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# Capillary electrophoretic analysis of alkaline phosphatase inhibition by theophylline

An analytical method for studying enzyme inhibition has been developed using capillary electrophoresis with laser-induced fluorescence detection. This technique is based on electrophoretic mixing of zones of enzyme and inhibitor in substrate-filled capillaries. Enzyme catalytic activity is measured by detecting the fluorescent reaction product as it migrates past the detector. Reversible enzyme inhibition is indicated by a transient decrease in product formation. The enzyme, alkaline phosphatase, has been studied using the fluorogenic substrate AttoPhos ([2,2′-bibenzothiazol]-6-hydroxy-benzthiazole phosphate). This assay has been used to quantify theophylline, a noncompetitive, reversible inhibitor of alkaline phosphatase. The detection limit for theophylline is estimated at 3 μM, and 8.6 amole of alkaline phosphatase are required for each assay. The calculated $K_i$ for theophylline is 90 μM for the capillary electrophoretic enzyme-inhibitor assays.

**Keywords:** Enzyme assay / Alkaline phosphatase / Capillary electrophoresis / Theophylline / Inhibition EL 3900

## 1 Introduction

Capillary electrophoresis has emerged as a versatile tool for enzyme analysis [1, 2]. Homogeneous on-column capillary electrophoretic enzyme assays, where the enzyme is injected into the capillary as a discrete zone, offer several advantages. These include simplicity, rapid analysis times, and consumption of extremely small amounts of enzyme [1, 2]. Single enzyme molecules have been detected and studied [3, 4], and separation and detection of isoenzymes from single cells have been reported [5]. Either the substrate or enzyme concentration can be quantified [1, 6–8], and Michaelis-Menten constants can be determined [9–13]. Peptide mapping using on-column enzyme digestion has also been demonstrated [14], and enzymatic digestion of oligonucleotides has been studied [15].

The first on-column capillary electrophoretic enzyme assay was described by Bao and Regnier [6]. In these experiments, the running buffer contained the substrate and coenzyme required for the catalyzed reaction. The enzyme was injected into the capillary, and product formation was monitored continuously at a downstream absorbance detector. A method which is often termed electrophoretically mediated microanalysis (EMMA) was developed as a technique for performing enzyme assays [1, 8, 16, 17]. EMMA utilizes electrophoretic mixing of zones in a capillary [8]. For enzyme assays, EMMA is performed by injecting a zone of enzyme and a zone of substrate separately. The zones are allowed to mix electrophoretically to form the product. The appropriate injection order for the enzyme and substrate is determined by their relative electrophoretic mobilities [8, 18].

Enzyme inhibition is important in many scientific areas including pesticide science and pharmacology. Enzyme inhibitors are developed as pesticides to protect crops from damage caused by insects [19, 20]. Compounds are designed to inhibit specific enzymes for medical applications [21, 22]. For example, metal complexes developed to inhibit enzymes are used as drugs to treat cancer, hypertension, and arthritis [23]. Many chemical analysis methods have been developed using enzyme inhibition reactions. Environmental pollutants, organophosphorous pesticides, and heavy metal ions have all been studied through inhibition of reactions catalyzed by various enzymes [24].

A variation of the EMMA technique has been applied to study enzyme inhibition [25]. In these experiments, the running buffer contained erythro-9-(2-hydroxy-3-nonyl) adenine, a competitive inhibitor of adenosine deaminase. The substrate was adenosine. The product, inosine, was formed when the individually injected enzyme and substrate zones mixed electrophoretically in the capillary. In these experiments, the same inhibitor concentration was

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**Abbreviations: AttoPhos**, (2,2′-bibenzodiazol)-6-hydroxy-benzthiazole phosphate; **EMMA**, electrophoretically mediated microanalysis; **ES**, enzyme-substrate

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used throughout, while the substrate concentration was varied. The substrate and the enzyme solutions were pre-incubated independently with the inhibitor for 10 min before injection. When the injected enzyme and substrate zones had mixed electrophoretically in the capillary, the enzyme was allowed to incubate with the substrate at zero potential for 5 min. The high voltage was applied again, and the product was separated and detected by absorbance at 254 nm. Lineweaver-Burk plots for the data were used to determine the $K_i$ for erythro-9-(2-hydroxy-3-nonyl)adenine.

Enzyme inhibition has also been studied using electrophoretic mixing in microchip devices [9–11]. The first report of this type of assay was for the enzyme, β-galactosidase [10]. The fluorogenic substrate, resorufin β-D-galactopyranoside, was used, and the competitive inhibitor, phenylethyl β-D-thiogalactoside, was studied. Substrate, buffer, enzyme, and inhibitor were contained in separate reservoirs on the microchip device and were mixed in the device by electrokinetic transport controlled by applied potentials. The substrate concentration was controlled by mixing substrate and buffer in the device under potential control. Enzyme and inhibitor solutions at fixed concentrations were subsequently mixed with the diluted substrate solution, and product formation was detected in the final reaction channel at a downstream LIF detector. The $K_i$ was determined for phenylethyl β-D-thiogalactoside, and inhibition by two additional inhibitors was detected.

A related microchip assay was developed for observing protein kinase A inhibition by H-89, a known competitive protein kinase A inhibitor [9]. The substrate was fluorescently labeled kemptide, a heptapeptide that is not fluorogenic. In these experiments, the inhibitor was diluted on the microchip device and mixed with fixed concentrations of enzyme and substrate. Aliquots of the sample stream containing enzyme, substrate, and inhibitor were injected on the microchip device into a separation channel after 75 s of incubation in an incubation channel. Then, the product and substrate were separated electrophoretically and detected by fluorescence. The $K_i$ value for the inhibitor was determined.

A microchip assay was recently applied to study inhibition of acetylcholinesterase using acetylthiocholine as a substrate [11]. Competitive and irreversible acetylcholinesterase inhibitors were examined using this technique. The enzyme was continuously pumped through the microchip device. Substrate was added to the enzyme stream. The product of the enzyme reaction is not fluorescent; therefore, coumarinylphenylmaleimide was added on-column after product formation to derivatize the product for LIF detection. Discrete zones of inhibitor were injected into the enzyme stream before the point of substrate addition, and inhibition was indicated by a decrease in fluorescent product formation as the inhibitor zone migrated to the detector. The $K_i$ was determined for the competitive inhibitor, tacrine. Using this method, reversible and irreversible inhibitors could be analyzed and distinguished. Separation and detection of a mixture of inhibitors was demonstrated.

In this paper, we report the development of an on-column capillary electrophoretic enzyme inhibition assay with fluorescence detection. In this assay, the capillary is filled with a fluorogenic substrate. Next, separate zones of the inhibitor and enzyme are injected. As the zones of inhibitor and enzyme migrate through the capillary at a constant applied potential, product is continuously formed. When the zones of inhibitor and enzyme mix, product formation decreases. Alkaline phosphatase inhibition by the noncompetitive reversible inhibitor, theophylline, has been studied using this technique.

## 2 Materials and methods

### 2.1 Reagents

Calf intestinal alkaline phosphatase (EC 3.1.3.1) was obtained from ICN Biomedicals (Aurora, OH). AttoPhos ([2,2′-bibenzothiazol]-6-hydroxy-benzthiazole phosphate) was purchased from JBL Scientific (San Luis Obispo, CA). Theophylline, 99%, was purchased from Aldrich (Milwaukee, WI). Chloroform was purchased from Acros (Pittsburgh, PA). Boric acid was supplied by J. T. Baker Chemical (Phillipsburg, NJ). All solutions and buffers were prepared in doubly distilled water.

### 2.2 CE-LIF instrumentation

The CE-LIF instrumentation was constructed in-house. The 457.9 nm line of a Coherent Innova 90C-5 argon ion laser (Santa Clara, CA) was used for excitation. The laser beam was focused onto the capillary by a fused-silica plano convex lens ($f$ = 15.0 mm) (Optosigma, Santa Ana, CA). The power of the laser beam at the capillary was 20 mW. The fluorescence was collected at 90° to the laser beam by a 20 × microscope objective (Edmund, Barrington, NJ) and was filtered by a 560 ± 10 nm bandpass filter (Oriel, Stratford, CT). A 1 mm diameter aperature (Oriel) was used as a spatial filter. Fluorescence was detected by a photomultiplier tube (PMT) (HC120; Hamamatsu, Bridgewater, NJ) at 800 V. The PMT output was filtered by a low-pass filter at 50 Hz, and then sent to an analog-to-digital board (Lab-PC-1200; National Instruments, Austin,TX). A LabVIEW program (National Instruments) was written in-house for data acquisition. The

sampling rate was 10 Hz. The data were analyzed using Excel (Microsoft) and Peakfit (SPSS, Chicago, IL). A Spellman high-voltage power supply (CZE1000R; Hauppauge, NY) was used for the electrophoresis system. Fused-silica capillaries with a 50 μm ID and 220 μm OD (SGE, Austin, TX) were utilized. The detection window was made by burning away the polyimide coating.

### 2.3 Electrophoretic conditions

The running buffer for each experiment contained 100 mM borate buffer at pH 9.5 and 1.0 mM AttoPhos, a fluorogenic alkaline phosphatase substrate. The pH of the running buffer was determined by a series of experiments to optimize the reaction conditions. The separation buffer was extracted three times with equal volumes of chloroform before use. AttoPhos is known to contain some AttoFluor, the fluorescent product, as an impurity, and the extraction with chloroform reduced the AttoFluor present [26]. The enzyme solution contained 1.7 nM alkaline phosphatase, 100 mM borate at pH 9.5, and 1.0 μM fluorescein. Fluorescein was used as an internal standard. All inhibitor solutions contained the inhibitor and 100 mM borate at pH 9.5. The applied electric field was 210 V/cm (15.0 kV) for all experiments. Injections were performed electrokinetically at 15.0 kV. Two electrodes were used for the injection of the solutions. One electrode was used only for the running buffer. The second electrode was used to inject the enzyme and inhibitor. This procedure reduced contamination of the running buffer with enzyme. The outside of capillary was rinsed with 100 mM borate buffer at a pH of 9.5 after each injection to help reduce contamination of the running buffer with enzyme, inhibitor, and fluorescein. All experiments were carried out at room temperature. All injection times of enzyme, theophylline, and fluorescein were 3.0 s.

### 2.4 Determination of electrophoretic mobilities

The electrophoretic mobilities of the substrate, inhibitors, and enzyme were determined by CE using a UV/Vis absorbance detector at 200 nm. The detector was a Linear UVIS 204 Detector (Linear Instruments, Reno, NV) with an on-column capillary cell. The applied electric field for the separations was 210 V/cm (15.0 kV), and injections were performed electrokinetically at 15.0 kV. All injections were 3.0 s. The data were collected with a strip chart recorder. Mesityl oxide was used as a neutral marker.

### 2.5 Microplate experiments

Microplate experiments analogous to the electrophoretic experiments for theophylline analysis were performed using a FLUOstar microplate fluorometer (BMG, Durham, NC). The concentration of AttoPhos was 1.0 mM, and the enzyme concentration was 1.7 nM. The concentration of theophylline was varied. The excitation filter was centered at 450 nm, and the emission filter was centered at 555 nm. The microplates used were 96-well plates by Nunc (V-96 Polypropylene MicroWell Plate; Roskilde, Denmark). The enzyme was injected into each well separately by the instrument. The product formation was observed at 0.1 s intervals in order to determine the initial velocity of the reaction. The results for each well were plotted, and the slope of each line was determined.

## 3 Results and discussion

### 3.1 On-column capillary electrophoretic assay of alkaline phosphatase

Alkaline phosphatase has been the subject of several studies using on-column capillary electrophoretic enzyme assays. EMMA has been used to observe the catalyzed reaction between alkaline phosphatase and the substrates, *p*-aminophenylphosphate and *p*-nitrophenylphosphate [27]. Michaelis-Menten constants for alkaline phosphatase have been determined using the EMMA technique [12]. Catalysis by single alkaline phosphatase molecules has been studied using CE [4].

Figure 1 presents the results of an alkaline phosphatase enzyme assay without inhibitor. This enzyme assay is similar to previously reported assays [6]. A 3.0 s zone of alkaline phosphatase (1.7 nM) is injected into the capillary. A potential of 210 V/cm is then applied, and the product is

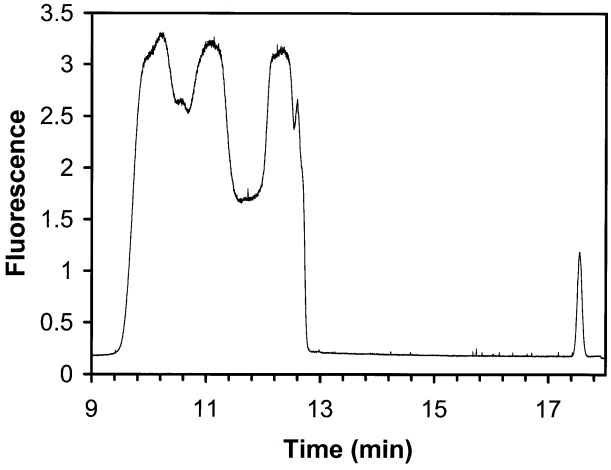


**Figure 1.** Electropherogram of an alkaline phosphatase enzyme assay with no inhibitor. A 3.0 s zone of 1.7 nM alkaline phosphatase was injected at 15.0 kV into a capillary filled with 1.0 mM AttoPhos in 100 mM borate buffer at pH 9.5. The applied electric field was 210 V/cm (15.0 kV). The current was 16 μA.

formed and detected continuously until the enzyme migrates past the detector. The 5.0 nL injection contains 8.6 amole of alkaline phosphatase. In this experiment, the enzyme-substrate (ES) complex migrates faster than the fluorescent product. As the enzyme migrates through the capillary, it leaves product behind. The first fluorescent product that reaches the detector is the product formed just before the enzyme passes the detector (9.5 min in Fig. 1). Product formed when the enzyme is first injected into the capillary reaches the detector last (12.8 min in Fig. 1). This phenomenon has been described previously [6].

The shape of the product peak in Fig. 1 is predicted to be a plateau as described by Bao and Regnier [6]. The shape of the observed plateau (Fig. 1) is irregular, but highly reproducible. The peak at the end of the plateau at 12.6 min is an artifact resulting from product formation after the enzyme has been injected into the capillary and before the high voltage has been reapplied. This has been described previously and does not interfere with the analysis [6]. The zero field incubation that results in this artifact is the basis of experiments designed to observe single enzyme molecules [3, 4]. The peak at 17.5 min is due to fluorescein (1.0 μM) that is added as an internal standard. At present, the nature of the other peaks and valleys on the plateau between 9.5 min and 12.6 min is unknown. Because the shape of this structure is reproducible from run to run and from day to day, it does not interfere with our initial analysis of enzyme inhibitors using this technique. However, future work will include efforts to understand this structure and to eliminate it in order to obtain the predicted plateau.

### 3.2 Inhibition of alkaline phosphatase

Theophylline is a noncompetitive, reversible inhibitor of alkaline phosphatase [28]. Reported $K_i$ values for theophylline with alkaline phosphatase range from 78 μM for bovine liver alkaline phosphatase to 135 μM for serum alkaline phosphatase [29, 30]. Theophylline is a clinically important compound that is used as a bronchodilator, respiratory stimulant, anti-inflammatory drug, and for apnea treatment [31–33].

Figure 2 presents the data from an enzyme assay to observe inhibition of alkaline phosphatase by theophylline. The experiment in Fig. 2 is identical to that in Fig. 1 except that the inhibitor, theophylline, is injected as well as the enzyme. Theophylline (0.10 mM, 3.0 s, 15.0 kV) is injected first. A constant potential (15.0 kV) is applied for 45 s. Then alkaline phosphatase (1.7 nM, 3.0 s, 15.0 kV) is injected. The alkaline phosphatase-AttoPhos complex has a greater electrophoretic mobility than theophylline.

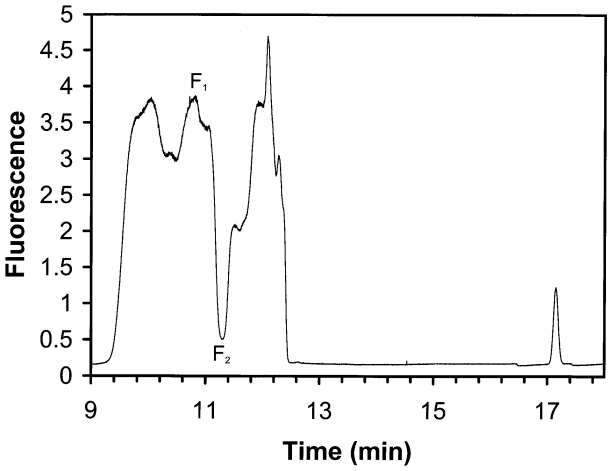


**Figure 2.** Electropherogram of an alkaline phosphatase-theophylline enzyme inhibition assay. First, a 3.0 s zone of 0.10 mM theophylline was injected. Next, high voltage (15.0 kV) was applied for 45 s. A 3.0 s zone of 1.7 nM alkaline phosphatase was then injected. Finally, high voltage was applied, and the assay was completed. All injections were performed electrokinetically at 15.0 kV. Running buffer, electric field and current were as in Fig. 1.

As alkaline phosphatase migrates through the capillary after injection, the zone of ES complex overtakes the theophylline zone, and inhibition occurs. When one compares Fig. 1, the control assay, to Fig. 2, the inhibition caused by the theophylline is evident at 11.3 min ($F_2$ in Fig. 2).

The injection order required to observe enzyme inhibition depends on the relative migration rates of the enzyme and inhibitor zones. In this case, theophylline migrates slower than the ES complex. Therefore, theophylline is injected first to ensure that the two zones will mix in the capillary. If the inhibitor migrates faster than the ES complex, the enzyme must be injected first to ensure mixing. Two experiments, at most, must be performed to detect an inhibitor of unknown electrophoretic mobility relative to the enzyme. If the inhibitor is injected first, and no inhibition is observed, a second experiment in which the enzyme is injected first should result in detection of inhibition. The exception to this would be in those rare instances where the inhibitor and enzyme have nearly identical electrophoretic mobilities.

### 3.3 Enzyme and inhibitor injection times

It is possible to control the time that inhibition is observed by controlling the injection times of the inhibitor and enzyme. In EMMA, the differences in the mobilities of the reagents make it possible to control reagent contact time and control the time that the reactions will occur in the

**Table 1.** Enzyme and inhibitor relative injection times and observed inhibition electrophoretic mobilities

| $T_I - T_E$ (s)[a)] | Inhibition response Mobility ($cm^2$/Vs) | Fluorescein Mobility ($cm^2$/Vs) |
|---|---|---|
| 10.0 | $-2.15 \times 10^{-4}$ | $-3.02 \times 10^{-4}$ |
| 40.0 | $-1.97 \times 10^{-4}$ | $-3.05 \times 10^{-4}$ |
| 70.0 | $-1.80 \times 10^{-4}$ | $-3.07 \times 10^{-4}$ |

a) $T_I - T_E$, the time that the separation voltage (15.0 kV) was applied between the injection of zones of inhibitor and enzyme.

capillary [8]. Table 1 shows the results of experiments in which the distance between injected zones of alkaline phosphatase and theophylline has been varied to control the time at which inhibition is observed. The time difference ($T_I - T_E$) indicates the time that the separation potential (15.0 kV) was applied between the injection of theophylline and the injection of alkaline phosphatase. The observation of inhibition is reported as an electrophoretic mobility. As the distance between the inhibitor injection and enzyme injection is increased, the electrophoretic mobility of the inhibition response (decrease in product formation compared to control) increases as expected. As the distance between the injection of the two zones is increased, the mixing of the two zones occurs later in the experiment and closer to the detector. Because the ES complex migrates faster than the fluorescent product, the inhibition response is observed at the detector earlier.

Tlhe electrophoretic mobility of the internal standard, fluorescein, is also included in Table 1. The migration time of the fluorescein peak is observed to drift by several seconds between experiments, but the electrophoretic mobility of fluorescein does not change significantly (< 2%). The changes in migration times suggest that alkaline phosphatase or theophylline may be adsorbing on the uncoated capillary surface, altering electroosmotic flow.

### 3.4 Theophylline quantitation

Theophylline is a noncompetitive, reversible inhibitor of alkaline phosphatase [28]. Using Michalis-Menten treatment of this system, the $K_m$ value will remain the same at any inhibitor concentration, but the apparent $V_{max}$ will change with the inhibitor concentration. The Michalis-Menten equation is as follows:

$$v/V_{max} = [S]/(K_m + [S]) \quad (1)$$

where $v$ is the initial reaction velocity, $K_m$ is the Michalis constant for the ES complex, and [S] is the substrate concentration [34]. A relative velocity equation can be derived for a noncompetitive inhibitor [34]:

$$V_{max}^*/V_{max} = K_i/(K_i + [I]) \quad (2)$$

where $V_{max}^*$ is the apparent $V_{max}$ at a given inhibitor concentration, $V_{max}$ is the maximum velocity of the enzyme in the absence of an inhibitor, $K_i$ is the equilibrium constant for inhibitor binding to the enzyme, and [I] is the inhibitor concentration. Equation (1) can be rearranged to give the following equation:

$$1/V_{max}^* = (1/V_{max}K_i)\,[I] + 1/V_{max} \quad (3)$$

A plot of $1/V_{max}^*$ *versus* inhibitor concentration should be linear if the Michalis-Menten treatment is appropriate.

An inhibitor response factor was defined in order to quantitate theophylline. The inhibitor response ($RI_{ce}$) for the capillary electrophoresis experiments is:

$$RI_{ce} = (F_1 - F_0)/(F_2 - F_0) \quad (4)$$

The fluorescence at $F_1$ (Fig. 2) is proportional to the rate of product formation without inhibitor and is used to normalize the data due to variability in the magnitude of the plateau from experiment to experiment. $F_i$ is chosen because it is a reproducible peak in the product plateau that is easily identified from experiment to experiment. $F_2$ (Fig. 2) is the time when minimum product formation is observed, and the fluorescence is proportional to the velocity of product formation ($dP/dt$) during maximum inhibition. In all experiments, a saturating substrate concentration is used. Under these experimental conditions, $dP/dt$ is $V_{max}^*$. The observed baseline signal for the experiment is $F_o$. Thus, $RI_{ce}$ is proportional to $1/V_{max}^*$. The $RI_{ce}$ values at different theophylline concentrations have been plotted, and the results are shown in Fig. 3.

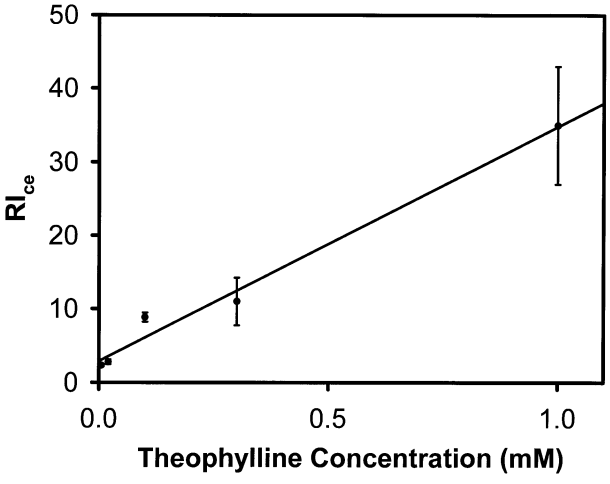


**Figure 3.** Plot of $RI_{ce}$ *versus* theophylline concentration. $RI_{ce}$ is proportional to $1/V_{max}^*$, the apparent $V_{max}$, at a given theophylline concentration.

The detection limit for theophylline is 3 μM, and the linear dynamic range spans 5.0 μM to 1.0 mM. The calculated $K_i$ for theophylline is 90 μM for the capillary electrophoretic enzyme-inhibitor assays and is in agreement with literature values [29, 30]. If a large concentration of theophylline (greater than 1.0 mM) is injected, a fourth peak in the electropherogram appears before the inhibition response. One hypothesis to explain this peak is that it results from a field amplification effect observed when the theophylline concentration becomes high enough to significantly alter the conductivity of the theophylline solution relative to the running buffer [35]. Theophylline is expected to have a charge of approximately –0.85 at pH 9.5.

Similar experiments were also performed using a microplate reader. Figure 4 shows a plot of an analogous response factor for the microplate fluorometer, $RI_{mp}$, *versus* theophylline concentration for the microplate reader. $RI_{mp}$ is equal to 1/slope where slope is defined from the data obtained from the microplate reader. These data were obtained by monitoring the product formation at 0.1 s intervals after the injection of alkaline phosphatase. The slope of the curve (fluorescence *vs.* time) was determined and is proportional to $V_{max}$*. The calculated $K_i$ for theophylline is 41 μM for the microplate enzyme-inhibitor assays. This analysis of the data assumes steady-state kinetics. The data in Fig. 3 suggest that the CE experiments exhibit steady-state behavior.

### 3.5 Interaction time of theophylline and alkaline phosphatase zones

Approximate interaction times for the alkaline phosphatase and theophylline zones have been calculated using the following equation:

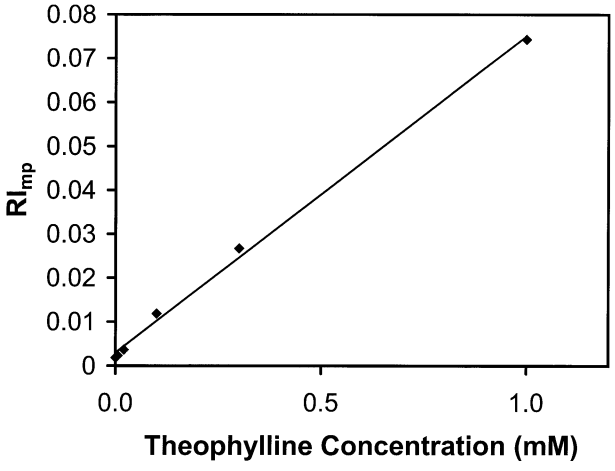


**Figure 4.** Plot of $RI_{mp}$ *versus* theophylline concentration. $RI_{mp}$ is a response factor for the microplate fluorometer experiments analogous to $RI_{ce}$.

$$T_I = L_Z/(V_{ap}-V_{th}) \quad (5)$$

where $T_I$ is the interaction time in seconds, $L_Z$ is the zone length of alkaline phosphatase in cm, $V_{ap}$ is the migration velocity of alkaline phosphatase in cm/s, and $V_{th}$ is the migration velocity of theophylline in cm/s. The approximate interaction time calculated for the experiments is 19 s. The experimental values for the interaction time were determined from the inhibition data. The reported experimental values for the interaction times are the peak width values that were calculated from the measured full width at half maximum for the observed inhibition peaks. In order to determine the experimental interaction times accurately, a double normalization procedure was developed, and the inhibition electropherograms (Fig. 2) were subtracted from the control electropherograms (Fig. 1). This procedure was necessary to better visualize the inhibition peaks, to account for variability in migration times between runs due to changes in electroosmotic flow, and to account for variability in plateau height from run to run. The first step in this method was to normalize the time scale so that the beginning of the product plateaus and the migration time of the internal standard peaks were the same in the control and inhibition experiments. The second step was to normalize the height of the product plateaus for the control and inhibition experiments. Finally, the inhibition electropherogram was subtracted from the detection electropherogram. Figure 5 is an example of a subtracted electropherogram produced using this procedure. The inhibition response now appears as a positive peak in the electropherogram at 11.9 min.

The observed interaction times are reported in Table 2. As the concentration of theophylline increases, the observed interaction time increases. Affinity capillary elec-

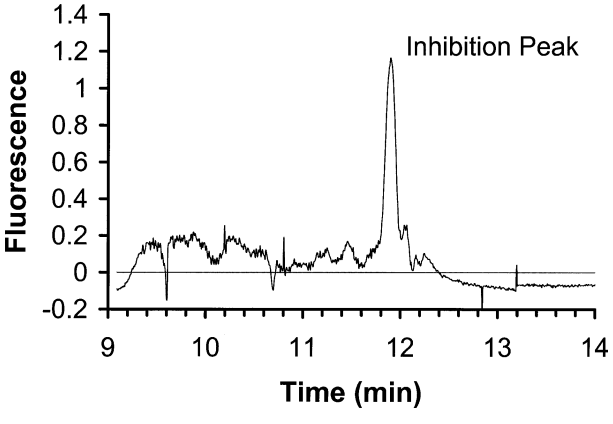


**Figure 5.** Example of a subtracted, normalized electropherogram (control minus inhibitor). The inhibitor concentration was 1.0 mM.

**Table 2.** Experimental interaction times of theophylline and alkaline phosphatase zones

| Theophylline Concentration (mM) | Peak width (s) |
|---|---|
| 0.0050 | 12.3 ± 1.8 |
| 0.020 | 16.9 ± 1.7 |
| 0.10 | 18.7 ± 2.4 |
| 0.30 | 20.8 ± 3.0 |
| 1.0 | 22.1 ± 1.7 |

trophoresis is used to study the interaction of proteins with ligands (*e.g.*, enzymes and inhibitors) [36, 37]. Changes in peak width corresponding to changes in the ratio of protein to ligand have been reported previously [38, 39]. Whitesides and co-workers [38] explored peak broadening for affinity CE studies of carbonic anhydrase and an arylsulfonamide inhibitor using computer simulations and experimental measurements. Their work showed that peak broadening occurred if the on and off rates for the protein-ligand reaction were comparable to the separation time. It is difficult to directly compare our results to this previous study. In our work, we observed the width of the inhibitor zone (ligand) as indicated by the transient decrease in product formation. In Whitesides' study, the authors directly observed the width of the carbonic anhydrase peak (enzyme) by absorbance detection. Also, the entire capillary was full of inhibitor in their work. Furthermore, the corresponding ratio of enzyme/ligand ranged from 0.05 to 6.25 in their work, while the corresponding ratio ranged from $7.4 \times 10^{-5}$ to $1.5 \times 10^{-3}$ in the present study. Their results support the hypothesis that our observed changes in interaction time as a function of inhibitor concentration are related to the affinity interaction between alkaline phosphatase and theophylline. This interesting observation will be the subject of future investigation.

## 4 Concluding remarks

The inhibition of alkaline phosphatase with theophylline was studied using CE. Only 8.6 amole of alkaline phosphatase were required for the CE experiment, whereas 340 fmole of alkaline phosphatase were required for analogous experiments using a microplate fluorometer (100 μL sample volume). The limit of detection for theophylline was estimated at 3 μM. The calculated $K_i$ for theophylline is 90 μM for the capillary electrophoretic enzyme-inhibitor assays and is in agreement with literature values. Future work will include studies with other enzymes and inhibitors and more detailed exploration of the effects of affinity interactions for the method. The effects of sample adsorption, temperature, and enzyme purity on this technique will be examined. This approach to the study of enzyme inhibition offers the ability to rapidly identify and quantify enzyme inhibitors while consuming minimal amounts of both enzyme and inhibitor.

*This research is supported by start-up funds from the University of Tennessee and by the NSF Research Experience for Undergraduates Program at UTK (Steven E. Johnston). The authors would like to thank Jason Pittman, Kim Schrum, and Joe Lancaster for their help with the instrumentation.*

Received December 20, 1999

## 5 References

[1] Bao, J. J., Fujima, J. M., Danielson, N. D., *J. Chromatogr. B* 1997, *699*, 481–497.

[2] Schultz, N. M., Tao, L., Rose, D. J., Kennedy, R. T., in: Landers, J. P. (Ed.), *Handbook of Capillary Electrophoresis*, CRC Press, Boca Raton, FL 1997, pp. 611–637.

[3] Xue, Q., Yeung, E. S., *Nature* 1995, *373*, 681–683.

[4] Craig, D. B., Arriaga, E. A., Wong, J. C. Y., Lu, H., Dovichi, N. J., *J. Am. Chem. Soc.* 1996, *118*, 5245–5253.

[5] Xue, Q., Yeung, E. S., *Anal. Chem.* 1994, *66*, 1175–1178.

[6] Bao, J., Regnier, F. E., *J. Chromatogr.* 1992, *608*, 217–224.

[7] Harmon, B. J., Patterson, D. H., Regnier, F. E., *J. Chromatogr. A* 1993, *657*, 429–434.

[8] Harmon, B. J., Patterson, D. H., Regnier, F. E., *Anal. Chem.* 1993, *65*, 2655–2662.

[9] Cohen, C. B., Chin-Dixon, E., Jeong, S., Nikiforov, T. T., *Anal. Biochem.* 1999, *273*, 89–97.

[10] Hadd, A. G., Raymond, D. E., Halliwell, J. W., Jacobson, S. C., Ramsey, J. M., *Anal. Chem.* 1997, *69*, 3407–3412.

[11] Hadd, A. G., Jacobson, S. C., Ramsey, J. M., *Anal. Chem.* 1999, *71*, 5206–5212.

[12] Xu, Y., Liu, X., Ip, M. P. C., *J. Liq. Chromatogr. Rel. Techn.* 1998, *21*, 2781–2797.

[13] Saevels, J., Schepdael, A. V., Hoogmartens, J., *Electrophoresis* 1996, *17*, 1222–1227.

[14] Chang, H.-T., Yeung, E. S., *Anal. Chem.* 1993, *65*, 2947–2951.

[15] Saevels, J., Huygens, K., Schepdael, A. V., Hoogmartens, J., *Anal. Chem.* 1997, *69*, 3299–3303.

[16] Avila, L. Z., Whitesides, G. M., *J. Org. Chem.* 1993, *58*, 5508–5512.

[17] Regnier, F. E., Patterson, D. H., Harmon, B. J., *Trends Anal. Chem.* 1995, *14*, 177–181.

[18] Harmon, B. J., Leesong, I., Regnier, F. E., *Anal. Chem.* 1994, *66*, 3797–3805.

[19] Gatehouse, A. M. R., Gatehouse, J. A., *Pestic. Sci.* 1998, *52*, 165–175.

[20] Hilder, V. A., Boulter, D., *Crop Protect.* 1999, *18*, 177–191.

[21] Palfreyman, M. G., McCann, P. P., Lovenberg, W., Joseph, G., Temple, J., Sjoerdsma, A., *Enzymes as Targets for Drug Design*, Academic Press, San Diego, CA 1989.

[22] Sandler, M., *Enzyme Inhibitors as Drugs*, The Macmillan Press Limited, London 1980.

[23] Louie, A. Y., Meade, T. J., *Chem. Rev.* 1999, *99*, 2711–2734.

[24] Krawczyk, T. K. V., *Chem. Anal.* 1998, *43*, 135–158.

[25] Saevels, J., Steen, K. V. D., Schepdael, A. V., Hoogmartens, J., *J. Chromatogr. A* 1996, *745*, 293–298.

[26] Craig, D. B., Wong, J. C. Y., Dovichi, N. J., *Anal. Chem.* 1996, *68*, 697–700.

[27] Wu, D., Regnier, F. E., Linhares, M. C., *J. Chromatogr. B* 1994, *657*, 357–363.

[28] McComb, R. B., George, N., Bowers, J., *Alkaline Phosphatase*, Plenum Press, New York 1979.

[29] Vinet, B., Zizian, L., Gauthier, B., *Clin. Biochem.* 1978, *11*, 57–61.

[30] Jourquin, G., Kauffmann, J. M., *J. Pharm. Biomed. Anal.* 1998, *18*, 585–596.

[31] Vassallo, R., Lipsky, J. J., *Mayo Clin. Proc.* 1998, *73*, 346–354.

[32] Page, C. P., *J. Clin. Pharmacol.* 1999, *39*, 237–240.

[33] Markham, A., Faulds, D., *Drugs* 1998, *56*, 1081–1091.

[34] Segel, I. H., *Enzyme Kinetics*, John Wiley and Sons, New York 1975.

[35] Chien, R.-L., Burgi, D. S., *Anal. Chem.* 1992, *64*, 489A–496A.

[36] Colton, I. J., Carbeck, J. D., Rao, J., Whitesides, G. M., *Electrophoresis* 1998, *19*, 367–382.

[37] Freitag, R., *J. Chromatogr. B* 1999, *722*, 279–301.

[38] Avila, L. Z., Chu, Y.-H., Blossey, E. C., Whitesides, G. M., *J. Med. Chem.* 1993, *36*, 126–133.

[39] Liu, J., Volk, K. J., Lee, M. S., Pucci, M., Handwerger, S., *Anal. Chem.* 1994, *66*, 2412–2416.