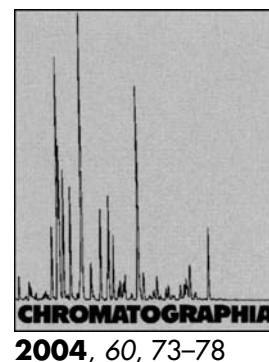


Use of a Dual-Marker Form of Analysis to Estimate Binding Constants Between Receptors and Ligands by Affinity Capillary Electrophoresis



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Abstract

This work describes the use of a dual-standard analysis approach termed the time-average ratio (TAR) in affinity capillary electrophoresis (ACE) to estimate binding constants of receptors to ligands. In this form of analysis the TAR is the migration time of the receptor divided by the average of the sum of the migration times of two non-interacting standards. This change in TAR as a function of the concentration of ligand yields a value for the binding constant. This concept is demonstrated using three model systems: carbonic anhydrase B (CAB, EC 4.2.1.1) and arylsulfonamides, vancomycin (Van) and ristocetin (Rist) from *Streptomyces orientalis* and *Nocardia lurida*, respectively, and D-Ala-D-Ala terminus peptides. Three ACE techniques are used to examine the three systems: standard ACE, flow-through partial-filling ACE (FTPFACE), and on-column derivatization coupled to ACE. The findings described here demonstrate that ACE data analyzed using the TAR form of analysis yield binding constants between receptors and ligands comparable to those estimated using other ACE forms of analysis. A comparison to three other forms of analysis is described.

Keywords

Affinity capillary electrophoresis
Use of a dual marker
Time-average ratio
Binding constants
Vancomycin and ristocetin

Introduction

During the past decade, advances in molecular biology have provided great insight into interactions between a myriad of biologically relevant molecules. Such interactions are critical in understanding the functions and molecular mechanisms of biological systems and the roles these interactions play in health and human disease. By determining how and

to what extent these species interact with each other, treatments for diseases including Alzheimer's, AIDS, and cancer might be expedited.

The development of new analytical techniques has made analysis of ever-decreasing amounts of material possible [1]. Capillary electrophoresis (CE) is a powerful separation technique that has gained widespread use in biological laboratories because of its versatility and

ease of use [2, 3]. CE separates molecules based on differences in electrophoretic mobilities (μ) under the influence of an applied field. Molecules migrate through the capillary column based on their charge-to-mass ratio.

One important application of CE is affinity CE (ACE) which has been used successfully to estimate binding parameters between ligands and receptors [4–32]. The past ten years has seen ACE emerge as one of the techniques of choice to study bimolecular noncovalent interactions. ACE uses the resolving power of CE to distinguish between free and bound forms of a receptor as a function of the concentration of free ligand in the electrophoresis buffer. In a typical form of ACE a sample of receptor and standard(s) is exposed to an increasing concentration of ligand in the running buffer causing a shift in the migration time of the receptor relative to the standard(s). Subsequent Scatchard analysis of the resultant change in migration time of the receptor is then utilized to estimate a binding constant. A number of interactions have been examined by ACE affording information on binding parameters. For example, Kaddis et al. have demonstrated the use of a competitive flow-through ACE assay to estimate binding constants of neutral ligands to proteins [33]. Dunayevskiy et al. have demonstrated that ACE can be used in combinatorial approaches to drug design by evaluating binding constants of a number of peptides to vancomycin [31]. Finally, ACE has also been used to

investigate an epitope on human immunodeficiency virus by a monoclonal antibody [20].

There are several forms of analysis that have been utilized to estimate binding constants using ACE [6, 23, 34–38]. Many of these forms require measurement of receptor electrophoretic mobilities (μ) in both complexed and uncomplexed states. Eq. (1) is but one example of an equation based on μ that has been

$$\Delta\mu_{R,L}/[L] = K_b\Delta\mu_{R,L}^{\max} - K_b\Delta\mu_{R,L} \quad (1)$$

used to estimate the K_b of ligands to receptors. In this equation, changes in the electrophoretic mobility μ_R of a receptor (R) on complexation with a ligand (L) present in the buffer can be correlated to K_b . Analysis of the magnitude of the change in mobility $\Delta\mu_{R,L}$ as a function of the concentration $[L]$ of ligand yields a value for the binding constant. The value for $\Delta\mu_{R,L}^{\max}$ is defined as the greatest change in $\Delta\mu_{R,L}$ upon full saturation of ligand to receptor.

Recently, mobility ratios (M) were used to estimate binding constants between ligands and receptors [39]. Here, M is defined as $(t_{eo}/t_r) + 1$ where t_{eo} and t_r are the migration times of a non-interacting standard and the receptor in question, respectively. Eq. (2) is subsequently used for

$$\Delta M_{R,L}/[L] = K_b\Delta M_{R,L} - K_b\Delta\mu_{R,L} \quad (2)$$

Scatchard analysis to estimate a value for $K_b\Delta M_{R,L}^{\max}$ is the greatest value for $\Delta M_{R,L}^{\max}$ obtained by full complexation of L to R .

We recently demonstrated the estimation of binding constants by ACE using a two-marker system form of analysis [6]. In this form of analysis K_b is estimated using the relative migration time ratio (RMTR) (Eq. 3). Here, t_r , t_s , and t_s' are the

$$RMTR = (t_r - t_s)/(t_s - t_s') \quad (3)$$

measured migration times of the receptor peak, and two non-interacting standard peaks, respectively. A Scatchard plot can be obtained using Eq. (4). Here, $\Delta RMTR_{R,L}$ is the

$$\Delta RMTR_{R,L}/[L] = K_b\Delta RMTR_{R,L}^{\max} - K_b\Delta RMTR_{R,L} \quad (4)$$

magnitude of the change in the relative migration time ratio as a function of the concentration of ligand. The value for $\Delta RMTR_{R,L}^{\max}$ determined by fully saturating

R with L causing the maximal shift in migration of the R - L complex. Eq. (4) allows for the estimation of K_b on a relative time scale using two non-interacting standards and compensates for fluctuations in electroosmotic flow (EOF) in the capillary column induced by electrophoresis.

In this paper, we extend our use of a dual-marker form of analysis by demonstrating that the use of a time average of two non-interacting standards also yields accurate binding constants. In this form of analysis termed the time average ratio (TAR), the value for TAR (Eq. (5)) is defined as the migration

$$TAR = (t_r)/((t_s + t_s')/2) \quad (5)$$

time of the receptor (t_r) divided by the average of the migration time of the two standards (t_s and t_s'). A Scatchard plot can be obtained using Eq. (6). Here, $\Delta TAR_{R,L}$ is the magnitude of the

$$\Delta TAR_{R,L}/[L] = K_b\Delta TAR_{R,L}^{\max} - K_b\Delta TAR_{R,L} \quad (6)$$

change in the time average ratio as a function of the concentration of ligand. $\Delta TAR_{R,L}^{\max}$ is defined as the maximum value for obtained upon full saturation of L to R .

To demonstrate the utility of the TAR form of analysis we examined three model systems employing three ACE techniques. In the first, carbonic anhydrase B (CAB, EC 4.2.1.1) and arylsulfonamides are examined using standard ACE. In the second, vancomycin (Van) from *Streptomyces orientalis* and D-Ala-D-Ala terminus peptides are examined using flow-through partial-filling ACE (FTPFACE). In the third, ristocetin (Rist) from *Nocardia lurida* and D-Ala-D-Ala terminus peptides are examined by on-column derivatization coupled to ACE. The general utility of the TAR form of analysis is compared to other forms of analysis in ACE.

Experimental

Chemicals and Reagents

All chemicals were analytical grade. 4-Carboxybenzenesulfonamide, 2, was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). *N*-Acetyl-D-

Ala-D-Ala, 5, *N*_α,*N*_ε-diacetyl-Lys-D-Ala-D-Ala, 6, vancomycin (Van) from *Streptomyces orientalis*, carbonic anhydrase B (CAB, EC 4.2.1.1, containing CAA and CAB isozymes, from bovine erythrocytes), and horse heart myoglobin (HHM) were purchased from Sigma Chemical Company (St. Louis, MO, USA) and used without further purification. Ristocetin was purchased from Bio Data Corporation (Horsham, PA, USA) and was used without purification. Mesityl oxide (MO) was purchased from Calbiochem (San Diego, CA, USA). [4-(Aminosulfonyl)phenyl]methylamino]-6-oxohexanoic acid, 1, [4-(Aminosulfonyl)phenyl]methylamino]-1,6-dioxohexylamino]-1,3-benzene-dicarboxylic acid, 3, and *N*-succinyl-D-Ala-D-Ala, 4, were synthesized based on literature procedures [5]. Stock solutions of Van (1 mg mL⁻¹), bovine CAB (1 mg mL⁻¹), and HHM (1–4 mg mL⁻¹) were each prepared by dissolving the lyophilized protein in buffer (192 mM glycine-25 mM Tris; pH 8.3). Stock solutions of NAD (1 mg mL⁻¹) and Rist (1 mg mL⁻¹), were each prepared by dissolving the samples in buffer (20 mM phosphate, pH 6.9). Stock solutions of acetic anhydride and succinic anhydride were prepared by dissolving the compounds in acetonitrile.

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 μm, length from inlet to detector of 50.5 (60.5 for FTPFACE and 40.5 for on-column derivatization coupled to PFACE) 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: Standard ACE, voltage, 25–30 kV; current, 5.2–5.8 μA; detection, 200 nm; temperature, 23 + 0.2 °C; FTPFACE, voltage, 25–30 kV; current, 5.2–5.8 μA; detection, 200 nm; temperature, 23 + 0.2 °C; for on-column derivatization coupled to PFACE, voltage, 20 kV; current, 22 μA; detection, 200 nm; temperature, 23 + 0.2 °C.

Procedures

For standard ACE: A sample (3.6 nL) of solution containing 0.2 mg mL^{-1} of carbonic anhydrase B, 0.1 mg mL^{-1} of horse heart myoglobin, and 0.08 mg mL^{-1} of mesityl oxide in buffer was introduced into the capillary by vacuum injection. The electrophoresis was carried out using a tris-glyc buffer and appropriate concentrations of the arylsulfonamide ligand ($0\text{--}120 \text{ }\mu\text{M}$). For Van, a sample (3.6 nL) of solution containing 0.14 mg mL^{-1} of Van, 0.2 mg mL^{-1} of CAB, and 0.17 mg mL^{-1} of MO in buffer was introduced into the capillary by vacuum injection. The electrophoresis was carried out using a sodium phosphate buffer and appropriate concentrations of 4 ($0\text{--}1150 \text{ }\mu\text{M}$). For FTPFACE: A sample of 4 was vacuum injected into the capillary for 0.10 min at high pressure followed by a sample (3.6 nL) of solution for 3 s containing 0.035 mg mL^{-1} of Van, 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-glyc buffer and increasing concentrations of 4 ($0\text{--}1150 \text{ }\mu\text{M}$) for 5.0 min. For on-column derivatization coupled to PFACE: The capillary was first equilibrated with buffer (20 mM phosphate; pH 6.9) at increasing concentrations of ligand. Plugs containing buffer (3.6 nL), Rist (1.2 to 2.4 nL), and acetic and succinic anhydride (1.2 to 2.4 nL), and buffer (3.6 nL) were then introduced by vacuum injection. Electrophoresis was carried out using increasing concentrations of the D-Ala-D-Ala ligands for 5.0 min.

Results and Discussion

To determine the efficacy of the TAR form of analysis, we examined the data obtained from three different types of ACE techniques: standard ACE, flow-through partial-filling affinity capillary electrophoresis (FTPFACE), and on-column derivatization coupled to ACE. In standard ACE a plug of receptor and non-interacting standards is injected and is subjected to increasing concentrations of ligand in the running buffer. Changes in the migration time between complexed and uncomplexed receptor are used for analysis and generation of the Scatchard plot. In our initial studies we examined the interaction of CAB and arylsulfonamides. CAB is a

zinc protein of the lyase class that catalyzes the equilibration of carbon dioxide and carbonic acid. It is strongly inhibited by sulfonamide-containing molecules.

In these experiments a plug of sample containing CAB, MO, and HHM were injected and electrophoresed. MO and HHM are non-interacting standards and are used in the analysis of the data. Neither standards adsorb onto the capillary column at the pH the study was conducted at nor degrade over the course of the ACE experiment. More importantly, they do not interact with either the receptor or ligand or cause perturbations in electroosmotic flow (EOF) that might occur due to changes in viscosity or joule heating. In these experiments, the concentration of 1 was successively increased from 0 to $120 \text{ }\mu\text{M}$.

Fig. 1 shows a representative series of electropherograms of CAB in buffer containing various concentrations of 1. Upon addition of increasing concentrations of 1 in the running buffer, the CAB peak shifts to the right for any concentration of ligand. CAB, when bound to 1, is more negative than in the unbound state, and is detected later than the uncomplexed form. Peak broadening was observed at intermediate concentrations and is caused by the retardation of migrating molecules due to their frequent interactions with the ligand in the region of intermediate status. The CAB peaks become sharper at the saturating concentrations of the ligand.

We then analyzed the migration time data using Eqs (1), (2), (4), and (6). Fig. 2A–D are Scatchard plots of the data according to the four equations. In the TAR form of analysis, a K_b value of 8.34×10^5 was obtained with a correlation coefficient of 0.98. Eqs (2) and (4) yielded similar values for K_b and the correlation coefficient. The use of Eq. (1) gave the lowest value for K_b and the poorest value for R^2 . Table 1 lists the binding constants obtained using Eqs (1), (2), (4), and (6). Two other ligands were analyzed the results of which are detailed in Table 1. The values for K_b obtained in Eq. (6) are similar to values obtained from previous ACE and other assay techniques.

To further prove the versatility of the TAR form of analysis we used FTPFACE to examine vancomycin (Van) and the small peptide *N*-succinyl-D-Ala-D-Ala (4). Van is a parenteral glycopeptide antibiotic that kills bacterial cells by

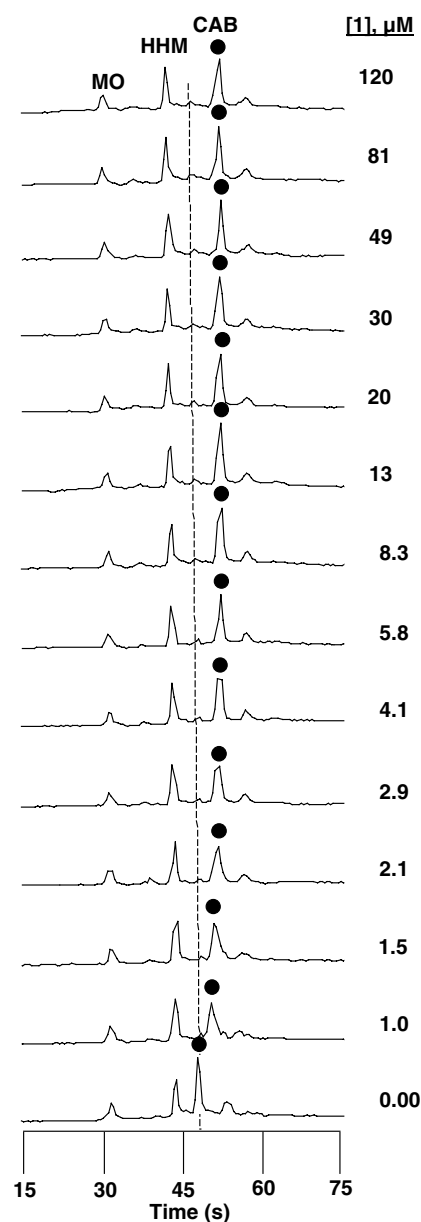


Fig. 1. A representative set of electropherograms of carbonic anhydrase B (CAB) in 0.192 M glycine- 0.025 M Tris buffer (pH 8.3) containing various concentrations of 1. The total analysis time in each experiment was 7.0 min at 30 kV (current: $5.2 \text{ }\mu\text{A}$) using a 60.5-cm (inlet to detector), $50\text{-}\mu\text{m}$ I.D. open, uncoated quartz capillary. Horse heart myoglobin (HHM) and mesityl oxide (MO) were used as internal standards

inhibiting peptidoglycan biosynthesis [40–48]. It functions by binding to the terminal D-Ala-D-Ala dipeptide of the bacterial cell wall precursors, thereby, impeding further processing of these intermediates into peptidoglycan. In FTPFACE, a plug of ligand is initially injected into the capillary followed by a small plug of sample containing receptor and two non-interacting standards. Upon electrophoresis, the sample plug flows

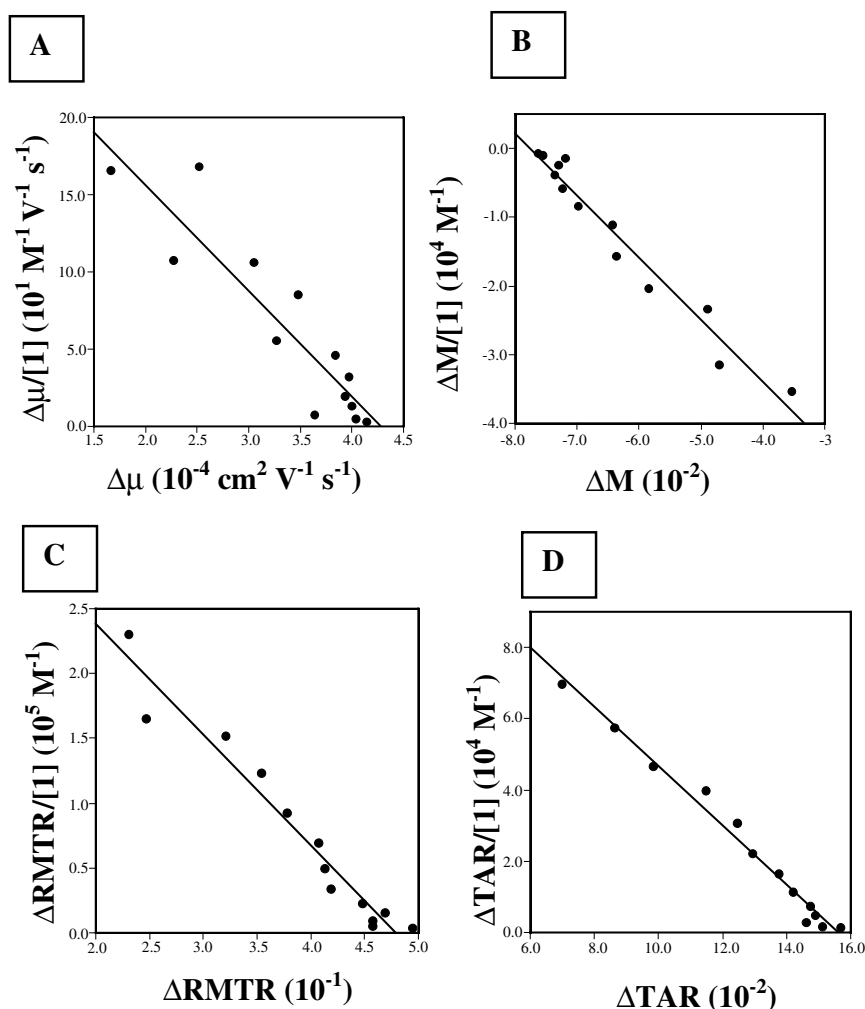


Fig. 2. Scatchard plots of the data for carbonic anhydrase B according to Eqs (1), (2), (4), and (6)

Table 1. Experimental values of binding constants K_b (10^6 M^{-1}) of ligands 1–3 and carbonic anhydrase B obtained by Eqs (1), (2), (4), and (6) using Standard ACE

Ligand	K_b (correlation coefficient)			
	Eq. (1)	Eq. (2)	Eq. (4)	Eq. (6)
1 ^a	6.85 (0.84)	9.05 (0.96)	8.53 (0.95)	8.34 (0.98)
2 ^b	5.8 (0.77)	9.59 (0.94)	11.16 (0.94)	10.33 (0.98)
3 ^c	7.83 (0.97)	7.70 (0.98)	7.10 (0.94)	7.40 (0.97)

^aPrevious estimates [6,9]: $K_b = 4.50\text{--}9.20 \times 10^5 \text{ M}^{-1}$.

^bPrevious estimates [6,9]: $K_b = 7.20\text{--}20.0 \times 10^5 \text{ M}^{-1}$.

^cPrevious estimates [6,9]: $K_b = 5.00\text{--}8.40 \times 10^5 \text{ M}^{-1}$.

into the domain of the ligand plug where a dynamic equilibrium is established between receptor and ligand. Continued electrophoresis enables the sample plug to flow-through the ligand plug where it is detected prior to the plug of ligand.

In these experiments, a plug of 4, at increasing concentrations, was vacuum injected into the capillary for 0.10 min followed by a plug of sample containing Van, CAB, HHM, and MO and electrophoresed for 5.0 min. In the present

experiment MO and HHM were used as non-interacting standards for the analysis. CAB can also be used in the analysis. The concentration of 4 was successively increased from 0 to 1150 μM .

Fig. 3 shows a representative series of electropherograms of Van in capillaries partially filled with increasing concentrations of 4. The complexation between 4 and Van results in an increasing negative charge and the complex is detected later than the uncomplexed form. A Scatchard

plot (not shown) of the data for Van using Eq. (6) realized a binding constant for the interaction. Table 2 summarizes the binding data for the two peptide ligands and Van obtained by Eqs (1), (2), (4), and (6). A stable EOF permits analysis of the data by all equations. The values obtained by Eq. (6) are in agreement with previous ACE studies on Van and arylsulfonamides and with those obtained from other techniques [39, 41].

Finally, we examined on-column derivatization coupled to PFACE using Rist. Like Van, Rist is a glycopeptide antibiotic that inhibits cell wall synthesis by impeding the action of transglycosylases and transpeptidases [40, 41]. In this final study, on-column acetylation and succinylation of Rist coupled to PFACE was examined. A plug containing increasing concentrations of 6 was injected to partially fill the capillary. A buffer plug was then vacuum injected into the capillary to separate the reagents from the ligand plug. The third injection contained a mixture of succinic and acetic anhydride while the fourth plug contained Rist, MO, and NAD. Upon electrophoresis, the separate zones of solutions overlap to yield the Rist derivatives. Fig. 4 shows a representative series of electropherograms of Rist in buffer plugs containing increasing concentrations of 6. Table 3 summarizes the binding data for the Rist derivatives to 6 obtained using Eqs (1), (2), (4), and (6). As can be seen by the data, Eq. (6) yields comparable binding constant information as the other forms of analysis. In the present case, since changes in EOF are small, all four forms of analysis can be used to effectively estimate a value for K_b .

In general, we found the TAR form of analysis to provide a better agreement in binding constant values to those obtained from other ACE studies and from assays using other analytical techniques. The use of μ , for example, has the greatest variance in binding constant values and in the values for the correlation coefficient. Both M and RMTR forms of analysis yield similar values of K_b as does TAR and with comparable values for the correlation coefficient.

There are several advantages in using the TAR form of analysis to estimate affinity constants of ligands to receptors. One, two non-interacting standards allow for a rapid assessment of binding as it is easy to physically observe a shift in the migration time of the peak in question.

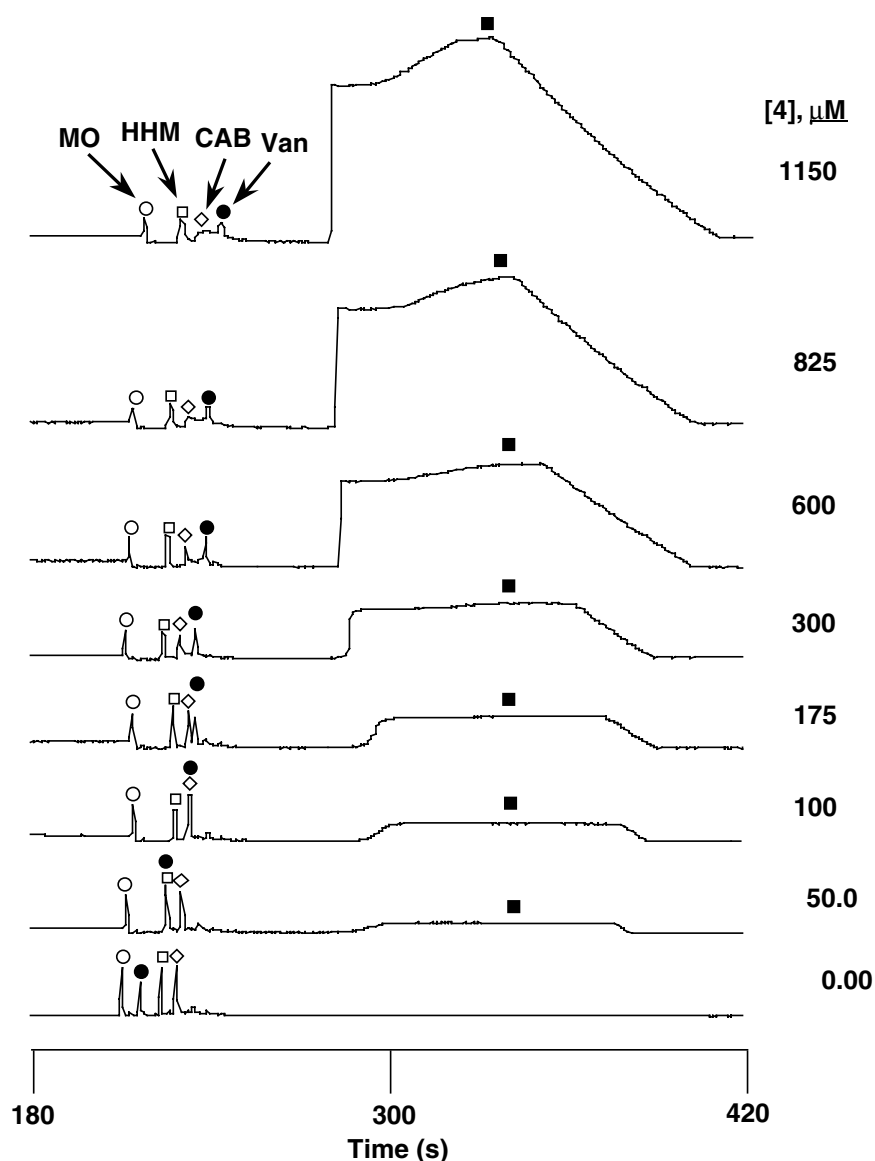


Fig. 3. A representative set of electropherograms of vancomycin (Van) in 0.192 M glycine-0.025 M Tris buffer (pH 8.3) containing various concentrations of 4. The total analysis time in each experiment was 7.0 min at 28 kV (current: 5.8 μ A) using a 60.5-cm (inlet to detector), 50- μ m I.D. open, uncoated quartz capillary. Horse heart myoglobin (HHM), carbonic anhydrase B (CAB), and mesityl oxide (MO) were used as internal standards. The solid square represents 4 partially-filled in the capillary column

Two, the use of multiple markers in ACE is better experimentally and provides for a choice in the analysis as negative peaks can arise in the vicinity of standards due to dilution of ligand during the binding event.

Conclusion

The results presented here demonstrate that binding constant data obtained by ACE can be estimated using two non-interacting standards and the time average ratio (TAR). We have shown this by the use of three model systems: CAB and

Table 2. Experimental values of binding constants K_b (10^3 M^{-1}) of ligands 4 and 5 to vancomycin obtained by Eqs (1), (2), (4), and (6) using the (FTPFACE) technique

Ligand	K_b (correlation coefficient)			
	Eq. (1)	Eq. (2)	Eq. (4)	Eq. (6)
4 ^a	12.93 (0.96)	12.32 (0.95)	9.37 (0.96)	10.49 (0.95)
5 ^b	2.29 (0.94)	2.91 (0.95)	3.79 (0.91)	3.98 (0.89)

^aPrevious estimates [6]: $K_b = 9.3\text{--}13.0 \times 10^3 \text{ M}^{-1}$

^bPrevious estimates [6]: $K_b = 3.9\text{--}8.7 \times 10^3 \text{ M}^{-1}$

arylsulfonamide ligands, and the glycopeptide antibiotics vancomycin and ristocetin to D-Ala-D-Ala terminus peptides. The use of TAR is an improvement over other forms of analyses for it yields accurate values for binding constants and

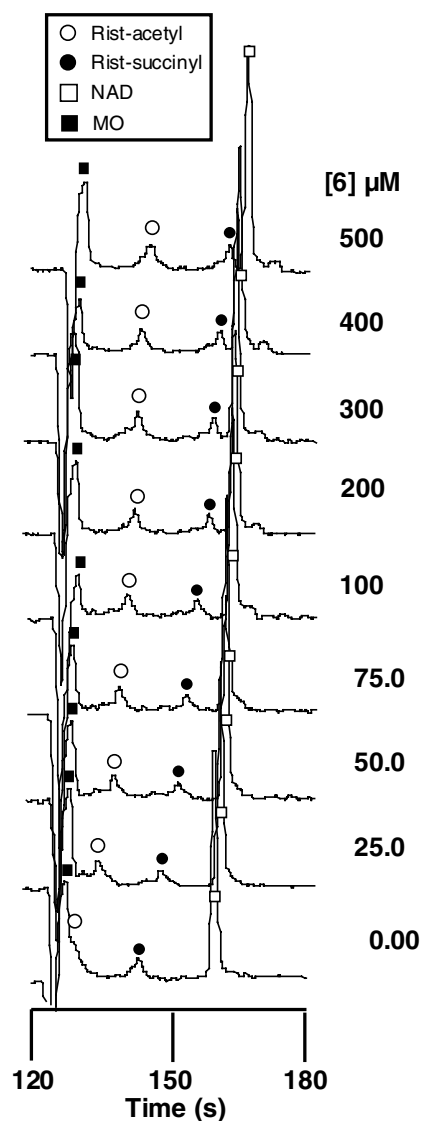


Fig. 4. A representative series of electropherograms of Rist derivatives in 20 mM phosphate buffer (pH 6.9) containing various concentrations of 6, using on-column receptor synthesis coupled to PFACE. The total analysis time in each experiment was 5.0 min. at 20 kV using a 46.5 cm (inlet to detector) 50 μ m I.D. open, uncoated quartz capillary

with higher correlation coefficients than found using alternative forms of analyses. Further studies to examine the versatility of TAR using other receptor-ligand combinations are in progress.

Table 3. Experimental values of binding constants K_b (10^3 M^{-1}) of ligand 6 to ristocetin obtained by Eqs (1), (2), (4), and (6) using the on-column/PFACE technique

Antibiotic	K_b (correlation coefficient)			
	Eq. (1)	Eq. (2)	Eq. (4)	Eq. (6)
Rist-acetyl	29.45 (0.97)	28.41 (0.98)	28.18 (0.99)	26.89 (0.99)
Rist-succinyl	16.97 (0.97)	14.62 (0.98)	15.27 (0.98)	14.46 (0.98)

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