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Oligomers-model building in protonation equilibria of sitagliptin

Research Article

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Abstract: Mixed protonation constants of sitagliptin phosphate at various ionic strengths *I* (mol kg^{-1}) in range 0.01 and 0.50 and at 298.15 K are determined using FBSTAC4 and HYPERQUAD nonlinear regression analyses of the potentiometric titration curve. At a low concentration c_L = 1.1 mmol kg^{-1} the monomers L, LH, LH_2, LH_3 and LH_4 dominate, while for a concentration range from c_L = 13.7 to 24.7 mmol kg^{-1} dimers L_2H_2, L_2H_3, L_2H_4 and L_2H are mainly present. The regression programme has almost no influence on the precision of the protonation constants. The accuracy of the protonation constants log β_{qr} depends on the accuracy of the group parameters. As two *group parameters* $c_{L,0}$, $c_{H,T}$ are ill-conditioned in a model, their determination is therefore uncertain: both can significantly cause a systematic error in the estimated *common parameters* $\log_{10}\beta_{qr}$. Fitness tests using regression diagnostics have proven the reliability of the parameter estimates.

Keywords: *Regression analysis • pH-titration • Curve fitting • Dimer • Sitagliptin phosphate • Protonation constant*

1. Introduction

Protonation equilibria of various drugs have been studied systematically in our laboratory. The protonation constants of sitagliptin phosphate L_qH_r are determined by nonlinear regression analysis of potentiometric pH-titration curves. While at a low concentration of about 10^{-6} mol kg^{-1} only monomers are formed, above 0.001 mol kg^{-1} concentrations some oligomers are supposed to be present. Sitagliptin phosphate of formula $C_{16}H_{15}F_6N_5O{\cdot}H_3{\bullet}PO_4$ and molecular weight 505.31 is known by the synonym Januvia on October 17, 2006 by Merck & Co. Chemically it is 4-Oxo-4-(3-(trifluoromethyl)-5,6-dihydro(1,2,4)triazolo-(4,3-a)pyrazin-7(8H)-yl)-1-(2,4,5-rifluorophenyl) butan-2-amine phosphate; (3R)-3-amino-1-(3-(trifluoromethyl)-6,8-dihydro-5H-(1,2,4) triazolo(4, 3-a)pyrazin-7-yl)- 4-(2,4,5-trifluorophenyl) butan-1-one phosphoric acid (Fig. 1).

It is stable under ordinary conditions. Sitagliptin works to inhibit competitively the enzyme dipeptidyl peptidase 4 (DPP-4). Sitagliptin phosphate is an oral antihyperglycemic (antidiabetic drug) of the dipeptidyl peptidase-4 (DPP-4) inhibitor class. This enzyme-inhibiting drug is used either alone or in combination with other oral antihyperglycemic agents such as metformin or a thiazolidinedione for treatment of diabetes mellitus type 2 [1-10]. This enzyme breaks down the incretins GLP-1 and glucose-dependent insulinotropic polypeptide GIP, gastrointestinal hormones released in response to a meal. By preventing GLP-1 and GIP inactivation, they are able to increase the secretion of insulin and suppress the release of glucagon by the pancreas. This drives blood glucose levels towards normal. As the blood glucose level approaches normal, the amounts of insulin released and glucagon suppressed diminishes, thus tending to prevent an “overshoot” and subsequent low

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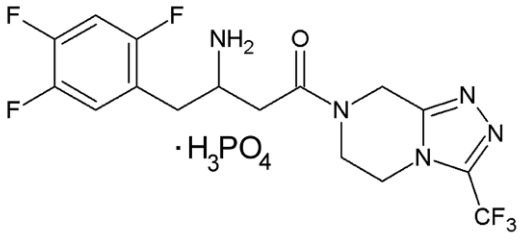

Figure 1. Sitagliptin phosphate structure.

blood sugar (hypoglycemia) which is seen with some other oral hypoglycemic agents. The benefit of this medicine is its fewer side effects (*e.g.* less hypoglycemia, less weight gain) in the control of blood glucose values. The DPP-4 enzyme is known to be involved in the suppression of certain malignancies, particularly in limiting the tissue invasion of these tumours. Inhibiting the DPP-4 enzymes may allow some cancers to progress. The hypothetical risk of cancer activation with DPP-4 down-regulation applies to all the DPP-4 inhibitors on the market (saxagliptin and vildagliptin) in addition to sitagliptin.

The aim of the present study was to determine some sitagliptin oligomers formed in solution equilibria and its protonation constants. The reliability of the found chemical model is discussed, together with the influence of the regression algorithm used, the ionic strength, the drug's concentration in the solution and the reproducibility of the titrations.

2. Computational methods

2.1. Potentiometric Data Analysis

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

$$rH + qL = L_qH_r \quad (\beta_{qr}) \tag{1}$$

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

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

and where the free, equilibrium 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. Two independent nonlinear regression approaches to a minimization of the sum of square residuals may be applied, programs ESAB [11-12] or FBSTAC4 [13-14] which use this strategy for treating pa_{H+} to find protonation/dissociation constants and the program HYPERQUAD [15] in which the objective function is given with $U = \mathbf{e}^T\mathbf{We}$, where $\mathbf{e}$ is a vector of residuals representing a measurement in pH and $\mathbf{W}$ is a matrix of weights.

2.2. Reliability of Estimated Protonation Constants

A number of protonation models, *i.e.* a number *m* of variously protonated species, 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 with experimental data test the adequacy of a proposed regression model with experimental data [17] and a reliability of found general parameter estimates, b_j, j = 1, …, *m, cf.* page 101 in [16] and was described previously in [18].

2.3. Errors in estimated formation constants

The analysis of variance ANOVA, can be applied to overall formation constants. They have been estimated using *k* identical titrations (tit) treated by *m* different software algorithms (sw) in order to examine the accuracy of the calculated formation constants. The dependence of β_{qr} on various errors [18] may be written as

$$\log \beta_{exp,qr} = \log \beta_{qr} + \varepsilon_{tit} + \varepsilon_{sw} + \varepsilon_i \tag{3}$$

where $\log \beta_{qr}$ is the "true" value of overall formation constants β_{qr}, ε_{tit} is the systematic error in the *k* titrations performed, ε_{sw} is the systematic error in the *m* software algorithms used and ε_i is the random error.

The following sample standard deviations are introduced: *the intra-titration* (or point-to-point) *standard deviation* s_i, *the inter-titration* (or titration-to-titration) *standard deviation* s_{tit}, and *the inter-algorithm* (or algorithm-to-algorithm) *standard deviation* s_{sw}. The resulting sample standard deviation in $\log \beta_{qr}$ can then be expressed by the relation

$$s_{qr} = \sqrt{s_i^2 + s_{tit}^2 + s_{sw}^2} \tag{4}$$

Two null hypotheses can be tested to find out if the effects of titrations and software algorithms are statistically significant: H_0: $\varepsilon_{tit} = 0$ *versus* $\varepsilon_{tit} \neq 0$ and H_0: $\varepsilon_{sw} = 0$ *versus* $\varepsilon_{sw} \neq 0$.

(i) If the variance ratio $F_{exp} = s_{tit}^2 / s_{res}^2 < F_{crit}(\alpha,(k-1),(k-1),(k-1)(m-1))$ where α is the significance level and s_{res}^2 is the sample variance coming from random errors then hypothesis H_0, *i.e.*, $\varepsilon_{tit} = 0$, is accepted and all experimental points belong to the same population

and there is no significant difference between titrations. (ii) In the same manner, if $F_{exp} = s_{sw}^2 / s_{res}^2 < F_{crit}(\alpha, (2-1),(k-1)(2-1))$, the hypothesis H_0, *i.e.*, ε_{sw} = 0, is accepted and all formation constants evaluated in each of *m* software algorithms belong to the same population and there is no significant difference among algorithms.

In order to establish if all titration points belong to the same data population, each titration is first treated separately to give (log β_{qr} ± *s*)$_i$, *i* = 1,..., *k*. Then all *k* titrations are used to calculate log $\beta_{qr,av}$ with the sample variance s_{av}^2 and (*k* - 1) degrees of freedom. Statistical testing is then carried out by setting $s_{av}^2 = s_i^2 / n_j + s_{tit}^2$, where n_j is the number of points in the *j*th titration. If $s_{av}^2 \approx s_i^2 / n_j$ then $s_{tit}^2 \approx 0$. The *F*-test is then used to assess the hypothesis at a given significance level α. If H_1 is accepted then $s_{tit}^2 > 0$ and the refinement of the constants must be performed separately for each titration.

A completely analogous analysis can then be performed for s_{sw}^2. For each algorithm there is (log β_{qr} ± *s*)$_i$, *i* = 1,..., *m*. All the data are used to obtain log $\beta_{qr,av}$ with the variance s_{av}^2 and (*m* — 1) degrees of freedom. Then one sets $s_{av}^2 = s_{tit}^2 / k + s_{sw}^2$. If H_0: $s_{sw}^2 \approx 0$, holds, there is no significant difference between the algorithms used and all log β_{qr} values belong to the same population. If hypothesis H_1 holds, then there is a significant difference between the algorithms used. If H_1 holds for both s_{tit}^2 and s_{sw}^2, the differences among titrations and among algorithms are significant and the search for causes of error with possible covariances becomes difficult.

3. Experimental procedure

3.1. Chemicals and solutions

Sitagliptin phosphate was donated by ZENTIVA GROUP, a.s. (Prague, Czech Republic) with declared purity checked by a HPLC method and alkalimetrically, was always >97%. This drug has been weighted straight to a reaction vessel to reach a resulting concentration of about 0.003 mol kg^{-1}. Other chemicals were described previously in [18].

3.2. Apparatus

The free hydrogen-ion concentration *h* was measured on the digital voltmeter Hanna HI 3220 with a precision of ±0.002pH using a combined glass electrode Theta HC 103-VFR and a standard calomel electrode in [18,20].

When the programs ESAB, FBSTAC4 or HYPERQUAD estimated $c_{H,T}$ and $c_{L,0}$ from an actual titration of a mixture of drug and hydrochloric acids with potassium hydroxide, some group parameters are given in the input data for ESAB [11-12] such as the Nernstian slope and pK_w, which are both accessible from [21]. 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, $c_{L,0}$ and $c_{H,T}$, were refined to give the best fit, and the fitness may be examined by the goodness-of-fit criteria.

4. Results and discussion

From the potentiometric titration curves of sitagliptin phosphate the total analytical concentration of the drug (*i.e.*, titrand) in solution c_L with the use of a second derivative method was firstly evaluated (Fig. 2). The total analytical concentration of titrand (drug) $c_{L,0}$ also was estimated as one of the unknown parameters of nonlinear regression model having been analysed by potentiometric titration curves.

Usually regression analysis of the residual-square sum function in the minimization process leads to several mathematical solutions which exhibit a sufficiently close fit of the calculated titration curve through experimental points but only one set of parameter estimates reaches a physical sense. The assumption of the physical meaning of the numerical estimates of unknown parameters, and hence their accuracy can be examined based on an agreement of both c_L. This criterion is therefore used to find the best chemical model, *i.e.*, the number of drug species of the protonation equilibria in solution, their stoichiometry, their equilibrium constants, as well as their equilibrium concentrations. A search for the chemical model of a protonation of sitagliptin phosphate is tested here using six various hypotheses about the number of species and their protonated stoichiometry (Table 1).

For a resolution test of a true chemical model, a degree-of-fit and physical sense of both estimated parameters is considered to prove their value. Moreover, in addition to a fitness test, the distribution diagram of the relative composition of the equilibrium mixture of differently protonated species of sitagliptin phosphate is also considered. Particular attention is also paid to the dominant and minority concentration species in an equilibrium mixture. The distribution diagram facilitates the design of other chemical hypotheses for numerical model testing in nonlinear regression. The search for the best chemical model tested from six proposed models when all concerning protonation equilibria of the sitagliptin phosphate at various concentrations in

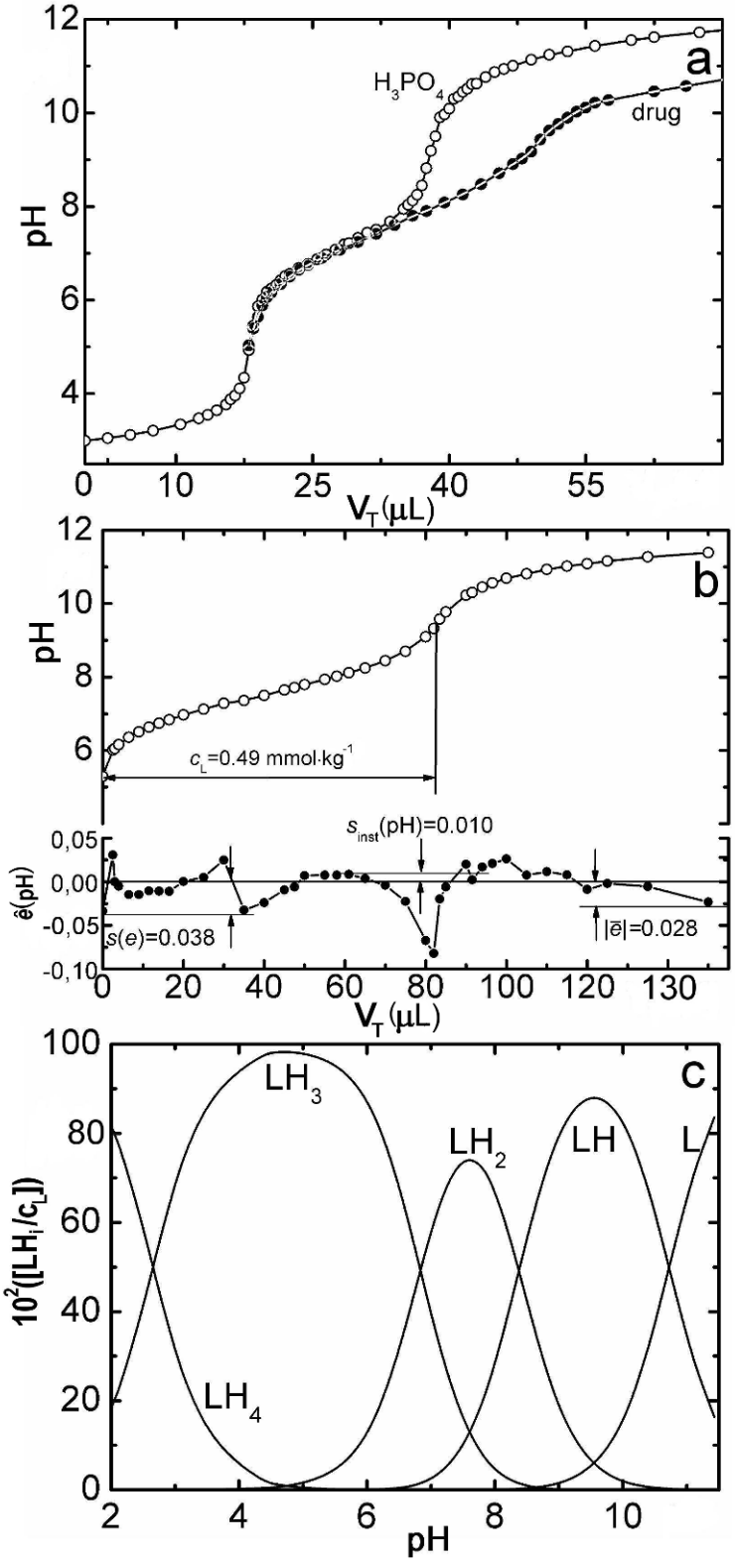

Figure 2. (a) Potentiometric titration curves of phosphoric acid (denoted H_3PO_4) and sitagliptin phosphate (denoted drug) with 0.876 mol kg^{-1} KOH in original (V_T, pH) co-ordinates. (b) The diagram of residuals examines a degree-of-fit of analysed titration curve. (c) The interpretation of a titration curve with distribution diagrams of relative population of all variously protonated species in the chemical model found using HYPERQUAD. c_L = 0.00048 mol kg^{-1}, I = 0.03 mol kg^{-1} (KCl), T = 298.16 K.

Table 1. Search for the best chemical model for the formation of oligomers in the system H^+-sitagliptin phosphate by regression analysis of one potentiometric titration curve (*cf.*, Fig. 1) using the program HYPERQUAD. In parentheses are given standard deviation in units of the last digit(s) in log β_{qr}.

Part a:

	Drug concentration c_L = 1.1 mmol kg^{-1}					
Hypothesis	**1st**	**2nd**	**3rd**	**4th**	**5th**	**6th**
log β_{11}	7.76(12)	10.79(07)	10.67(09)	10.72(08)	---	---
log β_{12}	---	18.17(11)	18.89(15)	19.10(13)	---	---
log β_{13}	---	---	25.31(19)	25.95(15)	---	---
log β_{14}	---	---	---	28.60(24)	---	---
log β_{21}	---	---	---	---	10.20(12)	13.52(11)
log β_{22}	---	---	---	---	---	21.97(12)
log β_{23}	---	---	---	---	---	28.75(18)
log β_{24}	---	---	---	---	---	31.39(28)
	Degree-of-fit by statistical analysis of residuals					
|ē|*100	31.5	22.4	22.3	11.4	23.3	14.8
s(e)*100	39.8	33.0	45.0	18.4	28.8	22.2
% c_L estimated	193.7	162.8	99.8	90.0	412.2	177.5
Sigma criterion	27.4	18.4	17.8	13.7	25.93	15.7
Model testing	Rejected	Rejected	Rejected	Accepted	Rejected	Rejected

Part b:

	Drug concentration c_L = 16.1 mmol kg^{-1}						
Hypothesis	**1st**	**2nd**	**3rd**	**4th**	**5th**	**6th**	**7th**
log β_{11}	7.56(04)	7.58(03)	11.60(08)	---	---	---	---
log β_{12}	---	9.34(12)	19.06(08)	---	---	---	---
log β_{13}	---	---	20.85(12)	---	---	---	---
log β_{14}	---	---	---	---	---	---	---
log β_{21}	---	---	---	9.03(02)	10.10(04)	13.79(10)	13.78(01)
log β_{22}	---	---	---	---	17.12(04)	21.94(10)	21.93(02)
log β_{23}	---	---	---	---	---	28.69(10)	28.82(02)
log β_{24}	---	---	---	---	---	---	30.90(02)
	Degree-of-fit by statistical analysis of residuals						
|ē|*100	28.1	28.0	21.8	16.8	23.1	17.4	1.3
s(e)*100	31.5	36.0	31.5	24.9	37.0	33.9	1.7
% c_L estimated	103.6	102.9	95.5	223.4	112.0	104.7	100.7
Sigma criterion	54.7	45.6	32.7	30.5	29.9	24.7	4.0
Model testing	Rejected	Rejected	Rejected	Rejected	Rejected	Rejected	Accepted

the solution has been carried out. (Fig. 2). Both titration curves for H_3PO_4 and the drug plotted in variables pH = $f(V_T)$ clearly show which part of the titration curve corresponds to the consumption of hydroxide on the strong phosphoric acid and which part corresponds to sitagliptin alone (Fig. 2a). For each data set, the distribution diagram of a relative population of variously protonated species can be plotted because in a certain concentration range only some actual species exist. In such a chemical model search three criteria are examined: (i) the degree-of-fit achieved; (ii) all the species found must have meaningful concentrations

different from the minor species being at 5% only; and (iii) the statistical significance of the standard deviations in log β_{qr} is examined. Tests have proven that at a low concentration of sitagliptin phosphate (for example 1.1 mmol kg^{-1}) the best chemical model contains monomers only L, LH, LH_2, LH_3 and LH_4, when charges on ions were omitted for simplicity.

For the test of the reliability of protonation constants estimates in a proposed chemical model the statistical analysis of residuals has been applied (Fig. 2b). It applies that as more group parameters are refined, the better fit of experimental data is achieved and therefore the protonation constants are more reliable. A quite sensitive criterion of reliability is the arithmetic mean of the absolute values of residuals $|\bar{e}|$ and the standard deviation of residuals $s(e)$. The degree-of-fit of experimental data can be assessed by comparing the statistical measures of residuals. The value of the instrumental noise $s_{inst}(y)$ can be represented with the standard deviation $s(e)$ calculated either from the added volume of titrant with declared value $s(V)$ = 0.0001 cm^3 or from the measured pH with declared value s(pH) = 0.01.

For a case of drug concentration 16.7 mmol kg^{-1} the presence of dimers has been proven. The arithmetic mean of the absolute values of residuals $|\bar{e}|$ = 0.013 pH units and the standard deviation of residuals $s(e)$ = 0.017 pH units are nearly the same as the value of the instrumental noise $s_{inst}(y)$ = 0.01 pH units. A set of residuals exhibits a normal distribution, which is confirmed by the Jarque-Berra normality test *cf.* page 67 in [21].

The first four models in Table 1 assume that no aggregates are formed and therefore the protonation constants of monomers log β_{11}, log β_{12}, log β_{13}, log β_{14} are calculated. When the minimisation process is performed for the first hypothesis supposing the protonation constants log β_{11} only, the model terminates with a poor fit to the experimental titration data, indicating that this model is inadequate. In the second hypothesis the protonation constants log β_{11} and log β_{12} and in the third hypothesis log β_{11}, log β_{12} and log β_{13} are estimated. In Table 1 other models are also tested. The search for the best model of the sitagliptin phosphate terminates the minimization process of the residual-square sum function with the best curve fitting using the 4th (Part a in Table 1) and 7th (Part b in Table 1) hypothesis.

In addition to statistical characteristics of residuals, the estimated percentage of sitagliptin phosphate concentration is also monitored which, for a wrong model, has no physical meaning being far from the chemically-analysed value, while for a true model is numerically correct. However, some found regression estimates of protonation constants even mathematically correct but leading to a local minimum can have no physical meaning and therefore the tested hypothesis of the 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 on Fig. 2c.

For the sitagliptin phosphate studied, several titrations were repeated and analysed with HYPERQUAD and FBSTAC4 and different chemical models were tried to fit the data. Five different concentrations c_L = 1.1, 2.3, 13.7, 16.7 and 24.7 mmol kg^{-1} were prepared and solutions containing the drug and hydrochloric acid were titrated with potassium hydroxide (Table 2). In this way for the smallest concentration c_L = 1.1 mmol kg^{-1} the monomers L, LH, LH_2, LH_3 and LH_4 were found to dominate while for a range from c_L = 13.7 to 24.7 mmol kg^{-1} mainly dimers L_2H_2, L_2H_3, L_2H_4 and L_2H without monomers are present. In the regression analysis pH was used as a dependent variable. In contrast, the differences between titrations are not significant as calculated by the *F*-test. An analysis of variance of the same data as those leading to Table 1, *i.e.,* for sitagliptin phosphate, 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 (c_L) are treated, because the fraction of aggregates increases with increasing c_L in agreement. It is therefore a confirmation of the fact that oligomers are formed with increasing drug concentration.

Fig. 3 brings the results of treating the data for sitagliptin phosphate under an influence of various concentrations of indifferent electrolyte potassium chloride adjusting an ionic strength. It is obvious that as a higher concentration of KCl as a better formation of dimers in solution. While in a solution without KCl in a mixture of dimers there are still some minor concentrations of monomers, in a solution with 0.493 mol kg^{-1} KCl there are only dimers present.

The analysis of variance was applied in order to investigate possible differences between the three various mathematical algorithms used (sw). Nonlinear regression analysis of potentiometric titration curves were performed with three different programs (Table 3) to examine the influence of the used mathematical algorithm. The program HYPERQUAD(pH) in which residuals are formulated with pH-dependent variable while in program FSTACO(V) residuals are incorrectly formulated using independent variable, *i.e.*, added volume of titrant *V*, and finally in program FBSTAC(pH) residuals are formulated again as the pH-dependent variable. The ANOVA test has proven that

Table 2. (a) An influence of drug concentration on a formation of dimers behind monomers in the system H^+-sitagliptin phosphate by regression analysis of potentiometric titration curve (*cf.*, Fig. 1) using the program HYPERQUAD. Standard deviations in units of the last digit(s) are given in parentheses. (b) Grand averages of formation constants of dimers and ANOVA testing of significance of influence of drug concentration with titration replication.

(a) An influence of drug concentration on a formation of dimers:

	1.1 mmol kg^{-1}	2.3 mmol kg^{-1}	13.7 mmol kg^{-1}	16.1 mmol kg^{-1}	24.7 mmol kg^{-1}
log β_{11}	10.72(08)	10.87(03)	8.95(09)	---	---
log β_{12}	19.10(13)	19.07(05)	13.65(14)	---	---
log β_{13}	25.95(15)	26.00(05)	15.86(05)	---	---
log β_{14}	28.60(24)	28.36(10)		---	---
log β_{21}	---	---	13.76(02)	13.78(01)	13.27(01)
log β_{22}	---	---	21.86(02)	21.93(02)	21.50(01)
log β_{23}	---	---	28.77(02)	28.82(02)	28.24(01)
log β_{24}	---	---	30.03(30)	30.90(02)	30.28(01)
	Degree-of-fit by statistical analysis of residuals				
$\lvert\bar{e}\rvert$*100	11.4	3.2	2.6	1.3	1.2
s(e)*100	18.4	5.2	3.9	1.7	1.7
% c_L estimated	90.0	68.9	100.0	100.7	95.7
Sigma criterion	13.7	7.1	3.3	4.0	3.0

(b) ANOVA Testing of an effect of the concentration of drug: H_0: $\varepsilon_{conc} \approx 0$ versus H_1: $\varepsilon_{conc} \neq 0$:

	log β_{21}	log β_{22}	log β_{23}	log β_{24}
Grand average log β_{qr}	13.49	21.64	28.48	30.55
F_{exp} versus $F_{0.95}(3-1, 15-3) = 4.256$	0.269, $p = 0.770$	0.622, $p = 0.558$	0.485, $p = 0.631$	0.668, $p = 0.537$
Accepted	H_0	H_0	H_0	H_0

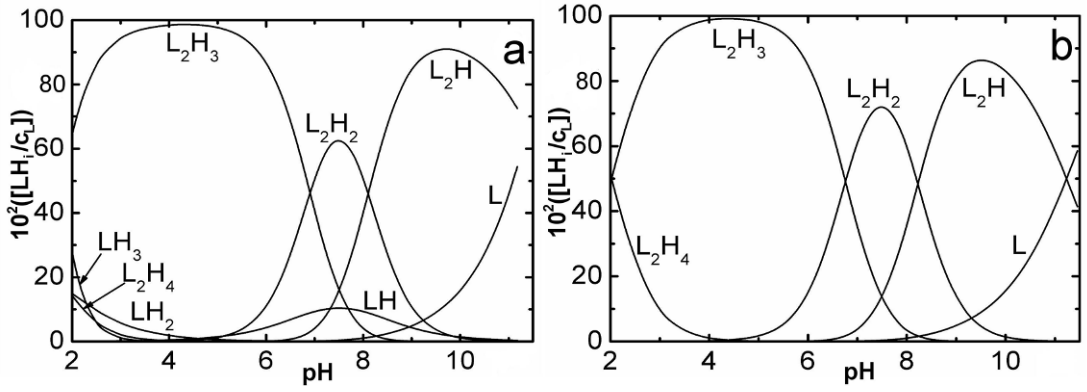

Figure 3. Two distribution diagrams of relative population of all variously protonated species in the chemical model in dependence on added concentration of KCl for adjustment of an ionic strength. $c_L = 0.0023$ mol kg^{-1}, $I = 0.03$ mol kg^{-1} (KCl), $T = 298.16$ K: (a) Without KCl, (b) 0.493 mol kg^{-1} KCl.

there is no statistically significant difference in the found estimates of protonation constants of dimers when using three different numerical approaches. It can be concluded that, regardless of the applied regression program, all programs lead to identical results.

5. Conclusions

The protonation constants of sitagliptin phosphate at a number of ionic strengths *I* [mol kg^{-1}] in range 0.01 and 0.50 and at temperatures of 298.15 K are determined

Table 3. (a) An influence of software algorithm used on the reliability of formation constant of dimers in the system H^+-sitagliptin phosphate by regression analysis of potentiometric titration curve (*cf.*, Fig. 1) using the program HYPERQUAD, FSTACO(V) and FBSTAC(pH). (b) Grand averages of formation constants of dimers and ANOVA testing of significance of influence of software algorithms with titration replication.

(a) An influence of software algorithm used on the formation constant of dimers:

	HYPERQUAD					FSTACO(V)					FBSTAC(pH)				
	Titration replication					Titration replication					Titration replication				
	1st	2nd	3rd	4th	5th	1st	2nd	3rd	4th	5th	1st	2nd	3rd	4th	5th
log β_{21}	13.47	13.41	13.43	13.48	13.52	13.46	13.39	13.41	13.49	13.50	13.50	13.43	13.44	13.50	13.54
log β_{22}	21.71	21.65	21.67	21.69	21.78	21.69	21.63	21.65	21.72	21.76	21.74	21.68	21.69	21.73	21.80
log β_{23}	28.50	28.46	28.46	28.48	28.57	28.49	28.44	28.44	28.51	28.55	28.53	28.48	28.46	28.51	28.59
log β_{24}	30.58	30.51	30.45	30.46	30.50	30.58	30.51	30.43	30.50	30.48	30.64	30.52	30.46	30.54	30.56
	Degree-of-fit by statistical analysis of residuals														
$\|\bar{e}\|$*100	0.9365	0.7298	1.3430	1.0019	0.8911	0.0344	0.0316	0.0140	0.0254	0.0161	0.8810	0.6071	1.1411	0.9254	0.8000
s(e)*100	1.3333	1.0849	2.1877	1.5130	1.2736	0.0790	0.0816	0.0375	0.0502	0.0443	1.1262	0.8016	1.5929	1.2515	1.0200
% c_L estimated	85.0	96.9	97.5	95.6	95.7	85.0	96.8	97.4	95.8	95.6	85.2	97.2	97.8	95.8	95.9
Sigma criterion	1.8320	2.0530	1.5710	1.4330	1.7030	0.0008	0.0008	0.0005	0.0005	0.0005	0.0110	0.0090	0.0160	0.0130	0.0100

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

	log β_{21}	log β_{22}	log β_{23}	log β_{24}
Grand average log β_{qr}	13.47	21.70	28.49	30.51
***F_{exp} versus $F_{0.95}$*(3 – 1, 15 - 3) = 3.885**	0.595	0.898	0.632	1.040
Accepted	H_0	H_0	H_0	H_0

using FBSTAC4 and HYPERQUAD nonlinear regression analysis of the potentiometric data. For the lowest concentration c_L = 1.1 mmol kg^{-1} the monomers L, LH, LH_2, LH_3 and LH_4 were found to dominate, while for a range from c_L = 13.7 to 24.7 mmol kg^{-1}only dimers L_2H_2, L_2H_3, L_2H_4 and L_2H without monomers were found. The fitness test is very sensitive on a selection of the chemical model (i.e. the stoichiometry of each species and a number of species in equilibria mixture). Therefore the goodness-of-fit test can be simply used in a search of the best chemical model. The programme has almost no influence on the precision of the protonation constants. Even two *group parameters* $c_{L,0}$, $c_{H,T}$ were ill-conditioned in a model and their determination is therefore uncertain. Parameters $c_{L,0}$, $c_{H,T}$ can significantly influence a systematic error in the estimated *common parameters* log β_{qr} .

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 block DOWNLOAD and block DATA.

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