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Thermodynamic dissociation constants of codeine, ethylmorphine and homatropine by regression analysis of potentiometric titration data

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Abstract

Concentration and mixed dissociation constant(s) of three drug acids H_JL, codeine, ethylmorphine and homatropine, at various ionic strengths I in the range of 0.03–0.81 have been determined with the use of regression analysis of potentiometric titration data when *common parameters* ($pK_{a,j}$, $j=1,\ldots,J$), and *group parameters* ($E^{0\prime}$, L_0, H_T) are simultaneously refined. Reliability of the dissociation constant(s) should be proven because three group parameters ($E^{0\prime}$, L_0, H_T) are ill-conditioned in the regression model and have influence on systematic error in the estimated pK_a. *Internal calibration* of the glass electrode cell in the concentration (stoichiometric) scale [H^+] performed during titration was used. The thermodynamic dissociation constant pK_a^T, an ill-conditioned ion-size parameter $\mathring{a}$ [10^{-8} m] and the salting-out coefficient C were estimated by nonlinear regression of $\{pK_a, I\}$ data. Goodness-of-fit tests provide various regression diagnostics enabling the reliability of parameters at 25°C to be proven. For codeine $pK_a^T=8.31\pm0.01$, $\mathring{a}=4\pm1$ [10^{-8} m], $C=0.45\pm0.04$; for ethylmorphine $pK_a^T=8.17\pm0.01$, $\mathring{a}=8\pm2$ [10^{-8} m], $C=0.54\pm0.03$; and for homatropine $pK_a^T=9.90\pm0.01$, $\mathring{a}=6\pm2$ [10^{-8} m], $C=0.51\pm0.03$.

Keywords: Dissociation constant; Protonation; Debye–Hückel; Ion-size parameter; Salting-out coefficient; Codeine; Ethylmorphine; Homatropine

1. Introduction

The transfer of drugs in solutions of gastrointestinal tract, through a membrane, into solution in the blood is affected by physical-chemical factors. The ultimate goal is to have the drug reach the site of action in a concentration which produces a pharmacological effect. For drugs which are weak acids or bases, the dissociation constant pK_a of the drug and the pH of the gastrointestinal tract fluid and blood stream will control the solubility of the drug. When a drug is ionized it will not be able to get through the lipid membrane, and only will when it is nonionized, and therefore has a higher lipid solubility.

The protonation constants of three drugs, codeine, ethylmorphine and homatropine, have been studied at various temperatures and ionic strengths [1]. However, only a few cases have the dependence of the protonation constants on ionic strength been systematically investigated. The dissociation constants of acid(s) can be estimated by analysis of acid–base titrations. The methods have been critically reviewed [2–4]: besides random errors, the systematic errors arise in instrumental measurements and the dissociation constants are obtained with limited precision and accuracy. Systematic errors are caused by limitations of (i) the apparatus and experimental technique, and

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(ii) the procedure of data treatment. Both limitations introduce bias into the quantities of dissociation constants. It is important that the systematic errors are taken into account and reduced as much as possible. If a systematic error is overlooked, the least-squares adjustment becomes meaningless, as the residual-square sum function becomes distorted. In regression model building, a source of problems may be found in examination of the *regression triplet* (data, model, method of estimation). Regression diagnostics concern identification of (a) the *data quality* for a proposed model, i.e. the critical examination of titration data and detection of the influential points (outliers and high leverages) which cause a shift of parameter estimates and also the procedure of calibration of the glass electrode cell. It was concluded [5] that an internal calibration of the glass electrode cell performed during titration is more accurate than an external calibration done separately. (b) The *model quality* for a given data set, i.e. the common and group parameters selected for refinement. (c) The *method of parameter estimation* selected on the basis of fulfillment of the conditions for the least-squares method. Besides ESAB [6,14] which seems to be the most powerful program because it permits refinement of group parameters and application of an internal calibration, another regression algorithm, the program PLUS, will be used.

Codeine or codeinium dihydrogenphosphoricum ($C_{18}H_{24}NO_7P$), also known as methylmorphine, codeisane or galcodine, has the systematic name (5α, 6α)-7,8-didehydro-4,5α-epoxy-3-methoxy-17-methylmorphinan-6α-ol-phosphate and has a molar weight of 299.37. Codeine is present in opium from 0.7 to 2.5%. When isolated it exists as monohydrate, orthorhombic sphenoidal rods and can be crystallized in water or diluted alcohol. The published dissociation constant is pK_a=8.21 at 25°C [1].

Ethylmorphine or ethylmorphinium chloratum ($C_{19}H_{24}NO_3Cl$), also known as dionine, ethomorphine, codethyline or 3-*o*-ethylmorphine, has the systematic name 7,8-didehydro-4,5α-epoxy-3-ethoxy-17-methylmorphinan-6α-ol-hydrochloride and has a molar weight of 313.40. Crystals can be isolated from ethanol. The published dissociation constant is pK_a=8.08 at 15°C [1].

Homatropine, or homatropinium bromatum ($C_{16}H_{22}NO_3Br$), also known as mandelyltropeine, has the systematic name endo-(±)-α-hydroxybenzeneacetic acid 8-methyl-8-azabicyclo[3.2.1]oct-3-yl ester and has a molar weight of 275.35. It exists in DL-form, being hygroscopic, only slightly soluble in water, but soluble in alcohol, benzene, chloroform and diluted acids. The published dissociation constant is pK_a=9.7 at 23°C [1].

All three drugs, codeine, ethylmorphine and homatropine, belong to a therapeutic category — analgesic, narcotic and antitussive. Codeine and ethylmorphine are narcotic analgesics which act on the central nervous system to relieve pain. When narcotics are used over a long period of time, the body becomes used to the narcotics and begins to require more of the drug to achieve the same relief. Homatropine belongs to anticholinergics and antispasmodics which are usually used to treat nausea, vomiting, abdominal cramps, and stool motility problems. A knowledge of dissociation constant of these drugs is important.

In this paper, we decided to investigate the dissociation constants of the three drugs at various ionic strengths at 25°C, to prove their reliability and also to estimate the thermodynamic dissociation constant pK_a^T and two parameters of the extended Debye–Hückel equation, an ion-size parameter $\mathring{a}$ and the salting-out coefficient *C*.

2. Theoretical

2.1. Determination of protonation/dissociation constants

An acid–base equilibrium of a drug studied is described in terms of protonation of the Brønsted base L^{z-1}

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

characterized by the protonation constant

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

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

$$L^{z-} + H^+ \rightleftarrows HL^{1-z}; \quad K_{H1}$$

$$HL^{1-z} + H^+ \rightleftarrows H_2L^{2-z}; \quad K_{H2}$$

The subscript to K_H indicates the ordinal number of the protonation step. Direct formation of each protonated species from the base L^{z-} can be expressed by the overall reaction

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

and by the overall constant $\beta_{Hj} = K_{H1}K_{H2}\ldots K_{Hj} = [H_jL^z]/(lh^j)$, where j denotes the number of protons involved in the overall protonation and l and h are the free concentrations of drug acid $[L^{z-1}]$ and hydrogen $[H^+]$, respectively. The mass balance equations are

$$L = l + \sum_{j=1}^{J}\beta_{Hj}lh^j \quad \text{and} \quad H = h + \sum_{j=1}^{J}j\beta_{Hj}lh^j$$

For dissociation reactions realized at constant ionic strength so called 'mixed dissociation constants' are defined as

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

These constants are found in experiments where pH values are measured with glass and reference electrodes, standardized with the practical pH(S)=pa_{H+} activity scale recommended internationally. The pH(S)=$p(a_{H+})_c$+log ρ_s where index c means molar concentrations and ρ_s is the density of the solvent. For aqueous solutions and temperatures up to 35°C this correction is less than 0.003 pH unit. The value of $[H_{j-1}L]/[H_jL]$ is determined by a potentiometric titration and a determination of 'concentration (stoichiometric) constants' pK_c is possible. If the protonation is studied at several ionic strengths or at a low value of ionic strength, the thermodynamic dissociation constant pK_a^T can be obtained by extrapolating to zero ionic strength (I=0), the reference state for the activity coefficient being an infinitely diluted solution.

For potentiometric *emf* titrations the following relationship holds for the total drug acid L and hydrogen ion H^+ concentrations

$$L = \frac{L_0V_0 + L_Tv_T}{V_0 + v_T} \quad \text{and} \quad H = \frac{H_0V_0 + H_Tv_T}{V_0 + v_T}$$

where H_0 (or L_0) is the total initial concentration of hydrogen ions (or drug acid) in the *titrand*, H_T (or L_T) is the total initial concentration of hydrogen ions (or drug acid) in the *titrant* (for hydroxide H_T is given), V_0 is the initial volume of the titrand and v_T is the volume of titrant added from burette. Potentiometric readings obtained with the proton-sensitive glass and reference electrodes cell can be described by the equation

$$E_{cell} = E^0 + \frac{RT\ln 10}{F}\log a_{H^+} + j_a a_{H^+} - \frac{j_bK_w}{a_{H^+}}$$
$$-E_{ref} = E^{0'} + S\log h \qquad (1)$$

where E^0 is the standard potential of a glass electrode cell plus other constants of the glass electrode such as the asymmetry potential, and $a_{H^+} = [H^+]y_{H^+} = h\,y_{H^+}$. A liquid-junction potential E_j is expressed by the term $E_j = j_a a_{H^+} - j_bK_w/a_{H^+}$, and S=$(RT\ln 10)/F$ is the slope of glass electrode for a Nernstian response, K_w is the operational ion product of water at temperature T. The term $E^{0'}$ in equation $E_{cell} = E^{0'} + S\log h$ is expressed

$$E^{0'} = E^0 + S\log y_{H^+} + j_a h y_{H^+} - \frac{j_bK_w}{hy_{H^+}} - E_{ref} \qquad (2)$$

with y_{H+} the activity coefficient of proton. 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 emf, $E_{cell,i}$, with the vector of unknown parameters ($\boldsymbol{b}$) being separated into the vector of *common parameters* ($\boldsymbol{K_a}$) and the vector of *group parameters* ($\boldsymbol{p}$)

$$v_i = f(E_{cell,i}; \boldsymbol{b}) = f(E_{cell,i}; \boldsymbol{K_a}, \boldsymbol{p}) \qquad (3)$$

Here the vector of common parameters $\boldsymbol{K_a}$=($K_{a,1}, \ldots, K_{a,m}$) contains m protonation constants of the acid H_jL while a vector of group parameters $\boldsymbol{p}$=($E^{0'}$, S, K_w, j_a, j_b, L_0, L_T, H_0, H_T) contains in addition to the two constants of Nernst equation, $E^{0'}$ and S, the total ligand concentration, L_0, and the hydrogen ion concentration, H_0 of titrand in vessel, and the corresponding quantities of titrant, L_T and H_T in burette [6]. In most cases, all these group parameters cannot be determined independently with sufficient accuracy. However, when working with media of constant high ionic strength, both K_w and j_a (with j_b) may be determined by separate experiments.

Group parameters $\boldsymbol{p}$ can be refined simultaneously with the common parameters $\boldsymbol{K_a}$. Since each

group parameter affects a part of the residual sum of squares $U(\boldsymbol{b})$ that comes from a single group, a certain economy can be achieved in computation. The least-squares method is the best in case of an additive model of measurement and independent normally distributed errors having constant variance. The least-squares strategy is quite fast and often gives good minima. On the other hand, if some group parameter(s) are uncertain and do not affect the residual sum of squares $U(\boldsymbol{b})$ this uncertainty causes large standard deviations in other parameters. Two independent regression approaches to a minimization of the sum of squared residuals have been applied:

1. The program ESAB [6] uses this strategy for treating *emf* data to find 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 emf E_{cell}, one could treat E_{cell} as the independent (error free) variable and minimize the residual sum of squares $(v_{exp}-v_{calc})^2$. 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 $U(\boldsymbol{b})$ is defined

$$U(\boldsymbol{b})=\sum_{i=1}^{n} w_i (v_{exp,i} - v_{calc,i})^2 = \sum_{i=1}^{n} w_i e_i^2 \qquad (4)$$

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

$$\frac{1}{w_i} = s_i^2 = s_E^2 + \left[\frac{dE_i}{dv_i}\right]^2 s_v^2 \qquad (5)$$

and with a good equipment, we have generally s_E=0.1–0.3 mV and s_v=0.001–0.005 ml.

2. The program PLUS [7] uses a similar strategy, but treats v_{exp} as the independent variable and minimizes the sum of residual squares $(E_{cell,\,exp} - E_{cell,\,calc})^2$. The residual e is formulated with the emf E_{cell} so that $e_i = (E_{cell,\,exp,\,i} - E_{cell,\,calc,\,i})$ and the resulting residual sum of squares $U(\boldsymbol{b})$ is defined by

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

where w_i is the statistical weight usually set equal to unity but in PLUS the relation (5) can also be used.

2.2. Determination of thermodynamic dissociation constant

Let us consider a dependence of the mixed dissociation constant $K_a = a_{H^+}[L^{z-1}]/[HL^z]$ on ionic strength when both ions $[HL^z]$ and $[L^{z-1}]$ have roughly the same ion-size parameter $\mathring{a}$ [10^{-8} m] in the dissociation equilibrium $HL^z \rightleftarrows L^{z-1} + H^+$ with the thermodynamic dissociation constant $K_a^T = a_{H^+}a_{L^-}/a_{HL}$ and that the overall salting-out coefficients is given $C = C_{HL} - C_L$. This dependence is expressed by the extended Debye–Hückel equation

$$pK_a = pK_a^T - \frac{A(1-2z)\sqrt{I}}{(1 + B\mathring{a}\sqrt{I})} + CI \qquad (7)$$

where A=0.5112 mole$^{-1/2}$ l$^{1/2}$ K$^{3/2}$ and B=0.3291 mole$^{-1/2}$ m^{-1} l$^{1/2}$ K$^{1/2}$ 10^{10} for aqueous solutions and 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 $\boldsymbol{b} = \{pK_a, \mathring{a}, C\}$ are to be estimated by a minimization of the sum of squared residuals

$$U(b) = \sum_{i=1}^{n} w_i [pK_{a,exp,i} - pK_{a,calc,i}]^2 = \sum_{i=1}^{n} w_i [pK_{a,exp,i} - f(I;\, pK_a^T,\, \mathring{a},\, C)]^2 = \text{minimum} \qquad (8)$$

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, $\mathring{a}$, and C are unknown variables to be estimated.

2.3. Reliability of estimated dissociation constants

The adequacy of a proposed regression model with experimental data and the reliability of determined parameter estimates, b_j, $j = 1, \ldots, m$, may be examined by the goodness-of-fit test also called the fitness test, cf. page 101 in [4].

1. *The quality of parameter estimates* b_j, $j=1, \ldots, m$, is considered according to their confidence intervals or according to their variances $D(b_j)$. Often an empirical rule is used: parameter b_j is considered to be significantly differing from 0 when its estimate is greater than three standard deviations, $3\sqrt{D(b_j)} < |b_j|, \quad j = 1, \ldots, m$. Higher parameter variances are also caused by termination of a minimization process before reaching a minimum.
2. *The quality of experimental data* is examined by the identification of influential points with the use of regression diagnostics, cf. page 62 in [8]. The most suitable diagnostics are the *likelihood distances* LD_i and *Jackknife residuals* $\hat{e}_{J,i}$. For linear regression models, all characteristics for the identification of influential points are functions of the residuals, $\hat{e}_i$, and diagonal elements, H_{ii}, of the projection matrix $\boldsymbol{H}=\boldsymbol{X}(\boldsymbol{X}^{\mathrm{T}}\boldsymbol{X})^{-1}\boldsymbol{X}^{\mathrm{T}}$. For nonlinear regression models, the situation is rather more complicated as the parameter estimates and residuals cannot be expressed so simply as the linear combination of experimental data, cf. page 292 in [8]. When the Taylor type linearization of original nonlinear model is used, all methods of identification of influential points in linear models can be used. The procedure starts from the one-step approximation of the parameter estimate computed without *i*th point

$$b_{(i)}^1 = b - (\boldsymbol{J}^{\mathrm{T}}\boldsymbol{J})^{-1} J_i \frac{\hat{e}_i}{1 - P_{ii}} \tag{9}$$

where P_{ii} are elements of a projection matrix, $\boldsymbol{P}=\boldsymbol{J}(\boldsymbol{J}^{\mathrm{T}}\boldsymbol{J})^{-1}\boldsymbol{J}^{\mathrm{T}}$, $\boldsymbol{J}$ is the Jacobian and other details may be found in [8].

The influential points may be easily identified on the basis of the one-step approximation of the Jackknife residuals $\hat{e}_{J,i}$ calculated by relation

$$\hat{e}_{J,i} = \frac{\hat{e}_i}{\hat{s}_{(i)}\sqrt{1 - P_{ii}}} \tag{10}$$

where $\hat{s}_{(i)}^2$ is the residual variance computed by using estimates $\boldsymbol{b}_{(i)}$

$$\hat{s}_{(i)}^2 = \frac{U(\boldsymbol{b}) - (\hat{e}_i^2/1 - P_{ii})}{n - 4}$$

Jackknife residuals higher than 3 indicate *highly influential points.*

A nonlinear measure of the influence of the *i*th point on the parameter estimates is represented by the regression diagnostic called the likelihood distance

$$\mathrm{LD}_i = 2[\ln L(b) - \ln L(b_{(i)})].$$

When $\mathrm{LD}_i > \chi^2_{(1-\alpha)}$ (2) is valid, the *i*th point is strongly influential. The significance level α is usually chosen to be equal to 0.05, then $\chi^2_{0.95}(2) = 5.992$.

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. If proposed model represents the data adequately, the residuals should form a random pattern having a normal distribution $N(0, s^2)$ with the residual mean equal to 0, $E(\hat{e}) = 0$, and the standard deviation of residuals $s(\hat{e})$ being near to noise i.e. experimental error ϵ. Systematic departures from randomness indicate that the model and parameter estimates are not satisfactory. Examination of residual plots may assist graphical analysis of residuals, cf. page 288 in [8]. The *overall diagram of residuals* gives an initial impression: detection of outliers, detection of a trend in the residuals, detection of a sign change, detection of an abrupt shift of level in the experiment. The following statistics of residuals can be used for a numerical goodness-of-fit evaluation, cf. page 290 in [8]: (1) The *residual bias* is the arithmetic mean of residuals $E(\hat{e})$ and should be equal to 0; all residual values lying outside the modified Hoaglin's inner bounds B_L^* and B_U^*, cf. page in [9] are considered to be outliers. (2) The *mean of absolute values of residuals* $E|\hat{e}|$, and the square-root of the residuals variance $s^2(\hat{e}) = U(\boldsymbol{b})/(n - m)$ 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*, $s_{\mathrm{inst}}(y)$. Obviously it is also valid that $s(\hat{e}) \approx s_{\mathrm{inst}}(y)$. (3) The *residual skewness*, $g_1(\hat{e})$, for symmetric distribution of residuals should be equal to 0. (4) The *kurtosis*, $g_2(\hat{e})$, for normal distribution should be equal to 3. (5) The *determination coefficient D* calculated from the relation

$$D = \frac{1 - U(\boldsymbol{b})}{\sum_{i=1}^{n}(y_{\exp,i} - \bar{y}_{\exp})^2}$$

multiplied by 100% is called the *regression rabat* and is equal to percentage of points which correspond to proposed regression model. (6) The *Hamilton R-factor of relative fitness* is often used, expressed by R-factor $= \sqrt{U(\boldsymbol{b})/\sum_{i=1}^{n} y_i^2}$. There is an empirical rule of a fitness classification with the use of Hamilton R-factor. For a good fitness, the Hamilton R-factor reaches a value $\leq$1%, and for excellent fitness is lower than 0.5%. (7) To distinguish between various models the Akaike information criterion (*AIC*) is suitable to apply, being defined by the relation $AIC = -2L(b) + 2m$, or $AIC = n\ln(U(b)/n) + 2m$, where n is a number of data and m is a number of estimated parameters. The best regression model is considered to be a model for which this criterion reaches a minimal value.

3. Experimental

3.1. Chemicals

Codeine, *ethylmorphine*, *homatropine* were weighed directly into a reaction vessel to reach a resulting concentration of about 0.015 M. Purity was checked with the use of the melting point: codeine, theoretical 235°C, found 233.5°C; ethylmorphine, theoretical ~170°C, found 161°C; homatropine, theoretical 212°C, found 214°C.

Hydrochloric acid, 1 M, was prepared by dilution of concentrated HCl (p.a., Lachema Brno) with redistilled water and standardization against HgO and KI with a reproducibility better than 0.2% according to equation $HgO + 4KI + H_2O \rightleftarrows 2KOH + K_2[HgI_4]$ and $KOH + HCl \rightleftarrows KCl + H_2O$.

Potassium hydroxide, 1 M, was prepared from the exact weight of pellets p.a., Aldrich Chemical Company with a carbon-dioxide free redistilled water. The solution was stored for several days in a polyethylene bottle. This solution was standardized against a solution of potassium hydrogen-phthalate using the Gran method in the MAGEC program [10] with reproducibility of 0.1%.

Mercury oxide, and *potassium iodide*, *potassium chloride*, p.a. Lachema Brno were not further purified.

Twice-redistilled water was used in preparation of solutions.

3.2. Potentiometric apparatus

The free hydrogen-ion concentration h was measured via *emf* (Eq. (1)) on a digital voltmeter OP-208/1 (Radelkis, Budapest) with a precision of ±0.1 mV with the use of a glass electrode G202B (Radiometer, Copenhagen) and a commercial SCE reference electrode OP-8303P (Radelkis, Budapest). Titrations were performed in a water-jacketed double-walled glass vessel of 100 ml, 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 *emf* measurements were carried out at 25.0°C±0.1.

During the titrations, a stream of argon gas was bubbled through the solution both for stirring and for maintaining an inert atmosphere. The argon was passed through aqueous ionic medium by prior passage through one or two vessels also containing the titrand medium before entering the corresponding titrand solution. The gas is best introduced under surface of the titrand. Sometimes the flow has to be stopped while *emf* is measured.

The burettes used were syringe micro-burettes of 1250 μl capacity (META, Brno) with a 25 cm micrometer screw [11]. 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 Sartorius 1712 MP8 balance with a precision of ±0.015% in added volume over the whole volume range.

3.3. Calibration of glass electrode cell

The potentiometric titrations of drugs with potassium hydroxide were performed using a hydrogen concentration scale where the hydrogen ion concentration $[H^+]=h$ was known from a preparation of solution and the emf E_{cell} in mV was measured. Using a set of experimental data $\{E_{cell}, h\}$ from a calibration titration of hydrochloric acid of known concentration with standard potassium hydroxide, the unknown group parameters $E^{0'}$ and S in Eq. (1) were evaluated.

The *internal calibration* of the glass electrode cell was used when the program ESAB estimated H_T, L_0 and $E^{0'}$ 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 such as the Nernstian slope and pK_w, which both are accessible from the literature [12]. 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 as well as silicate in the alkali.

With ESAB three group parameters, $E^{0'}$, L_0 and H_T, were refined to give the best fit and the fitness may be examined by the goodness-of-fit criteria, for example, the Hamilton *R*-factor of relative fitness and the mean of absolute values of residuals. Since $E^{0'}$ might change from one titration to another because of a change of the liquid-junction potential, the *internal calibration* of the glass electrode cell seemed to be more accurate and has been preferred.

3.4. Procedure for 'equilibrium titration'

To determine mixed dissociation constants and/or thermodynamic dissociation constants of protonation equilibria of drug acids the following steps were applied:

Step 1. *Standardization of hydrochloric acid* c_{HCl}: hydrochloric acid was standardized by HgO+KI titration and was evaluated by the Gran method (MAGEC [10]).

Step 2. *Calibration of glass electrode cell*, $E^{0'}$, *S*, pK_w, H_T: the hydrogen concentration $[H^+]=h$ is known from an initial concentration H_0 and measured emf, *E*. From equation $E = E^{0'} + S \log h$, for each point $\{E, h\}$ of titration curve of known concentration of hydrochloric acid H_0 with standard potassium hydroxide, the group parameters $E^{0'}$, *S* and H_T were refined.

Step 3. *Determination of the concentration of drug acid* L_0: to analyze an *emf* titration curve concerning a mixture of a drug acid and HCl with KOH by ESAB or PLUS programs, the content of drug acid L_0 was determined. A mixture of 20 ml containing $L_0^{(0)}$=0.015 M drug, $H_0^{(0)}$=0.100 M hydrochloric acid and 10 ml of indifferent solutions of KCl for an adjustment of ionic strength was titrated with standard $H_T^{(0)}$=1.0 M KOH at 25°C and about 30–40 titration points $\{v, E_{cell}\}$ were recorded.

Step 4. *Protonation equilibria of drug acid*, $K_{H,j}$, j=1, ..., *J*: to analyze a set of *emf* titration curves concerning a mixture of a drug acid and HCl with KOH by ESAB or PLUS programs when previously estimated values of group parameters $E^{0'}$, *S*, H_T, L_0 are used, the dissociation constant $K_{H,j}$, $j = 1, \ldots, J$ was determined.

Step 5. *Reliability of dissociation constant* $K_{H,j}$, $j = 1, \ldots, J$: the reliability of the dissociation constant $K_{H,j}$, $j = 1, \ldots, J$ was considered on the basis of goodness-of-fit tests performed by the statistical analysis of residuals.

3.5. Computer data treatment

Computations related to the determination of dissociation constants were performed by regression analysis of titration curves using the ESAB program, version ESAB2M [6] and the PLUS program [7]. The thermodynamic dissociation constant pK^T, an ion-size parameter *å* and the salting-out coefficient *C* were estimated with the nonlinear regression program MINOPT in statistical system ADSTAT (TriloByte Statistical Software, Ltd. Pardubice) [13].

4. Results and discussion

4.1. Estimation of dissociation constants

For the adjusted value of an ionic strength, the potentiometric titration of a mixture of HCl and drug acid with potassium hydroxide was carried out. The initial tentative value of the dissociation constant of the drug studied, corresponding to the midpoint value in each plateau of the potentiometric titration curve (Fig. 1a), was refined by ESAB and/or PLUS programs.

Table 1 gives the results of the ESAB regression analysis of one part of a particular titration curve when the minimization process terminates. Besides the original data $\{v, E_{cell}\}$ and $\log h$, the Bjerrum protonation function at each point is given. Both common and group parameters are refined and the best curve-fitting achieved is proven by the results of a statistical analysis of residuals in the goodness-of-fit test. Reliability of the protonation constant may be

Table 1
ESAB refinement of common and group parameters for a titration of ethylmorphine with KOH[a]

I	Volume (cm^3)	Residual (cm^3)	E_{cell} (mV)	$-\log h$	Protonation function
1	0.3530	0.0005	134.00	4.737	1.00
2	0.3535	0.0004	93.90	5.415	1.00
3	0.3540	0.0003	74.30	5.747	1.00
4	0.3545	0.0002	60.10	5.987	0.99
5	0.3550	0.0001	50.90	6.142	0.99
6	0.3555	0.0001	43.50	6.267	0.99
7	0.3560	0.0000	38.10	6.359	0.98
8	0.3566	0.0000	33.30	6.440	0.98
9	0.3571	0.0001	28.00	6.529	0.97
10	0.3576	0.0001	24.80	6.583	0.97
11	0.3586	0.0001	18.60	6.688	0.96
12	0.3596	0.0000	13.90	6.768	0.96
13	0.3606	0.0000	9.60	6.840	0.95
14	0.3621	−0.0001	4.70	6.923	0.94
15	0.3641	−0.0001	−1.30	7.025	0.92
16	0.3666	−0.0003	−7.00	7.121	0.91
17	0.3696	−0.0005	−12.90	7.221	0.88
18	0.3731	−0.0005	−19.00	7.324	0.86
19	0.3776	−0.0003	−25.80	7.439	0.82
20	0.3826	−0.0003	−32.00	7.543	0.78
21	0.3881	−0.0002	−38.00	7.645	0.74
22	0.3956	−0.0006	−44.80	7.760	0.69
23	0.4031	−0.0002	−51.60	7.875	0.63
24	0.4106	0.0002	−58.00	7.983	0.57
25	0.4157	−0.0001	−61.70	8.046	0.53
26	0.4207	−0.0002	−65.50	8.110	0.50
27	0.4257	−0.0001	−69.50	8.177	0.46
28	0.4307	0.0003	−73.70	8.248	0.42
29	0.4357	0.0004	−77.90	8.319	0.38
30	0.4407	0.0002	−81.90	8.387	0.34
31	0.4457	0.0001	−86.20	8.460	0.31
32	0.4507	0.0002	−91.10	8.543	0.27
33	0.4557	0.0005	−96.60	8.635	0.23
34	0.4607	0.0006	−102.60	8.737	0.19
35	0.4657	0.0004	−109.20	8.848	0.15
36	0.4697	0.0002	−115.40	8.953	0.12
37	0.4732	0.0000	−121.80	9.061	0.10
38	0.4757	0.0001	−127.80	9.163	0.08
39	0.4782	0.0001	−134.90	9.283	0.06
40	0.4803	0.0001	−142.10	9.405	0.05
41	0.4818	0.0000	−148.30	9.509	0.04
42	0.4833	−0.0003	−154.80	9.619	0.03
43	0.4848	−0.0006	−161.80	9.738	0.02
44	0.4858	−0.0009	−166.90	9.824	0.02

Reliability of parameters estimates as demonstrated by a statistical analysis of residuals	
Bias, $E(\hat{e})$	1.23E-20 cm^3
Lower and upper Hoaglin's limits	−0.00107 cm^3 and 0.00107 cm^3, no outliers
Mean of absolute values of residuals, $E\lvert\hat{e}\rvert$	0.0002 cm^3
Variance, $s^2(\hat{e})$	1.01E-07
Standard deviation, $s(\hat{e})$	0.0003 cm^3
Skewness, $g_1(\hat{e})$	−0.57 (not differing from 0)
Kurtosis, $g_2(\hat{e})$	3.32 (not differing from 3)
Residuals sum of squares, $U(\boldsymbol{b})$	4.36E-06
Jarque–Berra normality test of a residuals	Normality accepted
Regression rabat, 100D	99.996%
Akaike information criterion, AIC	−703.60
Hamilton R-factor of relative fitness	0.076%

[a] Common parameters refined: $\log K_{H1}$=8.104(2); group parameters refined: L_0=0.01116(1) mol dm^{-3}, H_T=0.8630(2) mol dm^{-3}, E^0=414.3(3) mV; constants: H_0=0.0297 mol dm^{-3}, t=25.0°C, pK_w=13.899, V_0=10.251 cm^3, $s(V)$=0.001 cm^3, $s(E)$=0.2 mV, j_a=0.0 mV, j_b=0.0 mV, I_0=0.0387 (in vessel), I_T=0.8636 (in burette). Standard deviation of parameter estimate in last valid digits are in brackets.

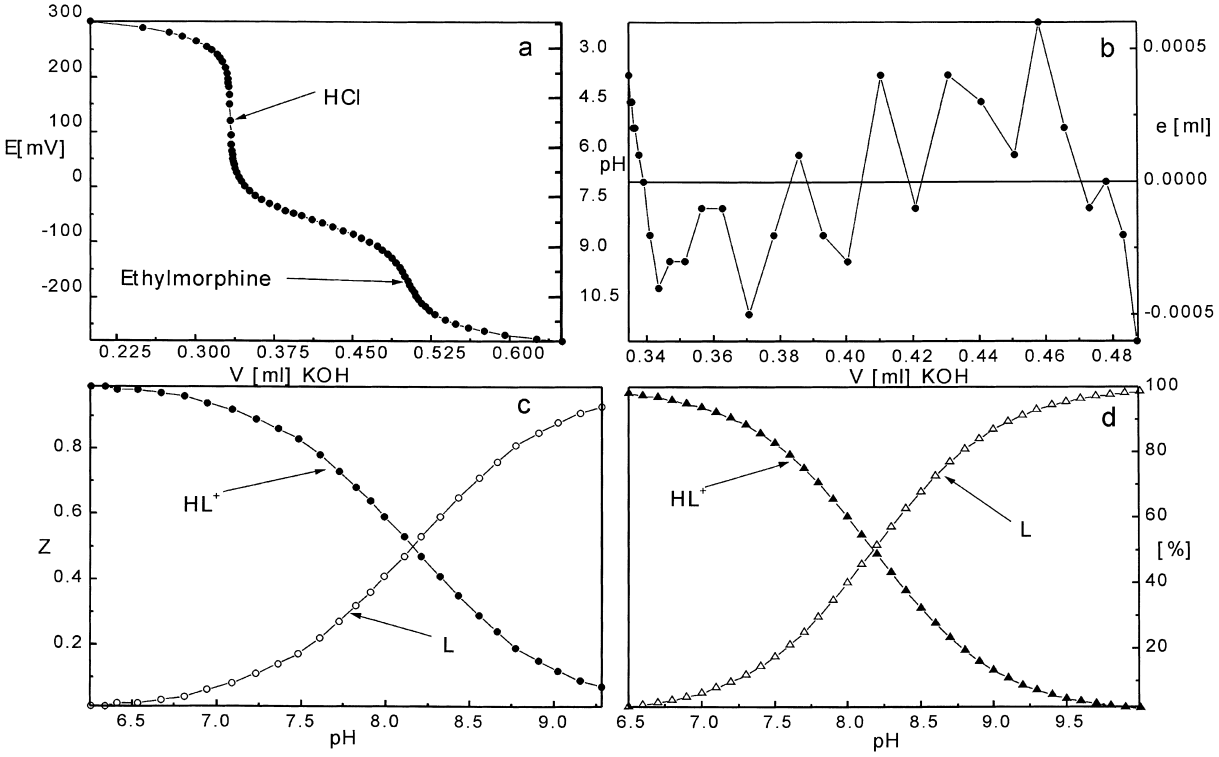

Fig. 1. Protonation equilibria of ethylmorphine analyzed with ESAB (a) Potentiometric titration curve of ethylmorphine with KOH; L_0=0.0146 M, L_T=0.8945 M, V_0=10.25 ml, I=0.029, T=25°C; (b) Plot of residuals; (c) Bjerrum formation function (ESAB); (d) Distribution diagram of relative presentation of all species of protonation equilibrium.

determined according to the goodness-of-fit. As more group parameters are refined, a better fit is achieved and therefore a more reliable estimate of protonation constants results. A quite sensitive criteria of the reliability of the protonation constant are the Hamilton *R*-factor of relative fitness and the mean of absolute values of residuals $E\left|\hat{e}\right|$. Comparing residuals with the instrumental noise, $s_{\text{inst}}(y)$, represented here by either $s(v)$=0.001 ml or $s(E)$=0.2 mV, an excellent fit is confirmed because the mean $E\left|\hat{e}\right|$ or the residual standard deviation $s(\hat{e})$ are nearly same or lower than the noise $s_{\text{inst}}(y)$. Here, $E\left|\hat{e}\right|$=0.0002 ml and $s(\hat{e})$=0.0003 ml are nearly the same and both values are lower than the instrumental error $s(v)$=0.0010 ml. As the bias $E(\hat{e})$ is equal to 10^{-20} which can be taken as 0, no systematic error in curve fitting is expected. All residuals oscillate between lower and upper Hoaglin's inner bounds and no residuals lay outside. Residuals exhibit a normal distribution as confirmed by the Jarque–Berra normality test for combined sample skewness and kurtosis (cf. page 80 in [9]), and also by the skewness $g_1(\hat{e})=-0.57$ (which is not significantly different from 0, inferring a symmetric distribution), and the kurtosis $g_2(\hat{e})$=3.32 (which is not significantly different from 3, inferring a normal distribution). The regression rabat, 100D=99.996%, indicates that a high percentage of titration curve points fulfill the regression model with the parameter estimates found; in fact, all points. With the use of Akaike information criterion, $AIC=-703.6$, a most suitable regression model among several plausible ones and the best estimates of common and group parameters have been found. As the Hamilton *R*-factor reaches a value of 0.076%, an excellent fitness is indicated and parameter estimates are considered sufficiently reliable.

Fig. 1 is a graphical presentation of regression analysis results showing the following: (a) The *potentiometric titration curve* of a mixture of HCl and ethyl-

Table 2
Concentration (pK_c) and mixed dissociation (pK_a) constant of codeine estimated by nonlinear regression program PLUS[a]

I	PLUS		
	pK_c	pK_a	R (%)
0.0298	8.322 (19)	8.264	0.235
0.0342	8.316 (20)	8.256	0.214
0.0400	8.316 (21)	8.251	0.231
0.0625	8.309 (37)	8.238	0.198
0.0900	8.314 (22)	8.237	0.182
0.1225	8.321 (24)	8.241	0.210
0.1600	8.336 (21)	8.255	0.212
0.2500	8.349 (17)	8.273	0.115
0.3600	8.423 (37)	8.356*	0.302
0.4900	8.417 (23)	8.365	0.170
0.6400	8.445 (15)	8.410	0.132
0.8100	8.482 (24)	8.467	0.209

[a] Standard deviation of parameter estimates in last valid digits is in brackets. Values denoted with asterisk are influential points (outliers) excluded from a regression analysis.

morphine shows the data at 25°C; (b) The *overall diagram* of classical residuals gives an initial impression of residuals. The true model and reliable parameter estimates are proven as the residuals exhibit a normal distribution with zero mean and also form a random pattern. Here are no systematic departures from randomness indicate a false model or false estimates of the parameter; (c) The *Bjerrum formation function* provides an overview of the dissociation of drug acid HL; (d) The *distribution diagram* of the relative abundance of all species of the protonation equilibrium of the drug acid seems to be more interesting than a numerical value of protonation constant only. The intersection of both curves gives a value of the protonation constant on pH-axis.

Table 2 provides concentration and mixed dissociation constant of codeine estimated by the nonlinear regression program PLUS when residuals $e_i = (E_{\text{cell, exp, }i} - E_{\text{cell, calc, }i})$ are minimized. Low values of the Hamilton R-factor reaching 0.1 or 0.2% prove an excellent fitness of calculated regression curve through experimental points. Fig. 2 shows the resulting dependence of the mixed dissociation constant pK_a of codeine on the square root of ionic strength.

Table 3 provides an estimate of the protonation constant of ethylmorphine determined at various values of ionic strength as a results of regression analysis with two mathematical approaches, by the program

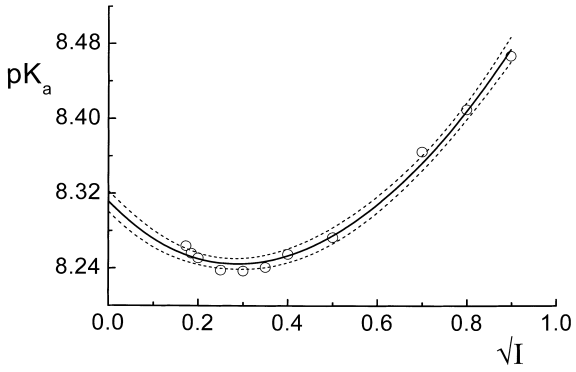

Fig. 2. Dependence of the mixed dissociation constant pK_a of codeine on the square root of ionic strength, which leads to parameter estimates pK_a^T=8.312(7), $\mathring{a}$=4(1) [10^{-8} m] and C=0.45(4) at 25°C (PLUS).

ESAB minimizing residuals $e_i = (v_{\text{exp, }i} - v_{\text{calc, }i})$ and by the program PLUS minimizing $e_i = (E_{\text{cell, exp, }i} - E_{\text{cell, calc, }i})$. Fig. 3 shows the resulting dependence of the mixed dissociation constant pK_a of ethylmorphine on the square root of ionic strength.

Table 4 provides results for the protonation constant of homatropine. Reliability criterion of protonation constant used for ESAB is the mean of absolute values of residuals $E\,|\hat{e}|$ reaching 0.2 or 0.3 μl. For PLUS the Hamilton R-factor proves a good fitness

Table 3
Concentration (pK_c) and mixed dissociation (pK_a) constants of ethylmorphine estimated by nonlinear regression programs ESAB and PLUS[a]

I	ESAB			PLUS		
	pK_c	pK_a	$\lvert\hat{e}\rvert$ (μl)	pK_c	pK_a	R (%)
0.0298	8.158 (2)	8.099	0.2	8.178 (8)	8.120	0.158
0.0342	8.104 (2)	8.044*	0.2	8.185 (5)	8.124	0.099
0.0400	8.150 (3)	8.077	0.3	8.186 (8)	8.121	0.163
0.0625	8.144 (2)	8.072	0.2	8.198 (9)	8.127	0.166
0.0900	8.176 (2)	8.098	0.2	8.199 (1)	8.122	0.241
0.1225	8.182 (3)	8.102	0.3	8.209 (19)	8.129	0.136
0.1600	8.217 (2)	8.136	0.3	8.228 (8)	8.147	0.144
0.2500	8.249 (3)	8.172	0.2	8.259 (8)	8.182	0.120
0.3600	8.319 (2)	8.252	0.2	8.316 (16)	8.249	0.300
0.4900	8.363 (2)	8.309	0.3	8.355 (8)	8.302	0.142
0.6400	8.422 (3)	8.387	0.2	8.418 (9)	8.383	0.144
0.8100	8.477 (3)	8.463	0.2	8.468 (7)	8.454	0.084

[a] Standard deviation of parameter estimates in last valid digits is in brackets. Values denoted with asterisk are influential points (outliers) and excluded from following regression analysis.

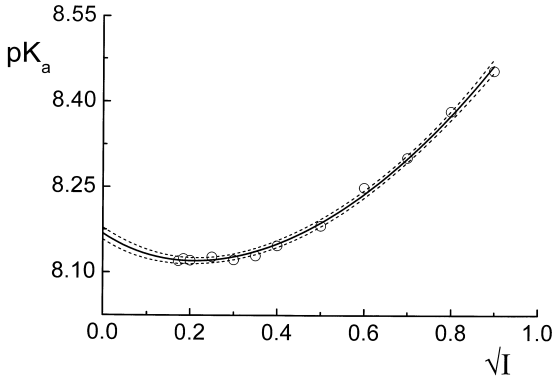

Fig. 3. Dependence of the mixed dissociation constant pK_a of ethylmorphine on the square root of ionic strength, which leads to parameter estimates pK_a^T=8.17(1), $\mathring{a}$=8(2) [10^{-8} m] and C=0.54(3) at 25°C (PLUS).

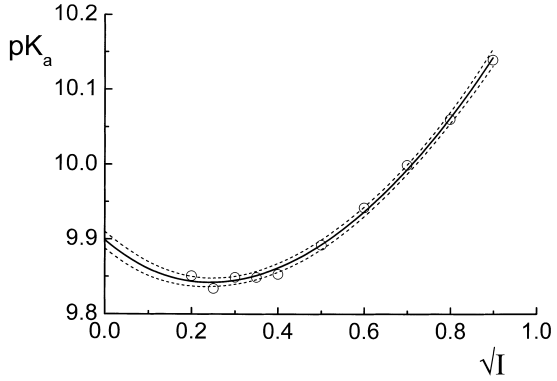

Fig. 4. Dependence of the mixed dissociation constant pK_a of homatropine on the square root of ionic strength, which leads to parameter estimates pK_a^T=9.90(1), $\mathring{a}$=6(2) [10^{-8} m] and C=0.51(3) at 25°C (ESAB).

achieved and therefore reliable estimates of protonation constants. Fig. 4 shows the resulting dependence of the mixed dissociation constant pK_a of homatropine on the square root of ionic strength.

4.2. Estimation of thermodynamic dissociation constant

Applying an extended Debye–Hückel, Eq. (7) on data from Tables 2–4 according to a regression criterion (8), three unknown parameters pK_a^T, $\mathring{a}$, and C have been estimated.

Table 4
Concentration (pK_c) and mixed dissociation (pK_a) constants of homatropine estimated by nonlinear regression programs ESAB and PLUS[a]

I	ESAB			PLUS		
	pK_c	pK_a	$\lvert\hat{e}\rvert$ (μl)	pK_c	pK_a	R (%)
0.0298	9.944 (3)	9.886*	0.2	9.859 (18)	9.801	0.258
0.0342	9.555 (3)	9.894*	0.2	9.860 (12)	9.799	0.182
0.0400	9.916 (4)	9.851	0.2	9.861 (11)	9.796	0.172
0.0625	9.906 (3)	9.834	0.2	9.853 (6)	9.781	0.097
0.0900	9.927 (2)	9.849	0.3	9.864 (12)	9.786	0.221
0.1225	9.917 (2)	9.849	0.3	9.863 (10)	9.783	0.086
0.1600	9.934 (1)	9.853	0.2	9.869 (8)	9.788	0.139
0.2500	9.985 (1)	9.892	0.3	9.897 (6)	9.819	0.092
0.3600	10.009 (2)	9.942	0.3	9.935 (14)	9.868	0.351
0.4900	10.052 (1)	9.999	0.2	9.958 (1)	9.905	0.091
0.6400	10.095 (1)	10.060	0.2	10.038 (7)	10.003	0.122
0.8100	10.153 (1)	10.139	0.2	10.066 (9)	10.052	0.154

[a] Standard deviation of parameter estimates in last valid digits is in brackets. Values denoted with asterisk are influential points (outliers) and excluded from a regression analysis.

Table 5 provides point estimates, calculated standard deviations of each parameter, and the absolute and relative biases obtained when the minimization process terminates. Two parameters, pK_a^T=8.166 and C=0.54, are estimated with very small bias 0.005 and 0.3%, and with small standard deviation s(pK_a^T)=0.007 and $s(C)$=0.027. This means that their estimation is quite reliable. The ion-size parameter $\mathring{a}$=7.6 [10^{-8} m] has a larger bias, about 3.5%, and a higher value of the standard deviation $s(\mathring{a})$=1.9 [10^{-8} m]. Linear parameters pK_a^T, and C in the regression model are well-conditioned and their estimation is sensitive. They have a strong influence on the residual-sum-of-squares function U. The nonlinear parameter $\mathring{a}$, being ill-conditioned in the regression model, has little influence on the residual-sum-of-squares function U and makes its numerical determination rather uncertain. Well-conditioned parameters pK_a^T, and C have great influence on an elliptic hyperparaboloid shape when variable U(pK_a^T, $\mathring{a}$, C) is plotted against the three parameters pK_a^T, $\mathring{a}$, C in (m+1)-dimensional space (here m=3). For well-conditioned parameters, such a shape exhibits an obvious, sharp minimum, the pit point. The ill-conditioned parameter $\mathring{a}$ leads to 'flat-bottomed-saucer shape' of hyperparaboloid U(pK_a^T, $\mathring{a}$, C) with no obvious minimum. Parameter $\mathring{a}$ with its larger standard deviation indicates that $\mathring{a}$ is

Table 5
ADSTAT refinement of the thermodynamic dissociation constant pK_a^T and parameters of extended Debye-Hückel equation $å$ and C for ethylmorphine at 25°C. Data are in Table 3 (PLUS)

Parameter	Point estimate	Standard deviation	Absolute Bias	Relative bias [%]		
(a) Point estimates of parameters						
pK_a^T	8.1660	0.00725	−0.0004	−0.005		
$å$	7.6249	1.9273	0.2659	3.487		
C	0.5399	0.0273	0.0017	0.307		
(b) Statistical analysis of residuals and indication of influential points (outliers)						
Point	$pK_{a,exp}$	$pK_{a,calc}$	$s(pK_{a,calc})$	Classical residual, e	Jackknife residual, e_J	Likelihood distance, LD_i
1	8.120	8.121	0.004	−0.001	−0.147	0.007
2	8.124	8.120	0.004	0.004	0.718	0.014
3	8.121	8.120	0.003	0.002	0.270	0.008
4	8.127	8.121	0.003	0.005	0.815	0.014
5	8.122	8.127	0.003	−0.006	−0.835	0.013
6	8.129	8.137	0.003	−0.008	−1.283	0.042
7	8.147	8.150	0.003	−0.003	−0.444	0.008
8	8.182	8.188	0.003	−0.006	−0.990	0.020
9	8.249	8.238	0.003	0.011	2.095	0.316
10	8.302	8.301	0.003	0.001	0.210	0.008
11	8.383	8.376	0.004	0.008	1.355	0.055
12	8.454	8.462	0.006	−0.008	−2.577	0.047

Reliability of parameters estimates as demonstrated by a statistical analysis of residuals	
Bias, $E(\hat{e})$	−0.0010
Mean of absolute values of residuals, $E\lvert\hat{e}\rvert$	0.0053
Variance, $s^2(\hat{e})$	4.9640E-05
Standard deviation, $s(\hat{e})$	0.0070
Skewness, $g_1(\hat{e})$	0.20 (not differing from 0)
Kurtosis, $g_2(\hat{e})$	1.86 (not differing from 3)
Residuals sum of squares, $U(\boldsymbol{b})$	4.4676E-04
Jarque–Berra normality test of a residuals	Normality accepted
Regression rabat, $100D$	99.70%
Akaike information criterion, AIC	−116.38
Hamilton R-factor of relative fitness	0.074%

ill-conditioned in model and therefore its determination is rather uncertain.

Table 5 provides the fitness of the calculated regression curve and an indication of influential points, outliers. Even though points no. 9 and 12 are suspicious, they are still not outliers because of both criteria, $\hat{e}_{J,9} < 3$ and $\hat{e}_{J,12} < 3$, $LD_9 < 5.992$ and $LD_{12} < 5.992$. The reliability of pK_a^T, $\mathring{a}$, and C estimates is proven by a goodness-of-fit test made here by a statistical analysis of classical residuals. As $s(pK_{a,i})$ oscillates from 0.002 to 0.003 (estimated by ESAB) and from 0.005 to 0.019 (estimated by PLUS), and reaches $E\left|\hat{e}\right| = 0.005$ and $s\left|\hat{e}\right| = 0.007$ what means that they are statistically of the same magnitude. Residuals exhibit symmetric, normal or rectangular distributions as the skewness, $g_1(\hat{e}) = 0.20$, does not significantly differ from 0, indicating a symmetric distribution, and the kurtosis, $g_2(\hat{e}) = 1.86$, also does not significantly differ from 3, indicating a normal distribution. The regression rabat, $100D$=99.70%, indicates that all points fulfill the regression model proposed with parameter estimates found. With the use of the Akaike information criterion, AIC=−116.38, several plausible regression models were examined but the model (7) gave the lowest value of AIC. The Hamilton R-factor proves an excellent fitness achieved and therefore reliable estimates of the parameters. Figs. 2–4 provide a graphical presentation of the dependence of the mixed dissociation constant on the square root of ionic strength.

5. Conclusions

The reliability of dissociation constants of three drug acids, (codeine, ethylmorphine and homatropine) should be proven when some parameters are ill-conditioned in model. Three *group parameters* $E^{0'}$, L_0, H_T were found to be ill-conditioned in model. Their determination is uncertain and might lead to false estimates of *common parameters* pK_a and therefore make the computational strategy important. These group parameters can have great influence on a systematic error in the estimated pK_a and they should be refined together with common parameters pK_a. Internal calibration of $[H^+]$ of the glass electrode cell performed during titration is more accurate than an external calibration of a_{H+}. Comparing two computational approaches, ESAB and PLUS programs, ESAB led to better fitness of potentiometric titration curve. The thermodynamic dissociation constant pK_a^T, an ill-conditioned ion-size parameter $\mathring{a}$ and the salting-out coefficient C were estimated by a nonlinear regression of a dependence of the mixed dissociation constant pK_a on an ionic strength I. Goodness-of-fit proved sufficient reliability of parameter estimates for three drugs at 25°C: for codeine pK_a^T=8.31±0.01, $\mathring{a}$=4±1 [10^{-8} m], C=0.45±0.04; for ethylmorphine pK_a^T=8.17±0.01, $\mathring{a}$=8±2 [10^{-8} m], C=0.54±0.03; and for homatropine pK_a^T=9.90±0.01, $\mathring{a}$=6±2 [10^{-8} m], C=0.51±0.03.

Acknowledgements

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