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Determination of protonation macro- and microconstants and octanol/water partition coefficient of the antiinflammatory drug niflumic acid

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Abstract: The drug niflumic acid is an amphoteric substance with overlapping pK_a values. The acid-base chemistry of the molecule has been characterized in terms of protonation macroconstants (with reference to stoichiometric ionizations) and microconstants (with reference to ionizations of individual species). The proton-binding sites were assigned using ^{1}H and ^{13}C NMR spectroscopy. Due to the very poor water solubility of niflumic acid, the aqueous pK_a values were determined from the apparent ionization constants in methanol–water solutions of various proportions by extrapolation to zero co-solvent using the Yasuda–Shedlovsky procedure. The k_z tautomerization microconstant of the equilibrium unionized form ⇄ zwitterionic form was determined from mixtures of organic solvent (dioxane or methanol) with aqueous buffer (at the pH of isoelectric point) by UV spectroscopy, and used for calculation of the other protonation microconstants. The zwitterionic form of the molecule predominates over the uncharged form, the concentration being maximal at the isoelectric pH. The apparent partition coefficients (P_{app}) of niflumic acid were measured in octanol/water solution by the shake-flask method over a wide pH range. The lipophilicity profile (logP_{app} vs pH) shows a parabolic shape near its maximum at the isoelectric point. A relationship derived between P_{app}, p_{XH^n}(micropartition coefficient of the uncharged microspecies) and P_{X^-}(partition coefficient of the anion) is valid for amphoteric drugs, in cases where the partition of the unionized form and the ion-pair partition of anion can be confirmed. The log$P$ values of microspecies indicate the high lipophilicity of niflumic acid, which is consistent with its good skin penetration and absorption.

Keywords: *Niflumic acid; protonation macro- and microconstants; pH profile of lipophilicity; macro-log*P*; micro-log*P.

Introduction

Niflumic acid is a potent drug used in the treatment of inflammatory and rheumatic disorders [1]. The compound has good pharmacokinetic properties: rapid absorption and distribution followed by extensive metabolism [2]. The clinical usage [3, 4] and pharmacology [5] of niflumic acid have been reported, as have various useful methods for quantitative determination of the substance in both pharmaceutical preparations and biological samples [6, 7]. Little has been published on the acid–base chemistry and lipophilicity of niflumic acid, yet such characterization is necessary for thorough understanding of the fate of the drug in the body (absorption, distribution, receptor binding, for example).

A spectroscopic study of the diffusion of niflumic acid across artificial lipid barriers related the physico-chemical properties of the drug to the diffusion rate, but assumed the substance to be monoprotic with a carboxylic pK_a of 4.73 [8]. The octanol/water distribution coefficients, D, at three different pH values were reported [8]. Apparently, the ampholytic nature of the molecule was not considered.

Niflumic acid is very poorly soluble in water. Bres *et al.* [9] reported data on the pH-dependent solubility of the drug; the minimum solubility was 24.54 mg l^{-1} at pH 3.6. From these data Asuero calculated the acidity constants (pK_a = 5.14 and 2.11) using a bilogarithmic evaluation method [10].

In light of the incompleteness of understanding of the acid-base and lipophilicity properties of niflumic acid, it was decided to re-investigate the molecule, using ^{1}H, ^{13}C NMR spectroscopy, UV spectrophotometry and potentiometry. The aims were: (1) to describe the acid–

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base properties of niflumic acid in terms of protonation macroconstants (stoichiometric protonation reactions) and microconstants (protonation reactions of distinct molecular species); (2) to investigate its pH partition profile over a wide pH range; (3) to characterize the microlipophilicity of actual species existing at physiological pH; and (4) to consider the extent of ion-pair partitioning and the effect of salt on such behaviour.

Experimental

Materials

Niflumic acid was generously supplied by its manufacturer (Gedeon Richter Chemical Works, Budapest) and was used without further purification. 1-octanol was HPLC grade (Aldrich); 1,4-dioxane and methanol were spectroscopic grade (Fluka). The Britton–Robinson buffer (acetic, phosphoric and boric acids, each at 0.04 M, treated with 0.2 M sodium hydroxide) was used for the pH range 2–7. For pH 0 and 1, 1 M and 0.1 M hydrochloric acid served as the aqueous phase in partitioning experiments. The preparation and standardization of 0.5 M HCl (Fisons) and 0.5 M NaOH (Volucon™, Rhône–Poulenc) titrants has been described elsewhere [11–13]. All other reagents were of analytical grade.

Determination of protonation macroconstants

Due to the poor water solubility of niflumic acid, the protonation macroconstants $\log K_1 (= pK_{a1})$ and $\log K_2 (= pK_{a2})$, were determined by a mixed-solvent method. All titrations were performed under an argon atmosphere (to exclude atmospheric CO_2) and at 25.0 ± 0.1°C. Five separate 20-ml semiaqueous solutions in 29.8–53.5 (%w/w) methanol of 0.9–1.3 mM niflumic acid and 0.15 M NaCl (to adjust ionic strength) were acidified by standardized HCl to pH 1.7. The solutions were then titrated with standardized NaOH to pH 10.5; after each titrant addition the pH was measured. The initial estimates of the p_sK_a values, which are the apparent ionization constants in the mixed-solvent, were obtained from the difference (Bjerrum) plots [11]. These values were then refined by a weighted nonlinear least-squares procedure [12]. The refined values were then extrapolated to zero co-solvent by the Yasuda–Shedlovsky procedure [13].

Determination of protonation microconstants

UV spectroscopy was used to determine the k_z tautomerization microconstant for the equilibrium: XH^0 (unionized form) ⇄ $XH^{\pm}$ (zwitterionic form). The method is based on the spectral differences between the zwitterionic form of the molecule (found predominantly at the isoelectric pH in aqueous solution) and the unionized form (found predominantly in organic solvent of low dielectric constant, such as dioxane). The spectra can be converted from one form to the other by changing the solvent mixture [14].

A stock solution (~5 mM) of niflumic acid was prepared in organic solvent (dioxane or methanol). A 0.10-ml aliquot of this solution was diluted to 10.00 ml with organic solvent or with Britton–Robinson buffer at pH 3.3 or with various mixtures of the buffer and organic solvent. The concentration of the organic solvent was changed from 0 to 100% in steps of 5% (dioxane) or 10% (methanol). The absorption spectra of these solutions were measured by a Hewlett–Packard 8452A diode array spectrometer. The tautomerization microconstant was calculated from the spectroscopic data with the aid of the relationship

$$k_{z(\%)} = \frac{A_{XH^0} - A_{\%}}{A_{\%} - A_{XH^{\pm}}} \qquad (1)$$

where: $k_{z(\%)}$ tautomerization constant in a given % solvent mixture; $A_{\%}$ absorbance of the compound in a given % solvent mixture; A_{XH^0} absorbance of the compound in pure organic solvent; and $A_{XH^{\pm}}$ absorbance of the compound in aqueous buffer solution at the isoelectric pH.

Investigation of the NMR spectra

Two ~0.1 M solutions of niflumic acid were prepared in either deuterated methanol or deuterated methanol acidified with 2M DCl. The ^1H and ^{13}C NMR spectra were recorded on a Bruker AC-400 NMR spectrometer at 400 and 100 MHz, respectively. TMS was used as internal standard.

Shake-flask determination of the partition coefficients

The apparent partition coefficients were measured by the shake-flask technique, as previously described [15]. In this work a shaking water-bath at 25.0 ± 0.1°C (Lauda, M20S) was used to equilibrate the organic and

the aqueous phases. Spectra were recorded by the HP 8452A spectrometer.

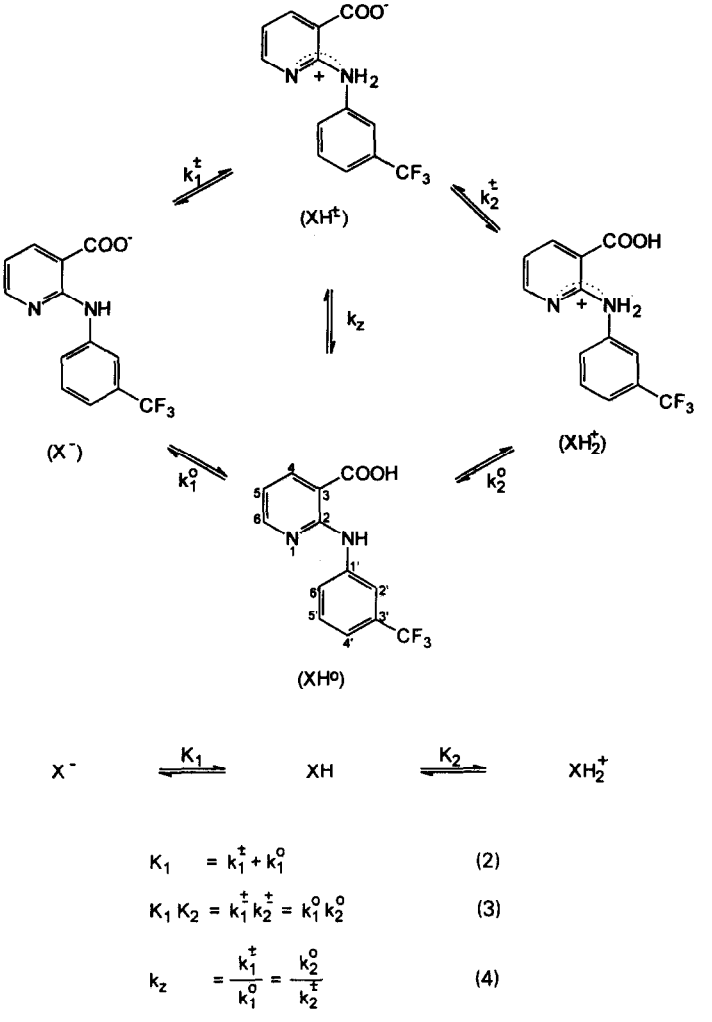
Potentiometric determination of the partition coefficients

Five separate alkalimetric titrations of 0.8–1.5 mM niflumic acid and 0.15 M NaCl, initially acidified to pH 1.7 with HCl, containing varying amounts of octanol (0.25 ml octanol/22 ml water to 5 ml octanol/10 ml water) were performed at pH 1.7–10.5. The titrations were conducted under argon at 25.0 ± 0.1°C. To test the effect of salt on the partitioning of niflumic acid, another five octanol/water titrations were performed under similar conditions as in the first series, except that no salt was added to adjust the ionic strength. The macroscopic log$P$ of the neutral XH compound and of the charged species XH_2^+ and X^- were estimated from difference (Bjerrum) plots [11], and refined by a weighted nonlinear least-squares procedure [12]. During the refinement, the aqueous pK_a values were included as unrefined contributions. Since these values had been determined only at 0.15 M ionic strength, the Debye–Hückel theory was used to correct the pK_a values to near zero ionic strength for the log$P$ determination where no salt was added [12].

Results and Discussion

Acid–base equilibria and proton-speciation

Niflumic acid contains two proton-binding sites and exists in four protonated forms in solution. The protonation equilibria, the relevant constants and their relationships are shown in Fig. 1. The protonation macro-



(2)

(3)

(4)

Figure 1
Protonation macro- and microequilibria of niflumic acid. Relationships between protonation macro- and microconstants.

constants (K_1 and K_2) describe the overall basicity of the molecule but generally cannot be assigned to individual functional groups when the constants are nearly overlapping. The protonation microconstants (k_1^+; k_1^0; k_2^+; k_2^0) provide information on the proton-binding sites and their interactions with other protonated groups of the molecule. These constants are useful in calculating the pH-dependent concentrations of the different protonation microspecies (microspeciation) [16, 17].

Beside the carboxylate group, the other proton-binding site in the molecule is the aminopyridinyl moiety. The protonation sites were studied by NMR spectroscopy. The chemical shifts of ^1H and ^{13}C NMR spectra are listed in Table 1. The chemical shifts caused by protonation are also shown. The upfield shift of C-1′ and the downfield shifts of the other carbon atoms of the phenyl ring are in agreement with published data for protonated anilines [18]. The downfield shift of C-6 atom of the pyridine ring cannot be the result of the protonation either of the carboxylate or of the amino group [19]. The protonation of the pyridine N atom resulted in a downfield shift of the α carbon [20]. However, the observed chemical shifts can be explained by a tautomeric equilibrium between the forms protonated on the exo- and the endocyclic nitrogen, which is shifted towards the protonated amino group.

Due to the low water solubility of niflumic acid, potentiometry in a purely aqueous medium cannot be used for pK_a determination. The other classical method, UV spectroscopy, has limited value in this instance; this is because it can only be used to determine the COOH pK_a since the protonation of the N atom does not result in measurable shifts of the UV spectrum. Consequently the Yasuda–Shedlovsky extrapolation procedure was used to determine the aqueous pK_a values from a series of methanol/water titrations. Table 2 contains the apparent pK_a values as a function of %w/w methanol. The aqueous pK_a values were calculated using the following linear regression equations:

$$p_sK_{a1} + \log[H_2O] = 7.441 - 98.12/\epsilon \quad (5)$$

$$p_sK_{a2} + \log[H_2O] = 4.808 - 62.56/\epsilon \quad (6)$$

These values are shown as stepwise protonation macroconstants in Table 3. Figure 2 shows the Yasuda–Shedlovsky plot. Compared with the cited literature data [10] the calculated logK_1 value is considerably different from that obtained using solubility data; this may be attributed in part to differences in experimental conditions (ionic strength and sample concentration). The results identify the isoelectric point of the molecule to be pH 3.3. As indicated by the macroconstant values, overlapping protonation is characteristic for niflumic acid. In such cases, the microconstants determine the dominant route of the protonation from the two possible forms (zwitterionic and unionized).

Experimental measurement of microconstants of niflumic acid is also problematic. The most frequently used methods, the pH-dependence of UV or NMR spectra are not suitable, because the protonation of both functional groups may influence on spectral

Table 1
^1H and ^{13}C NMR chemical shifts [ppm] of niflumic acid

Atom	MeOD	MeOD + DCl	Δδ
4-H	8.34	8.88	0.54
5-H	6.86	7.22	0.36
6-H	8.37	8.10	−0.27
2′-H	8.26	7.87	−0.39
4′-H	7.27	≈7.82	~0.5
5′-H	7.46	≈7.82	~0.4
6′-H	7.79	≈7.82	0
C-2	157.2	153.3	−3.9
C-3	109.6	114.8	5.2
C-4	142.1	142.2	0.1
C-5	115.4	115.0	−0.4
C-6	153.6	149.4	−4.2
COO	170.7	167.6	−3.1
C-1′	142.2	136.2	−6.0
C-2′	119.5	126.9	7.4
C-3′	132.1	133.8	1.7
C-4′	117.4	124.9	7.5
C-5′	130.5	132.9	2.4
C-6′	124.3	131.6	7.3
CF_3	125.8	125.0	−0.8

Table 2
Apparent dissociation constants in methanol–water mixtures

% w/w Methanol	p_sK_{a1}	p_sK_{a2}
53.5	4.31 ± 0.01	2.31 ± 0.06
43.3	4.31 ± 0.02	2.27 ± 0.08
39.0	4.33 ± 0.03	2.29 ± 0.10
34.0	4.38 ± 0.02	2.39 ± 0.05
29.8	4.40 ± 0.01	2.23 ± 0.06
0.0*	4.44 ± 0.03*	2.26 ± 0.08*

*Extrapolated by the Yasuda–Shedlovsky procedure (see text).

Table 3
Protonation macroconstants and microconstants of niflumic acid

Macroconstants	Microconstants
$\log K_1 = 4.44 \pm 0.03$	$\log k_z = 1.24 \pm 0.01$ (dioxane–water)
$\log K_2 = 2.26 \pm 0.08$	
isoelectric point = 3.3	$\log k_1^{\pm} = 4.42$
	$\log k_1^{0} = 3.18$
	$\log k_2^{\pm} = 2.28$
	$\log k_2^{0} = 3.52$
	$\Delta = \log k_1^{\pm} - \log k_2^{0} = \log k_1^{0} - \log k_2^{\pm} = 0.90$

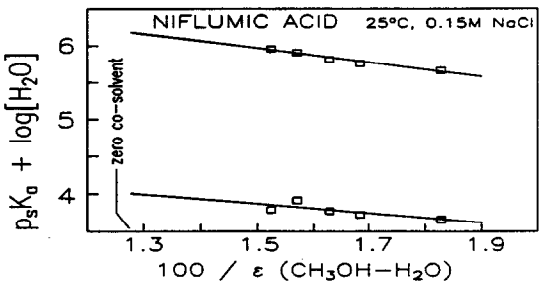


Figure 2
Yasuda–Shedlovsky plot in methanol–water for niflumic acid. The left end of the solid curves corresponds to zero co-solvent and the right end corresponds to about 55% w/w methanol.

changes. So a less commonly used method, the determination of the k_z tautomerization microconstant, was chosen. It was first described by Metzler and Snell for pyridoxine in a dioxane/water solvent system [21] and was successfully applied in a methanol/water solvent system by the present authors [14]. The spectrum of niflumic acid in dioxane and methanol was identified as that of the unionized form (XH^0) while the spectrum in aqueous buffer at pH of the isoelectric point (3.3) was assigned to that of the zwitterion ($XH^{\pm}$); these deductions were based on analogy to pyridoxine and on analysis of related compounds (anthranilic acid, nicotinic acid). As shown in Fig. 3 the spectra obtained at different organic solvent–water mixtures pass through isosbestic points, which are particularly sharp in the methanol–water system indicating the existence of only a single equilibrium, namely $XH^0 \rightleftarrows XH^{\pm}$. From the spectroscopic data $k_{z(\%)}$ values were calculated: the aqueous k_z values were obtained from the intercepts of the equations:

$$\log k_{z(\%)} = -0.053 \text{ dioxane } (\%\text{w/w}) + 1.239 \quad (n = 27;\ r = 0.9986) \quad (7)$$

$$\log k_{z(\%)} = -0.027 \text{ methanol } (\%\text{w/w}) + 1.439 \quad (n = 21;\ r = 0.9971) \quad (8)$$

That similar k_z values were obtained from different organic co-solvents supports the validity of our model. Furthermore, it is noteworthy that the formation of the unionized species is completed in 60% w/w dioxane (spectra unchanged 60–100% w/w dioxane) and 100% methanol, which can be well explained by the very similar dielectric constant of these solvents ($\epsilon_{60\%\text{w/w dioxane}} = 27.2$, $\epsilon_{\text{methanol}} = 32.6$).

The protonation microconstants calculated by equations 2–4 are summarized in Table 3. They are used to calculate the pH-dependent concentrations of microspecies, shown in Fig. 4. At two physiological pH values, pH 1 for gastric fluid and pH 7.4 for blood, the relative concentrations of microspecies of niflumic acid are given in Table 4.

The Δ value in Table 3 is an interactivity parameter. It quantifies the interaction between the two protonating sites. A value close to 1 indicates that protonation at one site decreases the basicity of the other site by almost one order of magnitude.

Lipophilicity

Since niflumic acid is a zwitterionic amphoteric molecule, it is capable of being ionized over the whole pH range. Thus in the shake-flask method, there is no obvious pH at which the measured apparent partition coefficient, P_{app}, is equal to the true partition coefficient, P (the experimental situation is somewhat different when partitioning is measured by potentiometry). Figure 5 shows the pH-partitioning profile of niflumic acid obtained by the shake-flask method, together with the actual values and standard deviations. The approximately parabolic shaped curve shows that maximum lipophilicity occurs at the isoelectric point.

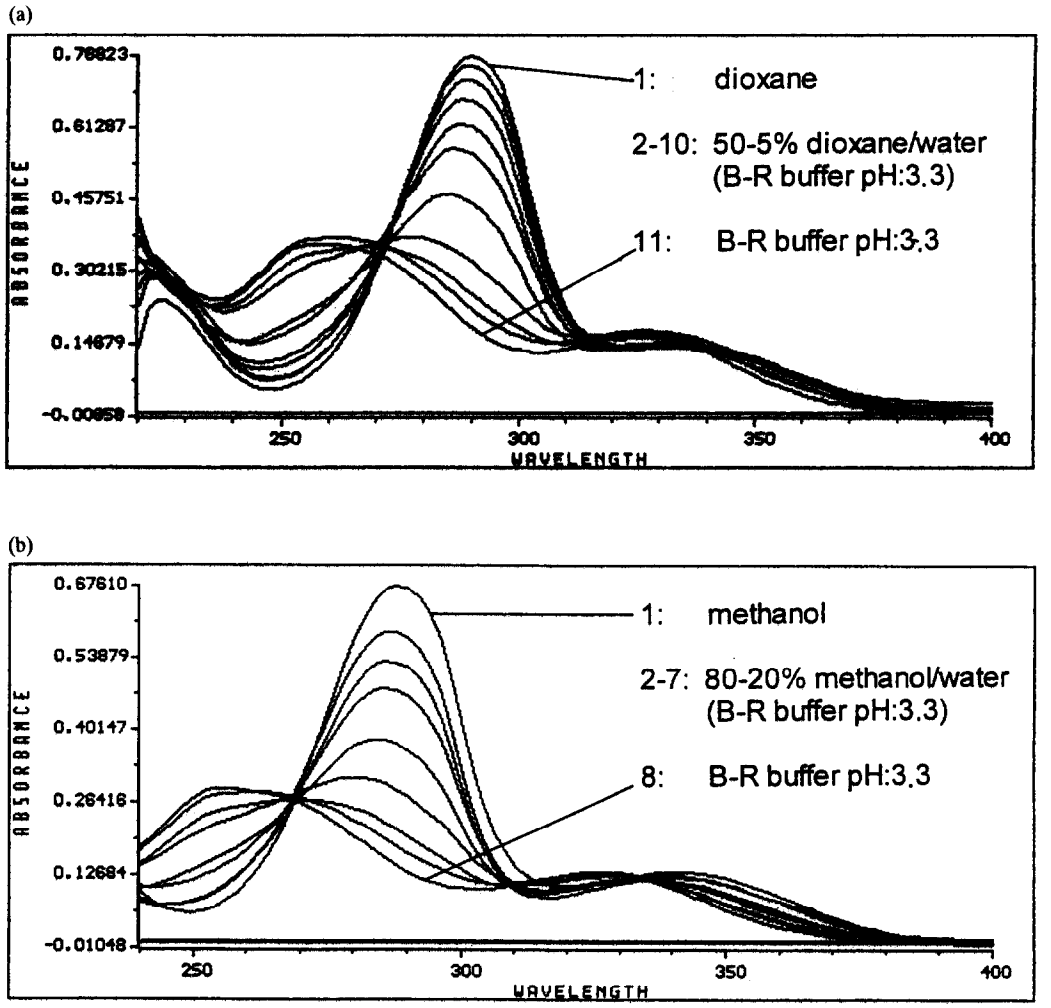


Figure 3
UV spectra of niflumic acid in the solvent system (a) dioxane–water (with Britton–Robinson buffer, pH 3.3), (b) methanol–water (Britton–Robinson buffer, pH 3.3).

Table 4
Relative concentrations of microspecies of niflumic acid

pH	X^- (%)	$XH^{\pm}$ (%)	XH^0 (%)	XH_2^+ (%)
1.0	0.002	5.92	0.34	93.80
7.4	99.92	0.08	0.005	4×10^{-7}

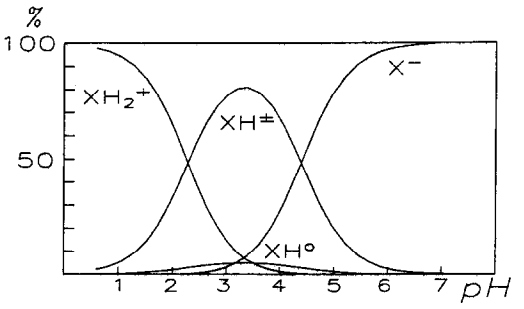


Figure 4
Distribution diagram of the microspecies.

Recently a relationship was derived for zwitterionic drugs between log*P* and $\log P_{app}$ [15] where the term "true" partition coefficient (log*P*) of the microspecies XH^0 is a micro-constant, which is known as micro-log*P* (or simple log*p*, by analogy to *K* versus *k* terminology with ionization constants).

$$\log P = \log p = \log P_{app} + \log(1 + (k_1^0 [H^+])^{-1} + k_2^0/k_2^{\pm} + k_2^0 [H^+]) \quad (9)$$

This model assumes that the zwitterion does

Table 5
Macro-log P values from potentiometric analysis

I (ionic strength)*	log$P$(XH)	log$P$(XH_2^+)	log$P$(X^-)
0.026 M	3.73 ± 0.01	2.48 ± 0.16	0.44 ± 0.03
0.151 M	3.88 ± 0.01	3.29 ± 0.05	1.23 ± 0.02

*Macro pK_a values from Table 3 were used as fixed contributions in the refinement of log$P$. For the low salt determinations, the Debye–Hückel theory [12] was used to "correct" the constants determined at 0.151 M to the expected values at 0.026 M:pK_a values were 4.55 and 2.26.

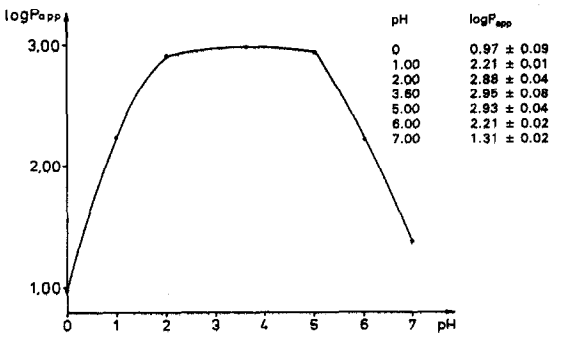


Figure 5
The pH profile of lipophilicity of niflumic acid obtained by the shake-flask method.

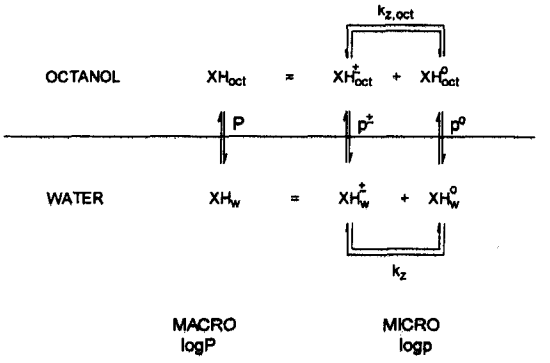


Figure 6
Macroconstant and microconstant partition equilibria between water and octanol. The octanol-phase tautomeric constant can be derived from the aqueous constants by: $k_{z,oct} = (p_{11}^0/p_{11}^+)/k_z$.

not participate in the actual partitioning. A general scheme illustrating the micro-log$P$ concept is shown in Fig. 6.

Application of equation 9 to the logP_{app} data of niflumic acid failed to yield a consistent value of log$p$ in the pH range 0–7. Up to pH 3.6, log$p$ = 4.48 ± 0.17, but the value progressively increased up to 5.15 when the pH was increased beyond 3.6. This suggested the need to include ion-pair partitioning in the model, in order to explain the observed trend.

A new model was set up assuming the partition of both the unionized microspecies and the anion, and a relation was derived as follows:

$$p_{XH^0} = [XH^0]_0/[XH^0]_w \qquad (10)$$

$$P_{X^-} = [X^-]_0/[X^-]_w \qquad (11)$$

$$P_{app} = \frac{[XH^0]_0 + [X^-]_0}{[X^-]_w + [XH^0]_w + [XH^\pm]_w + [XH_2^+]_w} \qquad (12)$$

Expressing the concentration of the equilibrium microspecies in the aqueous phase by the protonation microconstants yields:

$$P_{app} = \frac{[XH^0]_0 + [X^-]_0}{([XH^0]_w/k_1^0[H^+]) + [XH^0]_w + (k_1^\pm[XH^0]_w/k_1^0) + k_2^0[XH^0]_w[H^+]} \qquad (13)$$

By combination of equations 10, 11 and 13 the relationship between P_{app}, p_{XH^0} and P_{X^-} is given by a linear regression equation:

$$P_{app}\,(1 + (k_1^0[H^+])^{-1} + k_1^\pm/k_1^0 + k_2^0[H^+]) = P_{X^-}(k_1^0[H^+])^{-1} + p_{XH^0} \qquad (14)$$

where the slope gives the partition coefficient of anion, P_{X^-} and the intercept gives the partition coefficient of the uncharged microspecies, p_{XH^0}. In the case of niflumic acid, logp_{XH^0} = 4.81, logP_{X^-} = 1.07 were so derived. These results indicate the high lipophilic character of the molecule, which is consistent with its good absorption and skin penetration [2, 3].

Liphophilicity and the salt effect

Potentiometric titrations in the presence of octanol also produced information about the lipophilicity of niflumic acid. Table 5 summarizes the macroscopic log$P$ values derived from experiments with no added salt and with

0.15 M NaCl. Figure 7 illustrates the octanol-dependent difference plots, based on 10 separate titrations with octanol-containing solutions. The systematic pattern of dependency on the octanol–water volume ratio clearly supports not only anion partitioning but also cation partitioning, in addition to the predominant partitioning of the neutral species. A key feature in Fig. 7 which supports the partitioning of the cation is the evidence of titratable protons between pH 1.7 and 3.0. Symmetry arguments put forward in [11] can be applied. Data from all 10 titrations with octanol-containing solutions were subjected to a rigorous weighted nonlinear least-squares analysis, where several possible models were tested. The best set of partition coefficients, listed in Table 5, are those which satisfy the entire data structure, as graphically indicated in Fig. 7.

The lipophilicity profiles calculated from the macroconstants in Table 5 are shown in Fig. 8. The lower curve was based on the set of experiments with no added salt; the upper curve was based on the experiments with the physiological concentration of salt, 0.15 M

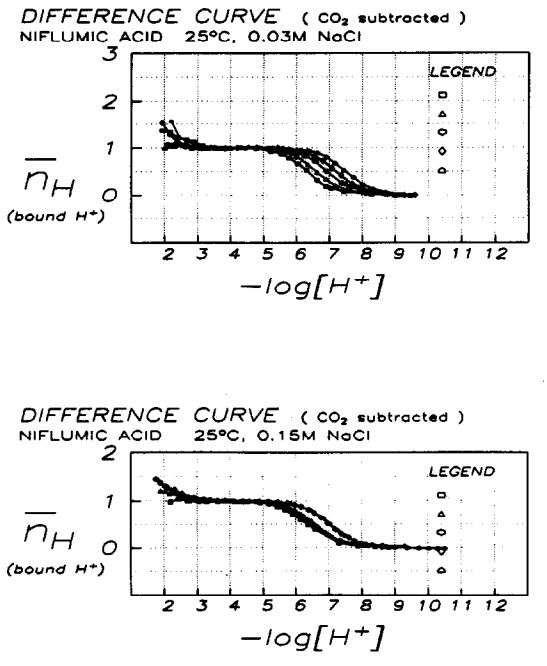


Figure 7
Difference (Bjerrum) curves for niflumic acid based on octanol–water titrations, in the absence of added salt to adjust the ionic strength (upper plot), and in the presence of 0.15 M NaCl (lower plot). The solid lines are used to connect adjacent points. The symbols square ..... pentagon corresponds to increasing amounts of octanol in both plots.

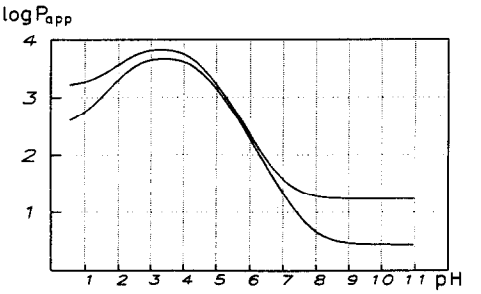


Figure 8
Lipophilicity profile, $\log P_{app}$ vs pH, calculated from the macroconstants determined by potentiometry. Lower curve, I = 0; upper curve, I = 0.15 M NaCl.

NaCl. Salt appears to be an important factor in the lipophilicity of niflumic acid. At pH 7.4, the apparent partition coefficient (P_{app} or D) is 12.0 at ionic strength 0.026 M and 27.5 at 0.15 M NaCl; there is more than a two-fold difference in the results. Akamatsu *et al.* [22] studied the lipophilicity of largely hydrophilic di- and tripeptides and found dramatic effects due to salt.

Values of macro-log$P$ can be converted to micro-log$P$ (or log$p$) if the relevant protonation microconstants are known. It can be shown that

$$P_{XH} = p_{XH^0}(k_1^0/K_1) + p_{XH^\pm}(k_1^\pm/K_1) \quad (15)$$

It is assumed that the zwitterion does not partition into octanol; therefore, the second term on the right-hand side of equation 15 can be neglected. From the resultant equation, the potentiometrically based calculation yields; $\log p_{XH^0} = \log P_{XH} + \log K_1 - \log k_1^0 = 5.14$ at 0.15 M NaCl; this value agrees with the shake-flask value of 4.81.

Conclusions

The results of the present work on niflumic acid strengthen the conviction that the description in terms of macroscopic pK_a values of the acid-base properties of a molecule having overlapping protonation properties is inadequate. Microspeciation provides indispensable information about submolecular protonation states. Furthermore, contrary to the present practice reflected in the literature, the lipophilicity of ampholytic drugs cannot be well characterized by a single $\log P_{app}$ (or log$D$) value, which is generally measured at pH 7.4.

It is necessary to deduce the true partition coefficient, which may be best represented as a micro-log*P* or simple log*p*. The latter may reveal anomalous behaviour and indicate ion-pair partitioning. It has also been shown that salt can have a significant effect on the lipophilicity of drugs. It is recommended that, in addition to buffering solutions in shake-flask determinations, NaCl be added to adjust the ionic strength in order to mimic physiological conditions.

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