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Optimization of conditions for flow-through partial-filling affinity capillary electrophoresis to estimate binding constants of ligands to receptors

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Abstract

This work details the determination of the minimal injection time of ligand required in flow-through partial-filling affinity capillary electrophoresis (FTPFACE) to estimate binding constants of ligands to receptors. Two model systems are examined in this study: carbonic anhydrase B (CAB, EC 4.2.1.1) and arylsulfonamides, and vancomycin from *Streptomyces orientalis* and D-Ala-D-Ala peptides. Using CAB, a minimal injection time of 0.07 min at high pressure was determined that provided for the accurate and reproducible measurement of binding constants. In the FTPFACE technique, the capillary is first partially filled with a zone of ligand followed by a sample plug containing receptor and non-interacting standards. Upon application of a voltage the receptor and standards flow into the zone of ligand where a dynamic equilibrium is achieved between receptor and ligand. Continued electrophoresis results in the receptor and standards flowing through the domain of the ligand plug prior to detection. Analysis of the change in the relative migration time ratio (RMTR) of the receptor, relative to the non-interacting standards, as a function of the concentration of ligand, yields a value for the binding constant. In the present study, variable injection times of 4-carboxybenzenesulfonamide (CBSA) were examined to determine the minimal injection time needed to establish an equilibrium between CAB and ligand. A mathematical relationship was derived that correlated injection time and ligand concentration to the change in RMTR and comparisons made between the experimental and calculated values. Binding constants were obtained for a series of arylsulfonamide ligands and D-Ala-D-Ala terminus peptides to CAB and Van, respectively. The results support the use of FTPFACE to estimate affinity constants under variable experimental conditions.

Keywords: Affinity capillary electrophoresis; Binding constants; Carbonic anhydrase B; Vancomycin

1. Introduction

The development of new molecular biology techniques has provided for the discovery of a myriad of biological interactions. To better understand the human body and its processes it is critical to ascertain the level and extent of interaction between biological entities. The finding of new biological interactions has far outpaced techniques that can easily quantitate the extent of interaction, hence, the development of novel analytical methods are highly warranted.

One technique that has shown great promise in quantifying receptor–ligand interactions is affinity capillary electrophoresis (ACE). ACE uses the resolving power and separation ability of CE to estimate the extent of binding between ligands and receptors. A number of biological interactions have been examined by ACE and include protein–protein, protein–DNA, protein–drug, protein–carbohydrate, peptide–peptide, peptide–carbohydrate, peptide–dye, carbohydr ate–drug, and antibody–antigen [1–33]. For example, Silverio et al. showed that antibiotics can be labeled using

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on-column techniques and their binding to ligands quantitated using ACE [1]. Qian and Tomer used ACE to investigate an epitope on human immunodeficiency virus by a monoclonal antibody [2]. Finally, Kiessig et al. used ACE to examine the interaction of the enzyme cyclophilin with the immunosuppressive drug cyclosporin A [3]. In a typical form of ACE a sample of receptor and a non-interacting standard(s) are injected onto the capillary column and are electrophoresed in an increasing concentration of ligand in the running buffer. A change in migration time of the receptor relative to the standard(s) is induced on formation of the receptor–ligand complex. This change in migration time is then used for the Scatchard analysis.

In instances when limited quantities of material are available, the use of traditional ACE techniques can be problematic. We recently developed the use of partial-filling ACE (PFACE) to examine receptor–ligand interactions [21–25]. In this technique the capillary is partially filled with ligand (or receptor) and a sample plug of receptor (or ligand) is introduced and electrophoresed. During the electrophoretic separation the plug of sample and zone of partially filled sample overlap within the capillary and an equilibrium is established between the two species. Analysis of the change in migration time of the receptor (or ligand) is then used in the analysis to obtain a value for the binding constant. As an extension to this technique we developed flow-through PFACE (FTPFACE) (Fig. 1) [22,25]. In FTPFACE a similar procedure as that used for PFACE is used except a smaller zone of ligand is partially filled into the capillary column. Upon electrophoresis, the sample plug flows through the partially filled zone. Subsequent analysis of the change in migration time of the receptor realizes a value for the binding constant.

In order for FTPFACE to become a more acceptable analytical technique to probe bimolecular non-covalent interactions, it is imperative that experimental conditions for the assay be optimized. Herein, we describe the determination of the minimal time of injection of ligand in FTPFACE using as model systems carbonic anhydrase B (CAB, EC 4.2.1.1) and vancomycin from *Streptomyces orientalis*.

2. Derivation of analysis

Least squares regression was used to obtain a predictive equation for the change in RMTR as a function of injection time and ligand concentration. Individual plots of the change in RMTR versus ligand concentration for each injection time suggested an asymptotic relationship of the form $\Delta\text{RMTR}_i = a(1 - e^{-sL_i})$, where ΔRMTR_i is the predicted change in RMTR and L_i is the ligand concentration for sample i. The parameter s is the initial slope of the curve as ΔRMTR_i increases with L_i and the parameter a is the horizontal asymptote for ΔRMTR_i as L_i becomes large. This asymptotic function was fit to the data for each injection time using nonlinear least squares regression in SPSS 11.5 (SPSS Inc., 2002). An examination of the estimated values of the initial slopes for the different injection times revealed no discernable trends. However, a similar analysis of the estimated asymptotes suggested a linear increase in the asymptote with an increase in the injection time. We then modified the regression model to account for injection time using the following mathematical expression:

$$\Delta\text{RMTR}_i = (\alpha + \beta T_i)(1 - e^{-sL_i}) \quad (1)$$

Here, T_i is the injection time and α and β are the intercept and slope, respectively, for the asymptote as a linear function of injection time. This three parameter model was fit to the entire data set using nonlinear least squares regression in SPSS. This resulted in the following parameter estimates and standard errors: $\alpha = 0.3415 \pm 0.0170$, $\beta = 0.8244 \pm 0.2515$, and $s = 4.534 \pm 0.373 \times 10^5$. The fitted regression model has a coefficient of determination of 0.871, indicating that 87% of the variation in the observed change in RMTR can be explained using injection time and ligand concentration as predictor variables.

3. Materials and methods

3.1. Chemicals and reagents

All chemicals were analytical grade. Carbonic anhydrase B (CAB, EC 4.2.1.1 containing CAA and CAB isozymes, from bovine erythrocytes), horse heart myoglobin (HHM), 4-carboxybenzenesulfonamide, **1**, *N*-acetyl-D-Ala-D-Ala, **9**, and vancomycin (Van) from *S. orientalis*, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mesityl oxide (MO) was purchased from Calbiochem (San Diego, CA, USA). Ligands (**2**–**8**) were synthesized based on literature procedures [34] (Fig. 2). *N*-succinyl-D-Ala-D-Ala, **10**, was synthesized based on literature procedures [26]. All chemicals were used without further purification. Stock solutions of CAB (1 mg mL^{-1}), HHM (1 mg mL^{-1}), vancomycin (4 mg mL^{-1}), and MO (0.15 mg mL^{-1}) were each prepared by dissolving the lyophilized proteins in buffer (0.192 mol L^{-1} glycine–0.025 mol L^{-1} Tris, pH 8.3). All Ligands **1**–**10** were dissolved in buffer.

3.2. Apparatus

The capillary electrophoresis (CE) system used in this study was a Beckman Model P/ACE 5510 (Fullerton, CA, USA). The capillary tubing (Polymicro Technology Inc., Phoenix, AZ, USA) used for the experiment was uncoated fused silica with an internal diameter of 50 μm, length from inlet to detector of 50.5 cm (60.5 cm for Van), and a length from detector to outlet of 6.5 cm. The conditions used in CE were as follows: for CAB, voltage, 20 kV; current, 5.8 μA; detection, 200 nm; temperature, 25.0 ± 0.1 °C; for Van, voltage, 28 kV; current, 5.2–5.8 μA; detection, 200 nm; temperature, 25 ± 2 °C. Data were collected and analyzed with Beckman System Gold software.

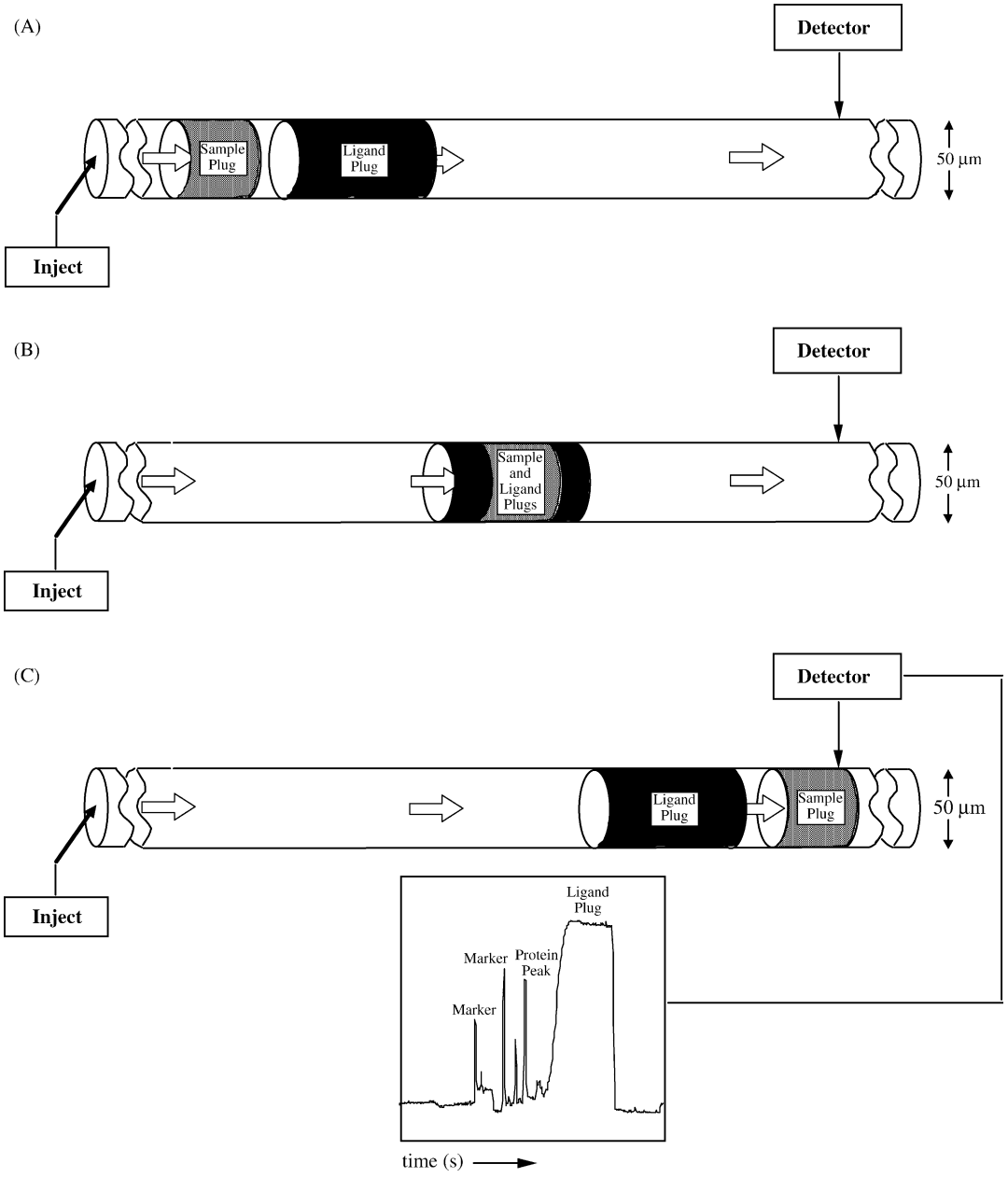

Fig. 1. Schematic of a flow-through partial-filling affinity capillary electrophoresis (FTPFACE) experiment.

3.3. Procedures

For CAB: a plug of increasing concentrations of ligand was vacuum-injected into the capillary under high pressure for an amount of time which varied between 0.02 and 0.10 min (**2**–**8** were injected for 0.10 min to allow for a standard of comparison), followed by a plug of sample (3.6 nL) of solution containing 1 mg mL^{-1} CAB, 1 mg mL^{-1} HHM, and 0.015 mg mL^{-1} MO in Tris–Gly buffer at low pressure for 3 s. The electrophoresis was carried out using a Tris–Gly buffer and repeated at increasing concentrations of the ligands for 5 min. For vancomycin, a sample of **10** was vacuum injected into the capillary for 0.18 min (0.10 min for 3) at high pressure followed by a sample (3.6 nL) of solution for 3 s containing 0.035 mg mL^{-1} of vancomycin, 0.14 mg mL^{-1} of CAB, 0.14 mg mL^{-1} of HHM, and 0.08 mg mL^{-1} of MO in buffer. The electrophoresis was carried out using a Tris–Gly buffer and increasing concentrations of **10**.

4. Results and discussion

To best determine the optimal conditions for FTPFACE we chose to study the interaction between CAB and arylsulfonamides. CAB is a zinc protein of the lyase class that catalyzes the equilibration of dissolved carbon dioxide and carbonic acid. It is strongly inhibited by sulfonamide-containing

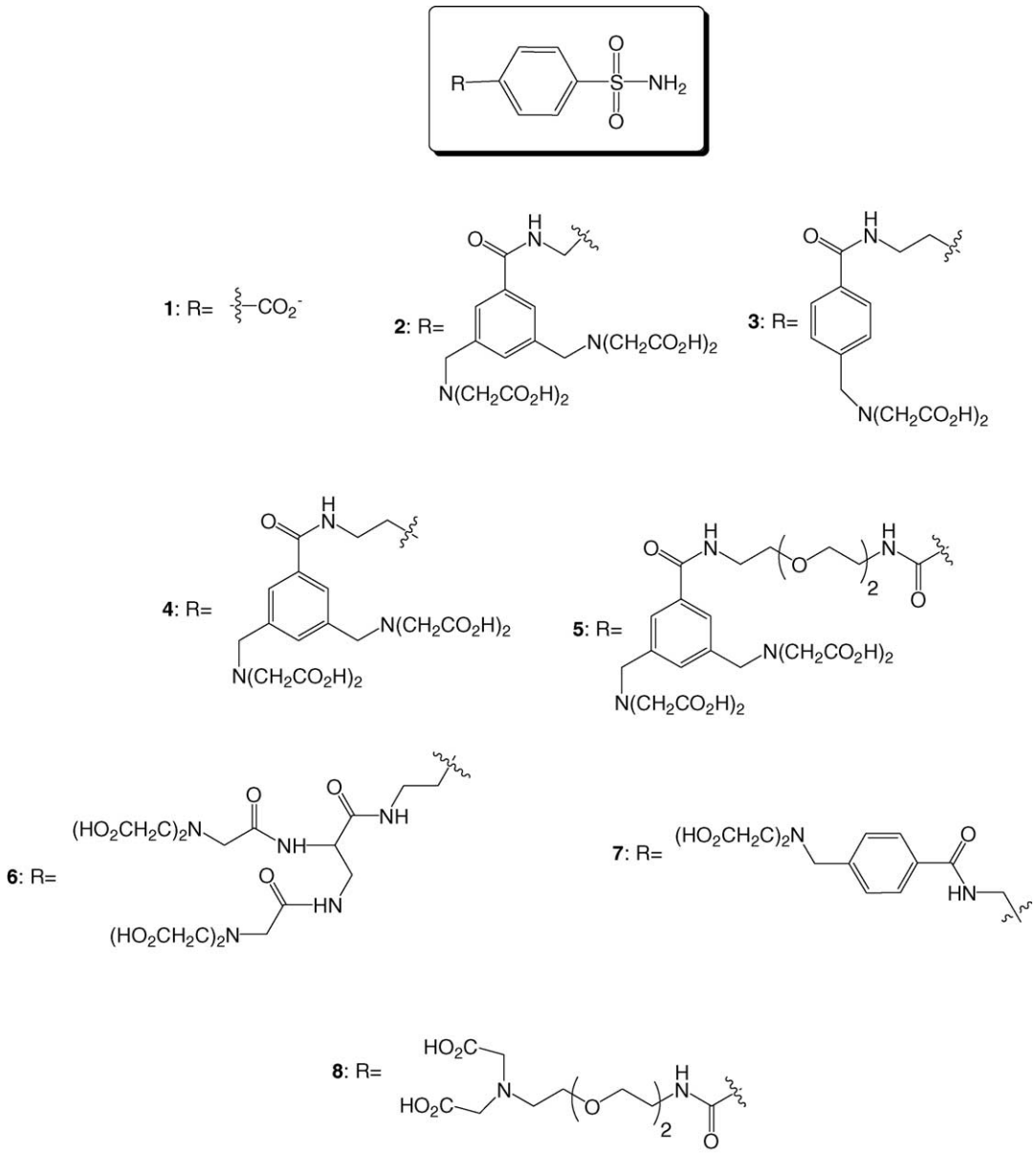

Fig. 2. Structures of compounds **1**–**8**.

molecules. In our initial studies we focused on determining the effect changes in injection time of ligand would have on the measurement of a binding constant (K_b). Using 4-carboxybenzenesulfonamide (**1**) a plug of **1** at increasing concentrations was vacuum-injected into the capillary at high pressure (20 psi) for 0.10 min, followed by a plug of sample containing CAB, HHM, and MO at low pressure for 3 s and electrophoresed. HHM and MO are non-interacting standards used in the data analysis and do not interact with either **1** or CAB. Upon electrophoresis, the sample plug enters the domain of **1** where a dynamic equilibrium is established between CAB and the ligand. Continued electrophoresis results in CAB and the non-interacting species flowing through the plug of ligand. The electrophoretic mobilities of CAB and the standards are greater than **1** and, hence, they all migrate through the capillary column at a grater rate than the zone of **1**.

Fig. 3 shows a representative series of electropherograms of CAB in capillaries partially filled with increasing concentrations (0–120 μM) of **1** in the running buffer. At the point of detection separate peaks for CAB, HHM, and MO are observed. The zone of **1** is seen as a box (not shown) at an increasing absorbance. The complex that forms between CAB and **1** is more negatively charged than CAB uncomplexed and, hence, the peak for the complex shifts to the right on increasing the concentration of **1** in the buffer. A fourth peak appears under the original CAB peak and is designated as inactive CAB a result of using an older sample of CAB in some of our studies. This inactive CAB does not effect the measurement of a binding constant.

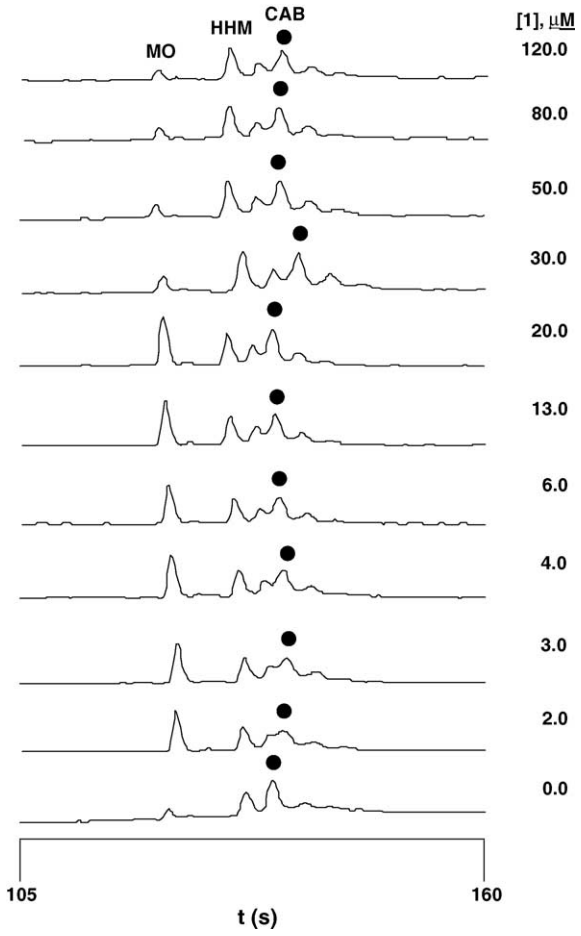

Fig. 3. A representative set of electropherograms of carbonic anhydrase B (CAB) in 0.192 M glycine–0.025 M Tris buffer (pH 8.3) containing various concentrations of **1** for an injection time of 0.10 min using the FTPFACE technique. The total analysis time in this experiment was 5.0 min at 20 kV (current 5.8 μA) using a 50.5 cm (inlet to detector), uncoated quartz capillary. Mesityl oxide (MO) and horse heart myoglobin (HHM) were used as internal standards.

Fig. 4 is a typical Scatchard plot of the data for CAB and **1**. In this form of analysis K_b is estimated using a dual-marker form of analysis termed the relative migration time ratio (RMTR) (Eq. (2)), whereby, CAB is referenced to two non-interacting standards (MO and HHM) [4]:

$$\mathrm{RMTR} = (t_r - t'_s)/(t'_s - t_s) \quad (2)$$

In Eq. (2) t_r, t_s, and t'_s are the measured migration times of CAB, and HHM and MO, respectively. Eq. (3) allows for the estimation of K_b on a relative time scale using the two non-interacting standards and compensates for fluctuations in electroosmotic flow (EOF) that may occur within the capillary column:

$$\Delta\mathrm{RMTR}_{R,L}/[L] = K_b\Delta\mathrm{RMTR}^{max}_{R,L} - K_b\Delta\mathrm{RMTR}_{R,L} \quad (3)$$

In Eq. (3), $\Delta\mathrm{RMTR}_{R,L}$ is the magnitude of the change in the relative migration time ratio as a function of the concentration of the ligand.

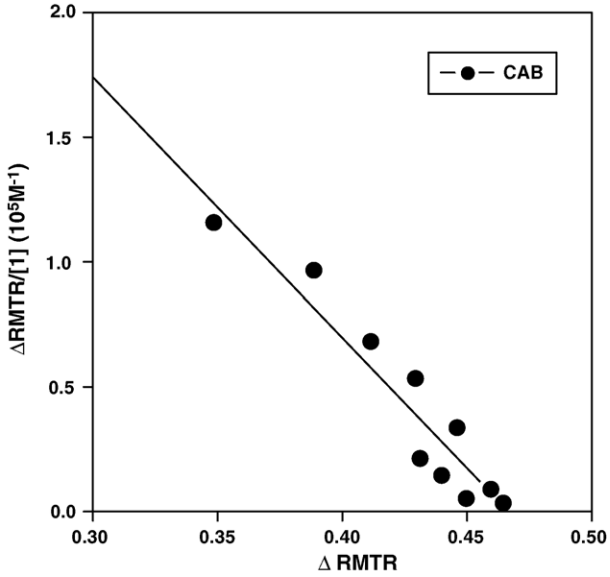

Fig. 4. Scatchard plot of the data for carbonic anhydrase B and **1** at a ligand injection time of 0.10 min according to Eq. (3).

Using Eq. (3) a K_b of 1.03×10^6 L mol^{-1} was obtained for the interaction between CAB and **1**. This value is comparable to our previous results using ACE and to values obtained using other assay techniques [4,14,21]. A correlation coefficient (R^2) of 0.949 was obtained for the fitness of the Scatchard plot. We then repeated the experiments with **1** but at injection times ranging from 0.02 to 0.08 min. On increasing the injection time for **1** from 0.02 to 0.08 min the value for K_b increased and approached the value obtained using an injection time of 0.10 min. Table 1 details the values for the binding constants obtained for CAB and **1** at different injection times of **1**. In addition, the value for the correlation coefficient (R^2) improved from 0.612 to 0.938. From these studies we determined the minimum time of injection of ligand to be approximately 0.07 min. In experiments where an injection time of less than 0.07 min was utilized, the calculated value for K_b was not representative of values obtained from our previous ACE studies and from other assay techniques and the value of R^2 decreased markedly.

Table 1
Experimental values of binding constants K_b (10^6 L mol^{-1}) of **1** to CAB measured by the FTPFACE technique

Injection time (min)	K_b[a] ($\times 10^6$ L mol^{-1})	S.D. ($\times 10^3$ L mol^{-1})	R^2
0.02	0.70	2.63	0.612
0.04	0.74	2.76	0.959
0.06	0.80	4.24	0.801
0.07	0.82	1.95	0.856
0.08	0.84	24.8	0.938
0.10	1.03	2.81	0.949

[a] Previous estimate [4,14,21]: K_b = 0.69–1.0 $\times 10^6$ L mol^{-1}.

Table 2
Correlation between injection time, volume of solution, and plug length using the FTPFACE technique

Injection time (min)	Time (s)	Volume of solution (nL)	Plug length (cm)
0.02	1.2	57.6	2.934
0.04	2.4	115.2	5.87
0.06	3.6	172.8	8.81
0.07	4.2	201.6	10.27
0.08	4.8	230.4	11.74
0.10	6.0	288.0	14.68

Previous estimate [4,14,21]: $K_b = 0.69–1.0 \times 10^6$ L mol^{-1}.

We then determined the exact volumes of solution injected and correlated that to the plug length in the FTPFACE techniques. Table 2 lists the values for injection time, volume of solution, and plug length for the ACE analysis using ligand **1**. As can be seen the amount of ligand injected is small (mm in dimension) and demonstrates that even though very small quantities of ligand are used in FTPFACE and equilibrium between receptor and ligand does occur above the minimum value of 0.07 min. The amount of ligand injected is 202 nL which equates to a zone width of 10.27 cm. The fact that an equilibrium is established between **1** and CAB with such a small zone of **1** is impressive. The extent of contact time between the two zones of solution is only a few seconds yet this is a sufficient amount of time for the **1**–CAB complex to form and to exist in equilibrium with unbound CAB (not seen due to fast k_{on} and k_{off} rates).

Using the data obtained for the different injection times we constructed a three dimensional mesh plot (Fig. 5) to demonstrate the relationship between the concentration of **1**, injection time, and ΔRMTR. From this data we derived Eq. (4) that correlated the experimental value of ΔRMTR to ligand concentration and injection time. We then recalculated the

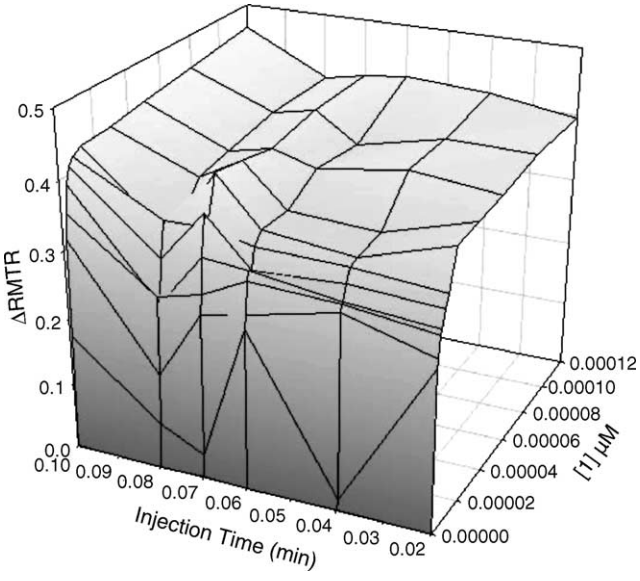

Fig. 5. Sigma Plot 3D mesh plot of [**1**] vs. injection time of ligand vs. ΔRMTR based on experimental data.

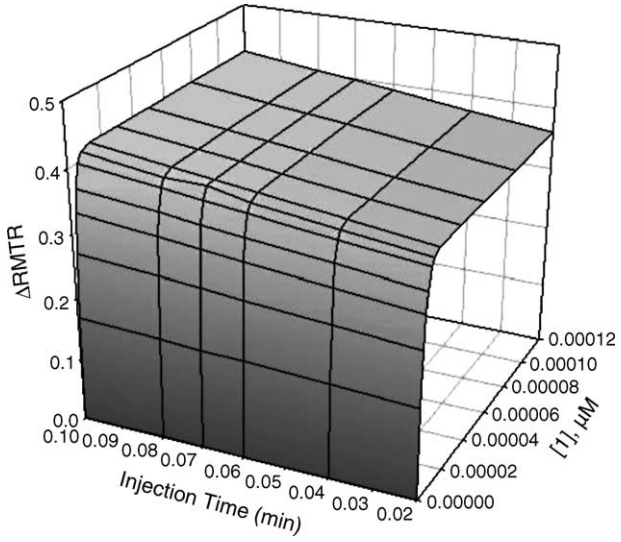

Fig. 6. Sigma Plot 3D mesh plot of [**1**] vs. injection time of ligand vs. ΔRMTR using results derived from Eq. (4).

values for ΔRMTR to generate (Fig. 6):

$$\Delta\text{RMTR} = (0.3451 + 0.7511\,\text{injection time}) \times (1 - e^{-504883.8[L]}) \quad (4)$$

A noticeable feature of both Figs. 5 and 6 is that the data for ΔRMTR levels off at approximately 8.0 μM signifying near complexation of CAB by **1**. A second feature of the graph is that the value for ΔRMTR slowly increases with increasing injection time. This result is expected as a dynamic equilibrium would be more easily achieved when the zones of CAB and **1** are overlapped for a longer period of time.

To determine the accuracy of Eq. (4) we ran a series of arbitrary injection times and concentrations of **1**. Table 3 details the values for ΔRMTR obtained experimentally and calculated using Eq. (4). As can be seen there is a good correlation between the experimental and theoretical values for ΔRMTR proving the efficacy of Eq. (4) to estimated ΔRMTR.

Upon determining the minimal injection time needed for FTPFACE to successfully obtain accurate values for K_b we

Table 3
Comparison of the values for ΔRMTR as obtained by experiment and by Eq. (4)

[**1**] (μM)	Injection time (min)	ΔRMTR		%Error
		Experimental	Theoretical	
4.0	0.02	0.3018	0.3123	3.51
8.0	0.02	0.3708	0.3538	4.59
13.0	0.02	0.3439	0.3596	4.56
22.0	0.04	0.3622	0.3751	3.58
2.0	0.05	0.2534	0.2629	3.73
3.0	0.05	0.3191	0.3139	1.64
2.0	0.05	0.2491	0.2629	5.50
5.0	0.05	0.3589	0.3432	4.39
7.0	0.05	0.3783	0.3690	2.47
1.5	0.06	0.2030	0.2072	2.06
9.0	0.09	0.4209	0.4071	3.28

Table 4
Experimental values of binding constants K_b ($\times 10^6$ L mol^{-1}) of ligands **2**–**8** to CAB measured by the FTPFACE technique[a]

Ligand	K_b ($\times 10^6$ L mol^{-1})	S.D. ($\times 10^3$ L mol^{-1})	R^2
2	19.70	47.2	0.877
3	4.83	2.22	0.970
4	76.1	262	0.952
5	1.30	9.65	0.999
6	1.11	442	0.788
7	0.512	112	0.853
8	0.07	1.41	0.981

[a] Two binding studies were conducted for each ligand and in triplicate.

used this value to analyze a series of inhibitors (**2**–**8**) of CAB. Although it was found that binding constants could be realized using a ligand injection time of 0.07 min we chose 0.10 min for the binding studies since the values for K_b obtained at this value were more representative of those found in our earlier ACE studies and because the values for R^2 were considerably higher at 0.10 min than at 0.07 min. Table 4 lists the binding constants obtained using the optimized FTPFACE conditions. These values for K_b are similar in order to those obtained with ligands of similar structure.

In our next series of experiments we examined the glycopeptide antibiotic vancomycin (Van) from *S. orientalis* and its binding to D-Ala-D-Ala terminus peptides [35–39]. Van inhibits the growth of Gram-positive bacteria by hindering cell wall peptidolglycan biosynthesis. These drugs bind to the D-Ala-D-Ala portion of peptidoglycan intermediates, inhibiting the transglycosylation reaction required from cross-linking of the cell wall. Using the same 0.1 min ligand injection time we examined two peptides: *N*-Ac-D-Ala-D-Ala (**9**) and *N*-succinyl-D-Ala-D-Ala (**10**). A similar series of electropherograms (Fig. 7) as that observed between CAB and its inhibitors was obtained. Fig. 8 is the Scatchard plot for Van and **10** using Eq. (3). Table 5 lists the binding constants obtained between Van and ligands **9** and **10**. The values obtained by FTPFACE are comparable to those estimated by conventional ACE techniques and by other assay methods.

A series of experiments were also conducted at voltages ranging from 20 to 30 kV to ascertain if the measurement of a K_b varied with voltage. We did not find any measurable change in K_b upon changing the voltage. At voltages less than 20 kV a greater duration of overlap between ligand and receptor occurs and, hence, little difference in K_b is observed to that found at voltages greater than 20 kV.

It is important to note that the sequence of injection of receptor, ligand, and standards, is dependent on the elec-

Table 5
Experimental values of binding constants K_b ($\times 10^3$ L mol^{-1}) of ligands **9** and **10** to Van measured by the FTPFACE technique[a]

Ligand	K_b ($\times 10^3$ L mol^{-1})
9	6.7
10	9.1

[a] The reported binding constants are the average values from six experiments for each ligand.

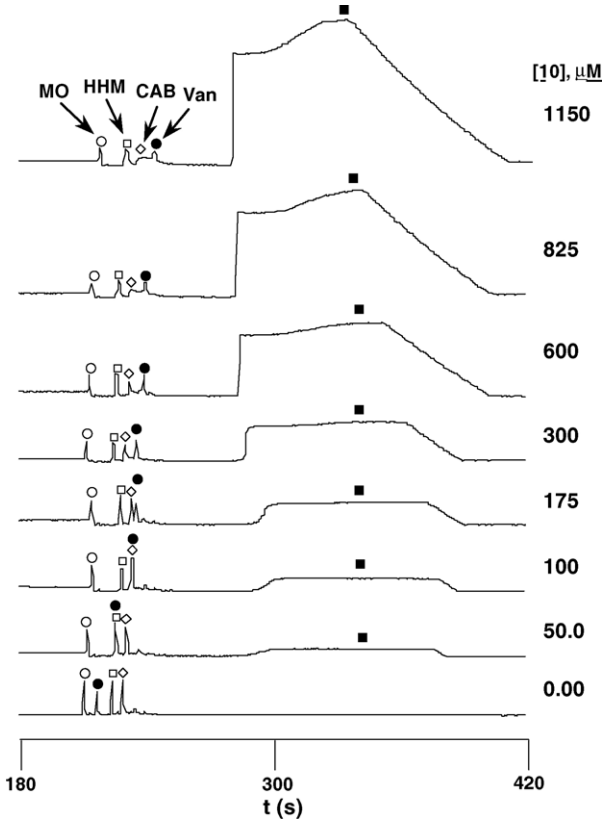

Fig. 7. A representative set of electropherograms of Van in 0.192 M glycine–0.025 M Tris buffer (pH 8.3) containing various concentrations of **10** for an injection time of 0.10 min using the FTPFACE technique. The total analysis time in this experiment was 7.0 min at 28 kV (current 5.8 μA) using a 60.5 cm (inlet to detector), uncoated quartz capillary. Mesityl oxide (MO), carbonic anhydrase B (CAB, and horse heart myoglobin (HHM) were used as internal standards.

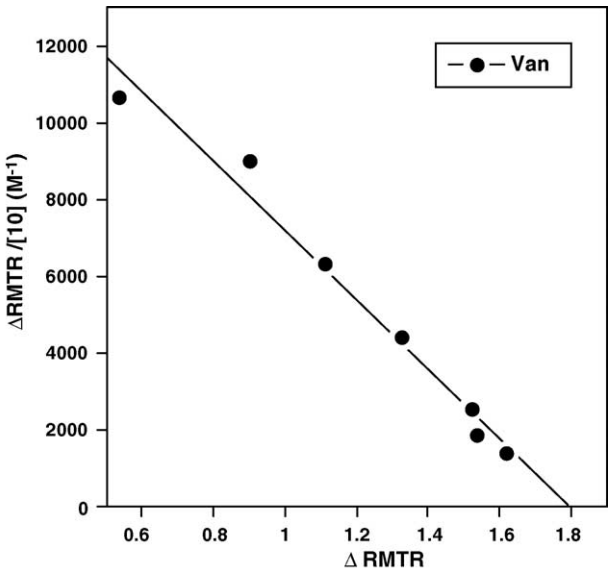

Fig. 8. Scatchard plot of the data for Van and **10** at a ligand injection time of 0.10 min according to Eq. (3).

trophoretic mobilities of the species. In the present study, both CAB and Van migrate faster through the capillary than their respective ligands and, hence, the ligand is first injected into the capillary. If, on the other hand, the order of migration was different the injection order of receptor and ligand would need to be switched to attain a dynamic equilibrium.

FTPFACE has several advantages as a method for measuring bimolecular non-covalent interactions. First, it requires even smaller quantities of receptor and ligand than in traditional ACE techniques. Second, it does not require radiolabelled or chromophoric ligands. Third, purification of the sample prior to injection is not necessary as long as the component to be analyzed can be separated from other species. Fourth, the commercial availability of automated instrumentation, and the high reproducibility of data, make it experimentally convenient.

5. Conclusion

The present study details experimental conditions needed to successfully utilize FTPFACE to determine binding constants between a receptor and ligand. Using as model systems CAB and Van a minimal time of injection of ligand was determined and used to obtain binding constants between receptors and ligands. In FTPFACE a zone of solution containing ligand is partially filled in the capillary column followed by a plug of receptor and standards and electrophoresed. The change in migration time of the receptor relative to the standards at increasing concentrations of ligand is used in the Scatchard analysis to yield a binding constant. Further work to expand the range of receptor–ligand combinations that can be examined using FTPFACE are in progress.

Acknowledgments

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