

# Use of secondary chemical equilibria in liquid chromatography to determine dissociation constants of leukotriene B<sub>4</sub> and prostaglandin B<sub>2</sub>

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## Abstract

The secondary chemical equilibrium of a monoprotic acid in reversed-phase high-performance liquid chromatography (RP-HPLC) is controlled both by the pH of aqueous component and the ratio of organic solvent to water in the mobile phase. The pH dependence of capacity factors of a monoprotic acid follows the titration curve of the acid. The midpoint of the sigmoidal curve corresponds to the  $pK_a$  value of the acid. Therefore, the  $pK_a$  of the acid can be determined by RP-HPLC. Capacity factors of prostaglandin B<sub>2</sub> and leukotriene B<sub>4</sub> were measured at several mobile phase pH values, and acetonitrile mobile phase compositions.  $pK_a$  values determined at various ratios of acetonitrile to water were plotted against percent acetonitrile. The straight line that resulted was extrapolated to 100% aqueous solution. The thermodynamic  $pK_a$  values of prostaglandin B<sub>2</sub> and leukotriene B<sub>4</sub> were determined to be 5.07 and 5.17, respectively.

## 1. Introduction

Leukotrienes and prostaglandins are two groups of biologically active compounds derived from arachidonic acid in normal biological processes such as the maintenance of blood pressure and body temperature, and in anaphylaxis [1].

The systematic name for leukotriene B<sub>4</sub> (LTB<sub>4</sub>) is 5(*S*),12(*R*)-dihydroxy-6*Z*,8*E*,10*E*,14*Z*-eicosatetraenoic acid. That for prostaglandin B<sub>2</sub> (PGB<sub>2</sub>) is 7-[2-(3-hydroxy-1-octenyl)-5-oxocyclopent-1-enyl]-5-heptenoic acid. These two compounds are carboxylic acids, and a knowledge of the acid–base dissociation constants of these metabolites can lead to a better understanding of their solution biochemistry.

This information can help to improve the analytical methodology for the separation of these metabolites from their biological environment, and for preparation for further analysis.

The dissociation constants of benzoic acid and phenylacetic acid were determined by this technique in order to have  $pK_a$  values to compare with literature values for well studied compounds.

## 2. Theory

The dissociation of weak acids in HPLC is considered to be a secondary chemical equilibrium because the primary equilibrium is the distribution of the solute between the mobile phase and the stationary phase [2]. For a mono-

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protic weak acid equilibrium,  $\text{HA} \rightleftharpoons \text{H}^+ + \text{A}^-$ , the observed chromatographic retention of the analyte in RP-HPLC will be the mol-fraction weighted average of the retention of the dissociated and undissociated forms. In terms of mol fractions ( $X$ ) and capacity factors ( $k'$ ), this can be expressed as

$$k'_{\text{obs}} = X_{\text{HA}}k'_{\text{HA}} + (1 - X_{\text{HA}})k'_{\text{A}} \quad (1)$$

Since the equilibrium can be shifted without changing the retention behavior of the two forms of the weak acid, the equilibrium constant and the limiting capacity factors for the two forms can be obtained from multiple values of the average retention behavior. From the equilibrium constant expression,  $K_{\text{a}} = [\text{H}^+][\text{A}^-]/[\text{HA}]$ , and Eq. 1, the expression

$$k'_{\text{obs}} = \frac{k'_{\text{HA}}[\text{H}^+] + k'_{\text{A}}K_{\text{a}}}{[\text{H}^+] + K_{\text{a}}} \quad (2)$$

can be derived. Eq. 2 predicts that a plot of  $k'_{\text{obs}}$  versus pH will be sigmoidal with a midpoint pH value which corresponds to the  $\text{p}K_{\text{a}}$  of the weak acid. Simply, the pH dependence of the capacity factor follows the dissociation curve [3]. This concept is reviewed and other pertinent literature is referenced in two papers by Foley and May [4,5], and one by Van de Venne et al. [6].

### 3. Experimental

#### 3.1. Chromatographic system

A Waters (Ventura, CA, USA) HPLC system consisting of two Model 510 pumps, U6K injector, Model 484 tunable UV detector, and Model 510 refractive index detector were 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 Phenomenex (Torrance, CA, USA) Spherex ODS, 5  $\mu\text{m}$ , 250  $\times$  4.6 mm. Isocratic elution was performed using a mobile phase flow-rate of 1.0 ml/min. The UV detector was set at 254 nm for benzoic acid and phenylacetic acid, and at 280 nm for LTB<sub>4</sub> and PGB<sub>2</sub>. All

work was performed at ambient temperature which was  $22 \pm 1^\circ\text{C}$ .

#### 3.2. Reagents

The mobile phase was prepared by mixing HPLC-grade acetonitrile with the appropriate aqueous buffer to obtain mixtures in the range of 40–70% (v/v) acetonitrile. The mobile phase buffer was prepared from sodium acetate for pH below 6, and from monobasic sodium phosphate for pH above 6. Buffers were prepared at a strength of 0.01 M. Sodium perchlorate (0.02 M) was used to maintain constant ionic strength independent of pH. The mobile phase solution was degassed and filtered through a 0.45- $\mu\text{m}$  nylon 66 membrane filter disc prior to use.

The samples of LTB<sub>4</sub> and PGB<sub>2</sub> were dissolved in acetonitrile at a concentration of approximately 10  $\mu\text{g/ml}$ , and 10  $\mu\text{l}$  of these solutions were injected. Benzoic acid and phenylacetic acid were injected at 0.01 M concentration in acetonitrile. Sodium nitrate (0.01 M) was used to determine void volume. Retention times were determined with three replicate injections for each sample at each pH and percent acetonitrile.

A Corning Model 215 pH meter was used to determine pH to within  $\pm 0.01$  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 did not change by passing through the chromatographic system.

#### 3.3. Data analysis

The experimental data were fitted to Eq. 2 using the non-linear least square program MINSQ (version 3.12) from MicroMath Scientific Software, Salt Lake City, UT, USA.

### 4. Results and discussion

Observed capacity factors were determined for each compound at each pH and percent acetonitrile. Computer-generated plots of  $k'$  versus pH

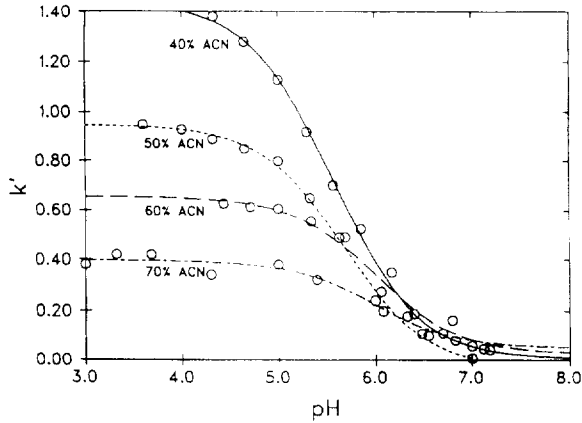


Fig. 1. Observed capacity factor ( $k'$ ) versus pH for benzoic acid in acetonitrile (ACN)-buffer mixture.

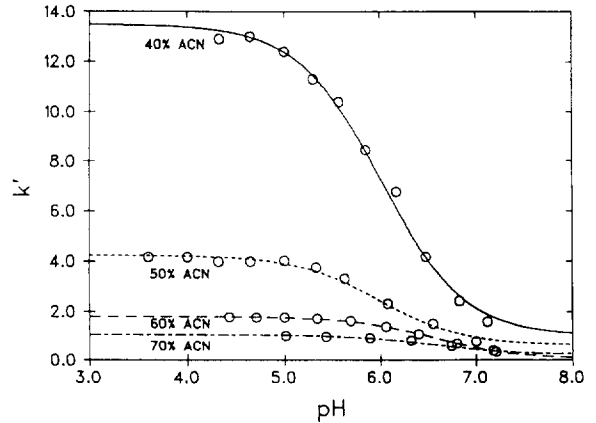


Fig. 3. Observed capacity factor ( $k'$ ) versus pH for LTB<sub>4</sub> in acetonitrile (ACN)-buffer mixture.

are shown in Figs. 1–4. The retention of the analyte decreased with increase in pH, and the sigmoidal relationship between  $k'$  and pH is very similar to a typical weak acid titration curve. The inflection point in a curve of this type, as determined by least square regression analysis, can be used to determine the acid dissociation constant.

The calculated  $pK_a$  values for the organic acids at different percent acetonitrile are given in Table 1. Plots of  $pK_a$  versus percent acetonitrile are linear, and linear regression analysis produced the following equations:

for benzoic acid

$$y = 4.63 + 0.0221x, R^2 = 0.935 \text{ and } R = 0.97;$$

for phenylacetic acid

$$y = 4.81 + 0.0220x, R^2 = 0.981 \text{ and } R = 0.99;$$

for LTB<sub>4</sub>

$$y = 5.01 + 0.0255x, R^2 = 0.939 \text{ and } R = 0.97;$$

for PGB<sub>2</sub>

$$y = 5.11 + 0.0259x, R^2 = 0.901 \text{ and } R = 0.99.$$

The intercept with the y-axis, in this case the  $pK_a$ -axis, gives the aqueous  $pK_a$  value. From the

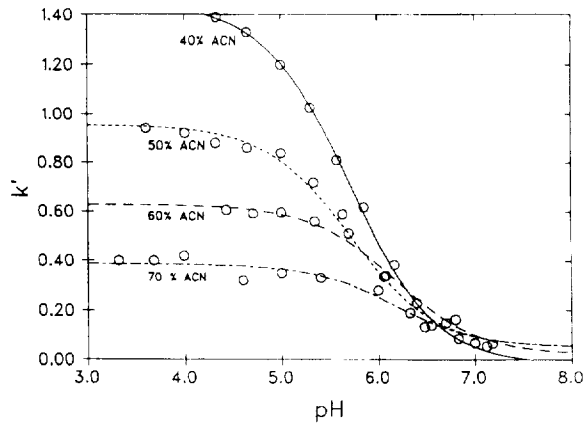


Fig. 2. Observed capacity factor ( $k'$ ) versus pH for phenylacetic acid in acetonitrile (ACN)-buffer mixture.

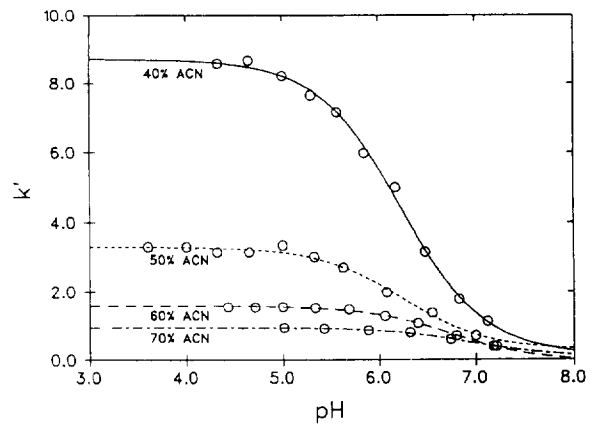


Fig. 4. Observed capacity factor ( $k'$ ) versus pH for PGB<sub>2</sub> in acetonitrile (ACN)-buffer mixture.

Table 1  
 $pK_a$  values determined in different percentages of acetonitrile

Acetonitrile (%)	Benzoic acid		Phenylacetic acid		LTB <sub>4</sub>		PGB <sub>2</sub>	
	$pK_a$	S.D.	$pK_a$	S.D.	$pK_a$	S.D.	$pK_a$	S.D.
40	5.57	0.03	5.71	0.03	6.11	0.03	6.23	0.04
50	6.63	0.03	5.86	0.02	6.17	0.05	6.25	0.08
60	6.01	0.04	6.17	0.02	6.56	0.02	6.74	0.02
70	6.18	0.03	6.34	0.03	6.83	0.01	6.93	0.02

S.D. = Standard deviation.

above equations, these values are 4.63 for benzoic acid, 4.81 for phenylacetic acid, 5.01 for LTB<sub>4</sub> and 5.11 for PGB<sub>2</sub>. Since the aqueous  $pK_a$  values determined here are influenced by salt effects, the Davies [7] modification of the Debye–Hückel equation was used to calculate thermodynamic  $pK_a$  values. The thermodynamic  $pK_a$  values are benzoic acid, 4.69; phenylacetic acid, 4.87; LTB<sub>4</sub>, 5.07; and PGB<sub>2</sub>, 5.17.

The  $pK_a$  values determined for benzoic acid and phenylacetic acid in this research effort are much higher than values reported in the literature. The major source of this discrepancy is the media effect. The extrapolation of the data from acetonitrile–water solutions to 100% water suffers from lack of data in the range of 0–40% acetonitrile. It could be possible to supplement this data for benzoic acid and phenylacetic acid in the 0–40% acetonitrile range. However, the eicosanoids (PGB<sub>2</sub> and LTB<sub>4</sub>) are strongly retained by the octadecyl-silica (ODS) column. At acetonitrile concentration below 40%, PGB<sub>2</sub> and LTB<sub>4</sub> could not be eluted from the C<sub>18</sub> column. The  $pK_a$  values determined for LTB<sub>4</sub> and PGB<sub>2</sub> are of a magnitude to be expected for compounds of this type of structure. Because of their similar structures, other monoprotic eicosanoids and most of the prostaglandins would be expected to have very similar  $pK_a$  values to those determined for LTB<sub>4</sub> and PGB<sub>2</sub>.

The advantage of HPLC can be exploited for such physico-chemical measurements when a chromatographic system with an eluent, which is a proper medium for the equilibrium constant to be evaluated, and yields conveniently measur-

able retention ratios, is available, and very low analyte concentrations in the effluent can be monitored by a sensitive detector.

Unlike all other methods, the use of HPLC affords measurements with impure samples without further purification because with properly chosen efficient chromatographic systems impurities are separated from the analyte. In our experiments, commercially available substances were employed without further purification and in all cases peaks representing impurities were present on the chromatogram.

This method is applicable for the measurement of dissociation constants only in those cases when the rate of equilibration in the mobile phase is rapid on the time scale of the chromatographic run. Otherwise poor peak shape and peak splitting render the evaluation of retention factors difficult without the computer.

Because of the low solute concentration in the eluent, the solubility does not impose constraints on determining dissociation constants by HPLC, whereas poor sample solubility often limits the employment of conventional techniques. Sub-microgram quantities of a substance may be adequate to measure equilibrium constants by HPLC, whereas conventional methods require larger quantities of the substance being analyzed.

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