ELSEVIER

Journal of Chromatography A, 763 (1997) 199–203

JOURNAL OF CHROMATOGRAPHY A

# Use of secondary equilibria in reversed-phase high-performance liquid chromatography for the determination of dissociation constants of polyprotic leukotrienes

J.E. Hardcastle*, R. Vermillion-Salsbury, K. Zhao, I. Jano

*Department of Chemistry and Physics, Texas Woman's University, Denton, TX 76204, USA*

**Abstract**

Observed retention factors were determined for leukotriene $B_4$, leukotriene $E_4$ and N-acetylleukotriene $E_4$ using mobile phases with different percentages of organic modifier in the pH range from 2 to 12. A two-stage least squares fitting procedure for fitting a general equation to the observed retention factors was used to calculate the dissociation constants at each percentage of organic modifier. The obtained dissociation constants were extrapolated to give $pK_a$ values in 100% aqueous solutions. The $pK_a$ values thus calculated for the leukotrienes were of a magnitude to be expected for compounds of this kind. The polyprotic leukotriene E4 has a $pK_{a_1}$ of 1.54, a $pK_{a_2}$ of 3.41, and a $pK_{a_3}$ of 10.16.

*Keywords:* Dissociation constants; Retention factors; Mobile phase composition; pH effects; Leukotrienes; Organic acids

## 1. Introduction

Leukotrienes are a group of complex biologically active compounds derived from arachidonic acid. They participate in a variety of biological processes such as maintaining blood pressure, maintaining body temperature, protecting organs from damage caused by trauma, disease and stress, and regulating parturition. An imbalance of these compounds has been implicated in a variety of conditions including arthritis, malignancy and allergic disorders [1]. Because of their importance in living systems, a thorough understanding of the chemical, physical and biological properties of these compounds is desirable. Each of the leukotrienes has at least one carboxylic acid group and, therefore, they may be considered as acids. The determination of the dissociation constants ($K_a$) of these compounds can lead to a better understanding of their chemistry in solution and allow better separation of these compounds from their biological media.

Leukotriene $E_4$ ($LTE_4$), a triprotic acid, is the compound of main interest in this study. The systematic name of $LTE_4$ is 5(*S*),6(*R*),7*E*,9*E*,11*Z*,14*Z*-5-hydroxy-6-(*S*-cysteinyl)-eicosatetraen-1-oic acid. A diprotic derivative of $LTE_4$, N-acetyl-$LTE_4$, and the monoprotic leukotriene $B_4$ ($LTB_4$) are included in this study. The structures of these compounds are shown in Fig. 1.

Leukotrienes are produced in very small quantities in the cell and are very expensive when purchased from specialty chemical suppliers. Nanogram quantities of leukotrienes can be used for the determination of dissociation constants by the HPLC technique herein described. The classical methods used for the dissociation constants require much larger

*Corresponding author.


*PII* S0021-9673(96)00738-8

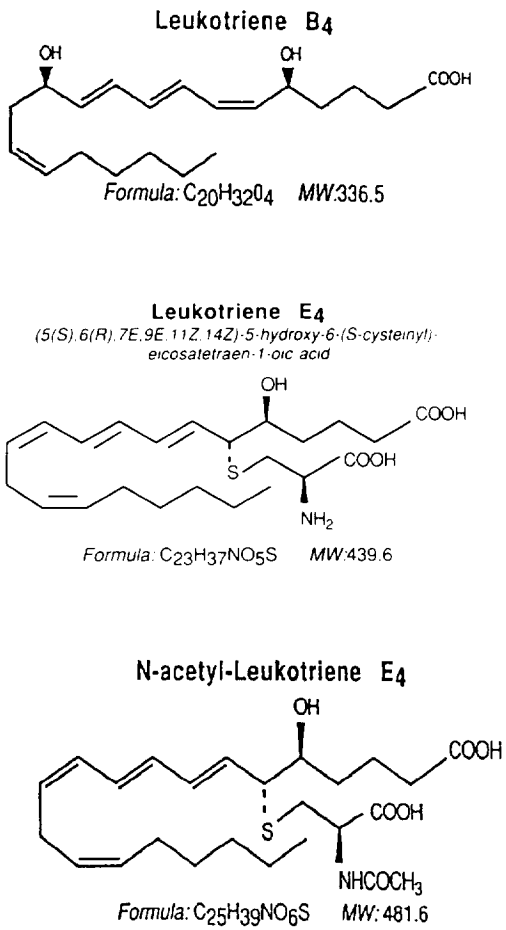


Fig. 1. Structures of the leukotrienes.

quantities of the analytical sample. Because of the low analyte concentration in the HPLC eluent, the analyte solubility does not impose constraints on the determination of dissociation constants by HPLC, whereas poor sample solubility may limit the use of conventional methods.

## 2. Theory

Weak acid dissociation equilibria in HPLC have been used to determine the acid dissociation constants of mono- and diprotic organic acids [2,3]. The theoretical basis for using secondary equilibria in HPLC has been discussed in several reports [4–7]. Jano et al. [8] have developed a general equation that can be applied to acids or bases containing many dissociable groups. It is written as:

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

where the $k_r$ values are the retention factors of the dissociated species and $K_a(r)$ is the product of the first $r$-dissociation constants. The variable, $x$, is related to the pH of the mobile phase: $x = 2.303 \cdot pH$ (see Ref. [8] for more details). This general equation has been used to determine the $pK_a$ values for the triprotic $LTE_4$ and the diprotic N-acetyl-$LTE_4$.

## 3. Experimental

### 3.1. Chromatographic system

A Waters (Ventura, CA, USA) HPLC system consisting of two model 510 pumps, a U6K injector, a model 484 tunable UV detector and a model 510 refractive index detector was used. The Waters Baseline 810 workstation and computer software (version 3.30) were used to control the system and to collect the data. The column used was a $C_8$ Alltech (Deerfield, IL, USA) Adsorbosil, 5 μm, 250×4.6 mm I.D. column. A $C_8$ silica base guard column was used. Isocratic elution was performed using a mobile phase flow-rate of 1.0 ml/min. The UV detector was set at 280 nm to read the characteristic absorbance of leukotrienes.

### 3.2. Reagents

The mobile phase was prepared by combining an aqueous buffer with an organic mixture of HPLC-grade acetonitrile–tetrahydrofuran (90:10, v/v) to obtain solutions in the range of 45 to 60% (v/v) organic. Aqueous buffers (0.01 $M$) in the pH range 2 to 12 were prepared from sodium acetate, sodium phosphate or sodium citrate. Sodium perchlorate (0.02 $M$) was used to maintain constant ionic strength, independent of the pH. The mobile phase solution was degassed and filtered through a 0.45-μm nylon 66 membrane filter disc prior to use.

Samples of $LTE_4$, N-acetyl-$LTE_4$ and $LTB_4$, purchased from Oxford Biomedical Research (Oxford, MI, USA), were dissolved in acetonitrile–water (60:40%, v/v) to a concentration of 5 μmol/ml. Retention times were determined with three replicate 10 μl injections of each leukotriene sample at each pH and percentage of organic modifier.

A Corning Model 215 pH meter was used to determine the pH to within 0.01 pH units. The apparent pH of the bulk mobile phase was measured and any adjustments were made before the mobile phase was used. Also, samples for pH determination were collected during solute elution. It was found that the apparent pH of the mobile phase did not change on passing through the chromatographic system.

## 4. Results

Observed retention factors were determined for each compound ($LTB_4$, $LTE_4$, N-acetyl-$LTE_4$) at each pH value and percentage of organic modifier. Plots of $k'$ versus pH for each compound at four different percentages (45, 50, 55 and 60%) of mobile phase modifier were computer-generated. Only those $k'$ versus pH plots obtained with 55% organic modifier are presented here (Figs. 2 –4). A two-stage least squares fitting procedure for fitting the general equation [8] to the observed retention factors was used to calculate the dissociation constants at each

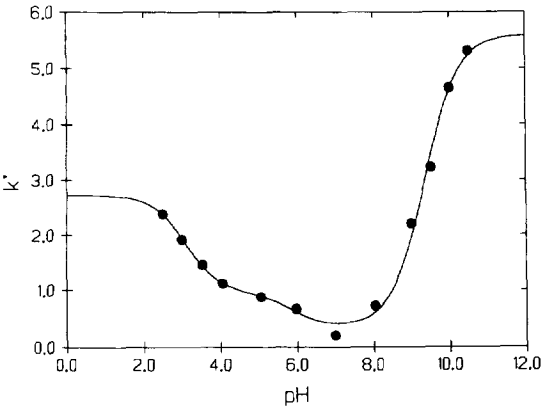


Fig. 3. Observed capacity factor ($k'$) versus pH for $LTE_4$ at 55% organic modifier.

percentage of organic modifier. The dissociation constants were found to depend on the composition of the mobile phase. Statistical analysis showed that the experimental data fitted the general equation [8] with a standard deviation that varied from case to case between ±0.02 and ±0.30. The fitted general equation was then used with the dissociation constants to calculate theoretical retention factors for each percentage of organic modifier. The correlation coefficient, $R$, between the observed and calculated retention factors was 0.98 or better for each leukotriene at the four different percentages of organic modifier (Table 1).

For the monoprotic acid, $LTB_4$, the $k'$ versus pH plot (Fig. 2) is sigmoidal and resembles an acid–

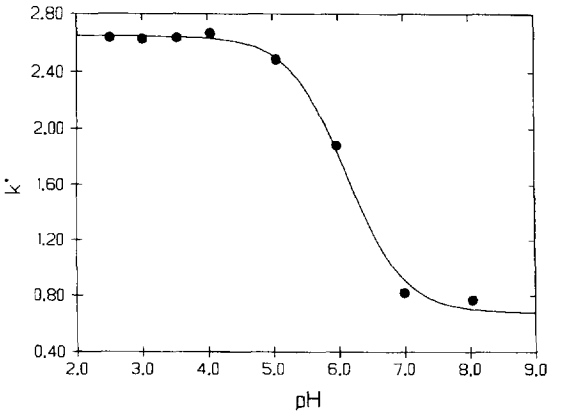


Fig. 2. Observed capacity factor ($k'$) versus pH for $LTB_4$ at 55% organic modifier.

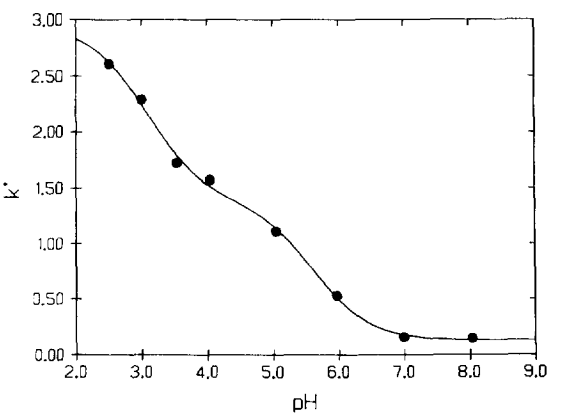


Fig. 4. Observed capacity factor ($k'$) versus pH for N-acetyl-$LTE_4$ at 55% organic modifier.

Table 1
Calculated $pK_a$ values for the leukotrienes at different percentages of mobile phase organic modifier

| % Organic modifier | $LTB_4$ | $LTE_4$ | | | N-Acetyl-$LTE_4$ | |
|---|---|---|---|---|---|---|
| | $pK_{a_1}$ | $pK_{a_1}$ | $pK_{a_2}$ | $pK_{a_3}$ | $pK_{a_1}$ | $pK_{a_2}$ |
| 45 | 5.92 | 2.60 | 5.45 | 9.66 | 2.98 | 5.45 |
| 50 | 6.04 | 2.82 | 5.20 | 9.42 | 3.04 | 5.47 |
| 55 | 6.13 | 3.09 | 5.74 | 9.44 | 3.08 | 5.60 |
| 60 | 6.29 | 2.93 | 5.96 | 9.44 | 3.13 | 5.75 |

The standard deviation for these calculated $pK_a$ values is ±0.02.

Table 2
Summary of aqueous $pK_a$ values[a] for the leukotrienes

| Compound | $pK_{a_1}$ | $pK_{a_2}$ | $pK_{a_3}$ |
|---|---|---|---|
| $LTB_4$ | 4.84±0.02 | | |
| $LTE_4$ | 1.54±0.11 | 3.41±0.17 | 10.16±0.07 |
| N-Acetyl-$LTE_4$ | 2.54±0.01 | 4.49±0.03 | |
| Cysteine [9] | 1.71 | | 10.78 |

[a] The standard error of estimate obtained from the linear regression fitting procedure varies from case to case. The last digit in the $pK_a$ value is not significant in some cases, but it is kept for the uniformity of results.

base titration curve. As the pH increases, the $k'$ decreases, indicating the presence of the rapidly eluting anion species. The inflection point in a curve of this type gives the $pK_a$ of the acid. This is true, however, only for monoprotic acids. In the case of polyprotic acids, the pH values of the inflection points do not necessarily coincide with the $pK_a$ values of the acid unless the $pK_a$ values are far enough apart from each other [8]. For this reason, the dissociation constants of leukotrienes were not obtained from the inflection points of the $k'$ versus pH plots. They were calculated, as mentioned above, by the least squares fitting procedure described in [8].

The $pK_a$ values at the different percentages of organic modifier were used to extrapolate to the aqueous $pK_a$ by a linear regression fitting procedure. The regression obtained for $LTB_4$ was:

$$pK_a = 4.84 + 2.40\varphi \quad SEE = \pm 0.02$$

where $\varphi$ is the fraction of the organic modifier. The $y$-intercept, 4.84, is the aqueous $pK_a$ of $LTB_4$. The standard error of estimate, SEE, for this regression is ±0.02. The correlation coefficient between the $pK_a$ values of the regression line and the input-$pK_a$ values is, in this case, 0.994. The same procedure was also used to determine an aqueous $pK_a$ value of 4.22±0.06 for benzoic acid, which compares favourably with literature values.

The $k'$ versus pH plot for $LTE_4$ (Fig. 3) shows that, as the pH increases, $k'$ decreases, passing through a minimum, and then increases. At low pH values, the carboxylic groups are protonated, the compound is less polar and the $k'$ values are higher. As the pH increases, carboxylate anions predominate, the compounds become more polar and elute from the column faster. Beyond pH 7, the protonated amino group begins to dissociate, the compounds become less polar and the $k'$ increases as the pH increases. $LTE_4$ has three dissociable groups and the $k'$ versus pH plot (Fig. 3) shows evidence of three apparent inflection points.

Linear regression fitting of the $pK_a$ values versus fraction of organic modifier for the three dissociable groups of $LTE_4$ yielded the following regression lines:

$$pK_{a_1} = 1.54 + 2.52\varphi \quad SEE = \pm 0.11$$

$$pK_{a_2} = 3.41 + 4.14\varphi \quad SEE = \pm 0.17$$

$$pK_{a_3} = 10.16 - 1.28\varphi \quad SEE = \pm 0.07$$

The $y$-intercept values correspond to the aqueous $pK_a$ values (Table 2).

The $k'$ versus pH plot for N-acetyl-$LTE_4$ (Fig. 4) has two inflection points, one for each carboxylic group. Plots of $pK_a$ versus percentage of organic modifier were fitted to the following linear regression equations:

$$pK_{a_1} = 2.54 + 0.98\varphi \quad SEE = \pm 0.01$$

$$pK_{a_2} = 4.49 + 2.06\varphi \quad SEE = \pm 0.03$$

The $y$-intercepts are the aqueous $pK_a$ values of the acid. These are calculated values within the corresponding standard error of estimate. $pK_{a_1}$ is for the cysteinyl carboxyl group and $pK_{a_2}$ is for the carboxylic group on the twenty-carbon chain.

## 5. Discussion

Table 2 represents a summary of the aqueous $pK_a$

values of the leukotrienes. The $pK_a$ values for the carboxyl group on the twenty-carbon chain, ($pK_{a_1}$ for $LTB_4$, and $pK_{a_2}$ for both $LTE_4$ and N-acetyl-$LTE_4$) are of a magnitude expected for long chain carboxylic acids. The carboxylic acid group on the twenty-carbon chain of $LTE_4$ is more acidic than might be expected and this may be due to the nearby dissociable groups. The cysteinyl side chain appears to increase the acidity of this carboxyl group of $LTE_4$ and N-acetyl-$LTE_4$, compared to $LTB_4$.

The cysteinyl carboxyl group of $LTE_4$ has a $pK_{a_1}$ value similar to free cysteine. However, the cysteinyl carboxyl group of N-acetyl-$LTE_4$ is less acidic than this group of $LTE_4$. Acetylation of the amino group reduces the inductive effect seen with the free amino group of $LTE_4$ and, thus, the cysteinyl carboxyl group of N-acetyl-$LTE_4$ is less acidic.

The amino group of $LTE_4$ appears to be a slightly weaker base (stronger acid) than this group in free cysteine. The reason for this is unclear at present. In water solutions, $LTE_4$ may assume a conformation that favours more interaction between various dissociable groups and this could affect the acid–base character of these groups. It is also possible that the limited number of points (four) used in the linear regression analysis is another reason for the difference between the $pK_a$ of $LTE_4$ and that of free cysteine.

In these experiments, column dissolution at high pH values was not observed and the column appeared to maintain good stability. Kirkland et al. [10] suggest that the use of a precolumn of silica support could enhance the column's lifetime at high pH values. Also, these authors reported that column life was prolonged when used at high pH values if the mobile phase contained a high percentage of acetonitrile, as was the case in our experiments. Additionally, the column used for the work reported herein was end-capped and was of the polymeric phase type, and this enhances stability at high pH values. Thus, we believe that the measurements of retention factors at high pH are reliable.

## Acknowledgments

This research was supported by a grant to the Department of Chemistry and Physics from the Robert A. Welch Foundation.

## References

[1] J. Ninneman, Prostaglandins, Leukotrienes and the Immune Response, Cambridge Univ. Press, New York, 1988.
[2] J.E. Hardcastle, M. He, B. Begum and R. Vermillion-Salsbury, J. Chromatogr. A, 691 (1995) 225.
[3] F. Szokoli, Z. Nemeth and J. Inczedy, Chromatographia, 29 (1990) 265.
[4] Cs. Horváth, W. Melander and I. Molnar, Anal. Chem., 49 (1977) 142.
[5] J.P. Foley and W.E. May, Anal. Chem., 59 (1987) 102.
[6] J.P. Foley and W.E. May, Anal. Chem., 59 (1987) 110.
[7] J.K.M. van de Venne, J.L. Hendrikx and R.S. Deelder, J. Chromatogr., 167 (1987) 1.
[8] I. Jano, J.E. Hardcastle, K. Zhao and R. Vermillion-Salsbury, J. Chromatogr. A, in press.
[9] H.A. Sober and R.A. Harte (Editors), Handbook of Biochemistry, Chemical Rubber Co., Cleveland, OH, 1968.
[10] J.J. Kirkland, M.A. van Straten and H.A. Claessens, J. Chromatogr. A, 691 (1995) 3.