-hyperglycemic agent, anti-diabetic drug, of the new dipeptidyl peptidase-4 (DPP-4) inhibitor class of drugs [1-5]. The most important incretin hormones are GLP-1 [6,7] and glucose-dependent insulinotropic polypeptide (GIP). These hormones, secreted in the human small intestine, are responsible for insulin release due to increased glucose levels. In contrast to the agents that promote insulin secretion via glucose-independent mechanisms, GLP-1's dependence on glucose concentration is considered beneficial due to a lower risk of hypoglycemia. The GLP-1 also inhibits glucagon secretion and increases beta cell mass by

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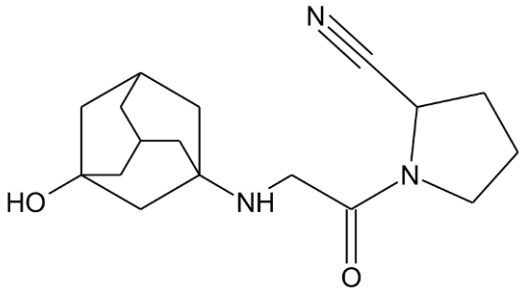

Figure 1. Chemical structure of vildagliptin.

stimulating proliferation and neogenesis. Vildagliptin inhibits the inactivation of GLP-1 [6,7] and GIP [7] by DPP-4, allowing GLP-1 [6,7] and GIP to potentiate the secretion of insulin in the beta cells and suppress glucagon release by the alpha cells of the islets of Langerhans in the pancreas.

It was proven by HPLC that vildagliptin oligomer is presented as a minor moity in pure vildagliptin substance [19]. In NMR spectra two different substances can be seen in pure vildagliptin (one with the chemical shift ^{1}H-NMR ($CDCl_3$) 4.70-4.81 (m, 0.8H) and the other with 4.83-4.91 (m, 0.2H)) [20].

The aim of this study was to determine some oligomers formed in solution of the vildagliptin and its protonation equilibria. The reliability of the chemical model is discussed, together with the influence of the used regression algorithm, concentration of the drug in solution and the reproducibility of the titrations.

2. Methodological procedure

2.1. Potentiometric data analysis

Assume that protons (H) and ligand (L) form various species according to the reaction

$$r\mathrm{H} + q\mathrm{L} = \mathrm{H}_r\mathrm{L}_q \quad (\beta_{qr}) \tag{1}$$

where the overall formation (protonation) constant of the protonated species, β_{qr}, may then be expressed as

$$\beta_{qr} = \frac{\left[L_q H_r\right]}{\left(\left[L\right]^q \left[H\right]^r\right)} = \frac{c}{l^q\, h^r} \tag{2}$$

and where the free concentration [L] = l, [H] = h and [L_qH_r] = c. For the pa_{H+} scale, h in Eq. 2 is substituted for a_{H+}, the constant β_{qr} now being a mixed protonation constant. Potentiometric readings obtained with the proton-sensitive glass and reference electrodes cell can be described by the equation

$$E_{cell} = E^0 + \frac{f.RT\ln 10}{F}\log_{10} a_{\mathrm{H}^+} + \\ + j_a a_{\mathrm{H}^+} - \frac{j_b K_{\mathrm{W}}}{a_{\mathrm{H}^+}} - E_{ref} = E^{0'} + S\log_{10} h \tag{3}$$

where E^o is the standard potential of a glass electrode cell containing some other constants of the glass electrode as the asymmetry potential, *etc*., and $a_{\mathrm{H}^+} = \left[\mathrm{H}^+\right] y_{\mathrm{H}^+} = h y_{\mathrm{H}^+}$, a liquid-junction potential E_j is expressed by the term $E_j = j_a a_{\mathrm{H}^+} - j_b K_{\mathrm{w}} / a_{\mathrm{H}^+}$, and $S = (f\,.\,RT\ln 10)/F$ is the slope of glass electrode for a Nernstian response, K_w is the operational ion product of water at temperature T [K], the correction factor f is taken as an adjustable parameter. For a constant ionic strength the activity coefficient does not change and the term $E^{0'}$ in the pH range from 3 to 11 is practically constant.

An explicit equation for the titration curve under a constant ionic strength expresses a dependence between the volume of *titrant* added from burette V_i and the monitored pa_{H+} or *emf* $E_{cell,i}$ with the vector of unknown parameters (**b**) being separated into the vector of *common parameters* ($\boldsymbol{\beta}_{qr}$) and the vector of *group parameters* (**p**), *i.e.*,

$$V_i = f(pH;\mathbf{b}) = f(pH;\boldsymbol{\beta}_{qr},\mathbf{p}) \tag{4}$$

where the vector of common parameters $\boldsymbol{\beta}_{qr}$ contains m protonation constants of the species $(\mathrm{L}_q\mathrm{H}_r)_i$, i = 1, …, m, while a vector of group parameters **p** = ($E^{0'}$, S, K_w, j_a, j_b, L_0, L_T, H_0, H_T) containing, besides two constants of Nernstian equation, $E^{0'}$ and S, the total ligand concentration L_0, and the hydrogen ion concentration H_0 of titrand in the vessel, and the corresponding quantities of titrant L_T and H_T in burette [8-10] and the total hydrogen ion concentration may be written as

$$H_{exp} = (H_0V_0 + H_TV_T)/(V_0 + V_T) \tag{5}$$

Group parameters **p** can be refined simultaneously with the common parameters $\boldsymbol{\beta}_{qr}$. Two independent regression approaches to a minimization of the sum of square residuals have been applied:

(1) The program ESAB [8,9] or FBSTAC4 [11,12] uses this strategy for treating pa_{H+} or *emf* data to find protonation/dissociation constants that give the "best" fit to experimental data. As primary data contains the total concentration H_T of proton from burette and the measured pa_{H+}, it is assumed pa_{H+} is correct and therefore the residual sum of squares $(V_{exp} - V_{calc})^2$ can be minimised. The residual e is formulated with

the volume of *added titrant V* from burette so that e_i = ($V_{exp,i}$ - $V_{calc,i}$) and the resulting residual sum of squares $U(\mathbf{b})$ is defined

$$U(\mathbf{b}) = \sum_{i=1}^{n} w_i (V_{\mathrm{T,exp},i} - V_{\mathrm{T},calc,i})^2 = \sum_{i=1}^{n} w_i e_i^2 \qquad (6a)$$

(ESAB, FBSTAC4)

or

$$U(\mathbf{b}) = \sum_{i=1}^{n} w_i (\mathrm{p}H_{\mathrm{exp},i} - \mathrm{p}H_{calc,i})^2 = \sum_{i=1}^{n} w_i e_i^2 \qquad (6b)$$

(FBSTAC4)

$$U(\mathbf{b}) = \sum_{i=1}^{n} w_i (E_{\mathrm{exp},i} - E_{calc,i})^2 = \sum_{i=1}^{n} w_i e_i^2 \qquad (6c)$$

(FBSTAC4)

where w_i is the statistical weight usually set equal to unity, while in ESAB or FBSTAC4 it may be equal to

$$\frac{1}{w_i} = s_i^2 = s_E^2 + \left[\frac{dE_i}{dV_i}\right]^2 s_V^2 \qquad (7)$$

In this analysis, generally s_E = 0.1 mV or 0.01 pH units and s_V = 0.0005 - 0.0010 cm^3.

(2) In the program HYPERQUAD [13] the objective function is given with Eq. 6b or in matrix notation $U = \mathbf{e}^T\mathbf{We}$, where **e** is a vector of residuals and represents a measurement in mV or pH and **W** is a matrix of weights. In order to minimize the objective function, the Gauss-Newton-Marquardt method is used. The *SIGMA* criterion of a goodness-of-fit is defined as

$$SIGMA = \sqrt{\frac{\sum_{i=1}^{n} w_i e_i}{n-m}} \qquad (8)$$

where the weights *w* are calculated from estimates of the error in pa_{H+} or *emf* and titre, the latter only being important in regions where the titration curve slopes more steeply. Sigma square is also a chi-squared statistic.

2.2. Reliability of estimated protonation constants

A number of protonation models, *i.e.*, a number of variously protonated species *m*, their stoichiometry *q* and *r* in $(L_qH_r)_i$, i = 1, …, *m*, and their formation constants β_{qr} may be examined by the goodness-of-fit test the adequacy of a proposed regression model with experimental data and the reliability of found general parameter estimates, β_j, j = 1, …, *m*, *cf.* page 101 in [14].

(1) *The quality of found parameter estimates* b_j, j = 1, …, *m*, is considered according to their confidence intervals or according to their variances $s^2(b_j)$. Higher parameter variances are also caused by the termination of a minimization process before reaching a minimum [14].

(2) *The quality of experimental data* is examined by the identification of influential points with the use of regression diagnostics, *cf.* page 62 in [15].

(3) *The quality of achieved curve fitting:* the adequacy of a proposed model and *m* parameter estimates found with *n* values of experimental data is examined by the goodness-of-fit test based on the statistical analysis of classical residuals. The following statistics of residuals can be used for a numerical goodness-of-fit evaluation, *cf.* page 290 in [15]: The *mean of absolute values of residuals* $E|\hat{e}|$, and the square-root of the residuals variance $s^2(\hat{e}) = U(b)/(n-m)$ known as the estimate of the *residual standard deviation*, $s(\hat{e})$, should be both of the same magnitude as the instrumental error of regressed variable *y*, *i.e.* $s_{inst}(y)$. Obviously, it is also valid that $s(\hat{e}) \approx s_{inst}(y)$.

2.3. Determination of enthalpy and entropy change

The enthalpy change ΔH^0 for the dissociation process was calculated using Van´t Hoff equation

$$d \ln K / dT = \Delta H^0 / RT^2 \qquad (9)$$

From the free-energy change ΔG^0 and ΔH^0 values, the entropy ΔS^0 could be calculated:

$$\Delta G^0 = -RT \ln K \qquad (10)$$

$$\Delta S^0 = \left(\Delta H^0 - \Delta G^0\right)/T, \qquad (11)$$

where *R* (ideal gas constant) = 8.314 J K^{-1} mol^{-1}, *K* is the thermodynamic dissociation constant and *T* is the absolute temperature.

2.4. Procedure

In order to determine mixed protonation/dissociation constants of protonation equilibria of drug acids, the following steps were applied:

Step 1. *Calibration of glass electrode cell*: The hydrogen activity scale pa_{H+} is used after standardization on 3 WTW standard buffers.

Step 2. *Determination of the concentration of drug acid* L_0: In order to analyse a *pH*-titration curve concerning a mixture of a drug acid and HCl with KOH by ESAB, FBSTAC4 or HYPERQUAD programs, the

content of drug acid L_0 was determined. A mixture of 15.00 cm^3 containing $L_0^{(0)}$ = 0.003 mol·dm^{-3} drug, $H_0^{(0)}$ = 0.019 mol·dm^{-3} hydrochloric acid and 3 cm^3 of different solutions of KCl leading to constant ionic strength of value 0.050 mol dm^{-3} was titrated with the standard $H_T^{(0)}$ = 0.896 mol dm^{-3} KOH at 25°C and about 80-100 titration points {*V,* pH} were recorded. Even the vildagliptin was chromatographically pure substance. The software used enables a more precise value of the optioned parameters in the regression model to be estimated. The refinement of the drug concentration is a great advantage of the advanced software used (FBSTAC4, HYPERQUAD, *etc.*).

Step 3. *Protonation equilibria of drug acid,* $\log_{10} \beta_{pq\,j}$, j = 1, ..., J: The protonation constant log $\beta_{pq\,j}$, $K_{a,\,j}$, j = 1, ..., J was determined to analyse a set of pH titration curves concerning a mixture of a drug acid and HCl with KOH by ESAB, FBSTAC4 and HYPERQUAD programs when previously estimated values of group parameters H_T, L_0 are used.

Step 4. *Reliability of protonation constant* $\log_{10} \beta_{pq\,j}$, j = 1, ..., J: The reliability of the dissociation constant $\log_{10} \beta_{pq\,j}$, j = 1, ..., J and a chemical model determination was considered on the basis of goodness-of-fit tests performed by the statistical analysis of residuals. The standard deviation of each log β_{pq} is in the brackets of the estimated value and enables a calculation of the resulting uncertainty.

3. Experimental procedure

3.1. Materials

Vildagliptin was donated by ZENTIVA k.s. (Prague) with declared purity checked by a HPLC method and alkalimetrically, which was always >99% and a phosphate interference was unambiguously excluded by sulphate ash analysis. The result < 0.05% clearly confirms the absence of any inorganic molecules and/or ions. This drug has been weighted straight to a reaction vessel to reach a resulting concentration of about 0.003 mol dm^{-3}. Because the stability of the drug was confirmed by the Zentiva k.s. it was not necessary to perform more than two titrations at each temperature and each concentration. *Hydrochlorid 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 better than 0.002 according to the equation HgO + 4 KI + H_2O ↔ 2 KOH + $K_2[HgI_4]$ and KOH + HCl ↔ KCl + H_2O. *Potassium hydroxide,* 0.876 mol dm^{-3}, was prepared from the exact weight of pellets p. a., Aldrich Chemical Company with carbon-dioxide free redistilled water. The solution was stored for several days in a polyethylene bottle in argon atmosphere. This solution was standardized against a solution of potassium biphthalate using the derivative method with reproducibility 0.001. All solutions were preserved from atmospheric CO_2 by means of soda lime traps. *Mercury oxide, potassium iodide* and *potassium chloride,* p.a. Lachema Brno were not extra purified. *Grade A glassware* and *twice-redistilled water* were employed in the preparation of all the solutions.

3.2. Apparatus

The free hydrogen-ion concentration *h* was measured on the digital voltmeter Hanna HI 3220 with a precision of ±0.002pH with the use of a combined glass electrode Theta HC 103-VFR and a standard calomel electrode. Titrations were performed in a water-jacketed double-walled 100 cm^3 glass vessel, closed with a Teflon bung containing the electrodes, an argon inlet, a thermometer, a propeller stirrer and a capillary tip from a micro-burette. All pH measurements were carried out at T = (298.15 ± 0.10) K. During the titrations a stream of argon gas was bubbled through the solution both for stirring and for maintaining an inert atmosphere to exclude any $CO_{2(g)}$ and $O_{2(g)}$ traces. The argon was passed through an aqueous ionic medium by prior passage through two vessels also containing the titrand medium before entering the corresponding titrand solution. The gas is best introduced under and also above the surface of the titrand. The flow under the surface was sometimes stopped while the pH was measured. If the gas-flow is too fast, the solution might be lost spray on the walls.

The burettes used were syringe micro-burettes of 1.250 cm^3 capacity (META, Brno) with a 25.00 cm micrometer screw [16]. The polyethylene capillary tip of the micro-burette was immersed into a solution when adding reagent, but pulled out after each addition in order to avoid leakage of reagent during the pH reading. The micro-burette was calibrated by weighing water on a Kern 770 balance with a precision of ±0.00015 in added volume over the whole volume range.

The potentiometric titrations of drugs with potassium hydroxide were performed using a hydrogen activity scale. Standardization of pH meter was performed using WTW standard buffers.

3.3. Computation and software

Computation relating to a determination of dissociation constants was performed by regression analysis of the titration curve using ESAB, FBSTAC4 programs and HYPERQUAD program. When the programs ESAB, FBSTAC4 or HYPERQUAD estimated H_T and L_0 from an actual titration of a mixture of drug and hydrochloric

Table 1. Search for the best chemical model for the formation of dimers in the system H^+-vildagliptin by regression analysis of one potentiometric titration curve (*cf.*, Fig. 2) using the program HYPERQUAD. Standard deviations in units of the last digit(s) are given in parentheses. *Experimental conditions:* H_0 = 0.019 mol dm^{-3}, H_T = 0.876 mol dm^{-3}, V_0 = 18.30 cm^3, (298.15 ± 0.20) K.

	Drug concentration c_L = 8.20 mmol dm^{-3}			
Hypothesis	1st	2nd	3rd	4th
$\log_{10}\beta_{11}$	7.98(03)	11.85(04)	---	---
$\log_{10}\beta_{12}$	---	19.76(04)	---	---
$\log_{10}\beta_{13}$	---	---	---	---
$\log_{10}\beta_{14}$	---	---	---	---
$\log_{10}\beta_{21}$	---	---	13.6(04)	13.66(01)
$\log_{10}\beta_{22}$	---	---	21.81(05)	21.86(01)
$\log_{10}\beta_{23}$	---	---	29.37(05)	29.45(01)
$\log_{10}\beta_{24}$	---	---	---	31.39(02)
	Degree-of-fit achieved by statistical analysis of residuals			
$\lvert\bar{e}\rvert$*100	16.62	12.48	12.12	1.67
s(e)*100	22.63	21.19	22.98	2.31
g_1(e)	0.20	-1.60	-2.22	1.11
g_2(e)	4.10	5.41	7.16	5.09
Lower Hoaglin's limit	-0.62	-0.54	-0.38	-0.07
Upper Hoaglin's limit	0.57	0.45	0.27	0.07
% c_L estimated	106.3	100.6	97.0	96.2
Sigma criterion	23.7	12.0	11.7	2.4
Tested model is	Rejected	Rejected	Rejected	Accepted

acids with potassium hydroxide, some group parameters were used from the input data for ESAB [8,9] such as the Nernstian slope and pK_w, which are also both accessible from the literature [17]. Group parameters can be estimated by a regression analysis of both segments of a titration curve or from the acid segment only if the basic one might be affected by some carbonate or silicate in the alkali. With ESAB, two group parameters, L_0 and H_T, were refined to give the best fit and the fitness may be examined by the goodness-of-fit criteria.

3.4. Supporting information

Complete experimental and computational procedures, input data specimens and corresponding output in numerical and graphical form for the programmes, ESAB, FBSTAC4 and HYPERQUAD are available free-of-charge on line at *http://meloun.upce.cz* and in the DOWNLOAD and DATA blocks.

4. Results and discussion

The aim of this study was to determine some of the oligomers formed in solution of vildagliptin and its protonation equilibria. Until now no equilibria study of vildagliptin have been recorded in the literature. The search for the best chemical model from several proposed ones concerning protonation equilibria of the vildagliptin when various concentrations of a drug were used has been performed with the use of the HYPERQUAD regression analysis of the potentiometric titration curve (Table 1, Fig. 2).

The titration curve clearly shows which part of a titration curve corresponds to the consumption of hydroxide on the vildagliptin alone (Fig. 2). Using the potentiometric titration curve of vildagliptin, the total analytical concentration of the drug in solution c_L with the use of the derivative method was first evaluated. The total analytical concentration of titrant L_0 was also determined as an unknown parameter of the nonlinear regression model analysed by potentiometric titration curves, and therefore the agreement of both values, c_L and L_0, can also be used as an accuracy criterion of the found chemical model. Usually, a regression analysis of the residual-square-sum function in the minimization process leads to several mathematical solutions which can exhibit a sufficiently close fit of the calculated titration curve through experimental points, but only one numerical solution may reach a physical sense. The condition of the physical meaning

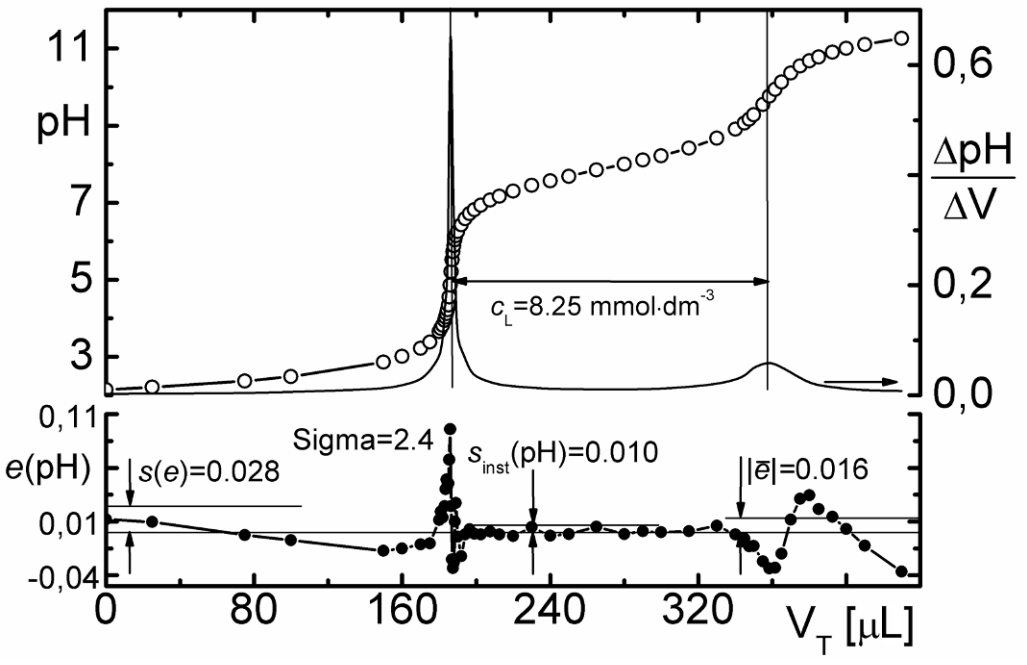

Figure 2. Determination of the analytical concentration of the vildagliptin c_L with the use of the derivative method of the potentiometric titration curve in original (V_T, pH) co-ordinates (upper part) and the goodness-of-fit statistics s_{inst}(pH), Sigma, $s(e)$, $|\bar{e}|$ after a nonlinear regression search of the chemical model using HYPERQUAD (lower part). *Experimental conditions*: titrand c_L = 8.25 mmol dm^{-3}, titrant 0.876 mol dm^{-3} KOH, I = 0.03 (KCl), T = 298.16 K, s_{inst}(pH) = 0.010 pH units, Sigma = 2.4, $s(e)$ = 0.028 pH units, $|\bar{e}|$ = 0.016 pH units.

of the numerical estimates of parameters, and hence an accuracy of both concentration estimates, is represented by an agreement of both, c_L and L_0. Therefore this is one of the criteria being used to find the best chemical model, *i.e.*, the number of species of protonation equilibrium in solution, their stoichiometry, their formation constants, as well as their equilibrium concentrations.

In addition to the fitness test, a distribution diagram of the relative population of the equilibrium mixture of differently protonated species of acid-base titration is also considered. During titration, particular attention is paid to the dominant and minor concentrations of the individual species of an equilibrium mixture. The distribution diagram also facilitates the design of other chemical hypotheses for the numerical model testing in a nonlinear regression. For each set of data, the distribution diagram of the relative population of variously protonated species can be plotted in the printout so that in a certain concentration range only such species are used. In this kind of hypothesis search, certain criteria have to be met: (i) the degree-of-fit achieved; (ii) all the species found must have meaningful concentrations different from the minor species; and (iii) the standard deviations in log β_{qr} are examined and tested. Tests to search for the best chemical model have proven that at a low concentration of vildagliptin (for example 3 mmol dm^{-3}) the best hypothesis of the chemical model contains only the monomers L, LH, LH_2 and LH_3 (ionised monomers were omitted for the sake of simplicity).

For the test of the reliability of protonation constants estimates in the proposed chemical model hypothesis, statistical analysis of residuals has been applied which examines the degree-of-fit achieved (Fig. 2). It is valid that as more group parameters are refined, the better the fit of the experimental data and therefore the protonation constants estimates are considered to be reliable. A quite sensitive criterion of reliability here is the arithmetic mean of the absolute values of residuals $|\bar{e}|$ and standard deviation of residuals $s(e)$. The degree-of-fit of experimental data can be assessed by comparing the statistical measures of residuals with value of the instrumental noise $s_{inst}(y)$ which is represented with the standard deviation $s(e)$ calculated either from the measured pH with s(pH) = 0.01. In the case of a drug concentration 8.2 mmol dm^{-3} and dimers in equilibria, an excellent fit has been proven because the arithmetic mean of the absolute values of residuals $|\bar{e}|$ is 0.016 pH units, the standard deviation of residuals $s(e)$ is 0.028 pH units and Sigma is 2.4 and both are nearly the same magnitude as the instrumental noise $s_{inst}(y)$ = 0.01 pH units. All residuals oscillate between the upper and lower Hoaglin´s confidence limits and

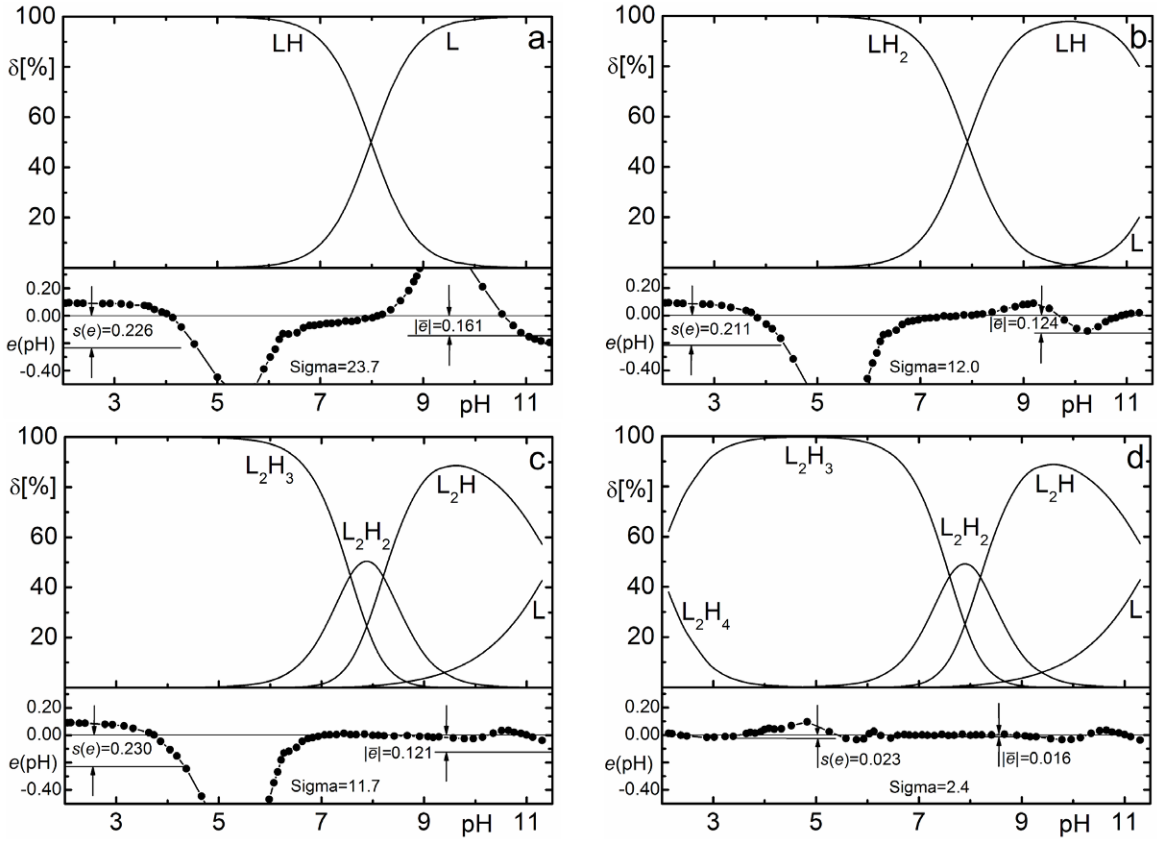

Figure 3. The search for the best chemical model for the formation of dimers in the system H^+-vildagliptin by regression analysis of one potentiometric titration curve of Fig. 2 (Table 1) and the resolution criteria in goodness-of-fit analysis s_{inst}(pH), Sigma, $s(e)$, $|\bar{e}|$ after a regression search of the chemical model using HYPERQUAD are in the lower parts of each figure: (a) The 1st hypothesis concerns L and LH is rejected, (b) The 2nd hypothesis L, LH and LH_2 is rejected, (c) The 3rd hypothesis L, L_2H, L_2H_2 and L_2H_3 is rejected, (d) The 4th hypothesis L, L_2H, L_2H_2, L_2H_3 and L_2H_4 is accepted. The charges are omitted for the sake of simplicity. *Experimental conditions*: titrand c_L = 8.25 mmol dm^{-3} and H_0 = 0.019 mol dm^{-3}, titrant H_T = 0.876 mol dm^{-3}, V_0 = 18.3 cm^3, T = 298.15 K.

no residuals lie outside these limits. A set of residuals exhibits a normal distribution, which is confirmed by the Jarque-Bera normality test for the combined test of skewness and kurtosis, *cf*. p. 67 in [18]. The first two models in Table 1 assume that no aggregates are formed and the protonation constants of monomers $\log_{10}\beta_{11}$ and $\log_{10}\beta_{12}$ are calculated.

In the minimisation process of the first model hypothesis supposing one only protonation constant $\log_{10}\beta_{11}$, the model terminates with a poor fit $s(e)$ = 0.226 pH units and Sigma 23.7, and thus indicates that the model is inadequate. In the second model hypothesis, two protonation constants $\log_{10}\beta_{11}$ and $\log_{10}\beta_{12}$ are supposed and the model hypothesis exhibits $s(e)$ = 0.211 pH units and Sigma is 12.0. When three dimers are supposed in the model hypothesis then the $s(e)$ is 0.229 pH units and Sigma is 11.7. The model with four dimers exhibits the best curve fitting $s(e)$ = 0.023 pH units and Sigma is 2.4. In the search for the best model, the minimization terminates with the best curve fitting at the 4th hypothesis. In addition to statistical characteristics of residuals, the percentage of vildagliptin concentration is also monitored; for a wrong model this mostly has no physical meaning, while for a true model it is numerically correct. However, some regression estimates found that are mathematically correct lead to a local minimum with no physical meaning and therefore the tested hypothesis of chemical model is rejected. The clear interpretation of the potentiometric titration pH-curve brings a distribution diagram of the relative population of all variously protonated species in Fig. 3.

Several titrations were repeated and analysed with HYPERQUAD for the vildagliptin studied and different chemical models were tried to fit the data. Three different concentrations c_L = 3.3, 5.1 and 6.3 mmol dm^{-3} were prepared and solutions containing drug and hydrochloric acid were titrated with potassium hydroxide (Table 2). In this way for the smallest concentration c_L = 3.3 mmol dm^{-3} the monomers L and LH were found to dominate, while for a higher concentration

Table 2. An influence of drug concentration on a formation of dimers behind monomers in the system H^+-vildagliptin by regression analysis of potentiometric titration curve (*cf.*, Fig. 2) using the programs FSTACO4 and HYPERQUAD. Standard deviations in units of the last digit(s) are given in parentheses *Experimental conditions:* H_0 = 0.019 mol dm^{-3}, H_T = 0.876 mol dm^{-3}, V_0 = 18.30 cm^3, (298.15 ± 0.20) K.

	FSTACO4(V)	HYPERQUAD	HYPERQUAD	HYPERQUAD
	3.30 mmol dm^{-3}		5.10 mmol dm^{-3}	6.30 mmol dm^{-3}
$\log_{10} \beta_{11}$	7.57(01)	7.62(00)	11.94(04)	---
$\log_{10} \beta_{12}$	---	---	19.65(04)	---
$\log_{10} \beta_{13}$	---	---	21.71(06)	---
$\log_{10} \beta_{14}$	---	---	---	---
$\log_{10} \beta_{21}$	---	---	---	13.69(04)
$\log_{10} \beta_{22}$	---	---	---	21.53(05)
$\log_{10} \beta_{23}$	---	---	33.18(20)	28.75(04)
$\log_{10} \beta_{24}$	---	---	---	30.77(07)
	Degree-of-fit by statistical analysis of residuals			
$\lvert\bar{e}\rvert$*100	0.02	1.19	7.64	3.60
s(e)*100	0.02	1.59	13.91	4.57
g_1(e)	---	0.44	-0.02	-0.54
g_2(e)	---	2.90	4.48	2.71
Lower Hoaglin's limit	---	-0.06	-0.30	-0.18
Upper Hoaglin's limit	---	0.05	0.25	0.14
% c_L estimated	99.5	99.3	100.5	96.8
Sigma criterion (HYPERQUAD)	---	0.9	8.4	7.3

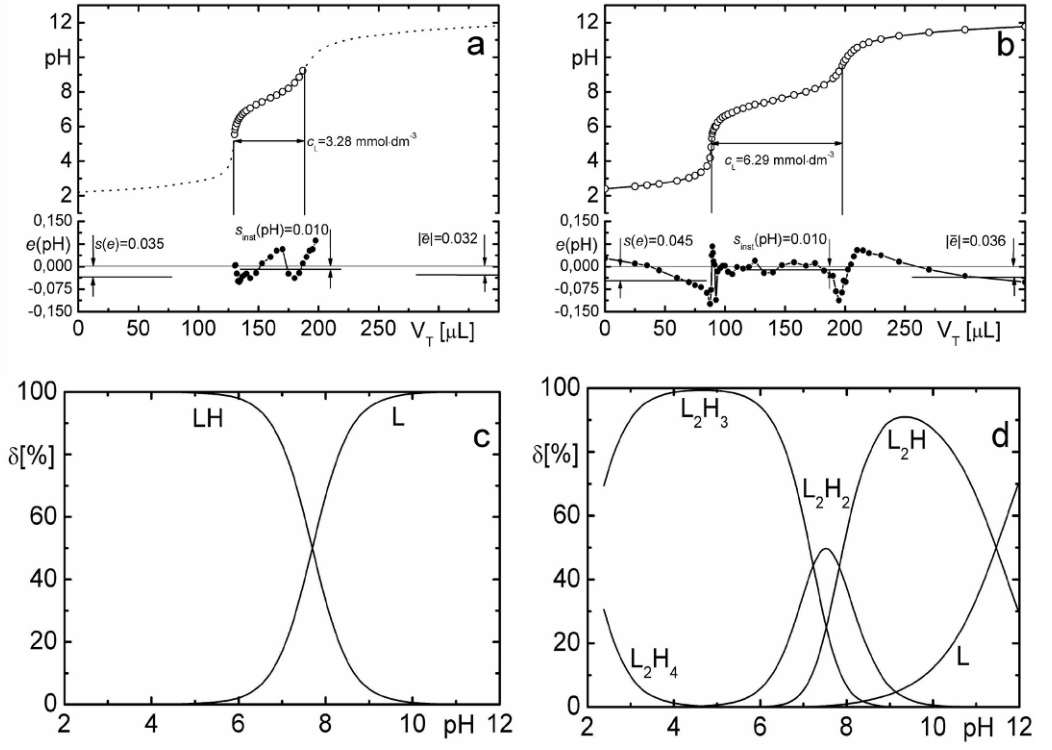

Figure 4. Potentiometric titration curve of vildagliptin for two different concentrations of titrand, c_L = 3.3 mmol dm^{-3} when monomers exist (a), and c_L = 6.3 mmol dm^{-3} when dimers prevail (b) when titrant is 0.876 mol dm^{-3} KOH. The titration curve is in original (V_T, pH) co-ordinates and its interpretation using distribution diagrams of the relative population of all variously protonated species was calculated for monomers (c), and for dimers (d). The resolution criteria in goodness-of-fit analysis s_{inst}(pH), Sigma, s(e), $\lvert\bar{e}\rvert$ after a regression search of the chemical model using HYPERQUAD are in the lower parts of each figure. *Experimental conditions:* T = 298.16 K, I = 0.13.

Table 3. (a) Influence of software algorithm used on the reliability of formation constant of dimers in the system H^+-vildagliptin by regression analysis of potentiometric titration curve (*cf.*, Fig. 2) using the program HYPERQUAD, FSTACO4(V) and FBSTAC4(pH). (b) Grand averages of formation constants of dimers and ANOVA testing of significance of influence of software algorithms with titration replication. *Experimental conditions:* H_0 = 0.019 mol dm^{-3}, H_T = 0.876 mol dm^{-3}, V_0 = 18.30 cm^3, (298.15 ± 0.20) K.

(a) *Influence of software algorithm used on the formation constant of dimers:*

	HYPERQUAD				FSTACO4(V)				FBSTAC4(pH)			
	Titration replication				Titration replication				Titration replication			
	1st	2nd	3rd	4th	1st	2nd	3rd	4th	1st	2nd	3rd	4th
$\log_{10}\beta_{21}$	13.86	13.95	13.77	13.94	13.86	13.89	13.76	13.96	13.82	13.89	13.70	13.89
$\log_{10}\beta_{22}$	21.93	21.99	21.88	22.03	21.93	21.95	21.85	22.05	21.91	21.95	21.84	22.01
$\log_{10}\beta_{23}$	29.30	29.37	29.26	29.42	29.29	29.30	29.22	29.44	29.27	29.30	29.13	29.38
$\log_{10}\beta_{24}$	30.90	30.66	31.29	30.18	30.91	30.71	30.28	31.20	30.90	30.71	30.35	31.13
	Degree-of-fit by statistical analysis of residuals											
$\lvert\bar{e}\rvert$*100	1.01482	2.59891	3.61415	1.69863	0.22679	0.26341	0.26415	0.23137	1.14107	2.07317	3.69434	2.0000
s(e)*100	1.72408	4.38904	5.96664	2.64473	0.27858	0.29341	0.30776	0.27699	1.52373	2.97856	5.38922	2.58505
% c_L estimated	97.8	97.6	99.5	98.2	97.7	97.4	99.3	98.2	97.7	97.4	99.3	98.2
Sigma criterion	1.437	1.400	1.299	1.912	0.000	0.000	0.001	0.001	0.016	0.032	0.057	0.027

(b) *ANOVA testing of an effect of the software algorithm:* H_0: $\varepsilon_{sw} \approx 0$ *versus* H_1: $\varepsilon_{sw} \neq 0$:

	$\log\beta_{21}$	$\log\beta_{22}$	$\log\beta_{23}$	$\log\beta_{24}$
Grand average $\log\beta_{qr}$	13.86	21.94	29.31	30.77
F_{exp} *versus* $F_{0.95}(4-1, 12-4) = 4.256$	0.455	0.168	0.572	0.002
Accepted	H_0: $\varepsilon_{sw} \approx 0$	H_0: $\varepsilon_{sw} \approx 0$	H_0: $\varepsilon_{sw} \approx 0$	H_0: $\varepsilon_{sw} \approx 0$

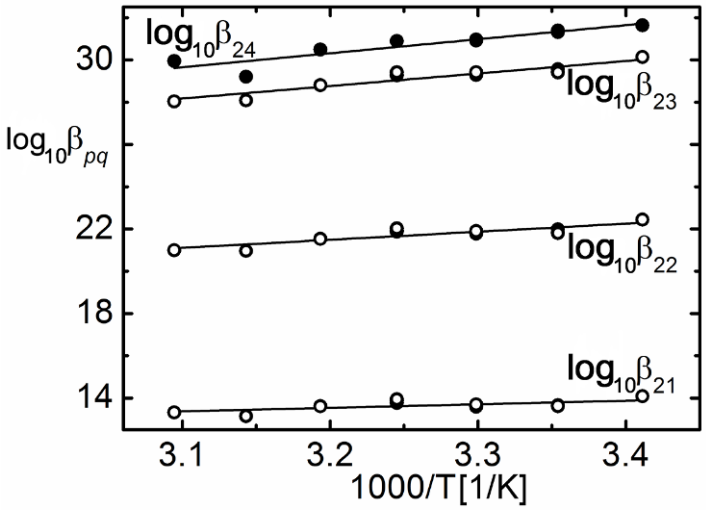

Figure 5. Dependence of protonation constants of four dimers $\log_{10}\beta_{qr}$ on temperature 1000/*T* (T in K) enables a calculation of ΔH^0 and ΔS^0.

than c_L = 6.3 mmol dm^{-3} mainly dimers L_2H_2, L_2H_3, L_2H_4 and L_2H without monomers were found (Fig. 4). In the regression analysis pH was used as a dependent variable. In contrast, the differences between titrations are significant as calculated by the *F*-test. Analysis of variance of the same data as those leading to Table 1, *i.e.,* for vildagliptin, shows that for all species formed the difference between titrations is larger than the variability within one titration. This is to be expected when data for different total drug concentrations (L) are treated,

Table 4. The dependence of protonation constants of four various dimers on temperature in the system H^+-vildagliptin by regression analysis of potentiometric titration curve (*cf.*, Fig. 2) using the program HYPERQUAD. Standard deviations in units of the last digit(s) are given in parentheses. *Experimental conditions:* H_0 = 0.019 mol dm^{-3}, H_T = 0.876 mol dm^{-3}, V_0 = 18.30 cm^3.

T /K	$\log_{10} \beta_{21}$	$\log_{10} \beta_{22}$	$\log_{10} \beta_{23}$	$\log_{10} \beta_{24}$
293.15	14.08(00)	22.45(00)	30.14(00)	31.64(05)
298.15	13.61(01)	21.81(01)	29.45(01)	31.39(01)
303.15	13.59(01)	21.79(01)	29.29(01)	30.92(02)
308.15	13.86(00)	21.93(00)	29.29(00)	30.90(01)
313.15	13.61(01)	21.54(01)	28.80(01)	30.49(01)
318.15	13.15(02)	20.96(02)	28.08(02)	29.21(03)
323.15	13.32(02)	20.99(02)	28.04(02)	29.95(03)

Table 5. Calculated thermodynamic parameters for protonation constants of dimers at temperature 298.15K in the system H^+-vildagliptin by regression analysis of potentiometric titration curves (*cf.*, Fig. 2) using the program HYPERQUAD. Standard deviations in units of the last digit(s) are given in parentheses. *Experimental conditions:* H_0 = 0.019 mol dm^{-3}, H_T = 0.876 mol dm^{-3}, V_0 = 18.30 cm^3, (298.15 ± 0.20) K.

	Estimation of $\log \beta_{qr}$	ΔH^0(kJ mol^{-1})	ΔG^0(kJ mol^{-1})	ΔS^0(J K^{-1} mol^{-1})
$\log_{10} \beta_{21}$	13.61(01)	-13.97	-33.74	66.29
$\log_{10} \beta_{22}$	21.81(01)	-31.72	-54.06	74.90
$\log_{10} \beta_{23}$	29.45(01)	-49.21	-72.88	79.40
$\log_{10} \beta_{24}$	31.39(01)	-55.12	-77.63	75.49

because the fraction of aggregates increases with increasing L, in agreement with the law of mass action. It is therefore a confirmation of the fact that oligomers are formed.

The analysis of variance was applied in order to investigate possible differences between the three mathematical approaches. Statistical testing by the Fisher-Snedecor *F*-test leads to the conclusion that there are no differences between the programs. Nonlinear regression analyses of potentiometric titration curves were performed with three different programs (Table 3) to examine the influence of used mathematical algorithm. In the program HYPERQUAD(pH) residuals are formulated with a pH-dependent variable, while in the program FSTACO(V) residuals are incorrectly formulated with the use of the independent variable added volume of titrant *V*, and finally in the program FBSTAC(pH) residuals are formulated again as the pH-dependent variable. The ANOVA test has shown that there is no statistically significant difference in the found estimates of protonation constants of dimers when using three different numerical approaches. It can be therefore concluded that regardless of the applied regression program all approaches lead to identical results.

The effect of the temperature on the dimer formation was investigated for vildagliptin (Table 4, Fig. 5) and the thermodynamic values ΔH^0 were calculated from the temperature dependence of formation constants. The calculated values show that the process is exothermic and takes place spontaneously (Table 5).

5. Conclusions

The formation of oligomers of the vildagliptin was studied potentiometrically. At concentrations higher than 6 mmol dm^{-3}, only dimers in solution are formed. The monomers L and LH were found to dominate for the smallest concentration c_L = 3.3 mmol dm^{-3}, while for a higher concentration than c_L = 6.3 mmol dm^{-3} mainly dimers L_2H_2, L_2H_3, L_2H_4 and L_2H without monomers were found. The algorithm used has very little influence on the precision of the formation constants in comparison with the reproducibility of the titration. Even two *group parameters* L_0, H_T were ill conditioned in a model and their determination is therefore uncertain. These group parameters L_0, H_T can significantly influence a systematic error in the estimated *common parameters* $\log_{10} \beta_{qr}$ and they should always be refined together. Goodness-of-fit tests for various regression diagnostics enabled the reliability of the parameter estimates to be found. Thermodynamic enthalpies ΔH^0 (kJ mol^{-1}) have been determined from the temperature variation

of protonation constants using Van´t Hoff´s equation. Negative values of ΔG^0 = -33.74, -54.06, -72.88 and -77.63 kJ mol^{-1} and positive value of ΔS^0 = 66.29, 74.90, 79.40 and 75.49 J K^{-1} mol^{-1} for $\log_{10} \beta_{21}$, $\log_{10} \beta_{22}$, $\log_{10} \beta_{23}$, and $\log_{10} \beta_{24}$ indicate that the protonation process is spontaneous. Negative values of ΔH^0 = -13.97, -31.72, -49.21 and -55.12 kJ mol^{-1} at 298.15 K indicate heat-releasing exothermic process.

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