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Research Article

The Thermodynamic Dissociation Constants of Clotrimazole, Terbinafine HCL, Acetylsalicylic Acid, Salicylic Acid, and Galanthamine by the Nonlinear Regression of Multiwavelength Spectrophotometric pH-Titration Data

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The mixed dissociation constants of five drugs—*clotrimazole, terbinafine HCl, acetylsalicylic acid, salicylic acid*, and *galanthamine*—at various ionic strengths I and at temperatures of 25°C and 37°C were determined with the use of multiwavelength and multivariate treatments of spectral data SPECFIT/32 nonlinear regression analysis and INDICES factor analysis. The factor analysis in the INDICES program predicts the number of components, 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: for clotrimazole $pK_{a,1}^T = 4.38(1)$ and 4.16(3); for terbinafine HCl $pK_{a,1}^T = 4.19(3)$ and 4.12(5); for acetylsalicylic acid $pK_{a,1}^T = 3.49(25)$ and 3.41(15); for salicylic acid $pK_{a,1}^T = 3.01(1)$ and 3.00(1) and for galanthamine $pK_{a,1}^T = 8.21(1)$ and 7.99(2) where in brackets the standard deviation is in the last significant digits. Goodness-of-fit tests for various regression diagnostics enabled the reliability of the parameter estimates found to be proven. Pharma Algorithms predicts pK_a being based on the structural formulae of drug compounds.

1. Introduction

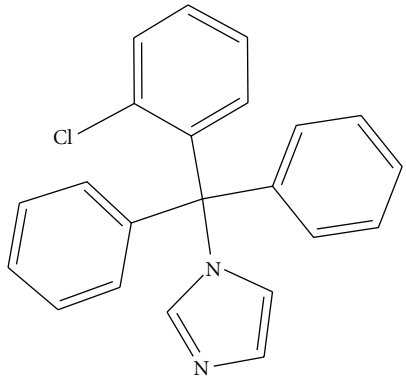
Acid dissociation constants (pK_a values) are key parameters to predict the extent of ionization of functional groups with respect to pH and also to explain chemical phenomena such as absorption, distribution, elimination of substances, biological activity, biological uptake, biological transport and environmental fate [1–3]. This information is important in drug discovery and development since the pharmacokinetic and pharmacodynamic properties of different protonation/dissociation forms of the drug molecules may vary considerably. There are several methods for the determination of dissociation constants. Traditionally, potentiometry and UV-VIS spectrometry have been the most useful techniques for the determination of equilibrium constants. Spectrophotometric methods in combination with suitable chemometric tools can be used for the determination of acid dissociation constants pK_a even for barely soluble drugs provided that the compound under consideration possesses chromophore(s) in proximity to the ionization centre, but the absorptivity should also change significantly on (de)protonation, indicating that the ionization centre is a part of the chromophore. The information can be extracted if multivariate spectrophotometric data are analyzed by means of an appropriate multivariate data analysis method. Hard-modelling methods, for example SQUAD(84) [4–11], include traditional least squares curve fitting approaches, based on a previous postulation of the chemical (here protonation) model. The postulations are a set of variously protonated species defined by their stoichiometric coefficients and protonation constants, which are refined by the least-squares minimization. However, a hard-modelling analysis cannot be applied if crucial information is missing. Soft-modelling or model-free approaches, for example SPECFIT [12–16], are based on much more general prerequisites, such as positive molar absorbance, positive concentration of all species, unimodality of concentration profiles, and closure, that is, a concentration of all species is the same for all

solutions. Naturally, if the strengths of hard-modelling and soft-modelling methodologies are combined, a much more powerful method of data analysis can be expected.

In addition, relevant software has been developed for the rapid estimation of pK_a values based on the chemical structure [17]. These techniques have certain advantages, for example, calculations can be performed on large virtual compound libraries. By entering the compound topological structure descriptors graphically, pK_a values of drug compounds are predicted using approximately hundreds of Hammett and Taft equations and quantum chemistry calculus. Still, erroneous data are often predicted for complex and flexible drug compounds containing several functional groups. Furthermore, these calculations are based on parameters in databases containing experimental data from the literature. Hence, sufficient data for new types of compounds, to give accurate predictions, may be missing.

An antifungal drug is a medication used to treat fungal infections such as athlete's foot, ringworm, candidiasis (thrush), serious systemic infection such as cryptococcal meningitis, and other. Such drugs are usually obtained by a doctor's prescription or purchased over-the-counter. Antifungals work by exploiting differences between mammalian and fungal cells to kill off the fungal organism without dangerous effects on the host. Unlike bacteria, both fungi and humans are eukaryotes. The basic structure of fungal cells and human cells is nearly identical. This means it is more difficult to find a target for an antifungal drug to attack that does not also exist in the infected organism. Consequently, there are often side-effects to some of these drugs. Some of these side-effects can be life-threatening if not used properly.

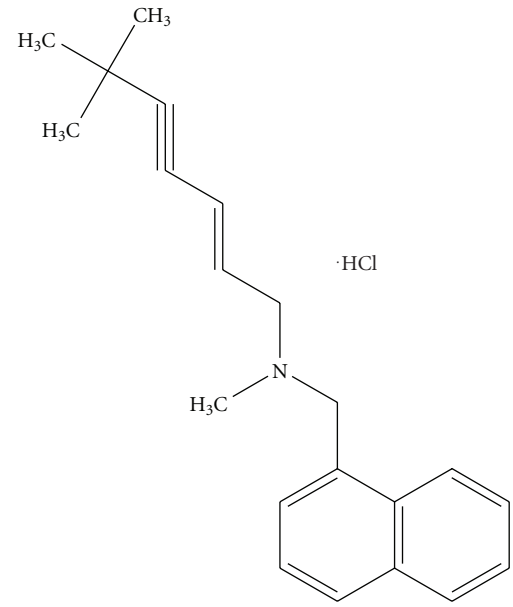
Clotrimazole (CLO) (chemically 1-[(2-chlorophenyl)-diphenyl-methyl]imidazole, CAS no. 23593-75-1, molecular formula: $C_{22}H_{11}ClN_2$, molecular weight: 344.837, description: a colourless, odourless, tasteless and crystalline solid, solubility: it is practically insoluble in water (<0.01 mg/mL), soluble in chloroform and methanol, in ethanol and in diethyl ether, it is freely soluble in acetone and methyl alcohol, is a weak base having a pK_a value of 4.7) is of the following structure:



Clotrimazole

Clotrimazole, an imidazole derivative with a broad spectrum of antimycotic activity, inhibits biosynthesis of the sterol ergostol, an important component of fungal cell membranes. Its action leads to increased membrane permeability and apparent disruption of enzyme systems bound to the membrane. Betamethasone and clotrimazole are used together to treat cutaneous tinea infections. It is a well-established drug used in dermatology and gynaecology, available in the form of solution formulations, tablet and cream [18–20].

Terbinafine hydrochloride (TER-HCl) (chemically (E)-*N*-(6,6-dimethyl-2-hepten-4-ynyl)-*N*-methyl-1-naphthalene methanamine hydrochloride, CAS no. 78628-80-5, molecular formula: $C_{21}H_{25}ClN$, molecular weight: 327.90, description: white to off-white finely crystalline powder, solubility: soluble in ethanol (45 mg/mL), DMSO (30 mg/mL), water (3 mg/mL)) is of the following structure:



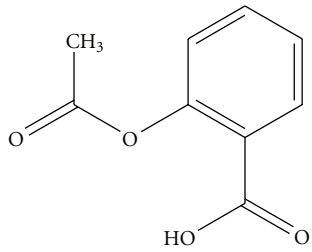
Terbinafine hydrochloride

Terbinafine hydrochloride is a new potent antifungal agent of the allylamine class that selectively inhibits fungal squalene epoxidase. The drug has broad-spectrum activity against yeast, fungi, moulds, and dermatophytes and is indicated for both oral and topical treatment of mycoses [21–23].

The salicylates are a group of analgesics, or painkilling drugs, that are derivatives of salicylic acid. The best known is acetylsalicylic acid, or aspirin. Now often made synthetically, they were originally derived from *salicin,* the active ingredient in willow bark, used for centuries in the treatment of pain and fever. Salicylates also occur naturally in many plants used as foods (e.g., strawberries, almonds, tomatoes). Methyl salicylate is the main component of wintergreen, sweet birch, gautheria, and betula oils; the compound is used in rubbing liniments to soothe muscular aches and as a flavouring. Sodium salicylate, traditionally used in the treatment of arthritis, is also used in dyes and as a non-edible preservative. In general, salicylates, especially aspirin, are used medically to reduce fever and inflammation and to relieve headache, menstrual pain, and pain in nerves, muscles, and joints. Because of the effects of salicylates on blood platelets and clotting, aspirin is often prescribed prophylactically for those at risk of stroke or heart attack. Salicylates are useful, relatively safe drugs, but normal doses

can cause gastrointestinal disturbances in sensitive patients and large doses can be toxic or fatal, especially to children.

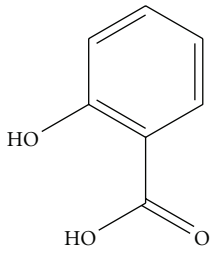
Acetylsalicylic Acid (ASA) (chemically 2-Acetoxybenzoic acid, CAS no. 50-78-2, description: colourless or a white crystalline powder, molecular formula: $C_9H_8O_4$, molecular weight: 180.16, melting point: 138–140°C, solubility: 1 in 300 of water, 1 in 5–7 in alcohol, 1 in 17 of chloroform and 1 in 20 of ether; soluble in solutions of acetates and citrates and, with decomposition, in solutions of alkali hydroxides and carbonates, pK_a is 3.49 at 25°C [24]) of the structure



Acetylsalicylic acid

is arguably the world's oldest and best known pharmaceutical product with accepted anti-inflammatory, anti-thrombotic, anti-pyretic, anti-oxidant and analgesic properties [25–27]. It has been suggested that daily use of aspirin can reduce the risk of some types of cancer [28], and even provide a means of extending human life [29]. Aspirin was the first-discovered member of the class of drugs known as nonsteroidal anti-inflammatory drugs (NSAIDs), not all of which are salicylates, although they all have similar effects and a similar mechanism of action.

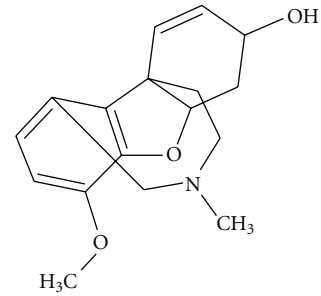
Salicylic Acid (SA) (chemically 2-Hydroxybenzoic acid, CAS no. 69-72-7, description: colourless or white crystalline powder, molecular formula: $C_7H_6O_3$, molecular weight: 138.12, melting point: 160°C, solubility: soluble 1 in 460 to 550 of water, 1 in 15 of boiling water, 1 in 3 to 4 in alcohol, 1 in 3 in ether, and 1 in 45 in chloroform, pK_a is 3.00 at 25°C) of the structure



Salicylic acid

is a beta hydroxy acid (BHA). This colourless crystalline organic acid is widely used in organic synthesis and functions as a plant hormone. It is derived from the metabolism of salicin. It is probably best known as a compound that is chemically similar but not identical to the active component of aspirin. It is widely used as kerotalic, antimicrobial and antifungal agent and as an external therapeutical agent (keratolytic) in many pharmaceutical preparations [30, 31].

Galanthamine (GAL) belongs to a class of acetylcholinesterase inhibitors approved for symptomatic treatment of Alzheimer's disease. Galanthamine (chemically (4aS,6R, 8aS)-4a,5,9,10,11,12-Hexahydro-3-methoxy-11-methyl-6H-benzofuro[3a,3,2-ef][2]benzazepin-6-ol, CAS no. 357-70-0, description: a white crystalline powder, molecular formula: $C_{17}H_{21}NO_3$, molecular weight: 287.358, melting point: 127-128°C, solubility: poorly soluble in water, pK_a is 8.2 at 25°C) of structure



Galanthamine

is a parasympathomimetic, specifically, a reversible cholinesterase inhibitor. It is indicated for the treatment of mild to moderate dementia of the Alzheimer's type. Galanthamine is postulated to exert its therapeutic effect by enhancing cholinergic function. This is accomplished by increasing the concentration of acetylcholine through reversible inhibition of its hydrolysis by acetylcholinesterase. If this proposed mechanism of action is correct, galanthamine's effect may lessen as the disease process advances and fewer cholinergic neurons remain functionally intact. There is no evidence that galanthamine alters the course of the underlying dementing process [32–36].

The procedure for the determination of the mixed protonation/dissociation constants has been described previously [37–40]. The details of the computer data treatment are collected in the *Supporting Information.* The nonlinear estimation of the thermodynamic dissociation constant $K_a^T = a_{H^+}a_{L^-}/a_{HL}$, is simply a problem of optimization in the parameter space in which pK_a and I are known and given values, while the parameters pK_a^T, a, and C are unknown variables to be estimated [41, 42]. The adequacy of a proposed regression model with experimental data and the reliability of parameter estimates p$K_{a,i}$ found, being denoted for the sake of simplicity as b_j, and ε_{ij}, $j = 1, \ldots, m$, may be examined by a goodness-of-fit test, cf. a previous tutorial [37–40]. Repeatability and reproducibility (R&R) are critical factors closely related to precision and accuracy. It helps to think of repeatability in terms of how capable the gage is of providing the same reading to a single user when measuring a specific sample. Repeatability is the variability of the measurements obtained by one person while measuring the same item repeatedly. This is also known as the inherent precision of the measurement equipment. Reproducibility is the variability of the measurement system caused by differences in operator behaviour. Mathematically, it is the variability of the average values obtained by several operators while measuring the same item.

The aim of the study was to determine the pK_a^T values of clotrimazole, terbinafine HCl, acetylsalicylic acid, salicylic acid, and galanthamine using pH–spectrophotometric

Table 1: Dependence of the mixed dissociation constants pK_a of clotrimazole on ionic strength using regression analysis of pH-spectrophotometric data with SPECFIT at 25°C and 37°C. The standard deviations of the parameter pK_a in the last valid digits are in brackets.

		Estimated pK_a at 25°C						
SPECFIT	Ionic strength	0.002	0.025	0.040	0.048	0.055	0.086	0.116
	pK_a	4.360(32)	4.325(24)	4.337(24)	4.318(13)	4.338(23)	4.333(24)	4.337(17)
	s(A) [mAU]	0.85	0.80	0.51	0.65	0.77	0.95	0.94
		Estimated pK_a at 37°C						
SPECFIT	Ionic strength	0.017	0.025	0.033	0.040	0.063	0.071	0.116
	pK_a	4.152(27)	4.072(26)	4.037(18)	4.073(11)	4.030(18)	4.066(21)	4.037(17)
	s(A) [mAU]	0.74	0.59	0.80	0.57	0.97	0.85	0.84

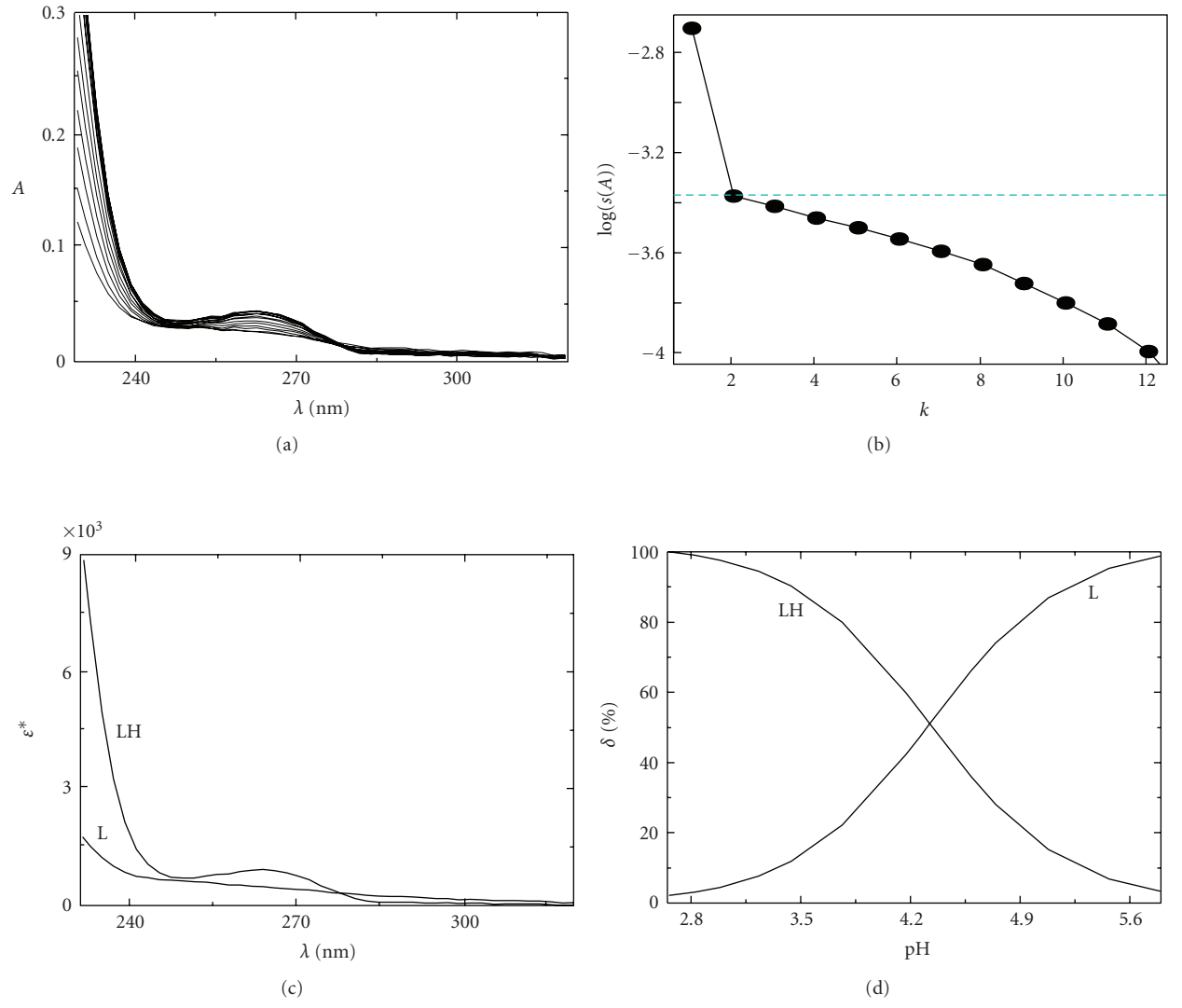


Figure 1: The nonlinear regression analysis of the protonation equilibria model and factor analysis of clotrimazole: (a) Absorption spectra in dependence on pH at 25°C, (b) Cattel's scree plot of the Wernimont-Kankare procedure for determination of the number of light-absorbing species in the mixture $k^* = 2$ leads to the actual instrumental error of the spectrophotometer used $s_{inst}(A) = 0.43$ mAU (INDICES in S-Plus), (c) Pure spectra profiles of molar absorptivities *versus* wavelengths for species L and LH, (d) Distribution diagram of the relative concentrations of species L and LH of clotrimazole in dependence on pH at 25°C. The charges of species are omitted for the sake of simplicity. (SPECFIT, ORIGIN).

Table 2: Dependence of the mixed dissociation constants pK_a of terbinafine hydrochloride on ionic strength using regression analysis of pH-spectrophotometric data with SPECFIT at 25°C and 37°C. The standard deviations of the parameter pK_a in the last valid digits are in brackets.

		Estimated pK_a at 25°C						
SPECFIT	Ionic strength	0.002	0.017	0.032	0.040	0.048	0.055	0.070
	pK_a	4.212(2)	4.347(4)	4.527(1)	4.617(2)	4.599(1)	4.768(2)	4.900(3)
	s(A) [mAU]	0.93	1.05	1.00	0.81	1.04	0.55	1.40
		Estimated pK_a at 37°C						
SPECFIT	Ionic strength	0.002		0.032		0.048	0.063	0.078
	pK_a	4.136(2)		4.276(1)		4.282(2)	4.470(1)	4.474(2)
	s(A) [mAU]	0.82		0.65		1.00	0.50	0.98

Table 3: Repeatability of measurements. The standard deviations of the parameter pK_a in the last valid digits are in brackets.

No.	Estimated pK_a at 25°C	s(A) [mAU]	No.	Estimated pK_a at 37°C	s(A) [mAU]
1	3.514(7)	0.83	1	3.440(15)	0.83
2	3.481(10)	0.95	2	3.413(11)	0.75
3	3.447(11)	0.96	3	3.400(13)	1.19
4	3.510(6)	1.00	4	3.422(14)	0.96
5	3.505(13)	0.98	5	3.403(7)	0.83
6	3.498(11)	0.88	6	3.405(11)	0.95
Sample mean	3.493(25)			3.414(15)	

titrations. The pK_a values were also calculated theoretically using computer program (Pharma Algorithms) making predictions based on the structural formula of drug compounds.

2. Experimental

2.1. Materials. *Clotrimazole* was purchased from AMOLI ORGANICS Ltd., with a purity of 100%. *Terbinafine HCl* was purchased from CHEMAGIS, with a purity of 100%. *Acetylsalicylic Acid* was purchased from Sigma-Aldrich Co., with a purity of ≥99%. *Salicylic Acid* was purchased from Sigma-Aldrich Co., with a purity of ≥99%. *Galanthamine* was purchased from Ivax-Pharmaceuticals s.r.o., with a purity of ≥99%. *Perchloric acid*, 1 M, was prepared from conc. $HClO_4$ (p.a., Lachema Brno) using redistilled 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 carbondioxidefree 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 [37–40].

2.2. Apparatus and Procedure. The apparatus used and the pH-spectrophotometric titration procedure described in [37–40] were applied. The microscale pH-titrimetric method generally decreases the amount of drug sample required but the duration of experiment is as long as that of a conventional titration. This method uses pH electrodes which requires the calibration of the pH electrodes cell every day. Calibration procedures normally take 20 minutes and the E^0 and slope of the electrode remain valid for less than 1 day. The free hydrogen-ion concentration $h = [H^+]$ was measured via *emf* on an OP-208/1 digital voltmeter (Radelkis, Budapest) with a precision of ±0.1 mV using a G202B glass electrode (Radiometer, Copenhagen) and an OP-8303P commercial SCE reference electrode (Radelkis, Budapest). Although spectrophotometric multiple-wavelength pH-titration has gained popularity in recent years, the method also requires precise pH measurement: an aqueous solution 20.00 cm^3 containing 10^{-5} mol.dm^{-3} drug, 0.100 mol·dm^{-3} hydrochloric acid and 10 cm^3 indifferent solution KCl for adjustment of ionic strength was titrated with standard 1.0 mol·dm^{-3} KOH at 298 K and from 20 to 50 absorption spectra were recorded. Titrations were performed in a thermostatic 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 pH measurements were carried out at 25.0°C ± 0.1° and 37.0°C ± 0.1°. As all methods involving the use of pH electrodes have the same limitation, namely, relatively extensive experimental time. During titration, the average pH meter responses time can be as long as 0.5 minute for each recorded point even with the best pH electrodes. Therefore, a typical titration process takes approximately 30 min to cover the whole pH range with 0.1 pH unit intervals necessary to provide the high quality data suitable for analysis. As the uniformity of the solution can be achieved quickly by vigorous stirring, and typical proton dissociation processes are less than 10^{-8} s, much time during pH titration experiments is wasted, waiting for the electrode to provide a stable response.

When the drug was titrated, a stream of argon gas was bubbled through the solution both to stir and to maintain an inert atmosphere. The argon was passed through an aqueous ionic medium by prior passage through one or two vessels also containing the titrand medium before entering the corresponding titrand solution. The burettes used were syringe micro-burettes of 1250 μL capacity (META, Brno) with a 2.50 cm micrometer screw, [43]. The polyethylene

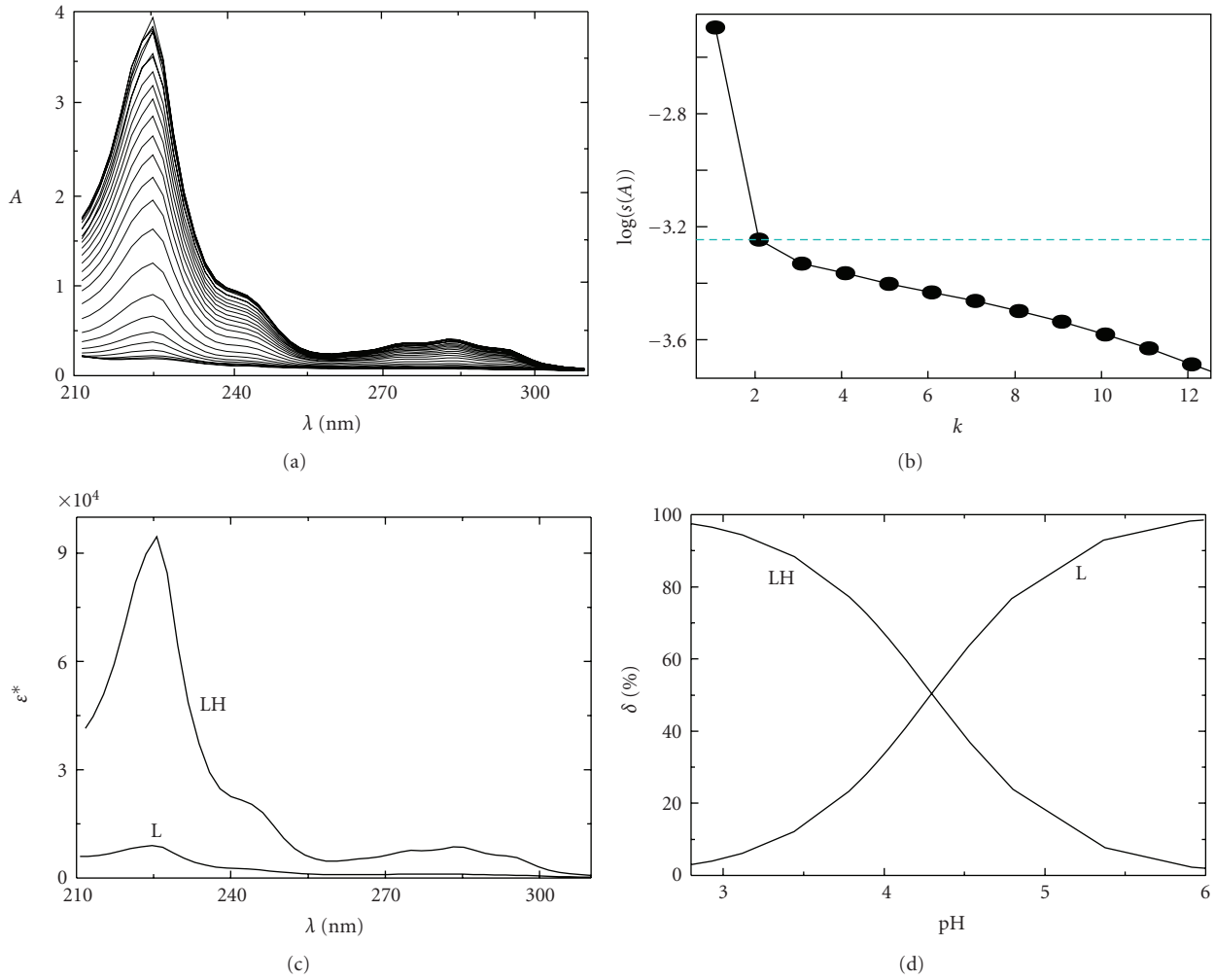


Figure 2: The nonlinear regression analysis of the protonation equilibria model and factor analysis of terbinafine hydrochloride: (a) Absorption spectra in dependence on pH at 25°C, (b) Cattel's scree plot of the Wernimont-Kankare procedure for determination of the number of light-absorbing species in the mixture $k^* = 2$ leads to the actual instrumental error of the spectrophotometer used $s_{\text{inst}}(A) = 0.56$ mAU (INDICES in S-Plus), (c) Pure spectra profiles of molar absorptivities versus wavelengths for species L and LH, (d) Distribution diagram of the relative concentrations of species L and LH of terbinafine hydrochloride in dependence on pH at 25°C. The charges of species are omitted for the sake of simplicity. (SPECFIT, ORIGIN).

capillary tip of the micro-burette was immersed into the solution when adding reagent, but withdrawn after each addition in order to avoid leakage of the reagent during the pH read out. The micro-burette was calibrated by ten replicate determinations of the total volume of delivered water by weighing on a Sartorius 1712 MP8 balance with results evaluated statistically, leading to a precision of ±0.015% in added volume over the whole volume range. The solution was pumped into the cuvette and spectrophotometric measurement was performed with the use of a Cintra 40 spectrophotometer (GBC, Australia).

The computation scheme for the determination of the protonation constants of the multicomponent system is taken from Meloun et al., cf. [1, page 226] and the five steps are described elsewhere [37–40, 44–48]: (1) instrumental error of absorbance measurements, $s_{\text{inst}}(A)$, (2) experimental design, (3) number of light-absorbing species with a factor analysis, (4) choice of computational strategy of the nonlinear regression, (5) diagnostics indicating a correct chemical (protonation) model: when a minimization process terminates, some curve-fitting diagnostics are examined to determine whether the results should be accepted: the physical meaning of parametric estimates, the physical meaning of the species concentrations, the goodness-of-fit test of spectra calculated, and the deconvolution of the experimental spectra set.

2.3. Computations. Computations relating to the determination of dissociation constants were performed by regression analysis of the UV/VIS spectra using the SPECFIT/32 [12–16] programmes. Most of graphs were plotted using ORIGIN 7.5 [49] and S-Plus [50]. The thermodynamic dissociation constant pK_a^T was estimated with the MINOPT nonlinear regression programme in the ADSTAT statistical system [51].

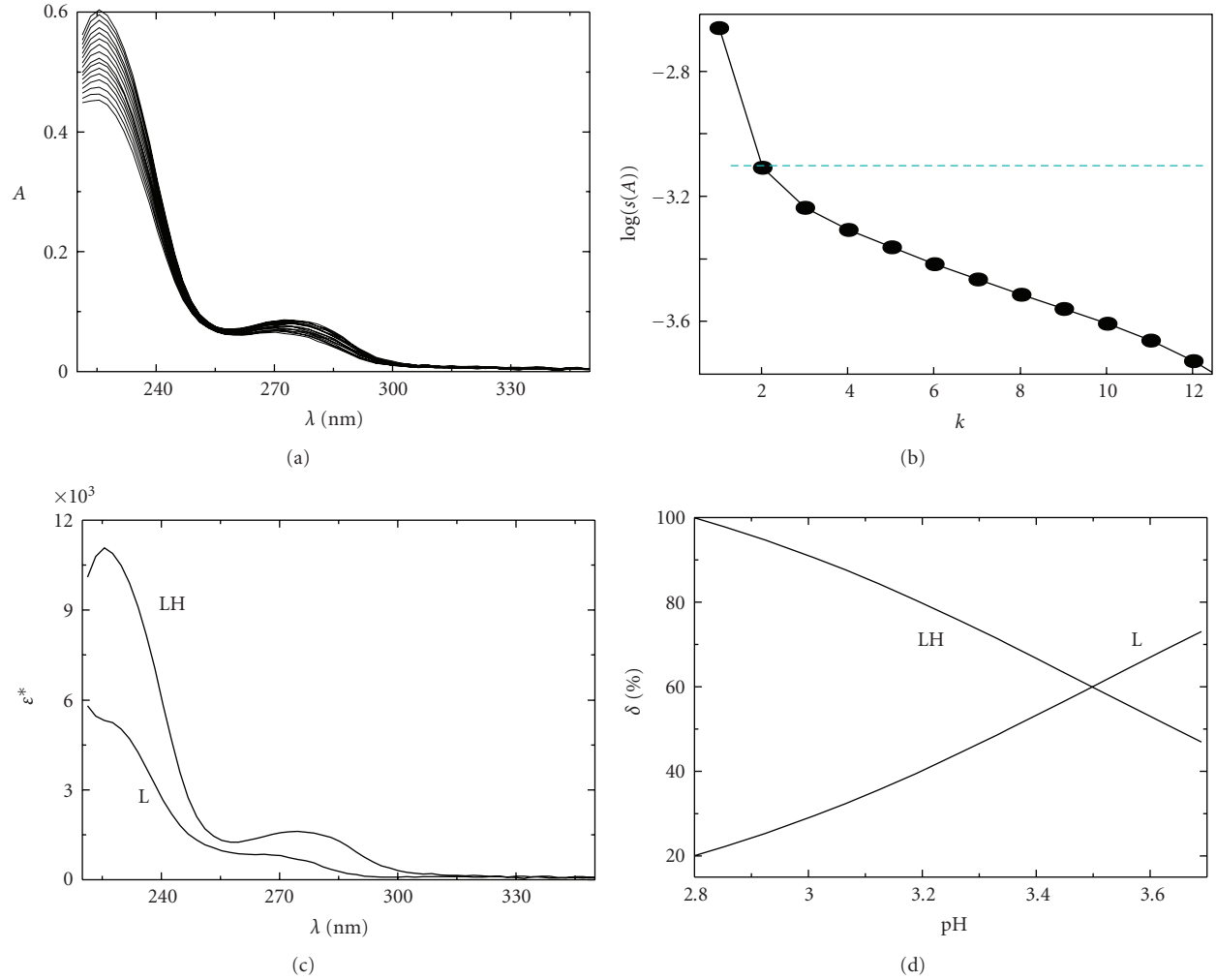


Figure 3: The nonlinear regression analysis of the protonation equilibria model and factor analysis of acetylsalicylic acid: (a) Absorption spectra in dependence on pH at 25°C, (b) Cattel's scree plot of the Wernimont-Kankare procedure for determination of the number of light-absorbing species in the mixture $k^* = 2$ leads to the actual instrumental error of the spectrophotometer used $s_{\text{inst}}(A) = 0.79$ mAU (INDICES in S-Plus), (c) Pure spectra profiles of molar absorptivities versus wavelengths for species L and LH, (d) Distribution diagram of the relative concentrations of species L and LH of acetylsalicylic acid in dependence on pH at 25°C. The charges of species are omitted for the sake of simplicity. (SPECFIT, ORIGIN).

A qualitative interpretation of the spectra with the use of the INDICES programme [52] aims to evaluate the quality of the dataset and remove spurious data, and to estimate the minimum number of *factors*, that is, contributing aqueous species, which are necessary to describe the experimental data and determine the number of dominant species present in the equilibrium mixture. Pharma Algorithms [17] is a programme 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.

2.4. Supporting Information Available. Complete experimental and computational procedures [37–40, 44–48], input data specimens, and corresponding output in numerical and graphical form for the programmes, INDICES, SQUAD(84), and SPECFIT/32 are available free of charge on line at http://meloun.upce.cz and in the block DOWNLOAD and block DATA.

3. Results and Discussion

3.1. Estimation of Dissociation Constants

3.1.1. Clotrimazole. A suggested strategy for effective experimentation in dissociation constants determination ensued from spectral data treatment is presented on the protonation equilibria of clotrimazole. The experimental spectra are acquired for the titration of an acid 4.29×10^{-5} M clotrimazole solution by a standard solution of 1 M NaOH to adjust pH value. As all variously protonated anions exhibit quite

Table 4: Dependence of the mixed dissociation constants pK_a of salicylic acid on ionic strength using regression analysis of pH-spectrophotometric data with SPECFIT at 25°C and 37°C. The standard deviations of the parameter pK_a in the last valid digits are in brackets.

		Estimated pK_a at 25°C						
SPECFIT	Ionic strength	0.001	0.017	0.047	0.054	0.062	0.069	0.077
	pK_a	2.997(20)	2.977(12)	2.976(15)	2.970(17)	2.971(12)	2.971(16)	2.970(16)
	s(A) [mAU]	1.02	0.92	0.80	0.86	0.72	0.83	0.79
		Estimated pK_a at 37°C						
SPECFIT	Ionic strength	0.001	0.009	0.017	0.032	0.039	0.054	0.062	0.077
	pK_a	2.995(13)	2.939(15)	2.964(11)	2.917(15)	2.935(14)	2.929(15)	2.930(17)	2.917(13)
	s(A) [mAU]	0.80	0.62	0.91	1.00	0.77	0.95	0.98	0.98

Table 5: Repeatability of measurements. The standard deviations of the parameter pK_a in the last valid digits are in brackets.

No.	Estimated dissociation constants pK_a at 25°C	s(A) [mAU]
1	2.914(13)	0.84
2	2.959(14)	0.91
3	2.965(15)	1.00
4	2.940(15)	0.89
5	2.935(18)	0.87
6	2.970(16)	0.79
7	2.925(21)	0.87
8	2.935(12)	0.87
Mean from the propagation-of-errors	2.943(6)	
Sample means	2.943(20)	

similar absorption bands, a part of the spectrum from 240–320 nm was selected as the most appropriate for an estimation of protonation constants. pH-spectrophotometric titration allows absorbance-response-surface data (Figure 1(a)) to be acquired for analysis with non-linear regression and the reliability of parameter estimates (pK_s' and ε_s') can be assessed on the basis of the goodness-of-fit test of residuals. In the first step of the spectra analysis, the number of light-absorbing species was estimated using the INDICES algorithm (Figure 1(b)). 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 coordinate $\log(s_k^*(A)) = -3.37$ which gives the value $s_k^*(A) = 0.43$ mAU and which also represents the actual instrumental error $s_{\text{inst}}(A)$ of the spectrophotometer used. Very low values of $s_{\text{inst}}(A)$ prove that quite reliable spectrophotometer and experimental techniques were used. In the first run, the dissociation constant and two molar absorptivities of clotrimazole by SPECFIT/32 are estimated. The reliability of the regression parameter estimates may be tested using the following diagnostic which have been described previously [37–40]. The goodness-of-fit establishes sufficiently reliable estimates of the dissociation constant and molar absorption coefficient. Figure 1(c) shows the curves of molar absorption coefficients of L^- and LH species in dependence on wavelength and Figure 1(d) shows 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 1 and Figure 6.

3.1.2. Terbinafine HCl. The experimental spectra are acquired for the titration of an acid 4.10×10^{-5} M terbinafine HCl solution by a standard solution of 1 M NaOH to adjust pH value. pH spectrophotometric titration allows absorbance-response data (Figure 2(a)) to be acquired for analysis by nonlinear regression, and the reliability of parameter estimates (pK_s' and ε_s') can be assessed 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 240–310 nm and p$K_a = 4.19$ ($s = 0.03$).

In the first step of the regression spectra analysis, the number of light-absorbing species is estimated by the INDICES algorithm (Figure 2(b)). 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 corresponding coordinate $\log(s_k^*(A)) = -3.25$ which gives the value $s_k^*(A) = 0.56$ mAU. The goodness-of-fit establishes sufficiently reliable estimates of the dissociation constant and molar absorption coefficient. Figure 2(c) shows the curves of molar absorption coefficients of L^- and LH species in dependence on wavelength and Figure 2(d) 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 and Figure 6.

3.1.3. Acetylsalicylic Acid. The estimation of thermodynamic dissociation constant pK_a^T of acetylsalicylic acid (ASA) is quite a major problem since during preparation it may contain its main degradation product salicylic acid (SA). So during the determination of ASA, the presence of SA must be considered. The experimental spectra are acquired for the fast titration of an alkaline 5.96×10^{-5} M acetylsalicylic acid solution by a standard solution of 1 M HCl (or $HClO_4$) to adjust pH value. pH spectrophotometric titration

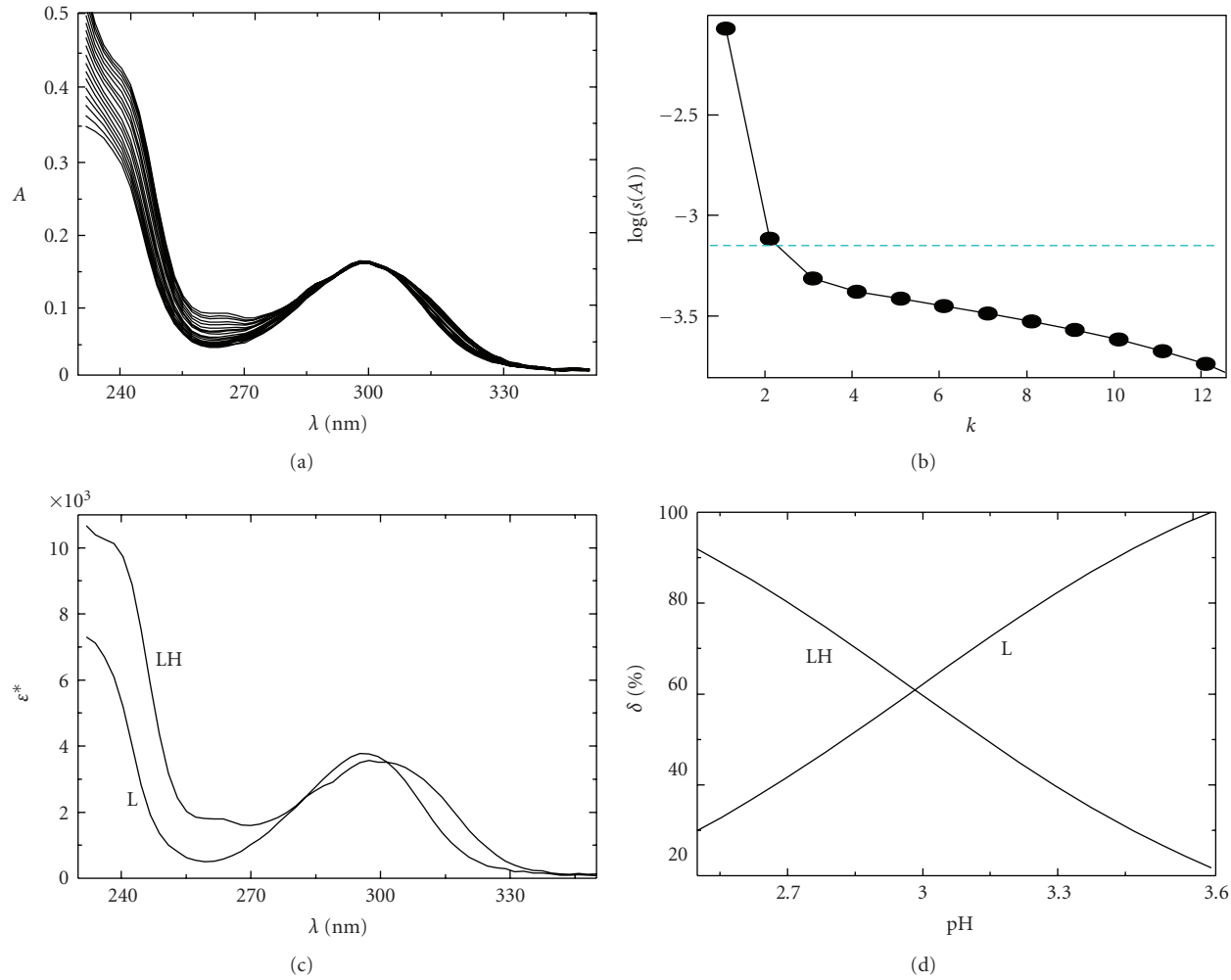


Figure 4: The nonlinear regression analysis of the protonation equilibria model and factor analysis of salicylic acid: (a) Absorption spectra in dependence on pH at 25°C, (b) Cattel's scree plot of the Wernimont-Kankare procedure for determination of the number of light-absorbing species in the mixture $k^* = 2$ leads to the actual instrumental error of the spectrophotometer used $s_{\text{inst}}(A) = 0.71$ mAU (INDICES in S-Plus), (c) Pure spectra profiles of molar absorptivities *versus* wavelengths for species L and LH, (d) Distribution diagram of the relative concentrations of species L and LH of salicylic acid in dependence on pH at 25°C. The charges of species are omitted for the sake of simplicity. (SPECFIT, ORIGIN).

Table 6: Dependence of the mixed dissociation constants pK_a of galanthamine on ionic strength using regression analysis of pH-spectrophotometric data with SPECFIT at 25°C and 37°C. The standard deviations of the parameter pK_a in the last valid digits are in brackets.

		Estimated pK_a at 25°C									
SPECFIT	Ionic strength	0.070	0.096	0.136	0.136	0.163	0.255	0.322	0.348	0.415	0.507
	pK_a	8.224(5)	8.241(6)	8.263(6)	8.291(4)	8.281(9)	8.309(4)	8.357(4)	8.330(6)	8.391(8)	8.409(4)
	s(A) [mAU]	0.90	1.07	1.12	0.87	1.74	0.93	0.84	1.03	1.43	0.83
		Estimated pK_a at 37°C									
SPECFIT	Ionic strength	0.017	0.030	0.083	0.110		0.176	0.229	0.269		0.362
	pK_a	7.989(11)	8.027(6)	8.049(5)	8.015(11)		8.109(7)	8.091(5)	8.147(5)		8.183(6)
	s(A) [mAU]	2.39	1.38	1.00	1.39		1.63	1.15	0.98		1.16

allows absorbance-response data (Figure 3(a)) to be acquired for analysis by nonlinear regression, and the reliability of parameter estimates (pK_s' and ε_s') can be assessed on the basis of the goodness-of-fit test of residuals. The best region of the spectrum seems to be 220–350 nm and pK_a = 3.49(25). Most of the selected methods of factor analysis by the INDICES algorithm lead to two light-absorbing components in the equilibrium mixture (Figure 3(b)). 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

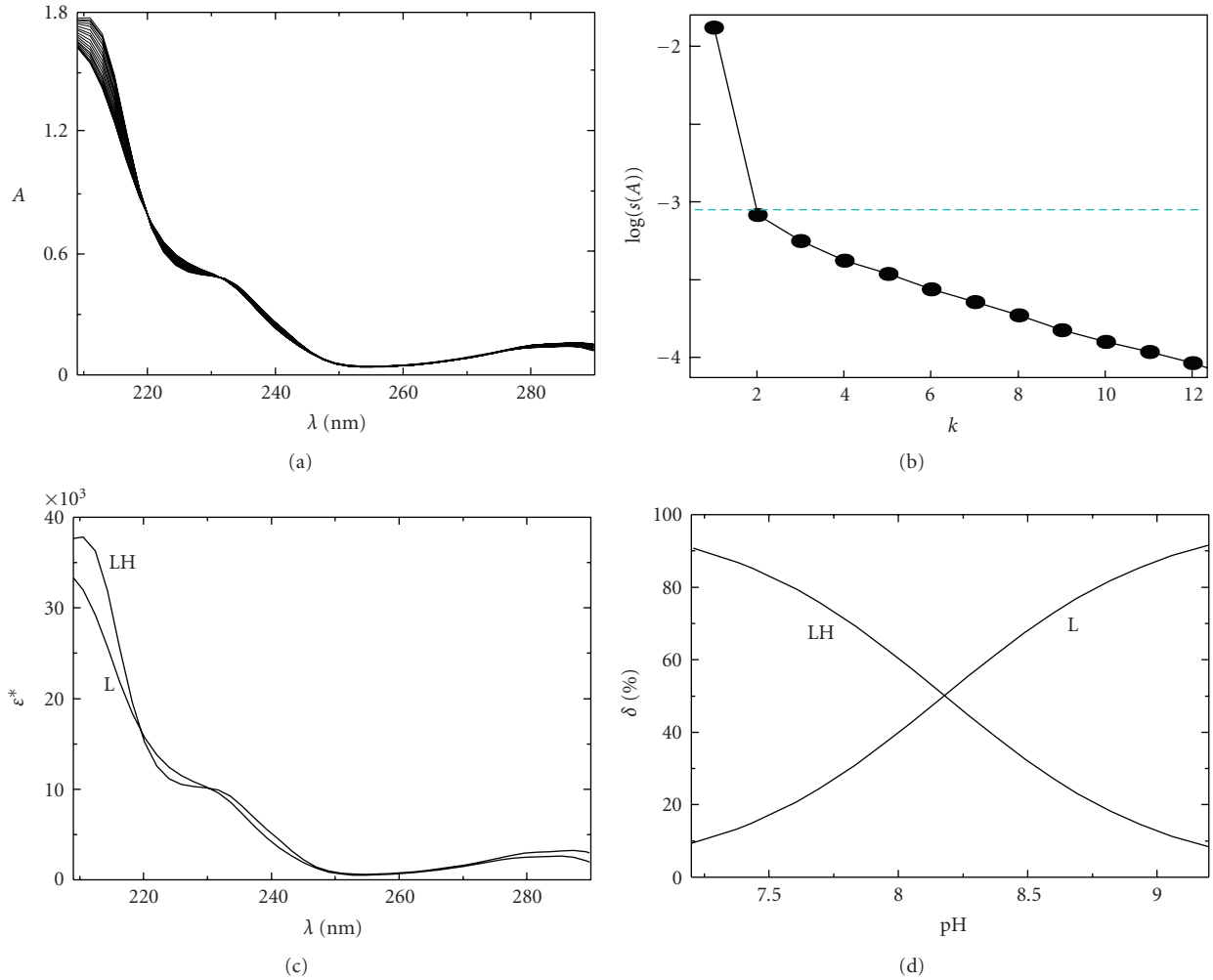


Figure 5: The nonlinear regression analysis of the protonation equilibria model and factor analysis of galanthamine: (a) Absorption spectra in dependence on pH at 25°C, (b) Cattel's scree plot of the Wernimont-Kankare procedure for determination of the number of light-absorbing species in the mixture $k^* = 2$ leads to the actual instrumental error of the spectrophotometer used $s_{\text{inst}}(A) = 1.00$ mAU (INDICES in S-Plus), (c) Pure spectra profiles of molar absorptivities versus wavelengths for species L and LH, (d) Distribution diagram of the relative concentrations of species L and LH of salicylic acid in dependence on pH at 25°C. The charges of species are omitted for the sake of simplicity. (SPECFIT, ORIGIN).

Table 7: Thermodynamic dissociation constants pK_a^T for clotrimazole, terbinafine HCl, acetylsalicylic acid, salicylic acid and galanthamine at two temperatures 25°C and 37°C. The standard deviations in the last valid digits are in brackets.

	pK_a^T at 25°C	pK_a^T at 37°C	pK_a^T (Pharma)
Clotrimazole	4.38(1)	4.16(3)	7.50 ± 0.50
Terbinafine HCl	4.19(3)	4.12(5)	7.00 ± 0.50
Acetylsalicylic Acid	3.49(25)	3.41(15)	3.50 ± 0.50
Salicylic Acid	3.01(1)	3.00(1)	3.00 ± 0.50
Galanthamine	8.21(1)	7.99(2)	8.60 ± 0.50

the corresponding coordinate $\log(s_k^*(A)) = -3.10$ which gives the value $s_k^*(A) = 0.79$ mAU. The goodness-of-fit establishes sufficiently reliable estimate of the dissociation constant and molar absorption coefficient. Figure 3(c) shows curves of molar absorption coefficients of L^- and LH species in dependence on wavelengths and Figure 3(d) shows the distribution diagram of L^- and LH species in dependence on pH. The repeatability of the estimated dissociation constant pK_a and its mean value at two temperatures 25°C and 37°C at one ionic strength $I = 0.001$ is in Table 3.

3.1.4. Salicylic Acid. The experimental spectra are acquired for the titration of an alkaline 4.18×10^{-5} M salicylic acid solution by a standard solution of 1 M HCl (or $HClO_4$) to adjust pH value. pH spectrophotometric titration allows absorbance-response data (Figure 4(a)) to be acquired for analysis by nonlinear regression, and the reliability of parameter estimates (pK_s' and ε_s') can be assessed 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

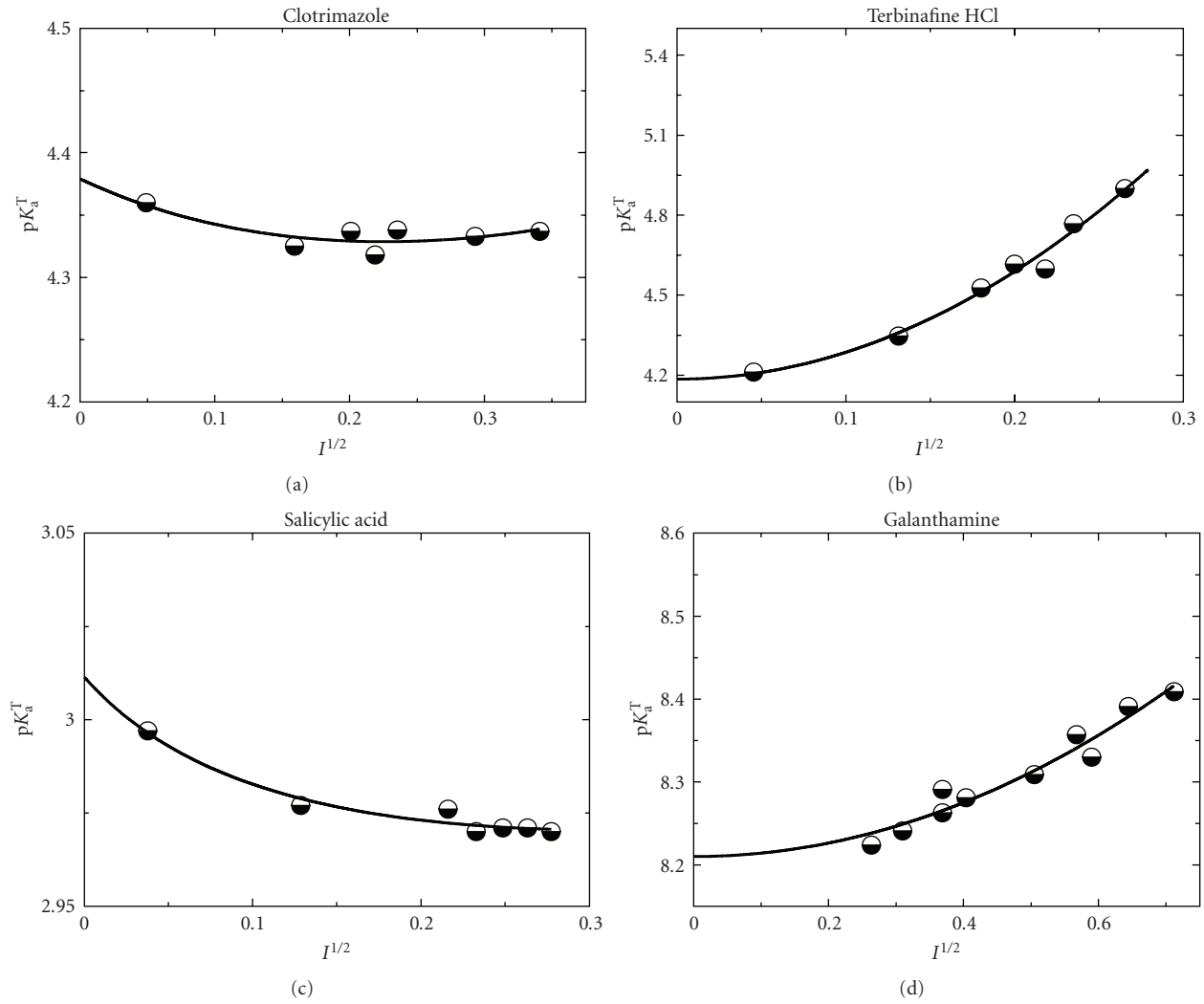


FIGURE 6: Dependence of the mixed dissociation constant pK_a of clotrimazole, terbinafine HCl, salicylic acid and galanthamine on the square root of the ionic strength, leading to the thermodynamic dissociation constant pK_a^T at 25°C, (ADSTAT, ORIGIN).

nearly similar absorption bands. In cases of small changes in spectra, a precise measurement of absorbance is necessary for a reliable detection of the deprotonation equilibrium studied. The best region of the spectrum seems to be 230–350 nm and $pK_a = 3.01$ ($s = 0.01$). Most of the selected methods of factor analysis by the INDICES algorithm lead to two light-absorbing components in the equilibrium mixture (Figure 4(b)). 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 coordinate $\log(s_k^*(A)) = -3.15$ which gives the value $s_k^*(A) = 0.71$ mAU and which may also be taken here as the actual instrumental error $s_{inst}(A)$ of the spectrophotometer used. The goodness-of-fit establishes sufficiently reliable estimate of the dissociation constant and molar absorption coefficient. Figure 4(c) shows curves of molar absorption coefficients of L^- and LH species in dependence on wavelengths and Figure 4(d) shows the distribution diagram of L^- and LH species on pH. The estimated dissociation constant pK_a at two temperatures 25°C and 37°C on an ionic strength I is in Table 4 and Figure 6. The repeatability of the estimated dissociation constant pK_a and its mean value at the temperature 25°C at one ionic strength $I = 0.001$ are in Table 5.

3.1.5. Galanthamine. The experimental spectra are acquired for the titration of an acid 4.71×10^{-5} M galanthamine solution by a standard solution of 1 M NaOH to adjust pH value. pH spectrophotometric titration allows absorbance-response data (Figure 5(a)) to be acquired for analysis by nonlinear regression, and the reliability of parameter estimates (pK_s' and ε_s') can be assessed 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. The best region of the spectrum seems to be 209–290 nm and $pK_a = 8.21$ ($s = 0.01$). Most of the selected methods of factor analysis by the INDICES algorithm lead to two light-absorbing components in the equilibrium mixture (Figure 5(b)). 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 coordinate $\log(s_k^*(A)) = -3.00$ which gives the value $s_k^*(A) = 1.00$ mAU. The goodness-of-fit establishes sufficiently reliable estimate of the dissociation constant and molar absorption coefficient. Figure 5(c) shows curves of molar absorption coefficients of L^- and LH species in dependence on wavelengths and Figure 5(d) shows 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 6 and Figure 6.

3.2. Thermodynamic Dissociation Constants. The thermodynamic dissociation constants as the unknown parameter pK_a^T were estimated by applying a Debye-Hückel equation to the data in Tables 1, 2, 4, 6, and Figure 6 according to the regression criterion; Table 7 shows point estimates of the thermodynamic dissociation constants of the five drugs (clotrimazole, terbinafine HCl, acetylsalicylic acid, salicylic acid, and galanthamine) at two temperatures. Because of the narrow range of ionic strengths the ion-size parameter and the salting-out coefficient C could not be estimated.

4. Conclusions

When drugs are very poorly soluble, pH-spectrophotometric titration may be used with non-linear regression of the absorbance-response-surface data instead of performing a potentiometric determination of the dissociation constants. The reliability of the dissociation constants of the five drugs (clotrimazole, terbinafine HCl, acetylsalicylic acid, salicylic acid, and galanthamine) may be proven with goodness-of-fit tests of the absorption spectra measured at various pH. The dissociation constant pK_a was estimated by non-linear regression of $\{pK_a, I\}$ data at 25°C: for clotrimazole $pK_{a,1}^T = 4.38(1)$, for terbinafine HCl $pK_{a,1}^T = 4.19(3)$, for acetylsalicylic acid $pK_{a,1}^T = 3.49(25)$, for salicylic acid $pK_{a,1}^T = 3.01(1)$, and for galanthamine $pK_{a,1}^T = 8.21(1)$, where in brackets is the standard deviation in last significant digits. Goodness-of-fit tests for various regression diagnostics enable the reliability of the parameter estimates to be determined.

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