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Flow-through partial-filling affinity capillary electrophoresis can estimate binding constants of neutral ligands to receptors *via* a competitive assay technique

This work evaluates the use of a competitive binding assay using flow-through partial-filling affinity capillary electrophoresis (FTPFACE) to estimate binding constants of neutral ligands to a receptor. We demonstrate this technique using, as a model system, carbonic anhydrase B (CAB, EC 4.2.1.1) and arylsulfonamides. In this technique, the capillary is first partially filled with a negatively charged ligand, a sample containing CAB and two noninteracting standards, and a neutral ligand, then electrophoresed. Upon application of a voltage the sample plug migrates into the plug of negatively charged ligand (L_-) resulting in the formation of a CAB- L_- complex. Continued electrophoresis results in mixing between the neutral ligand (L_0) and the CAB- L_- complex. L_0 successfully competes out L_- to form the new CAB- L_0 complex. Analysis of the change in the relative migration time ratio (RMTR) of CAB relative to the noninteracting standards, as a function of neutral ligand concentration, yields a value for the binding constant. These values are in agreement with those estimated using other binding and ACE techniques. Data demonstrating the quantitative potential of this method is presented.

Keywords: Affinity capillary electrophoresis / Binding constants / Competitive binding / Flow-through partial-filling
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1 Introduction

Capillary electrophoresis (CE) is a powerful separation technique that has gained widespread use in laboratories because of its versatility and ease of use [1–3]. It represents a merging of techniques derived from traditional electrophoresis and high-performance liquid chromatography (HPLC). CE separates molecules based on differences in their velocities under the influence of an electric field and provides for rapid analysis of water-soluble charged samples with high resolution. Separation of hydrophobic molecules can also be achieved in nonaqueous media. During the past decade, a number of versatile techniques have been developed to study complex mixtures of biomolecules. One technique that has been used with great success to estimate binding parameters of ligands to receptors is affinity capillary electrophoresis (ACE).

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Abbreviations: CAB, carbonic anhydrase B; FTPFACE, flow-through partial-filling affinity capillary electrophoresis; HHM, horseheart myoglobin; MO, mesityl oxide; RMTR, relative migration time ratio

ACE is a powerful technique for studying biomolecular noncovalent interactions and determining the binding and dissociation constants of formed complexes [4–35]. The ability to accurately and expeditiously study receptor-ligand interactions has made ACE a popular choice in rationale drug design and development. For example, Dunayevskiy *et al.* [4] have demonstrated that ACE can be used in combinatorial approaches to drug design by evaluating binding constants of peptides to vancomycin. Zhang *et al.* [5] have demonstrated a multiple-step ligand injection approach using ACE to determine binding constants of ligands to receptors. Finally, Novotny *et al.* [6] have used ACE to investigate the interactions of concanavalin A with various saccharide oligomers. ACE can only differentiate between the bound and free forms of a receptor as a function of the concentration of free ligand if the binding results in a sizeable change in the mass or charge-to-mass ratio. In a typical form of ACE, a sample of receptor and noninteracting standard is exposed to an increasing concentration of ligand in the running buffer causing a shift in the migration of the receptor relative to the standard. Subsequent Scatchard analysis yields a value for the binding constant.

The use of partial-filling strategies in ACE has recently been expanded upon yielding effective methods for measuring biochemical interactions [7–9]. We have demon-

strated the use of partial-filling strategies, termed flow-through partial-filling ACE (FTPFACE), to measure binding constants of charged ligands to receptors [10, 11]. In this technique, the capillary was first partially filled with a plug of ligand, a sample containing receptor and standards, then electrophoresed. Upon application of a voltage gradient, the ligand and sample zones overlap and a binding equilibrium between ligand and receptor is achieved. Continued electrophoresis results in the receptor and standards flowing through the domain of the ligand plug.

In these studies, the relative migration time ratio (RMTR) was used in the Scatchard analysis. The RMTR form of analysis involves analyzing the shift in the receptor peak relative to two noninteracting standards. Subsequent Scatchard analysis yields a value for the binding constant. In previous ACE studies we found the RMTR form of analysis to be more reliable for estimating binding constants than forms based on electrophoretic mobilities especially in cases where electroosmotic flow (EOF) was appreciable [5, 10]. Unfortunately, not all ligands for a given receptor are charged. In this case, analysis by ACE is problematic since a change in migration time for the receptor will not result during complex formation unless a sizeable change in mass is observed. Therefore, a technique that would allow for analysis of neutral ligands is warranted.

In this paper, we extend the use of FTPFACE by estimating binding constants of neutral ligands to carbonic anhydrase B (CAB, EC 4.2.1.1) via a competitive binding assay (Fig. 1). In this technique, the capillary is partially filled with a negatively charged ligand, a sample of receptor and noninteracting standards, and neutral ligand and electrophoresed. Analysis of the RMTR of the receptor relative to the noninteracting standards, as a function of the concentration of the neutral ligand, yields a value for the binding constant.

2 Materials and methods

2.1 Chemicals and reagents

All chemicals were analytical grade. CAB (EC 4.2.1.1, containing CAA and CAB isozymes, from bovine erythrocytes) and horseheart myoglobin (HHM) were purchased from Sigma Chemical (St. Louis, MO, USA) and used without further purification. 4-Nitrobenzenesulfonamide, **2**, *p*-toluenesulfonamide, **3**, and benzenesulfonamide, **4**, (Fig. 2) were purchased from Aldrich (Milwaukee, WI, USA). Compounds **1** and **5** were synthesized using methods from known literature procedures [12]. Mesityl oxide (MO) was purchased from Calbiochem (San Diego, CA,

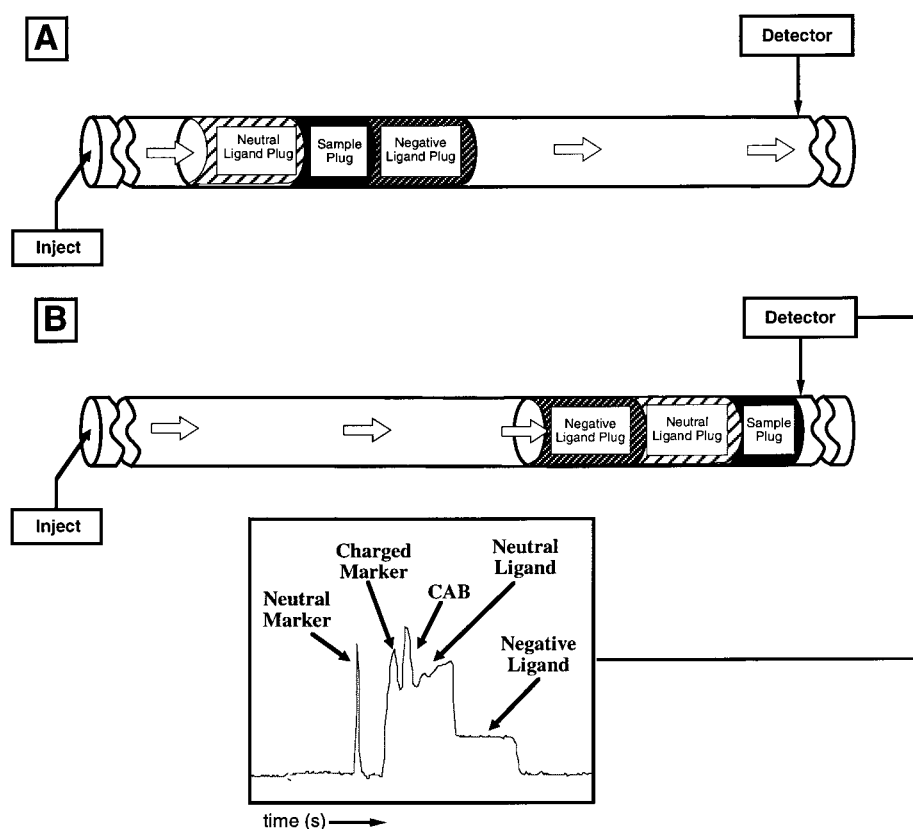
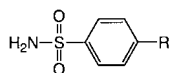


Figure 1. Schematic of a competitive binding FTPFACE experiment. The sample plug is enlarged to best pictorially represent the technique.



- 1 R = NHC(O)CH₂CH₂CH₂CH₂CO₂H
 2 R = NO₂
 3 R = CH₃
 4 R = H
 5 R = C(O)NHCH₂CH₂OH

Figure 2. Structures of compounds used in this study.

USA). Stock solutions (1 mg/mL) of bovine CAB and HHM (1 mg/mL) were each prepared by dissolving the lyophilized proteins in buffer (192 mM glycine-25 mM Tris, pH 8.3). Ligands **1–5** were also dissolved in buffer.

2.2 Apparatus

The capillary electrophoresis (CE) system used in this study was a Beckman Model P/ACE 5510 (Fullerton, CA, USA). The capillary tubing (Polymicro Technologies, Phoenix, AZ, USA) was of uncoated fused silica with an internal diameter of 50 μ m, a length from inlet to detector of 60.5 cm, and a length from detector to outlet of 6.5 cm. Data were collected and analyzed with Beckman System Gold software. The conditions used in CE were as follows: voltage, 28 kV; current, 5.8 μ A; detection, 200 nm; temperature, 25 \pm 0.1°C.

2.3 Procedures

A sample of **1** (20 μ M) was vacuum-injected into the capillary for 0.1 min at high pressure (20 psi), followed by a sample (3.6 nL) of solution containing 0.20 mg/mL of CAB, 0.313 mg/mL of HHM, and 0.001 mg/mL of MO in buffer for 3 s at low pressure (0.5 psi), followed by a sample of neutral arylsulfonamide for 2.5 min at low pressure. The electrophoresis was carried out using a Tris-Glyc buffer and repeated at increasing concentrations of the neutral arylsulfonamide ligand (0–64 μ M) for 5.85 min. The total analysis time of each experiment was 8.5 min.

3 Results and discussion

Using the FTPFACE technique, we examined the interaction between CAB and neutral arylsulfonamides **2–5**. CAB is an enzyme of the lyase class that catalyzes the equilibration of dissolved carbon dioxide and carbonic acid, speeding the movement of carbon dioxide from tissues to blood to alveolar air. It is a zinc protein found in kidney tubule cells and red blood cells and is strongly inhibited by a class of molecules called sulfonamides. In our initial studies a plug of **1** was vacuum-injected into the capillary under high pressure for 0.1 min, followed by a plug of sample containing CAB, HHM and MO at low pressure

for 3 s, and a plug of **2** for 2.5 min at low pressure and electrophoresed for 5.85 min. HHM and MO are noninteracting standards used in the data analysis and do not interact in the binding event. Upon electrophoresis, the sample plug penetrates the zone of **1** and a binding equilibrium is achieved between CAB and **1**. Continued electrophoresis permits the plug of **2** to enter the domain of the sample containing CAB, standards, and **1**, and a new equilibrium is achieved between it and CAB. The plug of **2** subsequently flows through both the sample and charged ligand plugs.

Figure 3 shows a representative series of electropherograms of CAB in capillaries partially filled with increasing concentrations (0–64 μ M) of **2** and electrophoresed at a constant concentration of **1**. At [**1**] = 20 μ M and [**2**] = 0 μ M, CAB is fully complexed to **1** and is approximately one unit more negative than when uncomplexed. Upon increasing the concentration of **2**, ligand **1** is displaced and the newly formed CAB-**2** complex becomes less negative and elutes at an earlier migration time than the CAB-**1** complex. The height of the ligand plateaus, as seen in the series of electropherograms, increases due to the increase

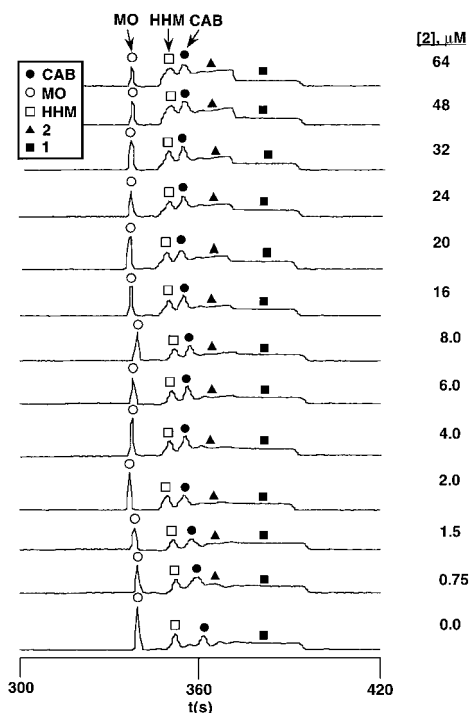


Figure 3. A representative set of electropherograms of CAB in 0.192 M glycine-0.025 M Tris buffer (pH 8.3) containing various concentrations of **2** using the competitive binding FTPFACE technique. The total analysis time in each experiment was 8.5 min at 28 kV (current, 5.8 μ A) using a 60.5 cm (inlet to detector), 50 μ m ID open, uncoated quartz capillary. MO and HHM were used as internal standards.

in concentration of **2** partially filled in the capillary. A sufficient injection time allows for an equilibrium to be established between it and CAB. Experiments conducted at ligand injection times greater than 0.10 min yield comparable binding constants. At injection times of less than 0.10 min an equilibrium cannot be established between the ligands and CAB and inaccurate values for the binding interaction are obtained. We do not understand why the plug of **2** does not begin to elute with the neutral marker MO. At pH 8.3 and in Tris-Gly buffer **2** is neutral and the ligand plateau should be realized adjacent to EOF. Still, we are confident compound **2** is neutral since upon increasing its concentration in the plug the migration time of the CAB complex shifts from right to left. At the highest concentration of **2** the resultant CAB-**2** complex elutes at the migration time where uncomplexed CAB elutes.

In the present work, we modified a Scatchard analysis used in previous ACE work [5, 10–15]. In earlier work, Colton *et al.* [15] showed that a dissociation constant between a receptor and a charged ligand could be estimated using Eq. (1) [15]:

$$R_f/[L] = (1/K_d) - (R_f/K_d) \quad (1)$$

Here, R_f was defined as the fraction of the total concentration of R ($[R]_T$) present as $R \cdot L$ where $R_f = [R \cdot L]/[R]_T$. In Colton's work $[L]$ is the concentration of a charged ligand. He showed that a similar analysis could be used to estimate binding between a receptor and a neutral ligand (L_0) in a competitive binding assay using Eqs. (2) and (3):

$$R_f = [R \cdot L_0]/[R]_T \quad (2)$$

$$R_f/[L_0] = (1 - R_f)/((K_d^0)(1 + [L_{\pm}]/[K_d^{\pm}])) \quad (3)$$

Here, K_d^{\pm} and $[L_{\pm}]$ are the known values for the dissociation constant of a charged ligand and concentration of charged ligand, respectively. Equation (3) was used to obtain binding constants of neutral ligands to a receptor. In the present work, we substituted $\Delta RMTR$ for R_f in Eq. (3) to estimate binding constants between neutral arylsulfonamides and CAB (Eq. 4):

$$RMTR = (t_r - t_s)/(t_s - t_s) \quad (4)$$

Here, t_r , t_s , and t_s' are the measured migration times of the receptor peak, and the two noninteracting standard peaks, respectively. In the present experiments, t_s and t_s' are the migration times of MO and HHM, respectively. We showed that by using Eq. (5) the value for K_{b0} could be obtained for the interaction of charged ligands and a receptor. Eq. (5) shows the general equation used for Scatchard analysis of this competitive binding system.

$$\Delta RMTR_{R,L}/[L_0] = (1 - \Delta RMTR_{R,L})/((K_{b0}^{-1})(1 + [L_{\pm}]/(K_{b_{\pm}}))) \quad (5)$$

Here, $K_{b_{\pm}}$ and L_{\pm} are the known values for the binding constant and concentration of the negatively charged ligand in the running buffer, respectively. In the present experiment, $K_{b_{\pm}}$ and L_{\pm} are $4.16 \times 10^6 \text{ M}^{-1}$ and $20 \text{ }\mu\text{M}$, respectively. L_0 is the concentration of the neutral ligand used in the experiment. Subsequent Scatchard analysis of the value of $\Delta RMTR$ measured by FTPFACE as a function of the concentration of L_0 gives the value for the binding constant (K_{b0}) of the neutral ligand to CAB. Figure 4 is a Scatchard plot of the competitive binding assay of **2** for CAB. The data was fit to a least squares fitting program (Kaleidograph).

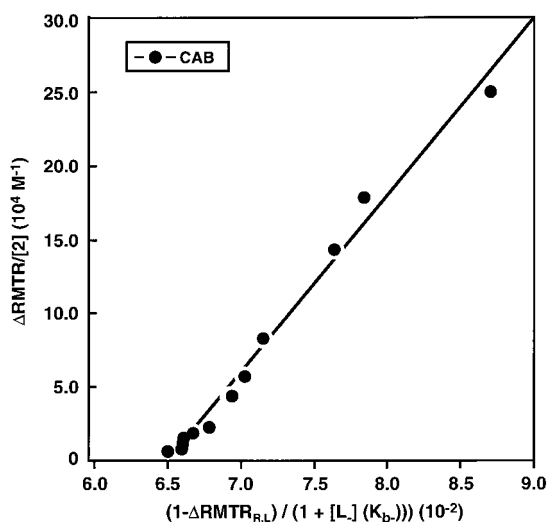


Figure 4. Scatchard plot of the data for CAB according to Eq. (5).

As can be seen by the linearity of the plot, Eqs. (4) and (5) allow for an accurate estimation of K_{b0} on a relative time scale using two noninteracting standards. Furthermore, the RMTR form of analysis compensates for fluctuations in current in the capillary column. Equation (5) could be used in this instance since the k_{on} and k_{off} rates of the CAB-ligand complex are fast relative to the length of the capillary column. Table 1 summarizes the binding data for ligands **2–5** and CAB obtained by Eq. (5). These values are in agreement with previous ACE studies on CAB and arylsulfonamides and with those obtained from other binding techniques. Using neutral ligands **2–5**, we demonstrated that a mathematical relationship can be obtained that can be used to estimate binding constants of neutral ligands to CAB. Figure 5 is a plot of the experimental data obtained for the binding of ligands **2–5** to CAB. From this plot we obtained Eq. (6) that relates $\Delta RMTR$, L_0 , and K_{b0} for ligands **2–5**.

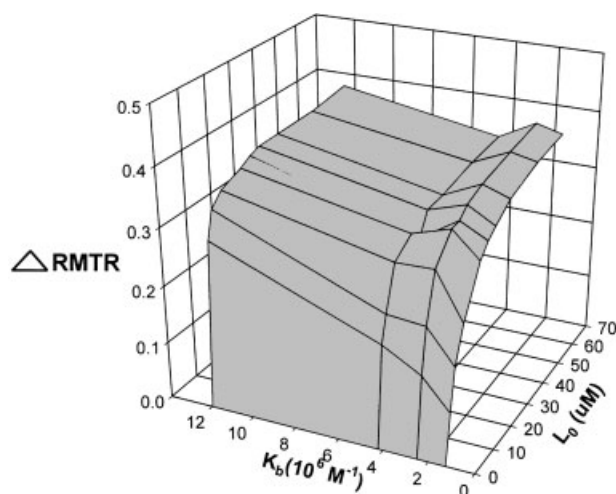
Table 1. Experimental values of binding constants K_{b0} ($10^6 M^{-1}$) of ligands 2–5 with CAB measured by the FTPFACE technique

Ligand	K_b
2	12.0 ^{a)}
3	2.5 ^{b)}
4	1.2 ^{c)}
5	4.0

a) Previous estimate [15]: $K_{b0} = 12.5 \times 10^6 M^{-1}$

b) Previous estimate [5, 15]: $K_{b0} = 2.0\text{--}2.5 \times 10^6 M^{-1}$

c) Previous estimate [5, 15]: $K_{b0} = 0.9\text{--}1.1 \times 10^6 M^{-1}$

**Figure 5.** Experimental binding data for ligands 2–5.

Equation (6) was obtained using the least-squares fitting method and the resulting matrix was solved using Mathematica. The use of Eq. (6) allows for the estimation of K_{b0} for other neutral ligands to CAB.

$$K_{b0}^{-1} = 0.054288 (0.40099 - \Delta RMTR) / [(\Delta RMTR) / (L_0) + 1031.4 - 128050 (0.40099 - \Delta RMTR)] \quad (6)$$

Table 2 lists the values of K_{b0} obtained experimentally and theoretically, and the percent error relative to the experimental data using Eq. (6) for a random series of experiments at varying neutral ligand concentrations. It is important to note that Eq. (6) can only be used for ligands with values of K_{b0} that lie between 1.2 and $12 \times 10^6 M^{-1}$. As can be seen, Eq. (6) provides an accurate means of estimating binding constants of neutral ligands to CAB. Although Eq. (6) is limited in scope the use of it does realize binding constants within small experimental error when compared to data obtained using more traditional ACE techniques. Competitive binding FTPFACE has several advantages as a method for measuring biomolecular noncovalent interactions. First, it requires smaller quanti-

Table 2. Comparison of the values of K_{b0} ($10^6 M^{-1}$) obtained by experimental data and by Eq. (6)

Ligand (L)	L_0 (μM)	$K_{b0} \text{exp.}$	$K_{b0} \text{calc.}$	% Error
2	20	12.0	12.9	8.0
2	32	12.0	11.7	2.6
3	2	2.5	2.5	0.05
3	20	2.5	2.5	1.3
4	32	1.2	1.4	9.4
4	64	1.2	1.3	7.6
5	2	4.0	4.8	13.3
5	8	4.0	4.0	7.0

ties of receptor and ligand than needed in traditional ACE techniques. Second, purification of the sample prior to injection is not required if the component to be analyzed can be separated from other species. Third, it does not require radiolabelled or chromophoric ligands thereby making derivatization of ligands unnecessary. Fourth, the commercial availability of automated instrumentation, and the high reproducibility of data, make it experimentally convenient.

4 Concluding remarks

This paper demonstrates the ease of estimating binding constants of neutral ligands to CAB using a competitive binding assay and FTPFACE. In these experiments, the capillary was first partially filled with a charged ligand, a sample containing CAB and two noninteracting standards, and finally a neutral ligand and electrophoresed. Upon application of a voltage, CAB and standards flow into the plug of charged ligand where equilibrium is established between CAB and the negative ligand. The neutral ligand then penetrates the plugs of charged ligand and sample and a new equilibrium is achieved between it and CAB. Continued electrophoresis results in the neutral ligand flowing through the plug of CAB and standards. The use of the relative migration time ratio yielded values for binding constants in agreement with those obtained by other assay methods and by other ACE techniques. Further work to demonstrate the versatility of this technique is in progress.

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