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The thermodynamic dissociation constants of four non-steroidal anti-inflammatory drugs by the least-squares nonlinear regression of multiwavelength spectrophotometric pH-titration data

Milan Meloun [a,*], Sylva Bordovská [a], Lubomír Galla [b]

[a] *Department of Analytical Chemistry, University of Pardubice, CZ 532 10 Pardubice, Czech Republic*
[b] *Bochemie Group, Herbacos Bofarma Ltd., Štrossova 239, CZ 530 02 Pardubice, Czech Republic*

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Abstract

The mixed dissociation constants of four non-steroidal anti-inflammatory drugs (NSAIDs) ibuprofen, diclofenac sodium, flurbiprofen and ketoprofen at various ionic strengths I of range 0.003–0.155, and at temperatures of 25 °C and 37 °C, were determined with the use of two different multiwavelength and multivariate treatments of spectral data, SPECFIT/32 and SQUAD(84) nonlinear regression analyses and INDICES factor analysis. The factor analysis in the INDICES program predicts the correct number of components, and even the presence of minor ones, when the data quality is high and the instrumental error is known. The thermodynamic dissociation constant pK_a^T was estimated by nonlinear regression of (pK_a, I) data at 25 °C and 37 °C. Goodness-of-fit tests for various regression diagnostics enabled the reliability of the parameter estimates found to be proven. PALLAS, MARVIN, SPARC, ACD/pK_a and Pharma Algorithms predict pK_a being based on the structural formulae of drug compounds in agreement with the experimental value. The best agreement seems to be between the ACD/pK_a program and experimentally found values and with SPARC. PALLAS and MARVIN predicted $pK_{a,pred}$ values with larger bias errors in comparison with the experimental value for all four drugs.

Keywords: Spectrophotometric titration; Dissociation constant; Protonation; Non-steroidal anti-inflammatory drugs (NSAIDs); Ibuprofen; Diclofenac sodium; Flurbiprofen; Ketoprofen; SPECFIT; SQUAD; INDICES; PALLAS; MARVIN; SPARC; ACD/pK_a; Pharma Algorithms

1. Introduction

Many drug compounds contain at least one acidic and/or basic functionality, and the ionization state of these groups plays an important role in determining the physicochemical properties of a compound. A number of approaches have been used to determine the pK_a values of organic molecules, including titrations based on potentiometry, spectrophotometry, solubility and liquid–liquid partitioning. Traditional potentiometric and spectrophotometric titrations, however, are still outstanding as the two most widely used techniques, because they are fast, accurate and reproducible [1].

1.1. Project aims

Since most drugs, and particularly the anti-inflammatories studied here, are sparingly soluble in water, the literature pK_a values were very often determined potentiometrically in mixtures of water and an organic solvent, to obtain a suitable solubility. Ràfols et al. [2] have published the dissociation constants of several non-steroidal anti-inflammatories, such as ibuprofen, diclofenac, flurbiprofen, ketoprofen and others, in a series of isopropyl alcohol–water mixtures. The organic solvents frequently used are methanol, ethanol, propanol, DMSO, DMFA, acetone and THF. Since the most literature data have been accumulated for MeOH–water solvent mixtures, and since it is generally accepted that methanol displays the solvatation effect closest to water, methanol is normally the organic solvent of choice, as noted in Ref. [3] and the references cited therein. The pK_a values have been successfully fitted to a general equation derived to explain the variation of the dissociation

* Corresponding author. Tel.: +420 466037026; fax: +420 466037068.
E-mail address: milan.meloun@upce.cz (M. Meloun).

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constant of an acid with solvent composition in binary solvent mixtures. The parameters of the regression model obtained allow us to study the effect of preferential solvation of the drugs on the pK_a values, and to estimate the aqueous pK_a value from pK_a values obtained in binary solvents of different composition.

Traditional potentiometric titrations, and even potentiometric mixed-solvent procedures, are typically limited to the analysis of compounds with a water solubility as low as 10^{-4} M, while spectrophotometry is convenient for pK_a determination in very dilute aqueous solutions (as low as 10^{-5} to 10^{-6} M); while the latter is the best tool it requires that the chromophore be in proximity to the ionization center to ensure sufficient spectral dissimilarities between the protonated and unprotonated forms of the drug [1]. When the components involved in the protonation equilibrium have distinct spectral responses their concentrations can be measured directly, and determination of the protonation constant is simple.

In previous work [4–24] the authors have shown that the spectrophotometric method, in combination with suitable chemometric tools, can be used for the determination of protonation constants β_{qr} or acid dissociation constants pK_a even for barely soluble drugs, and a tutorial is available in Ref. [25]. The hard modelling methods described include traditional least-squares curve fitting approaches, based on a previous postulation of a chemical model, i.e. the postulation of a set of species defined by their stoichiometric coefficients and formation constants, which are then refined by least-squares minimization. These mathematical procedures require the fulfilment of the mass-balance equations and the mass-action law. Among the most relevant algorithms is the SQUAD program [26–31]. On the other hand, soft modelling techniques such as SPECFIT [32–35] include multivariate curve resolution methods which are based on factor analysis, work without any assumption of a chemical model, and do not have the requirement of compliance with the mass-action law.

In this study, a critical comparative evaluation of both approaches, the experimental determination of pK_a and the computational prediction of p$K_{a,pred}$ on the basis of molecular structure have also been applied to series of four anti-inflammatory drugs which are most sparingly soluble in water, and for each of which the literature shows a dispersion of the aqueous pK_a values. Concurrently, the spectrophotometric determination of protonation constants was combined with a completely different approach based on the analysis of the chemical structure relative to a specific reactivity query. The procedure used in the PALLAS [36], MARVIN [37], SPARC [38], ACD/pK_a [39] and Pharma Algorithms [79] programs use algorithms predicting pK_a based on fundamental chemical structure theory. Due to their fragment-based approach, however, they are inadequate when the fragments present in the molecule under study are absent from the database. It is valid that such pK_a prediction only depends on the compounds very similar to those available in the training set. The MARVIN software developed by ChemAxon [37] and PALLAS software [36] are free of charge for academic use, and therefore, they are in an academic setting preferable to the advanced commercial software of ACD/Labs, provided that performance is also satisfactory. While the ACD/pK_a module [39] uses fragment methods to build a large number of equations with experimental or calculated electronic constants to predict pK_a values, the SPARC applies a mechanistic perturbation method to estimate the pK_a through a number of models that account for electronic effects, solvation effects, hydrogen bonding effects, and the influence of temperature. The user needs to know only the molecular structure of the compound to predict the property of interest. The REGDIA [40] regression diagnostics algorithm has previously been introduced to examine the accuracy of pK_a predicted with the four updated programs.

1.2. Non-steroidal anti-inflammatory drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs), and namely ibuprofen, diclofenac sodium, flurbiprofen and ketoprofen, are drugs with analgesic, antipyretic and anti-inflammatory effects [41,42]. They reduce pain, fever and inflammation. The term "non-steroidal" is used to distinguish these drugs from steroids, which have a similar eicosanoid-depressing, anti-inflammatory action. As analgesics, NSAIDs are unusual in that they are non-narcotic. NSAIDs are sometimes also referred to as non-steroidal anti-inflammatory agents/analgesics (NSAIAs). Since 1829 and the isolation of salicin from the folk remedy willow bark, NSAIDs have become an important part of the pharmaceutical treatment of pain and inflammation [43,44].

Most NSAIDs act as non-selective inhibitors of the enzyme cyclooxygenase, inhibiting both the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) isoenzymes. Cyclooxygenase catalyses the formation of prostaglandins and thromboxane from arachidonic acid (itself derived from the cellular phospholipid bilayer by phospholipase A_2). Prostaglandis act (amongst other things) as messenger molecules in the process of inflammation [42–46]. Most NSAIDs are weak acids, with pK_a of 3–5. They are absorbed well from the stomach and intestinal mucosa. They are highly protein-bound in plasma, usually to albumin, so that their volume of distribution typically approximates to the plasma volume. Most NSAIDs are metabolised in the liver by oxidation and conjugation to inactive metabolites, which are typically excreted in the urine, although some drugs are partially excreted in bile. Metabolism may be abnormal in certain disease states, and accumulation may occur even with normal dosage [47–50].

Recently Avdeef and coworkers have, in a series of papers about pK_a determination, summarized their work on the development and application of a new, automated analyzer for pK_a determination based on potentiometry [3–5]. A new, "four parameter" calibration approach has been proposed which provides very reliable pK_a determination in MeOH–water (and other) solvent systems. The precision of pK_a determination using the Yasuda–Shedlovsky extrapolation method has been presented for some examples and some extrapolated values of pK_a in zero organic solvent(%) for four anti-inflammantory drugs are shown in Table 1. This table also compares the potentiometrically measured pK_a values in an organic solvent–water mixture with the values determined by various instrumental methods.

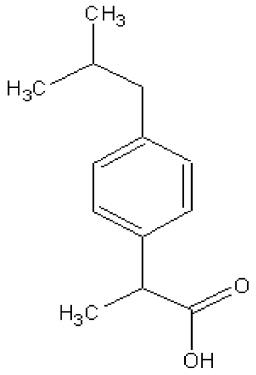
Table 1
Survey pK_a values of four non-steroidal anti-inflammatory drugs NSAIDs, i.e. ibuprofen, diclofenac sodium, flurbiprofen and ketoprofen found in the literature with the use of the extrapolated value of pK_a in pure water coming from a potentiometric mixed-solvent procedure and other instrumental methods such as spectrophotometry, capillary electrophoresis, the VAMCE technique (vacuum-assisted multiplexed capillary electrophoresis with ultraviolet detection) and capillary zone electrophoresis.

	Ibuprofen	Diclofenac sodium	Flurbiprofen	Ketoprofen
Ruiz et al. [52]	[b]4.54 (4), methanol/H_2O = 0/100	[b]4.19 (5), methanol/ H_2O = 0/100	[b]4.24, methanol/H_2O = 0/100	–
Oumada et al. [53]	[b]4.40, methanol/H_2O = 0/100	[b]4.05, methanol/H_2O = 0/100	[b]4.24, methanol/H_2O = 0/100	–
González et al. [54]	[b]4.55, *N*, *N*-dimethylformamid/H_2O = 0/100	–	–	[b]4.47, *N*, *N*-dimethylformamid/H_2O = 0/100
Ráfols et al. [55]	[b]4.30, butanol/H_2O = 0/100	[b]3.97, butanol/H_2O = 0/100	[b]4.13, butanol/H_2O = 0/100	[b]4.09, butanol/H_2O = 0/100
	[b]4.52, propanol/H_2O = 0/100	[b]4.16, propanol/H_2O = 0/100	[b]4.35, propanol/H_2O = 0/100	[b]4.36, propanol/H_2O = 0/100
Ráfols et al. [2]	[b]4.64, isopropylalcohol/H_2O = 0/100	[b]4.17, isopropylalcohol/H_2O = 0/100	[b]4.36, isopropylalcohol/H_2O = 0/100	[b]4.32, isopropylalcohol/H_2O = 0/100
Fini et al. [56]	[b]4.61, DMSO/H_2O = 0/100	[b]3.80, DMSO/H_2O = 0/100	[b]4.27, DMSO/H_2O = 0/1000	[b]4.45, DMSO/H_2O = 0/100
Herrador et al. [75]	–	–	–	[b]4.41, ACN/H_2O = 0/100
Tam et al. [57]	[a]4.24 (3), spectrophotometry	–	–	–
Takács-Novák et al. [4]	[b]4.51 (7), methanol/H_2O = 0/100	–	–	–
Pignatello et al. [62]	–	–	[b]4.27, ethanol/H_2O = 0/100	–
Somasundaram et al. [42]	–	–	[b]4.2, ethanol/H_2O = 0/100	–
Geiser et al. [76]	–	–	–	[a]4.22, capillary zone electrophoresis
Bouchard et al. [58]	[a]4.31, potentiometry, H_2O.	–	[a]4.21, potentiometry, H_2O	[a]4.25, potentiometry, H_2O
Avdeef et al. [3]	[b]4.31 (4), ACN/H_2O = 0/100	–	–	–
	[b]4.30 (5), dimethylformamide/H_2O = 0/100			
	[b]4.35 (3), DMSO/H_2O = 0/100			
	[b]4.33 (1), ethanol/H_2O = 0/100			
	[b]4.45 (4), methanol/H_2O = 0/100			
Zhou et al. [78]	–	[a]3.95, VAMCE technique	[a]3.82, VAMCE technique	[a]4.06, VAMCE technique
Poole et al. [77]	[a]4.14, capilary electrophoresis	–	–	[a]4.02, Capillary electrophoresis

The standard deviations of the pK_a in the last valid digits are in parentheses. The (0/100) value indicates the extrapolated pK_a value corresponding to pK_a in pure water.
[a] Means the activity scale.
[b] Means the concentration scale.

Avdeef [4] has established that Yasuda–Shedlovsky extrapolations for values wt.% up to 60 give bias errors in pK_a not greater than ±0.2 for weak acids and ±0.1 for weak bases.

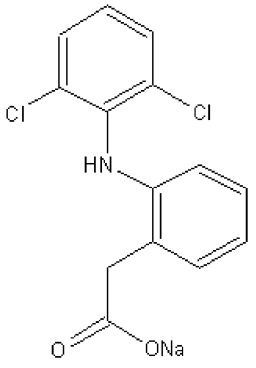
Ibuprofen (chemically 2-(4-isobutylphenyl)propionic acid, CAS No.: 15687-27-1, molecular formula: $C_{13}H_{18}O_2$, molecular weight: 206.3, description: a white, crystalline powder, or colourless crystals, solubility: practically insoluble in water, freely soluble in acetone, methanol and methylene chloride) is of the structure



Ibuprofen

and is a non-steroidal anti-inflammatory drug (NSAID) with analgesic and antipyretic properties. Ibuprofen has pharmacological actions similar to those of other prototypical NSAIAs, and is thought to be associated with the inhibition of prostaglandin synthesis [2,50,51]. To compare the pK_a values obtained from the potentiometric mixed/solvent procedures determined by various authors in Table 1 with those pK_a values measured by another methods, e.g. spectroscopy, even though the experimental conditions in the methods (concentration, temperature, and ionic strength) may differ considerably, leads to a sample, and the interval estimate of this sample mean 4.40 ± 0.10 gives a quite narrow range, thereby proving a quite good agreement of all the sample values.

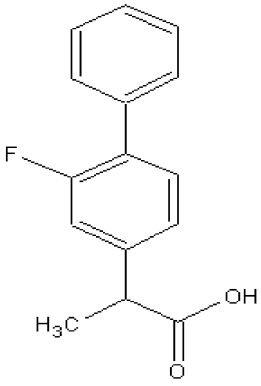
Diclofenac sodium (chemically 2-((2,6-dichlorophenyl) amino)-benzeneacetic acid monosodium salt, CAS No.: 15307-79-6, molecular formula: $C_{14}H_{10}Cl_2NNaO_2$, molecular weight: 318.14, description: a white or slightly yellowish crystalline powder, slightly hydroscopic, solubility: practically insoluble in water, free soluble in methanol, soluble in alcohol) is of the structure



Diclofenac sodium

and is an antipyretic, analgesic and well-tolerated non-steroidal anti-inflammatory agent with potent activity in the treatment of rheumatic diseases. As with other NSAIDs, its mode of action is not known; its ability to inhibit prostaglandin synthesis, however, may be involved in its anti-inflammatory activity, as well as contributing to its efficacy in relieving pain related to inflammation [2,59–61]. A comparison of the pK_a values obtained by various authors in Table 1 leads to a sample and the interval estimate of the sample mean 4.05 ± 0.14 proving a good agreement of all sample values.

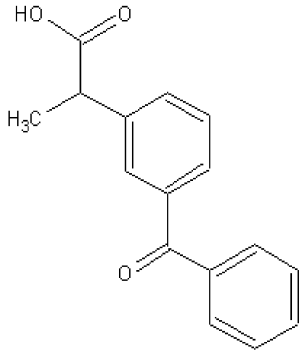
Flurbiprofen (chemically (±)-2-fluoro-α-methyl-4-biphenylacetic acid, CAS No.: 5104-49-4, molecular formula: $C_{15}H_{13}FO_2$, molecular weight: 244.26, description: is a white or slightly yellow crystalline powder, solubility: slightly soluble in water at pH 7.0 and readily soluble in most polar solvents) is of the structure



Flurbiprofen

and is an important NSAID which belong to the 2-arylpropionic acid class known as profens [41,62–64]. A comparison of the pK_a values obtained by various authors in Table 1 leads to a sample and the interval estimate of the sample mean 4.25 ± 0.10 proving a good agreement of all sample values.

Ketoprofen (chemically (*RS*)-2-(3-benzoylphenyl)propionic acid, CAS No.: 22071-15-4, molecular formula: $C_{16}H_{14}O_3$, molecular weight: 254.29, description: a white, crystalline powder, odourless or almost odourless, solubility: practically insoluble in water, freely soluble in acetone, alcohol and methylene chloride) is of the structure



Ketoprofen

and is a non-steroidal anti-inflammatory drug (NSAID) with analgesic and antipyretik properties [2,65–67]. A comparison of the pK_a values obtained by various authors in Table 1 leads to a

sample and the interval estimate of the sample mean 4.30 ± 0.12 proving a good agreement of all sample values.

2. Theoretical

2.1. Procedure for the determination of the mixed protonation/dissociation constants

An acid–base equilibrium of the drug studied is described in terms of the protonation of the Brönstedt base L^{z-1} according to the equation $L^{z-1} + H^+ \rightleftarrows HL^z$ 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:

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

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

The subscript to K_H indicates the ordinal number of the protonation step. The protonation equilibria between the anion L (the charges are omitted for the sake of simplicity) of a drug and a proton H are considered to form a set of variously protonated species L, LH, LH_2, LH_3, etc., which have the general formula L_qH_r in a particular chemical model and which are represented by n_c the number of species $(q, r)_i$, $i = 1, ..., n_c$ where index i labels their particular stoichiometry; the overall protonation (stability) constant of the protonated species, β_{qr}, may then be expressed as

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

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

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

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

$$A_{i,j} = \sum_{n=1}^{n_c} \varepsilon_{j,n} c_n = \sum_{n=1}^{p} (\varepsilon_{qr,j} b_{qr} l^q h^r)_n$$

where $\varepsilon_{qr,j}$ is the molar absorptivity of the L_qH_r species with the stoichiometric coefficients q, r measured at the jth wavelength [68]. The absorbance $A_{i,j}$ is an element of the absorbance matrix A of size (n_s H n_w) being measured for n_s solutions with known total concentrations of $n_z = 2$ basic components, c_L and c_H, at n_w wavelengths. Calculations related to the determination of the protonation constants may be performed by the regression analysis of spectra using versions of the SQUAD(84) program family [28] and SPECFIT/32 [35], and have been described previously [25]. The experimental and computational schemes for the determination of the protonation constants of the multicomponent system are taken from Meloun et al. [25,68]. The details of the computer data treatment are collected in the Section 3.4.

2.2. Determination of the thermodynamic protonation/dissociation constant

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

$$pK_a = pK_a^T - \frac{A(1-2z)\sqrt{I}}{(1 + Ba\sqrt{I})} + CI$$

where $A = 0.5112\ mol^{-1/2}\,l^{1/2}\,K^{3/2}$ and $B = 0.3291\ mol^{-1/2}\,m^{-1}\,l^{1/2}\,K^{1/2}\,10^{10}$ for aqueous solutions at 25 °C. The mixed dissociation constant pK_a represents a dependent variable while the ionic strength I stand for the independent variable. Three unknown parameters $b = (pK_a, a, C)$ are to be estimated by a minimization of the sum of the squared residuals [69]:

$$U(b) = \sum_{i=1}^{n} w_i [pK_{a,exp,i} - pK_{a,calc,i}]^2$$

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

The nonlinear estimation problem is simply a problem of optimization in the parameter space, in which the pK_a and I are known and given values while the parameters pK_a^T, a, and C are unknown variables to be estimated [69].

2.3. Reliability of the estimated dissociation constants

The adequacy of a proposed regression model with experimental data and the reliability of parameter estimates $pK_{a,i}$ found, being denoted for the sake of simplicity as b_j, and ε_{ij}, $j = 1, ..., m$, may be examined by a goodness-of-fit test, *cf.* a previous tutorial [25].

3. Experimental

3.1. Chemicals and solutions

Ibuprofen was purchased from Hubei Biocause Pharmaceutical Co. Ltd., with a purity of 100.2%. Diclofenac sodium was purchased from Amoli Organics Pvt. Ltd., with a purity of 100.01%. Flurbiprofen was purchased from Sigma–Aldrich Co., with a purity of 99%. Ketoprofen was purchased from Societa Italiana Medicinali Scandicci, with a purity of 99.7%. Perchloric acid, 1 M, was prepared from conc. $HClO_4$ (p.a., Lachema

Brno) using redestilled water and standardized against HgO and NaI with reproducibility of less than 0.20%. Sodium hydroxide, 1 M, was prepared from pellets (p.a., Aldrich Chemical Company) with carbondioxide free redistilled water and standardized against a solution of potassium hydrogen-phthalate using the Gran Method with a reproducibility of 0.1%. Mercuric oxide, sodium iodide, and sodium perchlorate (p.a. Lachema Brno) were not further purified. The preparation of other solutions from analytical reagent-grade chemicals has been described previously [25].

3.2. Apparatus and pH–spectrophotometric titration procedure

The apparatus used and the pH–spectrophotometric titration procedures have been described previously [25].

3.3. Software used

Computations relating to the determination of dissociation constants were performed by regression analysis of the UV-vis spectra using the SQUAD(84) [28] and SPECFIT/32 [35] programs. Most of graphs were plotted using ORIGIN 7.5 [70] and S-Plus [71]. The thermodynamic dissociation constant pK_a^T was estimated with the MINOPT nonlinear regression program in the ADSTAT statistical system (TriloByte Statistical Software Ltd., Czech Republic) [72]. A qualitative interpretation of the spectra with the use of the INDICES program [73] aimed to evaluate the quality of the dataset and remove spurious data, and to estimate the minimum number of factors, i.e. contributing aqueous species, which are necessary to describe the experimental data and determine the number of dominant species present in the equilibrium mixture. PALLAS, MARVIN [36,37], SPARC [38], ACD/pK_a [39] and Pharma Algorithms [79] are programs for making predictions based on the structural formulae of drug compounds. Entering the compound topological structure descriptors graphically, pK_a values of organic compound are predicted using approximately hundreds of Hammett and Taft equations and quantum chemistry calculus.

3.4. Supporting information available

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

4. Results and discussion

4.1. Estimation of dissociation constants

The 3D absorbance-response-surfaces representing the measured multiwavelength absorption spectra of four NSAID drugs: (a) ibuprofen, (b) diclofenac sodium, (c) flurbiprofen and (d) ketoprofen in dependence on pH at 25 °C are plotted in Fig. 1 and represent the input data of the regression programs SQUAD and SPECFIT.

Ibuprofen: the nonlinear regression analysis of spectra starts with data smoothing followed by a factor analysis using the INDICES program. The experimental spectra are obtained for the titration of an alkaline 5.54×10^{-5} M ibuprofen solution by a standard solution of 1 M HCl (or $HClO_4$) to adjust pH value. A comparison of both the SQUAD and SPECFIT regression program treatments, along with the proposed strategy for efficient experimentation in protonation constant determi-

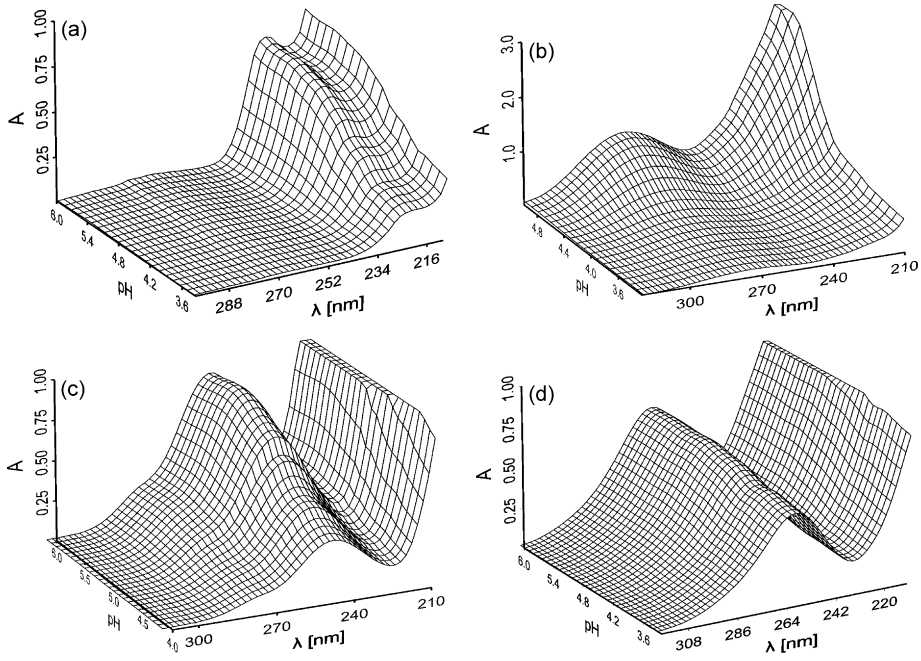


Fig. 1. The 3D absorbance response-surface representing the measured multiwavelength absorption spectra of four NSAID drugs: (a) ibuprofen, (b) diclofenac sodium, (c) flurbiprofen and (d) ketoprofen in dependence on pH at 25 °C (S-Plus).

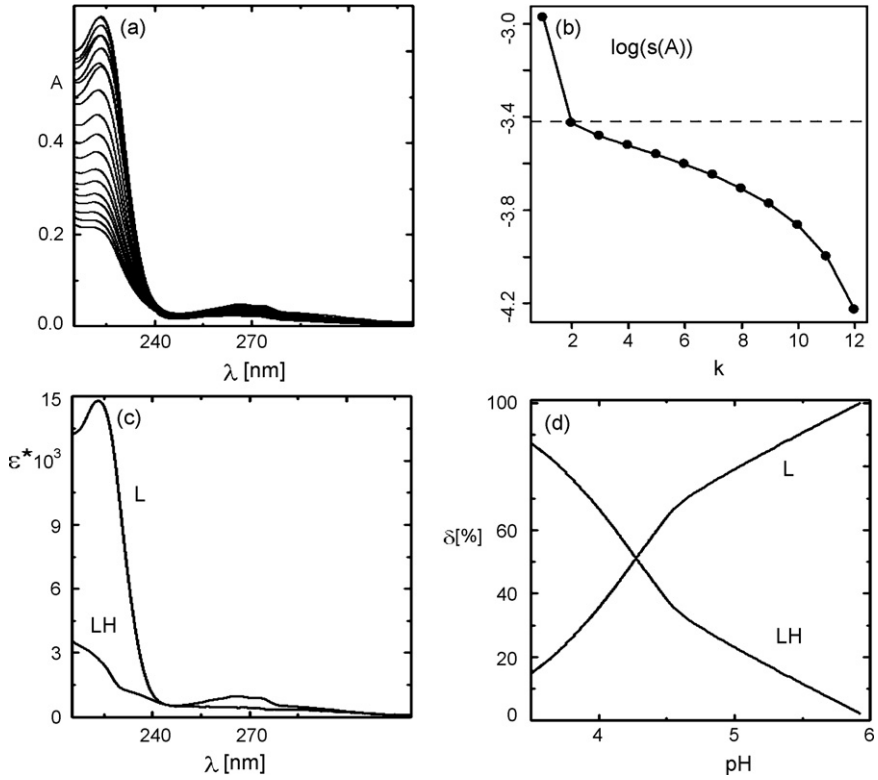


Fig. 2. The nonlinear regression analysis of the protonation equilibria model and factor analysis of ibuprofen: (a) absorption spectra in dependence on pH at 25 °C, (b) Cattel's scree plot of the Wernimont-Kankare procedure for determination of the number of light-absorbing species in the mixture $k^*=2$ leads to the actual instrumental error of the spectrophotometer used $s_{inst}(A)=0.38$ mAU (INDICES in S-Plus), (c) pure spectra profiles of molar absorptivities vs. wavelengths for species L and LH and (d) distribution diagram of the relative concentrations of species L and LH of ibuprofen in dependence on pH at 25 °C (SPECFIT, SQUAD and ORIGIN).

nation, is presented. pH-spectrophotometric titration enables the absorbance-response-surface data in Fig. 1 to be obtained for analysis with nonlinear regression. Even though the actual SQUAD version used has a limited dimension and input can contain 20 spectra only, an efficient spectra sample 20×39 ($n_s \times n_w$) was used (Fig. 2a) for regression analysis. As the changes in spectra are small within deprotonation in the range 240–300 nm, however, both of the variously protonated species L and LH exhibit partly similar absorption bands. When such small changes in absorbance spectra are available, a very precise measurement of absorbance is then neccessary for the reliable detection of the deprotonation equilibrium studied. In the first step of the spectra analysis, the number of light-absorbing species was estimated using the INDICES algorithm (Fig. 2b). The position of the break point on the $s_k(A)=f(k)$ curve in the factor analysis scree plot is indicated giving $k^*=2$ with the corresponding co-ordinate $\log(s_k^*(A))=-3.42$ which gives the value $s_k^*(A)=0.38$ mAU and which may also be taken here as the actual instrumental error $s_{inst}(A)$ of the spectrophotometer used. All of the other selected methods of modified factor analysis in the INDICES algorithm estimate the two light-absorbing components L and LH of the protonation equilibrium (where the ionic charges are omitted for sake of simplicity). The number of light-absorbing species p can be predicted from the index function values by finding the point $p=k$ where the slope of the index function $PC(k)=f(k)$ changes, or by comparing $PC(k)$ values to the instrumental error $s_{inst}(A)=0.38$ mAU. This is the common criterion for determining p. Very low values of $s_{inst}(A)$ prove that a sufficiently precise spectrophotometer and efficient experimental technique were used. The reliability of parameter estimates (pK's and ε's) can be evaluated on the basis of goodness-of-fit tests of the residuals.

The dissociation constant and two molar absorptivities of ibuprofen are estimated and refined by SQUAD(84) or SPECFIT/32 in the first run. The reliability of the regression parameter estimates may be tested using the following diagnostics:

- The first diagnostic indicates whether all of the parametric estimates β_{qr} (or pK_a) and ε_{qr} have physical meaning and reach realistic values for ibuprofen p$K_a=4.38$ ($s=0.03$) at 25 °C. As the standard deviations $s(\log \beta_{qr})$ of parameters $\log \beta_{qr}$ and $s(\varepsilon_{qr})$ of parameters ε_{qr} are significantly smaller than their corresponding parameter estimates, all the variously protonated species are statistically significant at a significance level of $\alpha=0.05$. The physical meaning of the protonation constant β_{qr}, molar absorptivities ε_{qr}, and stoichiometric indices q, r are examined. The absolute values of $s(\beta_j)$, $s(\varepsilon_j)$ gives information about the last U-contour of the hyperparaboloid in the neighbourhood of the pit, U_{min}. For well-conditioned parameters, the last U-contour is a regular ellipsoid, and the standard deviations $s(\beta_j)$ are reasonably low. High $s(\beta_j)$ values may be found with ill-conditioned param-

eters and a "saucer"-shaped pit. The relation $s(\beta_j)F_\sigma < \beta_j$ should be met where F_σ is equal to 3 for 99.9% statistical probability level. The set of standard deviations of ε_{qr} for various wavelengths, $s(\varepsilon_{qr}) = f(\lambda)$, should have a Gaussian distribution; otherwise, erroneous estimates of ε_{qr} are obtained. Fig. 2c shows the estimated molar absorptivities of all of the variously protonated species ε_L, ε_{LH} of ibuprofen dependence on wavelength are realistic.

- The second diagnostic tests whether all of the calculated free concentrations of the variously protonated species on the distribution diagram of the relative concentration expressed as a percentage have physical meaning, which proved to be the case (Fig. 2d). A distribution diagram makes it easier to judge the contributions of individual species to the total concentration quickly. Since the molar absorptivities will generally be in the range 10^3 to 10^5 l mol^{-1} cm^{-1}, species present at less than ca. 0.1% relative concentration will affect the absorbance significantly only if their ε is extremely high. The diagram shows the protonation equilibria of L and LH.
- The third diagnostic concerns the goodness-of-fit. The goodness-of-fit achieved is easily seen by examination of the differences between the experimental and calculated values of absorbance, $e_i = A_{exp,\,i,\,j} - A_{calc,\,i,\,j}$. Examination of the spectra and of the graph of the predicted absorbance response-surface through all the experimental points should reveal whether the results calculated are consistent, and whether any gross experimental errors have been made in the measurement of the spectra. One of the most important statistics calculated is the standard deviation of absorbance, $s(A)$, calculated from a set of refined parameters at the termination of the minimization process. This is usually compared to the standard deviation of absorbance calculated by the INDICES program

Table 2
Dependence of the mixed dissociation constants of four non-steroidal anti-inflammatory drugs on an ionic strength using regression analysis of pH-spectrophotometric data with SPECFIT and SQUAD, with the standard deviations of the parameter in the last valid digits in parentheses

(a) Ibuprofen

Estimated dissociation constants p$K_{a,1}$ at 25 °C							
Ionic strength	0.018	0.048	0.063	0.094	0.109	0.117	
p$K_{a,1}$	4.344(19)	4.287(26)	4.293(21)	4.321(21)	4.275(16)	4.322(33)	
$s(A)$ (mAU)	0.91	0.49	0.98	0.89	0.58	0.76	
Estimated dissociation constants p$K_{a,1}$ at 37 °C							
Ionic strength	0.003	0.003	0.018	0.064	0.079	0.086	0.124
p$K_{a,1}$	4.485(5)	4.503(8)	4.420(6)	4.320(8)	4.258(9)	4.278(16)	4.281(10)
$s(A)$ (mAU)	0.68	0.74	0.74	0.82	0.96	0.95	0.68

(b) Diclofenac sodium

Estimated dissociation constants p$K_{a,1}$ at 25 °C						
Ionic strength	0.003	0.048	0.079	0.094	0.117	0.155
p$K_{a,1}$	4.213(6)	4.261(3)	4.280(2)	4.307(2)	4.330(5)	4.414(1)
$s(A)$ (mAU)	0.53	0.65	0.93	0.84	0.34	0.55
Estimated dissociation constants p$K_{a,1}$ at 37 °C						
Ionic strength	0.003	0.018	0.034	0.048	0.071	0.109
p$K_{a,1}$	4.379(7)	4.479(5)	4.484(6)	4.580(5)	4.617(4)	4.677(4)
$s(A)$ (AU)	0.98	0.80	0.92	0.86	0.96	0.57

(c) Flurbiprofen

Estimated dissociation constants p$K_{a,1}$ at 25 °C							
Ionic strength	0.018	0.041	0.079	0.109	0.117	0.124	0.139
p$K_{a,1}$	4.227(11)	4.265(5)	4.358(8)	4.381(8)	4.451(5)	4.531(6)	4.521(6)
$s(A)$ (mAU)	0.41	2.25	0.83	0.97	2.27	0.66	0.97
Estimated dissociation constants p$K_{a,1}$ at 37 °C							
Ionic strength	0.003	0.026	0.048	0.049	0.079	0.094	
p$K_{a,1}$	4.373(6)	4.496(5)	4.508(5)	4.580(5)	4.729(4)	4.693(5)	
$s(A)$ (mAU)	0.80	0.94	1.00	0.89	0.71	0.97	

(d) Ketoprofen

Estimated dissociation constants p$K_{a,1}$ at 25 °C						
Ionic strength	0.033	0.048	0.064	0.079	0.117	0.155
p$K_{a,1}$	4.003(5)	4.005(5)	3.957(6)	3.948(9)	3.946(5)	3.974(7)
$s(A)$ (mAU)	0.85	0.76	0.81	0.89	0.74	0.81
Estimated dissociation constants p$K_{a,1}$ at 37 °C						
Ionic strength	0.033	0.064	0.078	0.094	0.109	0.140
p$K_{a,1}$	3.884(18)	3.848(5)	3.866(9)	3.922(11)	3.901(7)	3.941(8)
$s(A)$ (mAU)	0.88	0.72	0.92	0.87	0.93	0.94

[26], $s_k(A)$, and if it is valid that $s(A) \leq s_k(A)$, or $s(A) \leq s_{inst}(A)$, the instrumental error of the spectrophotometer used and the fit are considered to be statistically acceptable. Although this statistical analysis of the residuals [57,74] gives the most rigorous test of the degree-of-fit, realistic empirical limits must be used. After removal of outlying spectra, the statistical measures of all residuals e prove that the minimum of the eliptic hyperparaboloid U is reached: the residual standard deviation $s(e) = 0.69$ mAU (SQUAD(84)) or 0.58 mAU (SPECFIT32) always has sufficiently low values, below than 1 mAU proving a high reliability of parameter estimates. The estimated dissociation constant pK_a at two temperatures 25 °C and 37 °C in dependence on an ionic strength I is given in Table 2.

Diclofenac sodium: The experimental spectra are obtained for the titration of an alkaline 5.0×10^{-5} M diclofenac sodium solution by a standard solution of 1 M HCl (or $HClO_4$) to adjust pH value. pH-spectrophotometric titration enables absorbance-response data (Fig. 3a) to be obtained for analysis by nonlinear regression, and the reliability of parameter estimates (pK's and ε's) can be evaluated on the basis of the goodness-of-fit test of residuals. As the changes in absorbance spectra are significant within deprotonation, both of the variously protonated species L and LH exhibit sufficiently different absorption bands. The best region of the spectrum seems to be 240–330 nm and p$K_a = 4.24$ ($s = 0.02$). Most of the selected methods of factor analysis by the INDICES algorithm lead to two light-absorbing components in the equilibrium mixture (Fig. 3b). The position of the break point on the $s_k(A) = f(k)$ curve in the factor analysis scree plot is calculated and gives $k^* = 2$ with the corresponding co-ordinate log $(s_k^*(A)) = -3.25$ which gives the value $s_k^*(A) = 0.56$ mAU and which may also be taken here as the actual instrumental error $s_{inst}(A)$ of the spectrophotometer used. The goodnes-of-fit proves a sufficiently reliable estimates of the dissociation constant and molar absorption coefficient. Fig. 3c shows the curves of molar absorption coefficients of L and LH species in dependence on wavelength and Fig. 3d the distribution diagram of L and LH species in dependence on pH. The estimated dissociation constant pK_a at two temperatures 25 °C and 37 °C in dependence on an ionic strength I is in Table 2.

Flurbiprofen: The experimental spectra are obtained for the titration of an alkaline 4.4×10^{-5} M flurbiprofen solution by a standard solution of 1 M HCl (or $HClO_4$) to adjust pH value. A proposed strategy for efficient experimentation in dissociation constant determination followed by spectral data treatment is presented on the protonation equilibria of flurbiprofen (Fig. 4a). A part of the spectrum from 220 to 300 nm was selected, as the most convenient for an estimation of the protonation constant as there are sufficient changes

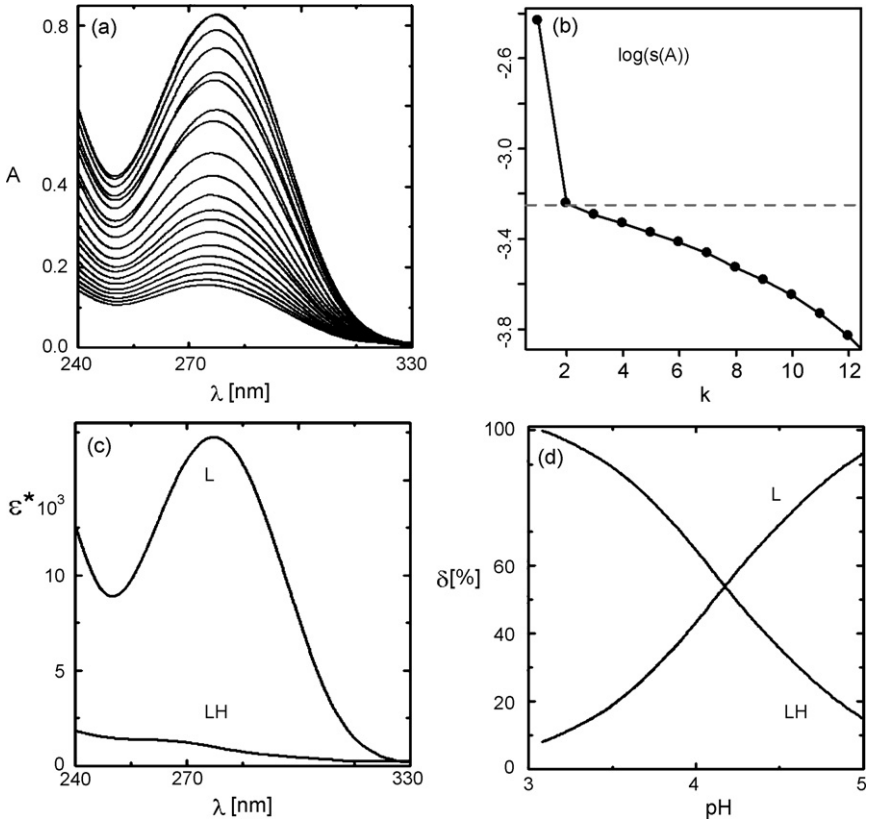


Fig. 3. The nonlinear regression analysis of the protonation equilibria model and factor analysis of diclofenac sodium: (a) absorption spectra in dependence on pH at 25 °C, (b) Cattel's scree plot of the Wernimont–Kankare procedure for determination of the number of light-absorbing species in the mixture $k^* = 2$ leads to the actual instrumental error of the spectrophotometer used $s_{inst}(A) = 0.56$ mAU (INDICES in S-Plus), (c) pure spectra profiles of molar absorptivities vs. wavelengths for species L and LH and (d) distribution diagram of the relative concentrations of species L and LH of diclofenac in dependence on pH at 25 °C (SPECFIT, SQUAD and ORIGIN).

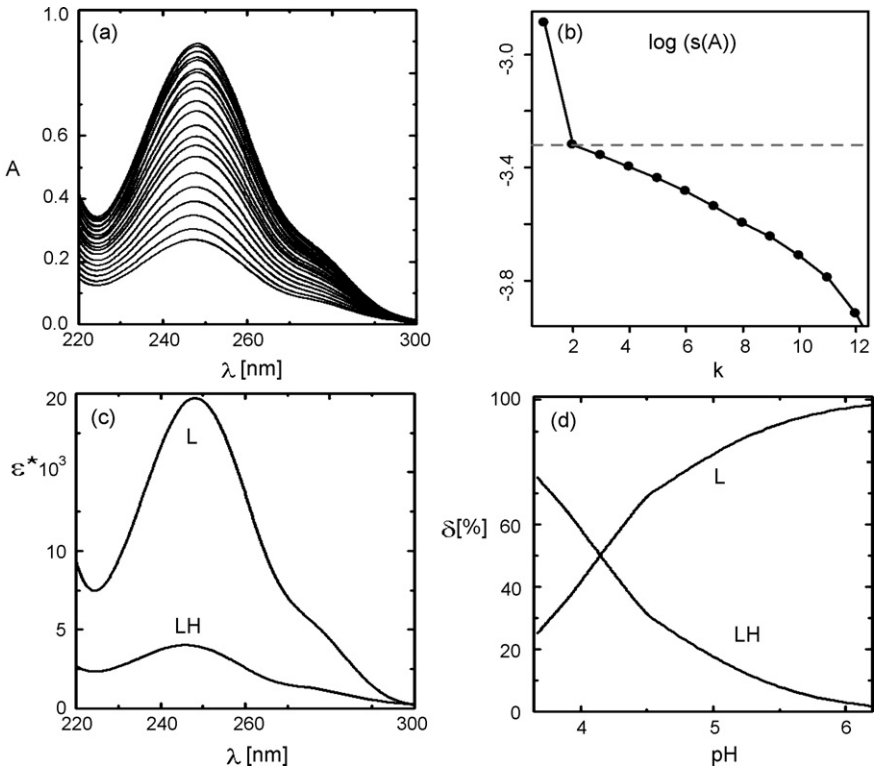


Fig. 4. The nonlinear regression analysis of the protonation equilibria model and factor analysis of flurbiprofen: (a) absorption spectra in dependence on pH at 25 °C, (b) Cattel's scree plot of the Wernimont–Kankare procedure for determination of the number of light-absorbing species in the mixture $k^*=2$ leads to the actual instrumental error of the spectrophotometer used $s_{inst}(A)=0.48$ mAU (INDICES in S-Plus), (c) pure spectra profiles of molar absorptivities vs. wavelengths for species L and LH and (d) distribution diagram of the relative concentrations of species L and LH of flurbiprofen in dependence on pH at 25 °C (SPECFIT, SQUAD and ORIGIN).

in the spectra. The number of light-absorbing species was estimated using the INDICES algorithm (Fig. 4b). The position of the break point on the $s_k(A)=f(k)$ curve in the factor analysis scree plot is calculated and gives $k^*=2$ with the corresponding co-ordinate log $(s_k^*(A))=-3.31$, which gives the value $s_k^*(A)=0.48$ mAU and which may also be taken here as the actual instrumental error $s_{inst}(A)$ of the spectrophotometer used.

The first diagnostic value indicates whether all of the parametric estimates β_{qr} and ε_{qr} have physical meaning and reach realistic values for flurbiprofen $pK_a=4.17$ $(s=0.04)$ at 25 °C. As the standard deviations $s(\log\ \beta_{qr})$ of parameters log β_{qr} and $s(\varepsilon_{qr})$ of parameters ε_{qr} are significantly smaller than their corresponding parameter estimates, all the variously protonated species are statistically significant at a significance level of $\alpha=0.05$. Fig. 4c shows the estimated molar absorptivities of all of the variously protonated species ε_L, ε_{LH} of flurbiprofen in dependence on wavelength.

The second diagnostic tests whether all of the calculated free concentrations of variously protonated species on the distribution diagram of the relative concentration expressed as a percentage have physical meaning, which proved to be the case (Fig. 4d). A distribution diagram makes it easier to judge the contributions of individual species to the total concentration quickly. The estimated dissociation constant pK_a at two temperatures 25 °C and 37 °C in dependence on an ionic strength I is shown in Table 2.

Ketoprofen: The experimental spectra are obtained for the titration of an alkaline 4.4×10^{-5} M ketoprofen solution by a standard solution of 1 M HCl (or $HClO_4$) to adjust pH value. pH-spectrophotometric titration enables absorbance-response data (Fig. 5a) to be obtained for analysis by nonlinear regression, and the reliability of parameter estimates (pK's and ε's) can be evaluated on the basis of the goodness-of-fit test of residuals. As the changes in spectra are quite small within deprotonation, however, both of the variously protonated species L and LH exhibit nearly similar absorption bands. In cases of small changes in spectra, a precise measurement of absorbance is neccessary for a reliable detection of the deprotonation equilibrium studied. The best region of the spectrum seems to be 210–330 nm and $pK_a=4.07$ $(s=0.02)$. Most of the selected methods of factor analysis by the INDICES algorithm lead to two light-absorbing components in the equilibrium mixture (Fig. 5b). The position of the break point on the $s_k(A)=f(k)$ curve in the factor analysis screen plot is calculated and gives $k^*=2$ with the corresponding co-ordinate log $(s_k^*(A))=-3.30$ which gives the value $s_k^*(A)=0.50$ mAU and which may also be taken here as the actual instrumental error $s_{inst}(A)$ of the spectrophotometer used. The goodnes-of-fit proves a sufficiently reliable estimate of the dissociation constant and molar absortion coefficient. Fig. 5c shows curves of molar absorption coefficients of L and LH

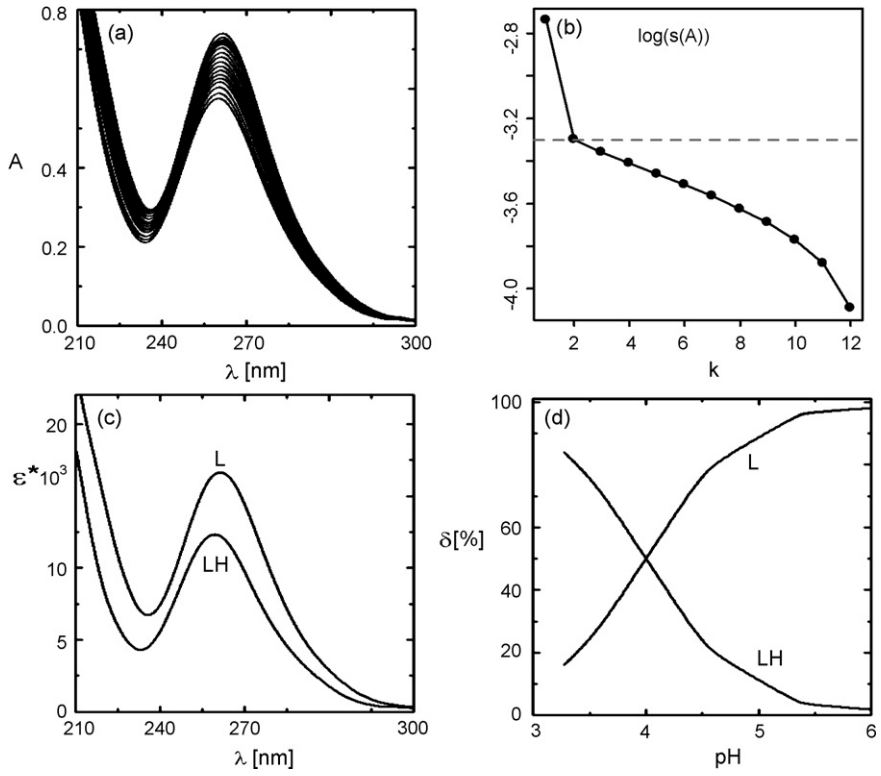


Fig. 5. The nonlinear regression analysis of the protonation equilibria model and factor analysis of ketoprofen: (a) absorption spectra in dependence on pH at 25 °C, (b) Cattel's scree plot of the Wernimont–Kankare procedure for determination of the number of light-absorbing species in the mixture $k^*=2$ leads to the actual instrumental error of the spectrophotometer used $s_{inst}(A)=0.50$ mAU (INDICES in S-Plus), (c) pure spectra profiles of molar absorptivities vs. wavelengths for species L and LH and (d) distribution diagram of the relative concentrations of species L and LH of ketoprofen in dependence on pH at 25 °C (SPECFIT, SQUAD and ORIGIN).

species in dependence on wavelengths and Fig. 5d the distribution diagram of L and LH species in dependence on pH. The estimated dissociation constant pK_a at two temperatures 25 °C and 37 °C in dependence on an ionic strength I is in Table 2.

4.2. Thermodynamic dissociation constants

The thermodynamic dissociation constants of the unknown parameter pK_a^T were estimated by applying a Debye–Hückel equation to the data of Table 2 and Fig. 6 [55]; Table 3 shows

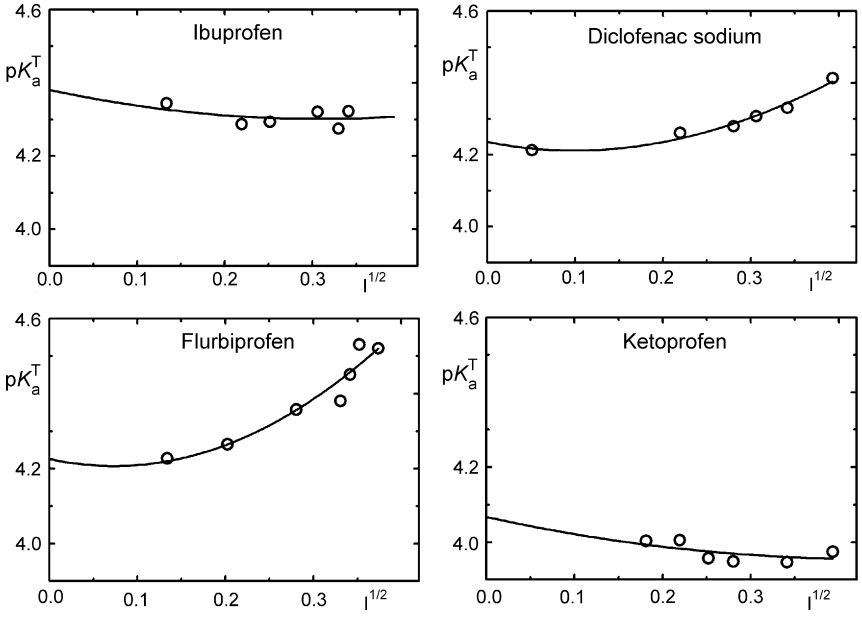


Fig. 6. Dependence of the mixed dissociation constant pK_a of four NSAIDs, ibuprofen, diclofenac sodium, flurbiprofen and ketoprofen on the square root of the ionic strength, leading to the thermodynamic dissociation constant pK_a^T at 25 °C.

Table 3
Comparison of the dissociation constants for four non-steroidal anti-inflammatory drugs, ibuprofen, diclofenac sodium, flurbiprofen and ketoprofen, determined at two temperatures, 25 °C and 37 °C, with the predicted value with the use of four algorithms, PALLAS, MARVIN, SPARC, ACD/pK_a and Pharma Algorithms, and with the literature values

	Ibuprofen	Diclofenac sodium	Flurbiprofen	Ketoprofen
pK_a^T at 25 °C (this work)	4.38 (3)	4.24 (2)	4.17 (4)	4.07 (2)
pK_a^T at 37 °C (this work)	4.51 (2)	4.41 (3)	4.38 (4)	3.87 (8)
p$K_{a,pred}$ (PALLAS) at 25 °C	4.08	4.48	3.03	3.49
p$K_{a,pred}$ (MARVIN) at 25 °C	4.85	4.00	4.37	3.88
p$K_{a,pred}$ (SPARC) at 25 °C	4.46	4.07	4.19	4.27
p$K_{a,pred}$ (ACD/pK_a) at 25 °C	4.41	4.18	4.14	4.23
p$K_{a,pred}$ (Pharma Algorithms) at 25 °C	4.30 ± 0.50	4.30 ± 0.50	4.30 ± 0.50	4.30 ± 0.50

The standard deviations in the last valid digits are in parentheses.

point estimates of the thermodynamic dissociation constants with standard deviation in brackets of the four non-steroidal anti-inflammatory drugs. Because of the narrow range of ionic strengths, the ion-size parameter a and the salting-out coefficient C could not be estimated. Estimated pK_a values are in agreement with those found in literature (Table 1). On the other hand, four prediction programs PALLAS, MARVIN, SPARC, ACD/pK_a and Pharma Algorithms were also used to compute the drugs aqueous pK_a and the results were compared with estimated thermodynamic dissociation constants. The reaction center is the carboxylic group. The computational algorithm involves simple combination of the perturbation potentials of perturber units with the reaction susceptibilities of the reaction center. The reaction parameters describing the reaction center are always the same, regardless of the appended molecular structures. The best agreement seems to be between the ACD/pK_a program and the experimentally found values or between the SPARC program and the experimentally found values, as for ibuprofen the estimated value at 25 °C is pK_a^T = 4.38 (s = 0.03) and that predicted with ACD/pK_a is p$K_{a,pred}$ = 4.41 while the value predicted with SPARC is p$K_{a,pred}$ = 4.46; for diclofenac sodium the value estimated at 25 °C is pK_a^T = 4.24 (s = 0.02) and that predicted with ACD/pK_a is p$K_{a,pred}$ = 4.18, while the predicted value with SPARC is p$K_{a,pred}$ = 4.07; for flurbiprofen is estimated value at 25 °C is pK_a^T = 4.17 (s = 0.04) and that predicted with ACD/pK_a is p$K_{a,pred}$ = 4.14, while the predicted value with SPARC is p$K_{a,pred}$ = 4.19; and for ketoprofen the estimated value at 25 °C is pK_a^T = 4.07 (s = 0.02), and that predicted with ACD/pK_a is p$K_{a,pred}$ = 4.23, while the value predicted with SPARC is p$K_{a,pred}$ = 4.27. Pharma Algorithms, PALLAS and MARVIN predicted p$K_{a,pred}$ values with larger bias errors in comparison to the experimental values found for all four drugs.

5. Conclusions

When drugs are very poorly soluble then pH-spectrophotometric titration may be used with nonlinear regression of the absorbance-response-surface data, instead of a potentiometric determination of the dissociation constants. The reliability of the dissociation constants of the four drugs (ibuprofen, diclofenac sodium, flurbiprofen and ketoprofen) may be proven with goodness-of-fit tests of the absorption spectra measured at various pH. Goodness-of-fit tests for various regression diagnostics enable the reliability of the parameter estimates to be determined.

Acknowledgments

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