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General equation for calculating the dissociation constants of polyprotic acids and bases from measured retention factors in high-performance liquid chromatography

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Abstract

A general equation relating the observed retention factor to the pH of the mobile phase, the dissociation constants, and the retention factors of the different ionic species has been derived. This equation is applicable to polyprotic weak acid and base dissociation events, that is, the secondary equilibria existing in the HPLC mobile phase. It is written as:

$$k_{obs} = \frac{k_o + \sum_{r=1}^{n} k_r \cdot K_a(r) \cdot e^{rx}}{1 + \sum_{r=1}^{n} K_a(r) \cdot e^{rx}}$$

where the k_r values are the retention factors of the dissociated species, and $K_a(r)$, the product of the first r-dissociation constants, $K_a(r) = \prod_{i=1}^{r} K_{a,i}$. x is related to the pH of the mobile phase: $x = 2.303 \cdot \mathrm{pH}$. The derived equation was used to calculatethe three dissociation constants of leukotriene E_4. Also, a formula is established for calculating the range of pH values where an ionic species is most likely to be predominant in the mobile phase.

Keywords: Dissociation constants; Retention factors; pH effects; Leukotrienes; Organic bases; Organic acids

1. Introduction

High-performance liquid chromatography (HPLC) has been shown to be an effective technique for the separation of weak acids and bases [1]. The interpretation of the physicochemical phenomena involved in the chromatographic process and the elucidation of the effect of solute ionization and solvation on chromatographic retention have been reported [2]. According to the theory, reversible associations take place between neutral and ionized solutes on the one hand and hydrocarbonaceous ligands bound to the surface of the stationary phase on the other hand. For weak acids and bases, a secondary equilibrium in the mobile phase is also established between the neutral solute molecule and its corresponding ionic species. A theoretical treatment based on this theory was developed and equations relating the retention factor of the solute to the dissociation constants and the pH of the mobile phase are derived for weak monoprotic, diprotic acids and zwitterions. An alternate equation was also reported for weak monoprotic bases [2]. In these equations the retention factors of the ionized species are considered as parameters. Their values are computed by a least-square fitting method which is utilized to fit the measured retention

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factors to the model equations. The importance of the theory stems from the fact that reversed-phase (RP) HPLC is a valuable technique for the separation of minute quantities of biologically important weak acids and bases [4]. The theory is helpful for finding the optimal conditions for such separations. The dissociation constants are usually measured by independent titration methods or calculated from thermodynamic data [3]. Some authors are, however, tempted to obtain the values of the dissociation constants of weak acids and bases from the same fitting procedure used for obtaining the retention factors [2,4,5]. Such a possibility is very important, especially when the amount of the compound is of the order of nanograms. However, obtaining the values of several parameters from fitting the retention factor, measured at different pH values, to the model equations raises a question about the accuracy of the values obtained. For example, the number of parameters in the equation increases rapidly with the number of carboxylic groups in weak acids. For n carboxylic groups in an acid, the number of parameters (as shown below) is $2n+1$, $n+1$ retention factors and n ionization constants. The question of accuracy of the values obtained for these parameters has not been examined.

In this work a general equation relating the observed retention factor to the pH of the mobile phase, the dissociation constants and retention factors of the different ionic species is derived. The equation is applicable to polyprotic weak acids and bases and amino acids. A relationship is also established for calculating the range of pH values where an ionic species is most likely to be predominant in the mobile phase.

A two stage, least-square fitting procedure is developed for fitting the general equation to the observed retention factors. The procedure handles cases where some elements of the Jacobi matrix (which appears in the formulation of the fitting procedure) are either too small or too large. A computer program implementing the procedure is developed. Actual calculations revealed that, in some cases, there may be a question about the uniqueness of the solution to the problem. Thus there may be more than one set of parameter values that reproduce the observed retention factors within a predetermined accuracy. Such cases may occur when the measurement of the retention factor of the solute is extended over large pH values (pH>8). Very high accuracy in numerical computation is required. Therefore caution has to be exercised when adopting the results of calculation. Apart from this, the method is promising, especially for calculating the dissociation constants in a nontraditional way. The method was applied in the calculation of the dissociation constants of leukotriene E_4 from the measured retention factor at various pH values of the mobile phase. The range of pH values for the predominance of each of the possible species was also calculated.

2. General treatment of a weak polyprotic acid

The original treatment developed by Horváth et al. [2] for the case of a diprotic acid, is generalized here. A weak polyprotic acid, H_nA, may ionize successively to produce several anions in the mobile phase as shown in Table 1.

$K_{ar,m}$ is the ionization equilibrium constant corresponding to the r^{th} ionization step, and m stands for mobile phase.

In the chromatographic process, the solute retention is, according to the theory, the result of reversible association between the dissociated and undissociated acid and hydrocarbonaceous ligand, L, of the stationary phase [2]. The following equilibria are assumed to take place as shown in Table 2, where s stands for the stationary phase. There are n ionization constants in the mobile phase, $K_{ar,m}$ ($r=1,2,...,n$) and $(n+1)$ binding equilibrium constants, $K_{LH_{n-r}A^{r-}}$ ($r=0,1,...,n$).

Table 1
Dissociation equilibria of polyprotic acid

Dissociation	Equilibrium-constant expression
$H_nA \rightleftharpoons H_{n-1}A^- + H^+$	$\frac{K_{a1,m}}{[H^+]_m} = \frac{[H_{n-1}A^-]_m}{[H_nA]_m}$
⋮	⋮
$H_{n-(r-1)}A^{(r-1)-} \rightleftharpoons H_{n-r}A^{r-} + H^+$	$\frac{K_{ar,m}}{[H^+]_m} = \frac{[H_{n-r}A^{r-}]_m}{[H_{n-(r-1)}A^{(r-1)-}]_m}$
⋮	⋮
$HA^{(n-1)-} \rightleftharpoons A^{n-} + H^+$	$\frac{K_{an,m}}{[H^+]_m} = \frac{[A^{n-}]_m}{[HA^{(n-1)-}]_m}$

Table 2
Acid–ligand association equilibria

Association	Binding equilibrium-constant expression
$H_nA + L \rightleftharpoons LH_nA$	$K_{LH_nA} = \frac{[LH_nA]_s}{[H_nA]_m[L]_s}$
⋮	⋮
$H_{n-r}A^{r-} + L \rightleftharpoons LH_{n-r}A^{r-}$	$K_{LH_{n-r}A^{r-}} = \frac{[LH_{n-r}A^{r-}]_s}{[H_{n-r}A^{r-}]_m[L]_s}$
⋮	⋮
$A^{n-} + L \rightleftharpoons LA^{n-}$	$K_{LA^{n-}} = \frac{[LA^{n-}]_s}{[A^{n-}]_m[L]_s}$

The retention factor, k, of the solute is, by definition given by:

$$k = \phi \frac{\sum_{r=0}^{n} [LH_{n-r}A^{r-}]_s}{\sum_{r=0}^{n} [H_{n-r}A^{r-}]_m} \quad (1)$$

where ϕ is the volume ratio of the stationary and mobile phases.The ratio ϕ is usually maintained constant. Successive multiplications of the equilibrium-constant expressions in the 2nd column of Table 1 lead to the general relation:

$$\frac{\prod_{i=1}^{r} K_{ai,m}}{[H^+]^r} = \frac{[H_{n-r}A^{r-}]_m}{[H_nA]_m} \quad (2)$$

from which the concentration of a given anion may be calculated in terms of the concentration of the undissociated acid:

$$[H_{n-r}A^{r-}]_m = [H_nA]_m \cdot \frac{K_a(r)}{[H^+]^r} \quad (3)$$

The constant $K_a(r)$ is the product of the first r equilibrium constants:

$$K_a(r) = \prod_{i=1}^{r} K_{ai,m} \quad (4)$$

(The subscript m is omitted from $[H^+]^r$.) It is important to recognize here that $K_a(r)$ is the equilibrium constant corresponding to the direct formation of the $H_{n-r}A^{r-}$ anion from the undissociated acid:

$$H_nA \rightleftharpoons H_{n-r}A^{r-} + rH^+ \quad (5)$$

From Eq. (3), the sum of concentrations of the anions, and the undissociated acid at equilibrium is calculated:

$$\sum_{r=0}^{n} [H_{n-r}A^{r-}] = [H_nA]_m \sum_{r=0}^{n} \frac{K_a(r)}{[H^+]^r} \quad (6)$$

where $K_a(0) = 1$ by definition.

Similarly, from the 2nd column of Table 2, we obtain the general relation:

$$[LH_{n-r}A^{r-}]_s = K_{LH_{n-r}A}[H_{n-r}A^{r-}]_m[L]_s \quad (7)$$

The retention factor of the anion $H_{n-r}A^{r-}$ is expressed as:

$$k_r = \phi \frac{[LH_{n-r}A^{r-}]_s}{[H_{n-r}A^{r-}]_m} \quad (8)$$

This allows the calculation of the concentration of the complex $LH_{n-r}A^{r-}$ in terms of the concentration of the corresponding anion in the mobile phase:

$$[LH_{n-r}A^{r-}]_s = \frac{k_r}{\phi} \cdot [H_{n-r}A^{r-}]_m \quad (9)$$

Summing over all the species yields:

$$\sum_{r=0}^{n} [LH_{n-r}A^{r-}]_s = \frac{1}{\phi} \sum_{r=0}^{n} k_r [H_{n-r}A^{r-}]_m \quad (10)$$

Substituting Eqs. (6,10) into Eq. (1) leads to the following general relation for the retention factor of the solute acid:

$$k = \frac{k_0 + \sum_{r=1}^{n} k_r \cdot K_a(r)/[H^+]_m^r}{1 + \sum_{r=1}^{n} K_a(r)/[H^+]_m^r} \quad (11)$$

k_0 is the retention factor of the undissociated acid H_nA, $(r=0)$, and k_r the retention factor of the anion $H_{n-r}A^{r-}$. The constants $K_a(r)$ are defined by Eq. (4).

The concentration of solvated proton may be expressed as a function of the pH of the mobile phase, $[H^+] = 10^{-pH}$. If however, a change of variable, $pH = x/\ln 10$, is made then: $[H^+] = e^{-x}$, and Eq. (11) is written as:

$$k = \frac{k_0 + \sum_{r=1}^{n} k_r \cdot K_a(r) \cdot e^{rx}}{1 + \sum_{r=1}^{n} K_a(r) \cdot e^{rx}} \quad (12)$$

This is the most general expression for the relation between the observed retention factor k of a weak polyprotic acid on the one hand, and the dissociation constants, retention factors of the anions, and the pH of the mobile phase on the other hand. This relation, as it will be seen in the following sections, is also applicable to weak polyprotic bases and to amino acids.

3. Probabilistic interpretation of Eq. (12)

Eq. (12) suggests that the retention factor, k, of the solute, is a weighted average of the retention factors, k_r, of the different species that may exist in the mobile phase. In fact, Eq. (12) may be written as:

$$k = X_0 k_0 + \sum_{r=1}^{n} X_r \cdot k_r \quad (13)$$

where

$$X_r = \frac{K_a(r)e^{rx}}{1 + \sum_{t=1}^{n} K_a(t)e^{tx}} \quad (14)$$

The quantity X_r has the properties of a normalized probability distribution function. It is evident, therefore, that the term X_r represents the probability that the observed retention factor k of the solute is equal k_r, and Eq. (12) represents the average of the probability weighted retention factors of the different species that exist in the mobile phase. We will exploit this concept later to calculate the range of pH-values in which a species $H_{n-r}A^{r-}$ may be predominantly present in the mobile phase.

It can also be readily shown that the term X_r is equal to relative concentration of the species $H_{n-r}A^{r-}$ at equilibrium:

$$X_r = \frac{[H_{n-r}A^{r-}]_m}{[H_nA]_m + \sum_{t=1}^{n} [H_{n-t}A^{t-}]_m} \quad (15)$$

The higher the relative concentration of the species, the larger its contribution to the average value of the retention factor of the solute.

4. The case of weak polyprotic base

A polyprotic (protonated) base BH_n^+ with n positively charged protons may lose protons successively as shown in Table 3.

The observed retention factor k of the base is expressed as a weighted average:

$$k = \sum_{r=0}^{n} X_r \cdot k_r = X_0 \cdot k_0 + \sum_{r=1}^{n} X_r \cdot k_r \quad (16)$$

where X_r is the relative concentration of species BH_{n-r}^+ in the mobile phase:

$$X_r = \frac{[BH_{n-r}^+]}{[BH_n^+] + \sum_{t=1}^{n} [BH_{n-t}^+]} \quad (17)$$

The successive multiplications of the equilibrium-constant expressions in the second column of Table 3 combined with Eq. (17) gives the general equation:

$$\frac{\prod_{i=1}^{r} K_{ai}}{[H^+]^r} = \frac{[BH_{n-r}^+]}{[BH_n^+]} = \frac{X_r}{X_0} \quad (18)$$

Therefore, the X_r may be expressed in terms of X_0:

$$X_r = X_0 \frac{K_a(r)}{[H^+]^r} \quad (19)$$

Table 3
Dissociation equilibria of a weak protonated base

Dissociation	Equilibrium-constant expression
$BH_n^+ \rightleftharpoons BH_{n-1}^+ + H^+$	$\frac{K_{a1}}{[H^+]} = \frac{[BH_{n-1}^+]}{[BH_n^+]}$
⋮	⋮
$BH_{n-(r-1)}^+ \rightleftharpoons BH_{n-r}^+ + H^+$	$\frac{K_{ar}}{[H^+]} = \frac{[BH_{n-r}^+]}{[BH_{n-(r-1)}^+]}$
⋮	⋮
$BH^+ \rightleftharpoons B + H^+$	$\frac{K_{an}}{[H^+]} = \frac{[B]}{[BH^+]}$

where:

$$K_a(r) = \prod_{i=1}^{r} K_{ai} \qquad (20)$$

Substituting Eq. (19) in Eq. (16) leads to a general expression for the retention factor of the weak base:

$$k = X_0 \left[k_0 + \sum_{r=1}^{n} \frac{k_r \cdot K_a(r)}{[H^+]^r} \right]$$

Since:

$$X_0 = \frac{[BH_n^+]}{[BH_n^+] + \sum_{r=1}^{n} [BH_{n-r}^+]} = \frac{1}{1 + \sum_{r=1}^{n} K_a(r)/[H^+]^r}$$

therefore:

$$k = \frac{k_0 + \sum_{r=1}^{n} k_r \cdot K_a(r)/[H^+]^r}{1 + \sum_{r=1}^{n} K_a(r)/[H^+]^r} \qquad (21)$$

Putting $[H^+] = e^{-x}$, then Eq. (21) takes the exact form of Eq. (12) which was derived for weak polyprotic acids. Attention must, however, be paid to the meaning of the parameter k_r values. Here k_0 is the retention factor of the protonated base BH_n^+, whereas in the case of a weak acid, k_0 is the retention factor of the neutral, undissociated molecule H_nA. A similar remark is also due to the dissociation constants. In case of a weak base, K_{a1} is the dissociation constant corresponding to the first dissociation of BH_n^+, while in the case of weak acid, K_{a1} is the first dissociation constant of the neutral acid molecule.

5. General treatment of amino acids

We now consider the case of amino acids where the general Eq. (12) is also applicable provided that the meaning of different parameters is properly specified.

The molecular formula for an amino acid may be written as $(H_2N)_\mu R_1 R_2 (COOH)_\nu$. Such a compound exists as $(H_3^+N)_\mu R_1 R_2 (COOH)_\nu$ at low pH values or as $(H_2N)_\mu R_1 R_2 (COO^-)_\nu$ at high pH values. To simplify notations, these extreme cases are denoted as $H_\mu^+ ABH_\nu$ and $AB^{\nu-}$, respectively.

Table 4
Dissociations of polyprotic amino acid

$$CH_\mu^+ ABH_\nu \rightleftharpoons H_\mu^+ ABH_{\nu-1}^- + H^+$$
$$\vdots$$
$$H_\mu^+ ABH_{\nu-(r-1)}^{(r-1)-} \rightleftharpoons H_\mu^+ ABH_{\nu-r}^{r-} + H^+$$
$$\vdots$$
$$H_\mu^+ ABH^{(\nu-1)-} \rightleftharpoons H_\mu^+ AB^{\nu-} + H^+$$

$$CH_\mu^+ AB^{\nu-} \rightleftharpoons H_{\mu-1}^+ AB^{\nu-} + H^+$$
$$\vdots$$
$$H^+ AB \rightleftharpoons AB^{\nu-} + H^+$$

When the pH of the mobile phase is gradually increased from low to high values, an amino acid undergoes successive dissociations where the carboxylic protons dissociate first and then protons from NH_3^+ groups dissociate, as shown in Table 4.

The number of dissociation steps is $\mu + \nu$, and the number of all possible species in the mobile phase is $\mu + \nu + 1$. Writing the corresponding dissociation constants and following a procedure similar to the one used in the treatment of a weak base leads to the following general expression for the observed retention factor:

$$k = \frac{k_0 + k_1 \cdot K_a(1)e^x + \ldots + k_{(\mu+\nu)} \cdot K_a(\mu+\nu) \cdot e^{(\mu+\nu)x}}{1 + K_a(1)e^x + \ldots + K_a(\mu+\nu)e^{(\mu+\nu)x}} \qquad (22)$$

where $K_a(r) = \prod_{i=1}^{r} K_{ai}$ and $[H^+] = e^{-x}$. K_{ai} is the dissociation constant of the i^{th} dissociation step. The retention factors correspond to different species as follows:

$$\begin{aligned} k_0 &= k(H_\mu^+ ABH_\nu) \\ k_1 &= k(H_\mu^+ ABH_{\nu-1}^-) \\ &\vdots \\ k_{\mu+\nu} &= k(AB^{\nu-}) \end{aligned} \qquad (23)$$

Eq. (22) takes the form of Eq. (12) by simply putting $n = \mu + \nu$.

6. The probability of the presence of a species as a function of the pH of the mobile phase

As indicated above, the term X_r, Eq. (14):

$$X_r = \frac{K_a(r)e^{rx}}{1+\sum_{t=1}^{n} K_a(t)e^{tx}}$$

represents the probability that the measured retention factor of the solute is equal to k_r, and Eq. (12) is, in fact, the probability-weighted average of the probable values, k_r, of the retention factor. Eq. (14) has the mathematical characteristics of a normalized probability distribution function.

Since k_r and $K_a(r)$ correspond to the reversible dissociation:

$$H_nA \rightleftharpoons H_{n-r}A^{r-} + rH^+$$

it follows that the term X_r is the probability of the presence of $H_{n-r}A^{r-}$ in the mobile phase expressed as a function of the pH. This function has a maximum at a certain value of pH of the mobile phase. To find the pH that corresponds to the maximum probability of $H_{n-r}A^{r-}$, we set the derivative of X_r, with respect to x ($x = \mathrm{pH}\cdot\ln 10$), equal to zero and solve for x or r. Setting $\delta X_r/\delta x = 0$ leads to the following equation:

$$r = \frac{\sum_{t=1}^{n} t \cdot K_a(t)e^{tx}}{1+\sum_{t=1}^{n} K_a(t)e^{tx}} \qquad (24)$$

This relation applies not only for weak acids,but also for weak bases and amino acids, where r defines the species under consideration as shown in Tables 1–4. r takes only integer values, but to simplify the mathematics, we study the variation of r as a continuous function of x, and then pick up the x values (or pH values) that correspond to integer values of r. In this manner, the pH values that correspond to optimal conditions for the existence of different species may be obtained.

7. Applications and discussion

In case of a monoprotic acid as an example, ($n = 1$), Eq. (24) takes the simple form:

$$r = \frac{K_a \cdot e^x}{1 + K_a \cdot e^x} = \frac{K_a}{e^{-x} + K_a} = \frac{K_a}{10^{-\mathrm{pH}} + K_a}$$

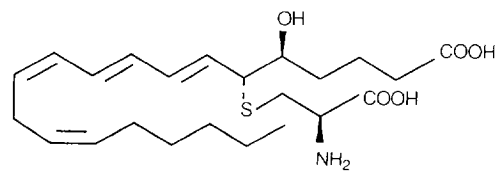
Table 5
The probability of the presence of the anion of benzoic acid in 45% acetonitrile solution

r	pH
$0.276722\cdot10^{-4}$	1.000
$0.276653\cdot10^{-3}$	2.000
$0.275966\cdot10^{-2}$	3.000
$0.269278\cdot10^{-1}$	4.000
0.216749	5.000
0.734558	6.000
0.965124	7.000
0.996399	8.000
0.999639	9.000
0.999964	10.000
0.999996	11.000
$0.100000\cdot10$	12.000
$0.100000\cdot10$	13.000

$K_a = 0.2767\cdot10^{-5}$.

which is, as expected, the percent ionization of the acid expressed in terms of the pH of the solution. Table 5 shows the variation of r of benzoic acid in a 45% acetonitrile solution. The K_a is obtained from fitting the measured retention factor of the acid to Eq. (12) by a least square fitting procedure described below. Experimental details are given elsewhere [6]. Maximum probability of finding the anion in the solution corresponds to complete ionization ($r = 1$). This occurs, as is seen in Table 5, when pH≥8 under the prevailing experimental conditions.

As another example, we report here the dissociation constants for leukotriene E_4, Fig. 1, which has two carboxylic groups and one NH_2. The measured retention factors in a 45% acetonitrile mobile- phase solution were fitted to Eq. (12), and the parameters were calculated. The dissociation constants found are: $K_{a1} = 2.51\cdot10^{-3}$, $K_{a2} = 3.57\cdot10^{-6}$ and $K_{a3} = 2.21\cdot10^{-10}$, corresponding to the following pK_a values: 2.60, 5.45 and 9.66, respectively. Details of



Formula: $C_{23}H_{37}NO_5S$ MW:439.6

Fig. 1. Structure of leukotriene E_4 (5(*S*),6(*R*),7E,9E,11Z,14Z)-5-hydroxy-6-(*S*-cysteinyl)-eicosatetraen-1-oic acid.

Table 6
The probability of prevalence of the different species of leukotriene E_4

r	pH
$0.245010 \cdot 10^{-1}$	1.000
0.200861	2.000
0.718493	3.000
0.996205	4.000
$0.125959 \cdot 10$	5.000
$0.178141 \cdot 10$	6.000
$0.197498 \cdot 10$	7.000
$0.201880 \cdot 10$	8.000
$0.218047 \cdot 10$	9.000
$0.268808 \cdot 10$	10.000
$0.295664 \cdot 10$	11.000
$0.299549 \cdot 10$	12.000
$0.299955 \cdot 10$	13.000

the experimental measurements are also presented elsewhere [6]. Table 6 shows the variation of r (Eq. (24)) as a function of the pH of the mobile phase. It is clear that a complete ionization of the first carboxylic proton ($r=1$) occurs at about pH=4.0, and the second carboxylic proton dissociates completely ($r=2$) at a pH between 7 and 8, while the proton of the NH_3^+ group dissociates ($r=3$) at pH≥ 12.

A relevant finding also became apparent from the data obtained from fitting the retention factor measured at various pH values into Eq. (12). Table 7 shows the observed retention factor of leukotriene E_4 at pH values corresponding to the maximum presence of the different species in the mobile phase and the calculated retention factors of these species.

The calculated retention factors k_r values are consistent with the observed factors and the corresponding values of r. The observed factors are,however, not exactly equal to the corresponding

Table 7
The measured retention factor k_{obs} of leukotriene E_4 at pH values corresponding to maximum presence of the different species in the mobile phase and the corresponding calculated retention factors k_r

pH	r	k_{obs}	k_r	Prevailing species	Net charge
1.0	0	6.40[a]	$k_0=6.10$	$(H_3N^+)R(COOH)_2$	+1
4.0	1	3.00	$k_1=3.28$	$(H_3N^+)R(COO)_2H^-$	0
8.0	2	0.65	$k_2=0.53$	$(H_3N^+)R(COO^-)_2$	−1
12.0	3	8.90[a]	$k_3=8.91$	$H_2NR(COO^-)_2$	−2

[a] Extrapolated value based on the experimental measurement in the pH range 2.00–11.68.

k_r values because of two reasons. First, the observed factor, k_{obs}, has a major contribution from the prevailing species and, in some cases, a minor contribution from other species which may exist in small concentrations at the pH under consideration. This will be more evident in the Section 8 where the variations of mole fractions of individual species are studied in detail. Second, the measured retention factors at various pH values have a certain range of scattering around the theoretical curve which is fitted by a least square fitting procedure. This may cause a deviation between the measured and calculated factors of an order of 0.2 to 0.3, depending on the level of precision of computation and the accuracy of the experimental measurements [6].

8. Variations of mole fractions of individual species

A better understanding of the secondary events in the mobile phase may be achieved by looking closely at the variations of mole fractions of the individual species during the variation of the pH. Eq. (14) is utilized for this purpose. X_r in this equation is the relative concentration (see also Eq. (15)) or the mole fraction. Fig. 2 shows the variations of mole fractions, X_r, of the leukotriene-species as functions of the pH of the mobile phase.

The curves denoted 0, 1, 2 and 3 represent the mole fractions of the undissociated, first-dissociated, second-dissociated and third-dissociated species, respectively. Three ranges of pH values may be distinguished. The range from pH=1 to 4 represents the first equilibrium between the undissociated acid ($r=0$) and the first-dissociated entity ($r=1$). As the mole fraction X_0 of the undissociated acid decreases, the mole fraction X_1 of the first-dissociated species increases and reaches a maximum ($X_1=0.9278$) at pH=4.01. This value corresponds to the maximum probability of the presence of species 1 as calculated in Table 6. It is also noted that at pH=4.01 there is a small amount of undissociated acid ($X_0=0.03668$) and another amount of species 2 ($X_2=0.03552$). Therefore, the main contribution to the observed retention factor at pH=4.01 comes from species 1 with minor contributions from species 0 and 2. The sum of these contributions is calculated by using Eq.

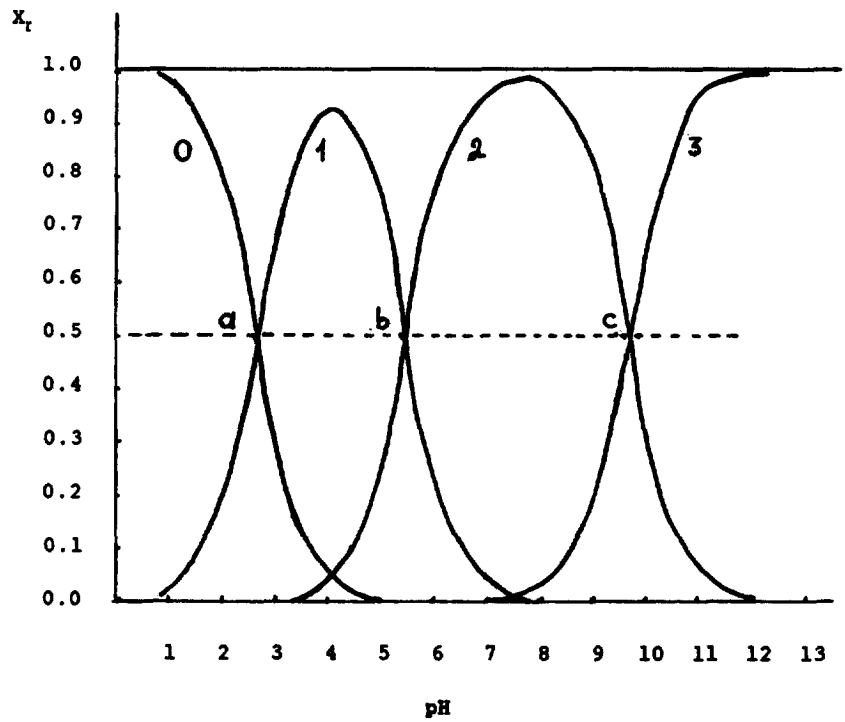


Fig. 2. Variations of mole fractions of leukotriene E_4-species. Curves 0, 1, 2, 3 (corresponding to $r=0$, 1, 2, 3, respectively) represent the variations of mole fractions of the leukotriene species as functions of the pH of the mobile phase.

(16), which yields $k=0.03668\cdot 6.10+0.9278+\cdot 3.28+0.03552\cdot 0.53=3.29$. This value is to be compared with the measured value $k=3.00$. The difference between the measured and calculated factors is within the limits of the standard error of estimate (0.30).

The second range of the pH values is from 4 to about 8 (Fig. 2). In this range there is an equilibrium between the first and second dissociated species. At about pH=8 the anion ($r=2$) becomes the predominant species in the mobile phase, where the measured retention factor ($k=0.65$) becomes practically equal to the factor $k_2=0.53$, specific to this anion.

The third range of pH values is extended from 8 to about 13. It represents the equilibrium between the second and third anions. The last ion becomes the only species existing in the liquid phase at pH ≥ 12. The listed value of the observed k ($k=8.90$) in Table 7 at pH=12 is an extrapolated experimental value. It has some uncertainty. Apart from this point, the agreement between the measured and calculated retention factors is within the limits of the standard error of estimate.

Fig. 2 also reflects other important facts. The points of intersection a, b, and c of the successive pairs of curves, occur at mole fractions $X_r=0.5$. The reason for this is that at an intersection point, there are only two acid species in the mobile phase at equilibrium with each other (Fig. 2). Since the sum of mole fractions equals 1, it follows that when the mole fraction of one species is 0.5, the mole fraction of the other species must also be equal 0.5. Therefore, the corresponding curves intersect at $X_r=0.5$.

It is also important to note that the pH values at the intersection points equal the respective pK_a values of the acid. The pH values at a, b and c (Fig. 2) are equal to the pK_a values of leukotriene, namely 2.60, 5.45 and 9.66, respectively. That this must be the case, can be proven as follows. Since the ratio of mole fractions of the two species at the intersection point is 1, and because the ratio of mole fractions is equal to the ratio of concentrations, it follows from Eqs. (3,4) that:

$$1 = X_{r+1}/X_r = [H_{n-(r+1)}A^{(r+1)-}]/[H_{n-r}A^{r-}]$$
$$= K_a(r+1)\cdot[H^+]^r/K_a(r)\cdot[H^+]^{r+1}$$
$$= \prod_{i=1}^{r+1} K_{a,i}/\prod_{i=1}^{r} K_{a,i}\cdot[H^+]$$
$$= K_{a,r+1}/[H^+]$$

Therefore, $K_{a,r+1}=[H^+]$ and $pK_{a,r+1}=pH$ at the intersection point of curves r and $r+1$.

9. Other considerations

It is also of interest to point out here that according to the theory, the retention-factor parameter k_r is related to the equilibrium constant $K_{L,r}$ of the reversible association between the species defined by r and the ligand L on the stationary phase:

$$k_r = \phi[L]_s K_{L,r} \tag{25}$$

This entails the relation:

$$\frac{k_r}{k_0} = \frac{K_{L,r}}{K_{L,0}} \tag{26}$$

where $K_{L,0}$ is the equilibrium constant of the reversible association between the ligand L and the undissociated acid. The ratio $K_{L,r}/K_{L,0}$ provides a relative measure of the strength of association between an ionic species and the ligand, as compared to the association of the undissociated acid with the ligand. Eq. (26) and the data in Table 7 allow arranging the different species resulting from leukotriene E_4 in the mobile phase in order of their relative strength of association with the ligand:

$$3 > 0 > 1 > 2$$

where we used the value of r to refer to the corresponding species as in Table 7. This order bears no significant correlation with the net charges on the different species (last column in Table 7). This order implies that structural factors are more important than mere net charge and molecular size in determining the strength of association of ions with the ligand on the stationary phase. We are currently looking into this question in more detail.

Regardless of this, however, it must be emphasized that the validity of Eqs. (12,24) is independent of the validity of the solute–ligand association mechanism. In fact, Eq. (12) may be readily derived on the basis of the equilibria taking place in the mobile phase only and considering the retention factors of different species as empirical parameters without recourse to the detailed mechanism of solute–ligand association.

Finally, it can be shown that in the case of monoprotic acids the inflection point on the curve of the observed retention factor as a function of the pH of the mobile phase corresponds to the pK_a of the acid. In fact, setting the second derivative of k (Eq. (12)) with respect to x equal zero:

$$\partial^2 k/\partial x^2 = 0 \tag{27}$$

leads to the equation $K_a = e^{-x} = [H^+]$, provided that $k_0 \neq k_1$. This shows that $pK_a = (pH)$ at the inflection point. This conclusion is supported by experimental findings (see [2]). However, Eq. (27) leads in the case of a polyprotic acid or base ($n>1$) to a complex equation whose roots do not, in general, correspond to the pK_a values of the solute. Only in case the pK_a values are far enough apart do they coincide with the pH values of the inflection points. This can be seen from Eq. (13) which, upon derivation, leads to:

$$\frac{\partial^2 k}{\partial pH^2} = \sum_{r=0}^{n} k_r \cdot \frac{\partial^2 X_r}{\partial pH^2}$$

The second derivative of k can be zero only if the second derivatives of all mole fractions, X_r, are zero at the same pH value. This evidently cannot occur except in the case where two X_r versus pH curves, corresponding to two species at equilibrium with each other, intersect at their mutual inflection points while other species have zero mole fractions (see Fig. 2 as an example). This condition can be satisfied if and only if the pK_a values of the acid are far apart from each other. In the case of a monoprotic acid, only two species exist in equilibrium, and the inflection point of the k versus pH curve occurs at $pH = pK_a$ provided $k_0 = k_1$. The experimental data and the fitted parameters confirm this conclusion [2,6].

10. Computational implementation

The general Eq. (12) may be used to obtain the

retention factors k_r and the dissociation constants K_a of polyprotic weak acids and bases and amino acids. This may be accomplished by fitting the set of measured values of the retention factor at various pH to Eq. (12).

It is clear from Eq. (12) that the measured retention factor k is a linear function of the k_r values of the different species in the mobile phase, while at the same time, it is a nonlinear function of the K_a values. Any attempt to transform Eq. (12) into a linear function of both kinds of parameters, k_r and K_a, will lead to the introduction of a new set of parameters which are not linearly independent and will most likely produce erroneous numerical results.

In this work we used an iterative least square fitting procedure that is carried out in two stages. In stage one, a set of initial values of k_r and K_a is used. The K_a values are kept constant while a new set of k_r values, is obtained by a linear-fitting procedure. In stage two, the values of k are kept constant and new dissociation constants are obtained by an iterative, non-linear least square fitting procedure. The two-stage cycle is repeated until convergence up to a predetermined precision is attained. The procedure is programmed in FORTRAN 77.

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