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Journal of Pharmaceutical and Biomedical Analysis
21 (2000) 1171–1182

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Multiwavelength spectrophotometric determination of acid dissociation constants Part V: microconstants and tautomeric ratios of diprotic amphoteric drugs

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Received 10 May 1999; received in revised form 20 August 1999; accepted 26 August 1999

Abstract

The acid-base equilibria of several diprotic amphoteric drugs, namely, niflumic acid, norfloxacin, piroxicam, pyridoxine and 2-methyl-4-oxo-3H-quinazoline-3-acetic acid have been characterized in terms of microconstants and tautomeric ratios. A multiwavelength spectrophotometric (WApH) titration method for determination of acid dissociation constants (pK_a values) of ionizable compounds developed previously was applied for this purpose. Microspeciation was investigated by three approaches: (1) selective monitoring of ionizable group by spectrophotometry, (2) deductive method and (3) k_z method for determination of tautomeric ratio from co-solvent mixtures. The formulation for (3) has been derived and found to invoke fewer assumptions than a reported procedure (K. Takács-Novák, A. Avdeef, K.J Box, B. Podányi, G. Szász, J. Pharm. Biomed. Anal., 12 (1994) 1369–1377). It has been shown that the WApH technique, for such types of ampholytes, is able to deduce the microconstants and tautomeric ratios which are in good agreement with literature data.

Keywords: Amphoteric drugs; Microconstant; Tautomeric ratio; Target factor analysis; Multiwavelength spectrophotometric titration

1. Introduction

Knowledge of acid-base properties of drugs has fundamental importance in pharmacy. To characterize the ionization (dissociation/protonation) ability of molecules, the pK_a/log K values are generally used. These classical, stoichiometric, macroscopic (or marcoconstant) parameters are adequate to quantitate the acidity/basicity of monoprotic drugs. For multiprotic compounds, they characterize the acid-base equilibria as a whole, but fail to provide information on specific ionizable sites. Dissociation/protonation microconstants (pk/log k) are useful to depict the site-

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PII: S0731-7085(99)00238-1

specific ionization of individual functional groups, which can be applied for calculating the pH-dependent distribution of species (microspeciation) [1].

For a diprotic amphoteric drug with pK_a values of comparable magnitude, the (de)protonation of one group affects the other. Depending on the pH values of the media, the two ionizable sites, such as an acidic (–COOH) and a basic (–N) groups, may exist in four different microforms, namely cation (H_2X^+), zwitterion ($HX^{\pm}$), neutral species (HX^0, chargeless) and anion (X^-) which are shown in Fig. 1. It is generally accepted that the neutral form is more lipophilic and hence more effective in diffusing through the cellular barrier as compared with the charged form(s). The pK_a values disclose no information about the equilibrium that generates HX^0, which is probably the predominant species traversing the lipid bilayer [2]. On the other hand, the microconstants (pk_1 through pk_4) and the tautomeric ratio (k_z) describe the amount of various microforms as a function of pH. These parameters are especially useful in research areas such as pharmaceutical drug discovery and development, where the ionization state of each functional group plays an important role in the absorption of drug molecules into therapeutic target(s) and in interactions with specific receptor binding sites.

Traditionally, the pH-metric titration method is employed to measure the pK_a values of ionizable compounds which are sufficiently soluble in water

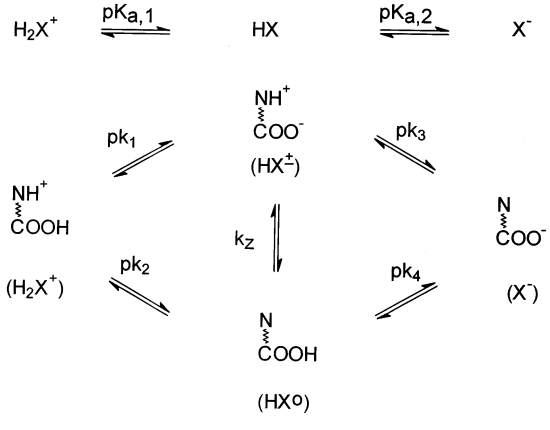

Fig. 1. Ionization scheme of a diprotic amphoteric molecule.

(≥ 0.5 mM). The determination of microconstants needs the combination of at least two experimental approaches. Beside pH-metry, which is used to obtain the macroconstants, the second method is typically spectroscopic (UV, NMR, CD, etc.) [3,4]. These approaches involve selective monitoring of one (or some) of the ionizable group(s), for molecules where the ionization of other group(s) causes negligible spectroscopic change. It has been pointed out that some of these treatments require several assumptions which may not always be valid [5]. If selective monitoring is hampered by large numbers, close proximity or high similarity of the functional groups, the deductive method could be employed. This method makes use of a close derivative of the parent molecule which contains a reduced number of ionizable groups. Assuming the masking of one or more functional groups would leave the acid-base properties of the other(s) unaltered, the pK_as of the derivative can be regarded as the relevant microconstants of the parent molecule.

For those amphoteric molecules where selective spectroscopic determination of one (or some) of the microconstants is not possible and the appropriate derivatives are not readily available, the micro-equilibria may be unravelled by measuring the tautomeric ratios spectrophotometrically (at a single wavelength) in mixtures of organic solvent and water at the pH of isoelectric point [6]. Extrapolation of the tautomeric ratios to zero organic solvent content could yield the aqueous k_Z value, from which other unknown microconstants could be calculated. The success of this method depends very much on the availability of an appropriate analytical wavelength channel with sufficient dissimilarity between the molar absorptivity coefficients of the zwitterion and neutral species. This is because unambiguous spectral assignment for the zwitterion and neutral species is necessary in the calculation [7]. In this work, we have described a new procedure for extracting the k_z value from the multiwavelength data obtained from several titrations of varying water/co-solvent ratios, without requiring those assumptions adopted when measurements are made only at the isoelectric point. We refer to those measurements in co-solvent mixtures as the k_z method.

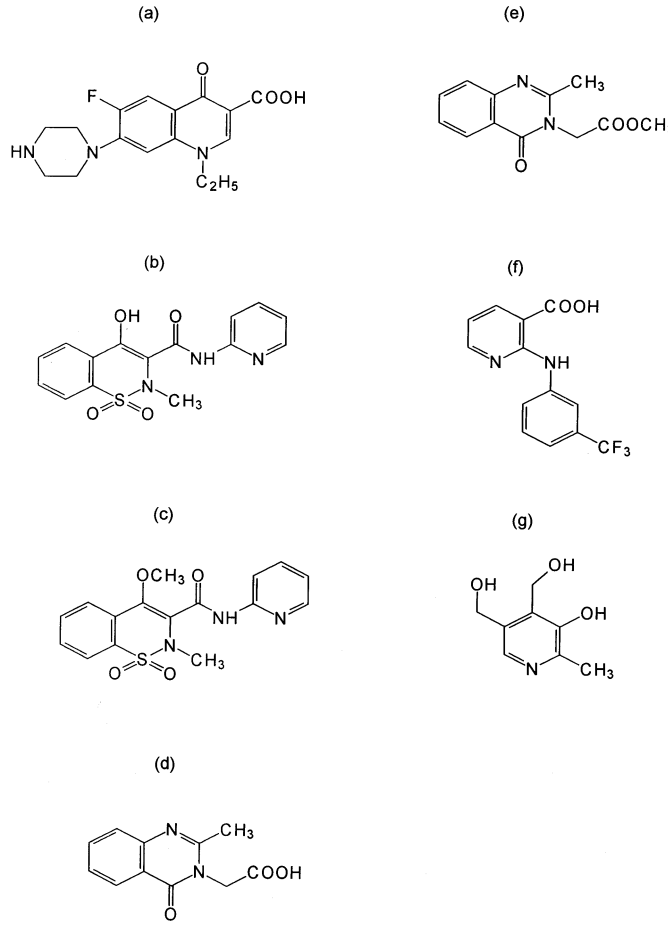

Fig. 2. Structures of (a) norfloxacin, (b) piroxicam, (c) *O*-methyl piroxicam, (d) quinazolone-3-CH_2COOH, (e) quinazolone-3-CH_2COOCH_3, (f) niflumic acid and (g) pyridoxine.

Using the aforementioned methods, a number of amphoteric drug molecules have been studied in one of our laboratories [6–12]. Despite these and other efforts, the interrogation of the microspeciation of amphoteric drug substances has not received wide attention [2]. This is probably because the determination of microconstants is time-consuming and requires skill and experience in both potentiometry and spectroscopy. It is envisaged that an automated method for measuring these parameters would greatly facilitate the study of microspeciation.

In our previous study, we developed a multi-wavelength spectrophotometric (WApH) titration approach to measure the pK_a values of monoprotic and diprotic drug compounds [13]. Specifically, we employed a fibre optics dip probe, a UV light source and a photodiode array detector in conjunction with a commercially available titrator (Sirius PCA101) to automatically capture the absorption spectra of the sample in the course of a pH-metric titration. Target factor analysis (TFA) has been utilized to deduce the pK_a values of ionizable drugs and resolve the absorption spectra of various species, without prior knowledge of their optical properties. In another study, we have shown that the TFA method outperforms the established first derivative technique in terms of obtaining pK_a results [14]. Moreover, the WApH technique has been applied successfully to

determine the pK_a values of several multiprotic ionizable drugs [15] and water-insoluble pyridine derivatives [16].

In this work, we extend the WApH technique in conjunction with pH-metric titration to determine the microconstants of several diprotic amphoteric drugs, namely, niflumic acid (anti-inflammatory), norfloxacin (anti-bacterial), piroxicam (anti-inflammatory), pyridoxine (vitamin) and 2-methyl-4-oxo-3H-quinazoline-3-acetic acid (= quinazolone-3-CH_2COOH, compound under development as a potential NMDA antagonist) [12]. We deliberately selected the following three types of compounds to exemplify the aforementioned approaches:

1. Compound which allow selective monitoring of ionizable group by spectrophotometry norfloxacin (Fig. 2a).
2. Compounds where deductive method can be applied because derivative molecules with fewer ionizable groups are available:
   2.1. Piroxicam and *O*-methyl piroxicam (Fig. 2b, c).
   2.2. Quinazolone-3-CH_2COOH and quinazolone-3-CH_2COOCH_3, (Fig. 2d, e).

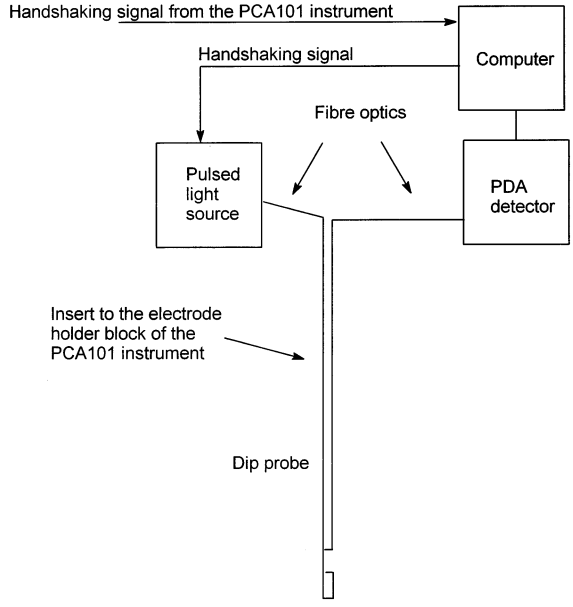

Fig. 3. Schematic for the experimental set-up utilized in the WApH titration.

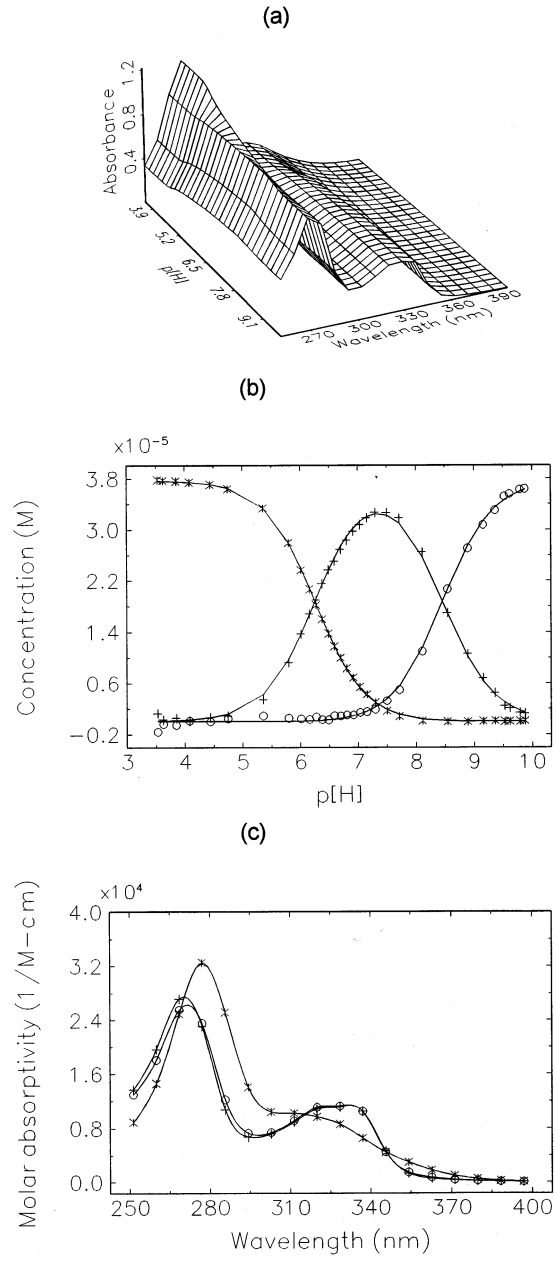

Fig. 4. (a) Absorption spectra of norfloxacin, (b) Distribution of species for norfloxacin as a function of pH with the symbols (* H_2X^+, + HX (HX$^{\pm}$ + HX^0), o X^-) represent the $\underline{C}_p$ matrix and solid lines denote the $\underline{C}_t$ matrix, (c) Molar absorptivity coefficients of norfloxacin. The symbols (see b) represent the elements in matrix $\underline{E}_p$. Solid lines are generated using the cubic spline interpolation method.

3. Compounds where k_z method can be applied:
   3.1. Niflumic acid (Fig. 2f).
   3.2. Pyridoxine (Fig. 2g).

In contrast to the conventional methods in which analyses were usually based on a single wavelength channel, the WApH technique utilizes more spectral information and the results could be more reliable. It is noted that the micro-equilibria of molecules examined in this study have already been established [6–9,12], which permits a direct comparison of results to test the validity of our method. In the subsequent discussion, a brief account on the WApH and TFA methods will be given. It will be shown that the microconstants determined using the WApH method are in good agreement with literature data.

2. Experimental section

2.1. Apparatus

A schematic diagram of the WApH titration is given in Fig. 3. The optical system consists of a pulsed deuterium lamp (Cathodeon, Cambridge, UK) with pre-aligned fibre optics output and an UV-VIS 256-element photodiode array (PDA) detector (Carl Zeiss, Herts., UK). This combination offers a spectral range of 200–735 nm with blaze wavelength at 220 nm. A bifurcated fibre optics dip probe (Custom Sensor and Technology, Missouri, USA) with optical path length of 1 cm is connected to the deuterium lamp and the PDA detector.

2.2. UV/pH titrations

All titrations were carried out by using a PCA101 automated titrator (Sirius, East Sussex, UK) [17]. A 3 mm hole was drilled through the electrode holder of the titrator to accommodate the dip probe such that it could be situated next to the pH electrode. Synchronization of the titrator, the pulsed deuterium lamp and spectrum acquisition by the PDA detector was accomplished using a terminate-and-stay-resident system [18]. The pH change per titrant addition was limited to about 0.1 pH units. The spectral data were acquired after each pH measurement, when the drift was less than 0.01 pH units per minute. About 20–30 pH readings and absorption spectra were collected from each titration. The pH elec-

Table 1
Acid dissociation macroconstants, microconstants and tautomeric equilibrium constants[a,b,c]

Type	I		II			III	
	Norfloxacin		Piroxicam	Quinazolone-3-CH_2COOH		Niflumic acid	Pyridoxine
	WApH	pH-metric	WApH	WApH	pH-metric	WApH	WApH
$pK_{a,1}$	6.25 ± 0.01	6.26 ± 0.01	1.88 ± 0.01	–	2.25 ± 0.04	2.28 ± 0.08	4.90 ± 0.05
$pK_{a,2}$	8.50 ± 0.03	8.37 ± 0.01	5.29 ± 0.02	–	3.30 ± 0.01	4.86 ± 0.05	8.91 ± 0.04
pk_1	6.28 ± 0.01	–	1.91	2.65		2.29	4.92
pk_2	7.43		3.00 ± 0.02[d]	2.47 ± 0.01[e]	–	4.23	5.86
pk_3	8.47		5.26	2.90		4.86	8.86
pk_4	7.32		4.18	3.08		2.92	7.95
log k_z	1.15		1.09	−0.18		1.94 ± 0.03[f]	0.91 ± 0.01[g]

[a] Constants of norfloxacin, piroxicam, quinazolone-3-CH_2COOH, niflumic acid and pyridoxine as determined using the WApH and the pH-metric techniques at 25°C and an ionic strength of 0.15 M
[b] Uncertainties equal to the standard deviation of the parameter obtained from at least three experiments.
[c] Those parameters without uncertainties quoted were calculated with the experimentally obtained parameters using Eqs. (5)–(7).
[d] pK_a value of *O*-methyl piroxicam (see Fig. 2c).
[e] pK_a value of quinazolone-3-CH_2COOCH_3 (see Fig. 2e).
[f] W, -0.044 ± 0.004 (see Eq. (11)).
[g] W, -0.021 ± 0.001 (see Eq. (11)).

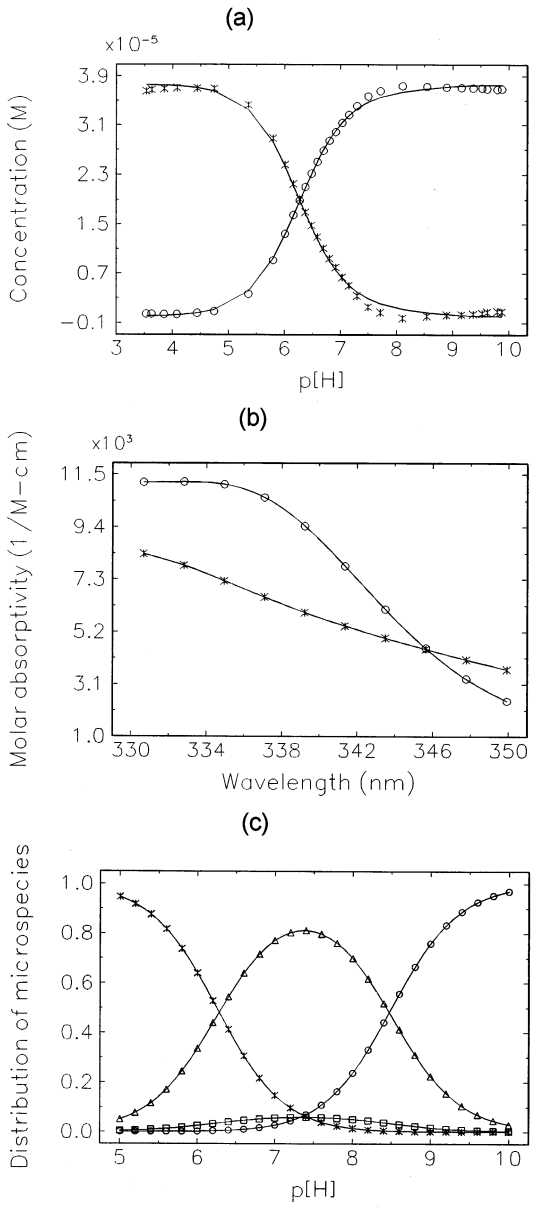

Fig. 5. (a) Distribution of species as a function of pH for the carboxylic acid (*) and the carboxylate (o) forms of norfloxacin. The symbols represent the $\underline{C}_p$ matrix and solid lines denote the C_t matrix, (b) Molar absorptivity coefficients for the carboxylic acid and carboxylate forms of norfloxacin with the symbols (see a) represent the elements in matrix $\underline{E}_p$. Solid lines are generated using the cubic spline interpolation method, (c) Distribution of microspecies ($*H_2X^+$, $\triangle HX^{\pm}$, $\square HX^0$ and oX^-) for norfloxacin as calculated using Eq. (8) with the microconstants listed in Table 1.

trode (Orion, Ross™ type, Beverly, MA, USA) was calibrated titrimetrically in the pH range of 1.8–12.2 [19]. All experiments were performed in aqueous solutions or methanol-water mixtures (up to 60 wt.%) with 0.15 M KCl under argon atmosphere at 25 ± 0.5°C using standardized 0.5 M HCl or 0.5 M KOH titrants. Solutions were made up of deionized water of resistivity $> 10^{14}$ Ω cm.

Sample concentrations of $1 \times 10^{-5} - 1 \times 10^{-4}$ M and $5 \times 10^{-4} - 5 \times 10^{-3}$ M were employed, respectively, in WApH and pH-metric titrations. For WApH titration of samples with mid range pK_as, experiments were carried out in the presence of 2.5×10^{-4} M potassium dihydrogen phosphate to allow sufficient spectra to be collected in the un-buffered region of the titration curve [15]. Typically, sample solutions were pre-acidified to a reasonably low pH value (1.8–3.0) then titrated alkalimetrically to an appropriate high pH value (7.0–11.0). Calculations of pK_a values from pH-metric data were performed using pK_aLOGP™ software (v5.01, Sirius).

2.3. Materials

Pyridoxine (HCl salt, Reanal, Budapest, Hungary) was of pharmacopoeial grade (Pharmacopoeia Hungary VII.). Some other compounds were generously supplied by the manufacturers: niflumic acid by Gedeon Richter Chemical Works (Budapest, Hungary); norfloxacin by Chinoin Pharmaceutical Works (Budapest, Hungary); piroxicam by EGIS Pharmaceutical Works (Budapest, Hungary) and used as received. Quinazolone-3-CH_2COOH, quinazolone-3-CH_2COOCH_3 and *O*-methyl piroxicam and were synthesized at the Semmelweis University of Medicine, Institute of Pharmaceutical Chemistry and published elsewhere [7,12].

3. Results and discussion

In a WApH titration, the spectral data obtained is a series of spectra acquired at different pH values. The absorbance data matrix, $\underline{A}$, can be expressed as follows:

$$\underline{A} = \underline{C}\ \underline{E} \quad (1)$$

where $\underline{C}$ and $\underline{E}$ represent, respectively, the concentration-pH profile of the ionization system and the molar absorptivity matrix with the inclusion of the optical path length. The principal component analysis [20,21] is first applied to $\underline{A}$ to calculate an abstract solution for $\underline{C}$ and $\underline{E}$, namely, $\underline{C}_{abs}$ and $\underline{E}_{abs}$, which contain only the primary eigenvalues ($\underline{\lambda}_r$) and eigenvectors ($\underline{Q}_r$). The residual standard deviation [21], IND function [20,21], eigenvalue ratio [22] and reduced eigenvalue ratio [23,24] are adopted to identify the number of principal components (independent light absorbing species). In the TFA treatment, $\underline{C}_{abs}$ and $\underline{E}_{abs}$ are rotated to the solution with physical significant, $\underline{C}_p$ and $\underline{E}_p$, via a transformation matrix $\underline{T}$ as defined below [21,25,26]:

$$\underline{T} = \underline{\lambda}_r^{-1}\ \underline{C}_{abs}^{T}\ \underline{C}_t \quad (2)$$

$$\underline{A} \approx \underline{C}_{abs}\ \underline{T}\ \underline{T}^{-1}\ \underline{E}_{abs} \quad (3)$$

$$\approx \underline{C}_p\ \underline{E}_p \quad (4)$$

where the superscripts -1 and T denote, respectively, inverse and transpose operations. The test matrix $\underline{C}_t$ in Eq. (2) contains the concentration-pH profiles of the ionization system which are generated theoretically. In this study, the proton concentration is related to the operational pH reading by a multi-parametric equation [19]. The spoil function as derived by Malinowski [21,26] is utilized to determine whether the target transformation has been successful. For a particular $\underline{A}$ matrix, the spoil function depends only on $\underline{C}_t$ which in turn is a function of the sought pK_a values [13] or microconstants as shown below. The TFA computation optimizes the unknown parameters for a global minimum of the spoil function. The simplex method can be used for this purpose [27].

It is not possible to resolve the spectral data from the zwitterion ($HX^{\pm}$) and the neutral species (HX^0) in a single titration experiment if both ionizable functional groups introduce significant spectral changes. This is because the concentrations of $HX^{\pm}$ and HX^0 are linearly dependent on each other. A summation of these two quantities is equivalent to the concentration of HX. In this sense, the spectral contributions from $HX^{\pm}$ and HX^0 degenerate to one principal component (see upper equation of Fig. 1). Thus, the pK_as instead of the microconstants are obtained in the TFA calculations. To characterize the ionization processes in terms of microconstants, the knowledge of the pK_a values and any one of the four microconstants or tautomeric ratio are necessary. This can be rationalized by Eqs. (5)–(7):

$$K_{a,1} = k_1 + k_2 \quad (5)$$

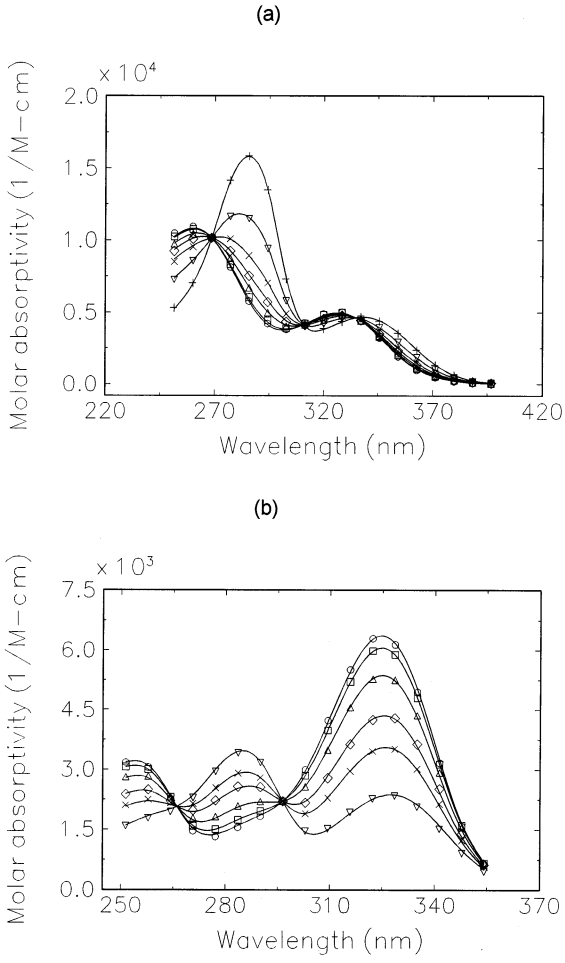

Fig. 6. The molar absorptivity spectra of HX for (a) niflumic acid (methanol content: o 0.2%, □8.3%, △21.0%, ◇27.6%, × 34.1%, ∇43.2%, + 56.3%) and (b) pyridoxine (methanol content: o 0.0%, □8.5%, △21.8%, ◇36.2%, × 46.3%, ∇62.5%) where lines indicate the â matrix and symbols denote the a matrix.

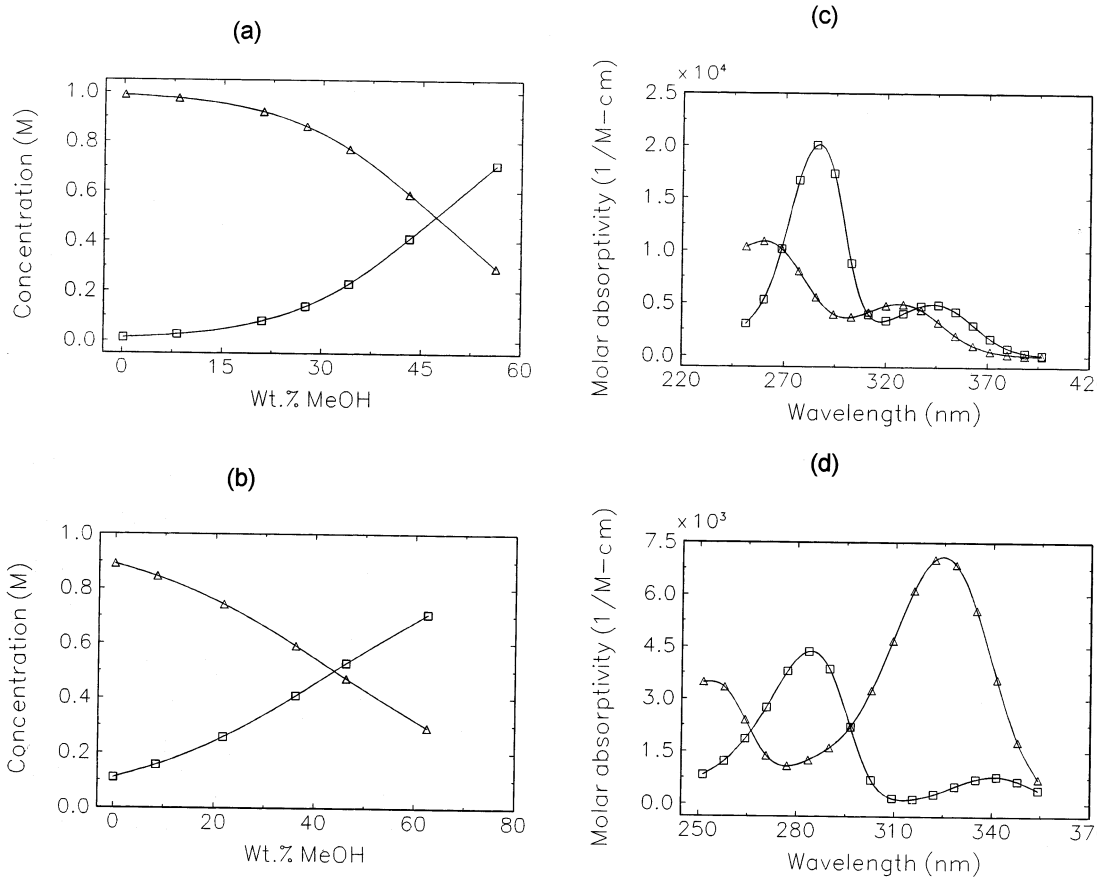

Fig. 7. Distribution of zwitterion (△) and neutral species (□) as a function of methanol content for (a) niflumic acid and (b) pyridoxine Molar absorptivity spectra of zwitterion (△) and neutral species (□) for (c) niflumic acid and (d) pyridoxine.

$$K_{a,1}\ K_{a,2} = k_2\ k_4 = k_1\ k_3 \tag{6}$$

$$k_Z = \frac{k_1}{k_2} = \frac{k_4}{k_3} = \frac{C\mathrm{HX}^{\pm}}{C\mathrm{HX}^{0}} \tag{7}$$

In this study, five diprotic amphoteric drugs were utilized to illustrate how we obtained the unknown microconstants and tautomeric ratio from the WApH experiments. We classified the compounds into three types (I, II and III, see above).

3.1. Type I compound

Norfloxacin contains two ionizable groups, namely a carboxylic group and a secondary amino group in piperazine ring. It has been reported that within the spectral region of 320–350 nm, the shape of the spectrum is independent of the ionization state of the piperazine moiety, but it is heavily influenced by that of the carboxylic group [8]. Thus, the ionization of the carboxylic acid group could be selectively monitored using this spectral region. Fig. 4a shows the absorption spectra of norfloxacin. Principal component analysis on this data matrix (250–400 nm) confirmed that three independent light absorbing species are involved in the ionization process. Table 1 lists the pK_a values of norfloxacin as determined by using the TFA method, which are in excellent agreement with literature and those determined pH-metrically. Fig. 4b, c show the distribution of species and the resolved molar absorptivity coeffi-

cients of norfloxacin. It can be seen that the molar absorption spectra of the monoprotic and the fully deprotonated species are overlapped in the spectral range of 330–350 nm. This implies that the ionization of the piperazine group does not display significant spectral shifts such that the other site may be selectively monitored. To this end, the microconstants were deduced by applying the TFA method on the absorbance data matrix in the region of 330–350 nm. The $\underline{C}_t$ matrix can be generated as below.

3.1.1. Distribution of microspecies

The distribution of microspecies is calculated by solving the following matrix equation [13]:

$$\begin{bmatrix} Y \\ 0 \\ 0 \\ 0 \end{bmatrix} = \begin{bmatrix} 1 & 1 & 1 & 1 \\ k_1 & -\mathrm{H} & 0 & 0 \\ 0 & k_3 & -\mathrm{H} & 0 \\ 0 & 0 & -\mathrm{H} & k_4 \end{bmatrix} \begin{bmatrix} C(\mathrm{H_2X^+}) \\ C(\mathrm{HX^\pm}) \\ C(\mathrm{X^-}) \\ C(\mathrm{HX^0}) \end{bmatrix} \quad (8)$$

where Y denotes the initial concentration of the sample used. The symbols k_3 and k_4 can be expressed in terms of k_1, $K_{a,1}$ and $K_{a,2}$ by using Eq. (5) and/or Eq. (6).

3.1.2. Formation of $\underline{C}_t$ matrix

The principal components to form the $\underline{C}_t$ matrix are established

$$\underline{C}_t^T = \begin{bmatrix} C(\mathrm{H_2X^+}) + C(\mathrm{HX^0}) \\ C(\mathrm{X^-}) + C(\mathrm{HX^\pm}) \end{bmatrix} \quad (9)$$

where the first row denotes the carboxylic acid species (H_2X^+ and HX^0) and the second row indicates the carboxylate species (X^- and $HX^\pm$). From Eqs. (2), (8) and (9), the TFA computation renders an optimization of k_1. It was found that the calculations successfully converged with a spoil value < 3.0. As shown in Table 1, the microconstants of norfloxacin agree well with the reported values of 6.3 (pk_1), 7.2 (pk_2), 8.5 (pk_3) and 7.6 (pk_4) obtained at 25°C and an ionic strength of 0.2 M [8]. Fig. 5 depicts the distribution of species and molar absorptivity coefficients for the carboxylic acid and carboxylate and the distribution of microspecies for norfloxacin as calculated using Eq. (8). As shown in Fig. 5c, the zwitterion is the predominant microform between pH 6.5 and 8.0.

3.2. Type II compounds

We now turn to piroxicam and quinazolone-3-CH_2COOH. As a result of the poor aqueous solubility of piroxicam, the pK_a values were deduced using the WApH technique. However, the pK_a values of quinazolone-3-CH_2COOH were measured pH-metrically. Next, a deductive method was applied to determine the microconstants. For piroxicam, pk_2 can be approximated by the pK_a value of the *O*-methyl derivative (Fig. 2c). Likewise, the pk_2 of quinazolone-3-CH_2COOH was obtained by measuring the pK_a value of the methyl ester (Fig. 2e). These derivative molecules are generally more water-insoluble than the parent compounds because some of the ionizable groups have been replaced by less polar methyl substituents. Here, WApH technique was used to determine the pK_a values of the derivative compounds. The pK_a values, microconstants and tautomeric ratios of piroxicam and quinazolone-3-CH_2COOH are given in Table 1. It is noted that the microconstants are in line with literature [7,12].

3.3. Type III compounds

Next, attention is directed to niflumic acid and pyridoxine. As shown in Fig. 2f, g, all ionizable groups are connected to the chromophores. Selective spectrophotometric monitoring is not applicable since the ionization of any one of these groups would result in measurable shifts of the UV spectrum. For this type of molecule, it has been proposed that the microconstants could be determined by measuring the tautomeric ratios spectrophotometrically from mixtures of organic

solvent and water at the pH of isoelectric point, with the tautomeric ratio calculated from the spectral data at an analytical wavelength using the following relationship [6]:

$$k_Z(\%) = \frac{A(XH^0) - A(\%)}{A(\%) - A(XH^{\pm})} \quad (10)$$

where: k_z (%) represents the tautomeric ratio in a given %(wt.) solvent mixture; $A(XH^{\pm})$ represents the absorbance of the compound in aqueous buffer; $A(XH^0)$ represents the absorbance of the compound in pure organic solvent; $A(\%)$ represents the absorbance of the compound in a given % solvent. The aqueous k_z value can be obtained from the intercept of the following equation:

$$\log\ k_z(\%) = W\ R + S \quad (11)$$

where R symbolizes wt.% solvent. W and S represent, respectively, the slope and the intercept.

It can be seen that the validity of Eq. (10) depends very much on the following assumptions:

1. Spectral contributions from H_2X^+ and X^- are neglected. This may not always true for compounds with heavily overlapping pK_as.
2. $A(XH^{\pm})$ and $A(XH^0)$ correspond, respectively, to the optical data obtained from 100% of $HX^{\pm}$ and 100% HX^0. In some amphoteric systems, complete formation of $HX^{\pm}$ or HX^0 may be difficult regardless of solvent composition. For instance, if k_z approaches unity, the correctness of $A(HX^{\pm})$ is questionable.
3. The molar absorptivity coefficients of $HX^{\pm}$ and HX^0 at the analytical wavelength should either be: $E(HX^{\pm}) \gg E(HX^0)$, or $E(HX^{\pm}) \ll E(HX^0)$.

Since the above assumptions are not always true, we have re-formulated Eq. (10) to deduce the aqueous k_z. In this new approach, several WApH titration experiments were performed in different wt.% of methanol. For each titration, the TFA method was applied to resolve the molar absorptivity spectra of species H_2X^+, HX and X^-. It is noted that the molar absorptivity spectrum of HX thus obtained is a linear combination of the molar absorptivity spectra of HX^0 and $HX^{\pm}$, which provide useful information to derive the unknown k_z value. The optical data of HX can be cast into a matrix $\underline{a}$ as follows:

$$\underline{a} = \underline{E}\ \underline{c} \quad (12)$$

where $\underline{a}$, $\underline{E}$ and $\underline{c}$ denote, respectively, the molar absorptivity spectra of species HX obtained at various wt.% methanol, the molar absorptivity spectra and the concentration-methanol content profiles of HX^0 and $HX^{\pm}$.

We first construct a $\underline{c}_t$ matrix which contains the theoretical concentration-methanol content profiles of HX^0 and $HX^{\pm}$:

$$\underline{c}_t = \begin{bmatrix} c(HX^0) \\ c(HX^{\pm}) \end{bmatrix} \quad (13)$$

Since $\underline{a}$ symbolizes the molar absorptivity spectra of HX, we can write,

$$c(HX^0) + c(HX^{\pm}) = 1 \quad (14)$$

From Eqs. (7), (11) and (14), $c(HX^0)$ and $c(HX^{\pm})$ can be expressed as below:

$$c(HX^0) = \frac{1}{1 + 10^{W\ R+S}} \quad c(HX^{\pm}) = \frac{10^{W\ R+S}}{1 + 10^{W\ R+S}} \quad (15)$$

Then, approximate molar absorptivity matrices for HX^0 and $HX^{\pm}$ ($\hat{\underline{E}}$) as well as HX ($\hat{\underline{a}}$) are calculated as follows:

$$\hat{\underline{E}} = \underline{a}\ \underline{c}_t^T(\underline{c}_t\ \underline{c}_t^T)^{-1} \quad (16)$$

$$\hat{\underline{a}} = \hat{\underline{E}}\ \underline{c}_t \quad (17)$$

In this study, an error function, err, is defined as below [21]:

$$err = \sum\sum(a - \hat{a})^2 \left(1 + \frac{\sum\sum|\hat{E} < 0|}{\sum\sum|\hat{E}|} \right),$$

$$0 < P \leq 100 \quad (18)$$

It can be seen that $\underline{c}_t$ depends only on W, S and R (wt.% methanol). Our computation renders to an optimization of W and S for a minimum err value.

In our previous study, we have reported the pK_a values and molar absorptivity spectra of niflumic acid and pyridoxine [13]. The pK_a values of these two compounds are included in Table 1 for clarification. Fig. 6 shows the molar absorptivity spectra of HX for niflumic acid and pyridoxine, in

which the lines indicate the $\underline{\hat{a}}$ matrix and the symbols denote the $\underline{a}$ matrix. Note the good agreement between theory and experiment. Table 1 gives the optimized log k_z values for niflumic acid and pyridoxine, which are consistent with the reported values of 1.24 for niflumic acid [6] and 0.90 for pyridoxine [28] obtained from dioxane-water mixtures. Fig. 7 depicts the distribution of zwitterion and neutral species as a function of methanol content ($\underline{c}_t$ matrix) and the molar absorptivity spectra of zwitterion and neutral species ($\underline{\hat{E}}$ matrix) for niflumic acid and pyridoxine. As shown in Fig. 7a, b, the concentrations of the zwitterionic forms of niflumic acid and pyridoxine increase as the methanol content decreases, suggesting that the formation of charged species is relatively favorable in aqueous media of high dielectric constants. On the other hand, the neutral species exhibit opposite behavior. Moreover, the molar absorptivity spectra for the neutral species and the zwitterions of niflumic acid and pyridoxine (see Fig. 7 c, d) agree well with the reported spectra of those compounds obtained in aqueous solution at the pH of isoelectric point (zwitterion) and in octanol (neutral species) [9]. These observations validate the suggested method for the determination of the tautomeric ratio of diprotic amphoteric substances.

With multiprotic amphoteric molecules, a comprehensive treatment is required to resolve the microscopic acid-base equilibria. Further work on this aspect is being carried out in our laboratories and results will be reported in due course.

4. Conclusions

We have extended the WApH technique in conjunction with TFA method to determine the microconstants and the tautomeric ratios of several diprotic amphoteric compounds, namely, niflumic acid, norfloxacin, piroxicam, pyridoxine and quinazolone-3-CH_2COOH. Three established approaches: (1) selective monitoring of ionizable group by spectrophotometry [8], (2) deductive method [7], and (3) k_z method for the determination of tautomeric ratio from co-solvent mixtures [6] have been utilized. It can be seen that the applicability of these methods depends very much on the nature of the molecule. Each of these approaches has limitations. For instance, the first method requires the presence of a suitable spectral region such that one of the ionizable groups does not cause measurable spectral shifts in the course of the titration experiments. The second method needs suitable derivative molecules which may be difficult to obtain in some occasions. Although we have re-formulated the mathematical procedure for the third method with fewer assumptions involved, the success of this treatment relies on the fact that the molar absorption spectra of zwitterion and neutral species should remain unchanged as the solvent content varies. While we note that the above methods should be applied with caution, particularly, with respect to the spectral properties of the compounds under investigation, we have demonstrated that the microconstants and tautomeric ratios as determined in this study are consistent with literature data. Moreover, with the aid of multivariate data treatment methodology, we have shown that different approaches for measuring microconstants can be accomplished by WApH technique in a highly automated fashion. This new application of WApH method may contribute to the wider use of microspeciation in medicinal chemistry.

Acknowledgements

We thank Mr J.E.A. Comer and Mr C. Peake for helpful discussion. K.T.-N. thanks for support by OTKA T 022623.

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