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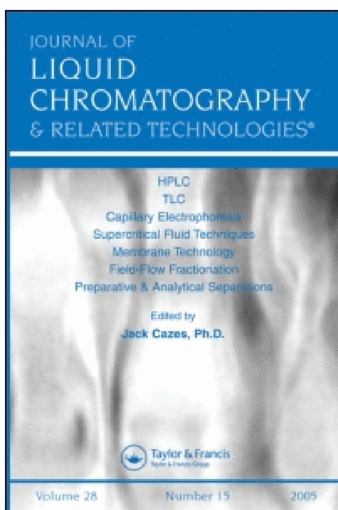


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# DETERMINATION OF THE $pK_a$ VALUES OF POLYCYCLIC AROMATIC HYDROCARBON METABOLITES BY CAPILLARY ZONE ELECTROPHORESIS

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# DETERMINATION OF THE $pK_a$ VALUES OF POLYCYCLIC AROMATIC HYDROCARBON METABOLITES BY CAPILLARY ZONE ELECTROPHORESIS

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## ABSTRACT

Capillary zone electrophoresis (CZE) was used to determine the $pK_a$ values of six environmentally and metabolically important PAH metabolites. The $pK_a$ values obtained from CZE were close to the $pK_a$ values reported in the literature which were obtained by spectroscopy. In this work, it was found that CZE is very suitable for the determination of the $pK_a$ values of the weakly acidic, large ring PAH metabolites. A $pK_a$ value acquired for one of the model compounds by spectrometry gave supporting evidence for the reliability of CZE in obtaining $pK_a$ values for relatively large molecular weight hydroxyl compounds.

## INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a significant class of pollutants that are ubiquitous in the environment. The control of the level of PAHs is receiving more and more attention because of their potential carcinogenicity. The most extensively studied PAH is benzo[a]pyrene (B[a]P).



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The potential toxicity of B[a]P depends in large part on its metabolic fate.[1] In a cellular environment, B[a]P is metabolically activated to a variety of products which include epoxides, hydroxyl aromatics, quinones, dihydrodiols, dioepoxides, tetrols, and water-soluble conjugates.[2] Pyrene and benz[a]anthracene (B[a]A) are also important PAH compounds. The PAH metabolites 1-hydroxypyrene (1-OH-Py), 3-hydroxybenz[a]anthracene (3-OH-B[a]A), and 3-hydroxybenzo[a]pyrene (3-OH-B[a]P) have been used as biomarkers for environmental and occupational PAH exposures.[3]

Various analytical methods, such as high performance liquid chromatography (HPLC),[4,5] gas chromatography (GC),[6] and micellar electrokinetic capillary chromatography (MECC)[7,8] have been developed to separate PAH metabolites. We previously reported a capillary zone electrophoresis (CZE) method for the separation of nine PAH metabolites.[9] In order to better understand the migration characteristics of these metabolites, the knowledge of their ionization constants is essential.

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In contrast to the numerous papers published on the biological monitoring of PAHs and their metabolites,[10-15] $pK_a$ data for PAH metabolites are very scarce in the literature. Among the $pK_a$ data, most of the $pK_a$ values were obtained by fluorescence spectrometry. For example, the $pK_a$ value for 1-OH-Py was determined by Van der Donckt et al. as early as 1969.[16] Later on, Capomacchia et al.[17,18] did a complete fluorescence spectrometry investigation of the ionization constants for all the isomeric monohydroxybenzo[a]pyrenes and some of the dihydrodiol derivatives. In order to understand the migration order of the metabolites in CZE, it is desirable to have the $pK_a$ values of the PAH metabolites obtained under the same conditions as in a CZE separation.

Recently, CZE has emerged as a convenient and precise method for the determination of ionization constants because of its high sensitivity and selectivity relative to potentiometric titration and ultraviolet spectroscopy.[19] Unlike potentiometric titration, CE requires small amounts of sample at low solute concentration. This is especially important for certain biological and environmental compounds because the standards could be very expensive and because of small quantities isolated from biological and environmental samples. Also, CE is advantageous because it can be automated. Several researchers have shown that ionization constants can be determined by CZE. For example, the ionization constants of three aromatic amines were determined by Cai et al.[20] using CZE. Cleveland's group has done a thorough $pK_a$ investigation for a variety of compounds with $pK_a$ values ranging from 2.43 to 9.99 with CZE.[19,21,22] Gluck et al.[23] extended the method to $pK_a$ values between 1.5 and 3.4. Furthermore, determination of ionization constants of water-insoluble (sparingly soluble) compounds by CZE has been reported.[24-26] Very recently, dissociation constants of cephalosporins were determined by CZE.[27]

The aim of this work was to investigate the feasibility of using CZE to determine the $pK_a$ values of the weakly acidic, large aromatic ring PAH metabolites. The relationships between electrophoretic mobility and thermodynamic $pK_a$ values have been introduced in detail previously.[19,21,22] The key equations are as follows:

$$pK_a = pH - \log\left[\frac{\mu_e}{\mu_{A^-} - \mu_e}\right] + \frac{0.5085 z^2 \sqrt{I}}{1 + 0.3281 a \sqrt{I}} \text{ (acids)} \quad (1)$$

$$pK_a = pH + \log\left[\frac{\mu_e}{\mu_{BH^+} - \mu_e}\right] - \frac{0.5085 z^2 \sqrt{I}}{1 + 0.3281 a \sqrt{I}} \text{ (bases)} \quad (2)$$

where $\mu_e$ is the effective mobility at a given experimental pH, $\mu_{A^-}$ the electrophoretic mobility of the fully ionized acid, $\mu_{BH^+}$ the electrophoretic mobility of the fully protonated base, $z$ the valence of the ion, $I$ the buffer ionic strength, and $a$ the ion size parameter, generally unknown and assumed to be 5 Å.

The compounds used in this work were all weak acids and therefore, non-linear regression of Eq. 1 was applied since it is normally the simplest and most precise approach for determining the $pK_a$ value of a compound by CE.[22]

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## EXPERIMENTAL

### Apparatus

CZE was performed with a Beckman P/ACE 5000 (Beckman, Fullerton, CA) instrument equipped with a UV detector. A fused-silica capillary with a 75 μm i.d. (375 μm o.d.) and a 57 cm total length (50 cm from inlet to detector) was used.

### Reagents

The benzo[a]pyrene metabolites and the benz[a]anthracene metabolite were purchased from the National Cancer Institute (NCI) repository at Midwest Research Institute (Kansas City, MO), and they were used without further purification. The 1-OH-Py sample was obtained from Aldrich (Milwaukee, WI). All buffer reagents were purchased from Aldrich. Methanol (MeOH) and water were HPLC grade and purchased from J.T.Baker Inc. (Phillipsburg, NJ).

**Methods**

Stock solutions containing 48 μg/mL of PAH metabolites were prepared in MeOH/water (7:3, v:v). Working solutions were prepared by diluting these stock solutions with the same solvent. The final concentration of each compound was 12 μg/mL.

All sample solutions were stored in the dark at 4°C. Solid samples were kept in a freezer at -10°C. All glassware was wrapped in aluminum foil to protect sample solutions from light.

The buffer concentration used throughout this study was 25 mM. These run buffers were prepared by dissolving the individual buffer reagent in HPLC water and adjusting to the appropriate pH with 1 M NaOH. The buffer pH was determined with an Orion research digital ionalyzer/501 equipped with a Orion 910200 combination electrode (errors in pH measurements were of ± 0.01 pH unit at room temperature). The pH values of the buffers were double checked before the running of each buffer to get the most accurate results.

Before use, all buffer solutions were filtered through a 0.22-μm Supor Acrodisc filter (Gelman, Ann Arbor, MI) and sonicated ten minutes to degas. At each pH value, sample solutions were run at least three times, and the average migration times were used to calculate the mobility values.

The CZE system was operated in a conventional mode with the cathode at the detector end. A 2s hydrodynamic injection was performed. The temperature was maintained under ambient conditions. Since the methanol content in the sample solution was relatively high, a separation potential of 25 kV was applied using a linear ramp of 2.5 min. At this voltage, currents of less than 60 μA were observed. Ultraviolet absorbance detection was used at 254 nm.

At the beginning of each working day, and whenever necessary, the capillary was rinsed sequentially with 1 M NaOH, 0.1 M NaOH, water, MeOH, water and then running buffer. Between each analysis, the capillary was washed with 0.1 M NaOH and buffer. MeOH in the sample solution was used as the neutral marker for the measurement of electroosmotic flow (EOF). Eletropherograms were recorded and analyzed on a IBM 350-P90 personal computer using Beckman's System Gold electrophoresis software (Beckman).

Pairs of data points for pH and $\mu_e$ were imported into Prism 2.0 (Intuitive Software, San Diago, CA, USA) where $\mu_{A^-}$ and p$K_a$ were determined by performing a non-linear fit to Eq. 1.

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**Spectroscopic $pK_a$ Determination of 1-OH-Py**

The spectroscopic $pK_a$ value of 1-OH-Py was determined by taking UV spectra with a Perkin Elmer Lambda 9 spectrometer using 1-cm cuvet at 23-24°C. All sample solutions had a concentration of $4 \times 10^{-5}$ M and were prepared in a series of 0.01 M buffers as follows: 3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid (CAPSO) (pH 9.42), 3-[(1,1-dimethyl-2-hydroxy-ethyl)amino]-2-hydroxypropane sulfonic acid (AMPSO) (pH 9.19, 8.98, 8.72), N-tris[hydroxymethyl]methyl-3-amino-propanesulfonic acid (TAPS) (pH 8.57, 8.33), and N-tris[hydroxymethyl]methylglycine (Tricine) (pH 8.19). A 1 M NaOH solution was used to adjust the buffer pH. The final ionic strength was 0.02. All of the absorbance values were obtained at 404 nm, where the absorbance of the neutral molecule and that of the anion of 1-OH-Py showed the largest difference. The $pK_a$ value at each pH was calculated according to the following equation:

$$pK_a = pH + \log \frac{A_I - A}{A - A_M} \qquad (3)$$

where A is the absorbance at a certain pH, $A_I$ the absorbance of anion (measured at pH 12 with 0.01M NaOH), $A_M$ the absorbance of the neutral molecule (measured at pH 5.98 with 0.01 M 2-[N-morpholino]ethanesulfonic acid (MES) buffer). The final $pK_a$ value was determined by taking average of the individual $pK_a$ value at each pH.

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## RESULTS AND DISCUSSION

**Buffer pH Range**

The buffers used in the CZE experiments were 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) and CAPSO. Table 1 shows the pH values, concentrations and the ionic strengths of the buffer series used and the amount of 1M NaOH added in order to adjust the pH. The equally spaced pH buffers (pH interval: 0.2-0.3 pH units) were run in order from high to low pH. For the $pK_a$ experiments with 3-OH-B[a]A, 3-OH-B[a]P, 7-OH-B[a]P and 9-OH-B[a]P, CAPS was used with a pH range from 10.6 to 9.7. For 12-OH-B[a]P and 1-OH-Py, CAPS was used from pH 10.6 to pH 9.7, and CAPSO was used to reach the even lower pH values.

Ideally, when measuring an unknown $pK_a$ value, it is important to cover a wide pH range.[22] The typical way is to equally space the buffer pH around the $pK_a$ value of the solute. In our work, we did not go beyond pH 10.6 since high

**Table 1**

**Buffer Series Used in the Study**

| Buffer | $pK_a$ | pH | Conc. (nM) | NaOH (Drops) | Ionic Strength | log $\gamma$[a] |
|---|---|---|---|---|---|---|
| CAPS | 10.4 | 10.63 | 25 | 13 | 0.04 | 0.08 |
| CAPS | 10.4 | 10.37 | 25 | 9 | 0.04 | 0.07 |
| CAPS | 10.4 | 10.10 | 25 | 7 | 0.03 | 0.07 |
| CAPS | 10.4 | 9.94 | 25 | 5 | 0.03 | 0.07 |
| CAPS | 10.4 | 9.74 | 25 | 4 | 0.03 | 0.07 |
| CAPSO | 9.6 | 9.53 | 25 | 8 | 0.04 | 0.07 |
| CAPSO | 9.6 | 9.25 | 25 | 4 | 0.03 | 0.07 |
| CAPSO | 9.6 | 9.01 | 25 | 3 | 0.03 | 0.07 |

[a] $$\log \gamma = \frac{0.5085 z^2 \sqrt{I}}{1 + 0.3281 a \sqrt{I}}$$

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alkaline condition favored decomposition of the monohydroxyl PAH metabolites. Also, an effort was made to obtain a buffer pH value as low as possible. However, we could not reach a pH low enough to bracket the $pK_a$. This is because with pH values lower than the lower pH limits mentioned above, there was not a suitable buffer to obtain well-shaped electropherograms with reproducible migration times. Since it is not always necessary for the buffer series used in this technique to bracket $pK_a$,[19] we decided to use the above buffer pH range.

## Buffer Concentration

It is very important to keep the ionic strengths of the buffer series constant throughout the $pK_a$ measurement because $pK_a$ depends on the ionic strength of the background electrolytes. In our experiments, all the buffers had an equal concentration of 25 mM and a 1 M NaOH solution was used to adjust the pH. In order to keep the ionic strength of the buffer constant, the contribution of the ionic strength from NaOH has to be minimized. Therefore, a high buffer concentration is preferred. Concentrated buffers also provide higher buffering capacity. On the other hand, dilute buffers have their own advantages, such as short running time and low Joule heating. By compromising these considerations, 25 mM was chosen as the buffer concentration.

**Table 2**

**$pK_a$ Values Determined by CZE Versus Literature Values**

| Molecule | $pK_a$ (lit.)[a] | $pK_a$ (CZE)[b] | Difference |
|---|---|---|---|
| 9-OH-B[a]P | 9.5[17] | 9.33 ± 0.13 | -0.17 |
| 7-OH-B[a]P | 9.2[18] | 9.06 ± 0.04 | -0.14 |
| 3-OH-B[a]A | nf[c] | 9.04 ± 0.15 | --- |
| 12-OH-B[a]P | 9.0[18] | 8.99 ± 0.26 | -0.01 |
| 1-OH-Py | 8.7[16] | 8.81 ± 0.10 | +0.11 |
| 3-OH-B[a]P | 8.6[17] | 8.79 ± 0.10 | +0.19 |

[a] The literature values from references 17 and 18 were measured at 24 ± 2°C.
[b] CZE-determined $pK_a$ values were obtained at an ionic strength of 0.03-0.04 and at room temperature, 23 ± 1°C.

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It is clear from Table 1 that, although different amounts of NaOH were added to the buffer solutions to obtain certain pH values, the ionic strengths of the overall buffer series were controlled at 0.03-0.04, which we consider to be essentially constant.

## $pK_a$ Determinations

Figure 1 gives two examples of the plots of effective mobilities against pH. Curve fits obtained by non-linear regression using Eq.1 are shown by the solid lines. Our CE-determined $pK_a$ values and $pK_a$ values from the literature are shown in Table 2. As shown in Table 2, the absolute differences ranged from 0.01 to 0.19. The dissimilarities in the experimental temperatures and ionic strengths could be the main reasons for the differences between our values and the literature values. For example, the $pK_a$ values from references 17 and 18 were obtained at 24 ± 2°C, while our experimental temperature was 23 ± 1°C. Moreover, there was nothing mentioned in references 17 and 18 about the control of ionic strength, while in our case, the ionic strengths of the buffer series were very well controlled.

The differences in the literature $pK_a$ values and our CZE results are somewhat larger compared with what some other research groups have reported.[19-22] However, most of the model compounds previously investigated had small molecular weights and were more acidic (or basic). These types of compounds are relatively easy to work with in CE. The metabolites we investigated are weakly acidic, large ring hydroxyl aromatics. It is very difficult

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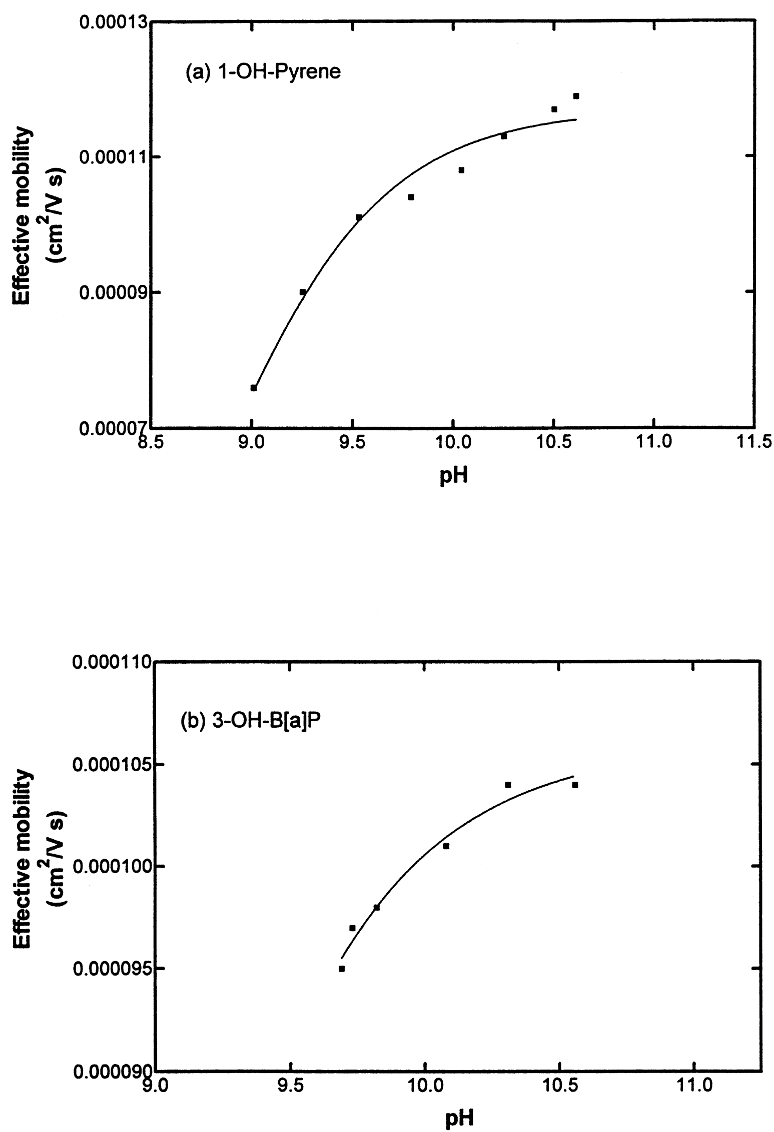


**Figure 1**. Dependence of effective mobilities of 1-OH-Pyrene and 3-OH-B[a]P on pH.

to find suitable buffers over a wide pH range to run these compounds by CZE. Schmitt et al.[26] measured the ionization constants of 12 hydroxytriazines by CZE. The differences between their results and the $pK_1$ values in the literature ranged from 0.06 to 0.20. This range is essentially within the range that we have

in Table 2. The curve shapes in Figure 1 are somewhat unique in that they are only part of the "S" shaped titration curves. As mentioned above, the pH ranges in Figure 1 are the largest possible distributions we could attain for these PAH metabolites. With the non-linear regression analysis of our data and the agreement of our $pK_a$ results with the literature values, we concluded that it is certainly possible to use CZE to determine the $pK_a$ values of the weakly acidic PAH metabolites.

To test the accuracy of our CZE results, the $pK_a$ value of 1-OH-Py was determined spectroscopically. (Due to the high cost of the other samples, this was the only compound whose $pK_a$ value was measured by spectroscopy.) The $pK_a$ value obtained spectroscopically was $8.73 \pm 0.03$ at 23-24°C and at an ionic strength of 0.02. The literature value for 1-OH-Py was 8.7 and our CZE value was 8.81. It is seen that the spectroscopically determined $pK_a$ value lies in between the literature value and the CZE value determined in this work.

The $pK_a$ values that we obtained by CZE would be very useful in explaining the migration orders of the PAH metabolites in a previous CZE method developed by us[9] because essentially the same experimental conditions were used as in the CZE separation. We are presently using the $pK_a$ values acquired by CZE to explain the migration characteristics of the PAH metabolites.

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## CONCLUSIONS

Previously, the determination of $pK_a$ values by CZE was limited to compounds either with small molecular weight or with a reasonably low $pK_a$ value ($\alpha$ 7). In this investigation, $pK_a$ values of six weakly acidic, large ring hydroxyl aromatic PAH metabolites were acquired by CZE. The $pK_a$ values determined by CZE agreed well with the literature values. A spectroscopic method was also used to further test the accuracy of the CZE result for 1-OH-Py. The spectroscopic $pK_a$ value was in between the literature value and the CZE value, indicating the reliableness of the $pK_a$ value determined by CZE. Thus, the results of this study showed that CZE is suitable for the determination of the $pK_a$ values of sparingly soluble, weakly acidic, and relatively high molecular weight PAH metabolites.

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