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Multiple-step ligand injection affinity capillary electrophoresis for determining binding constants of ligands to receptors

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Abstract

This work demonstrates the use of multiple-step ligand injection affinity capillary electrophoresis (ACE) using two model systems: vancomycin from *Streptomyces orientalis* and carbonic anhydrase B (CAB, EC 4.2.1.1). In this technique a sample plug of receptor and non-interacting standards is injected by pressure and electrophoresed in a buffer containing a given concentration of ligand. The sequence is repeated for all concentrations of ligand generating a single electropherogram containing a series of individual sample plugs superimposed on environments of buffer containing increasing concentrations of ligand. Analysis of the change in the relative migration time ratio, RMTR, relative to the non-interacting standards, as a function of the concentration of the ligand, yields a value for the binding constant. A competitive assay using the technique is also demonstrated using neutral ligands for CAB. These values agree well with those estimated using other binding and ACE techniques. Data demonstrating the quantitative potential of this method are presented.

Keywords: Multiple-step ligand injection; Binding constants; Receptor–ligand interactions; Carbonic anhydrase; Vancomycin; Proteins

1. Introduction

Accurate and expeditious analysis of receptor–ligand interactions is critical to rational drug design and development. The importance of the former goes without saying. Clinical trials are based on reliable and reproducible chemical analyses. The latter is problematic given the dramatic advances in chemical syntheses and the great number of potential drugs and precursors that have been achieved using combinatorial approaches to drug development. The increased output of potential drugs compared to traditional techniques of drug design has made expeditious and facile analysis of potential drugs a must in any new analytical technique.

Affinity capillary electrophoresis (ACE) is a versatile technique for studying biomolecular non-covalent interactions and has been shown to be an effective means of determining binding and dissociation constants of formed complexes [1–38]. For example, Kiessig et al. has used ACE to examine the interaction of the enzyme cyclophilin with the

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immunosuppressive drug cyclosporin A [3]. Heintz et al. has demonstrated the use of a partial-filling technique in ACE to estimate the binding of receptors for ligands [31]. Finally, Gao et al. has used ACE and protein charge ladders to determine the contribution of electrostatics to the values of measured binding affinities [20]. The technique uses the resolving power of CE to differentiate between the free and bound forms of a protein as a function of the concentration of free ligand. In a typical form of ACE a sample of receptor and non-interacting standard is exposed to an increasing concentration of ligand in the running buffer thereby causing a shift in the migration of the receptor relative to the standard. Subsequent analysis yields a value for the binding constant K_b.

Estimation of receptor–ligand interactions are increasingly requiring the use of smaller quantities of material. Methods that utilize minute quantities of material and which yield accurate estimation of a receptor–ligand interaction in a timely fashion are at a premium. Although ACE has been shown to be effective in estimating binding parameters of ligands to receptors, in cases where only small quantities of material are available, analysis by traditional ACE techniques is made difficult. In addition, expeditious analysis of the interaction in question may also be desirable particularly when combinatorial approaches to drug design are utilized. In cases where both conditions are needed modifications in the technique are warranted.

In this paper, we describe the use of multiple-step ligand injection ACE by estimating binding constants of ligands to receptors (Fig. 1) using two model systems: vancomycin (Van) from *Streptomyces orientalis* and carbonic anhydrase B (CAB, EC 4.2.1.1). In this technique a plug of receptor and non-interacting standards is injected and electrophoresed in a buffer containing a given concentration of ligand. The sequence is repeated at increasing concentrations of ligand. Analysis of the change in the relative migration time ratio, RMTR, relative to the non-interacting standards, as a function of the concentration of the ligand, yields a value for the binding constant. A competitive assay using neutral ligands for CAB is also demonstrated. The major advantages of the technique are the small quantities of material needed for the study and the shorter length of experiment compared to both traditional ACE techniques and other forms of binding assays.

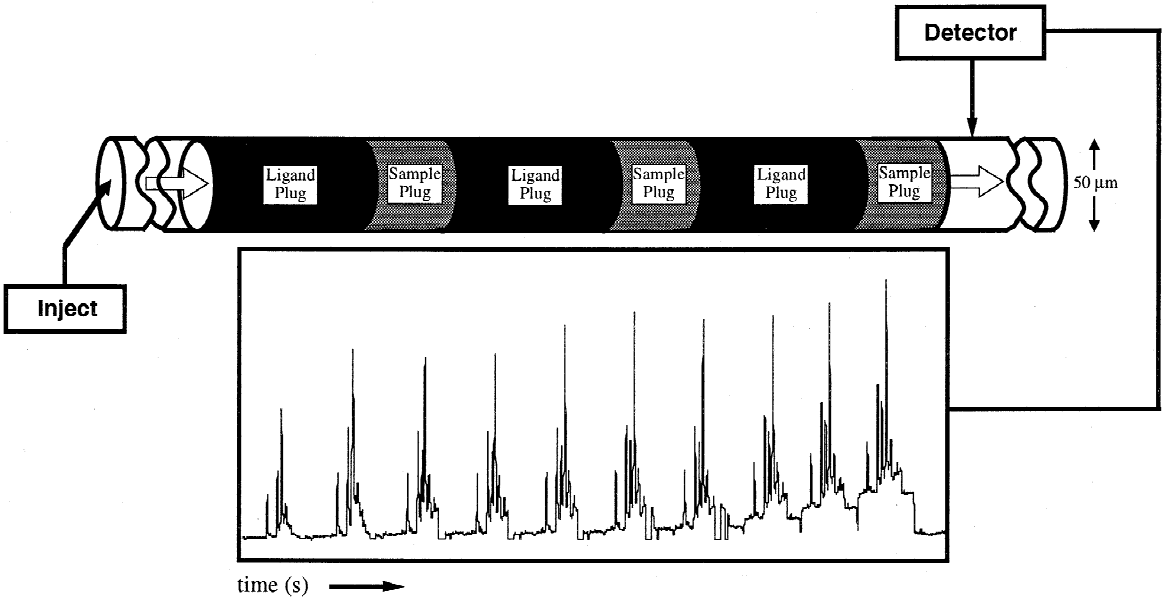


Fig. 1. Schematic of a multiple-step ligand injection ACE experiment.

2. Experimental

2.1. Chemicals and reagents

All chemicals were analytical grade. *N*-Acetyl-D-Ala-D-Ala **1**, vancomycin from *Streptomyces orientalis*, 4-toluenebenzenesulfonamide **5**, benzenesulfonamide **6**, and carbonic anhydrase B (CAB, EC 4.2.1.1, containing CAA and CAB isozymes, from bovine erythrocytes) were purchased from Sigma (St. Louis, MO, USA) and used without further purification. 4-Carboxybenzenesulfonamide **3**, was purchased from Aldrich (Milwaukee, WI, USA), mesityl oxide (MO) was purchased from Calbiochem (San Diego, CA, USA). *N*-Succinyl-D-Ala-D-Ala, **2**, and {([4-(aminosulfonyl)phenyl]methyl)amino}-6-oxohexanoic acid, **4**, were synthesized based on literature procedures [15]. Stock solutions of vancomycin (4 mg/ml), bovine carbonic anhydrase B (1 mg/ml), and horseheart myoglobin (HHM) (1 mg/ml) were each prepared by dissolving the lyophilized protein in buffer (192 m*M* glycine–25 m*M* Tris, pH 8.3).

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 μm, length from inlet to detector of 80.5 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, 24 kV; current, 4.0 μA; detection, 200 nm; temperature, 25±2°C.

2.3. Procedures

For Van, the capillary was first filled with buffer solution not containing ligand (solution A) followed by a sample (7.2 nl; a 1 s time of injection equates to 1.2 nl of volume of solution) of solution (solution B) containing 0.10 mg/ml of Van, 0.17 mg/ml of MO, and 0.33 mg/ml of CAB. The sample was subjected to electrophoresis in a solution (solution C) containing the first concentration (50 μ*M*) of derivatized D-Ala-D-Ala ligand for 2.0 min at 24 kV. A second sample of solution (14.4 nl) (solution B) containing Van, MO and CAB was injected for 12 s and subjected to electrophoresis in the next higher concentration of derivatized D-Ala-D-Ala ligand (100–1200 μ*M*) for 2.0 min at 24 kV. The process of sample injection and ligand electrophoresis was repeated until all concentrations of ligand were run. For CAB, the capillary was first filled with buffer not containing ligand (solution D) followed by a sample (7.2 nl) of solution (solution E) containing 0.33 mg/ml of CAB, 0.17 mg/ml of MO and 0.67 mg/ml of HHM. The sample was subjected to electrophoresis in a solution (solution F) containing the first concentration (1.0 μ*M*) of arylsulfonamide ligand for 2.0 min at 24 kV. Subsequent samples of solution (14.4 nl) (solution E) containing CAB, MO and HHM were injected for 12 s and subjected to electrophoresis in increasing concentrations of arylsulfonamide ligand (2–80 μ*M*) for 2.0 min at 24 kV similar to the Van experiment. For CAB competition experiments the same procedure was followed except that the neutral arylsulfonamide ligand concentrations (0–120 μ*M*) contained 20 μ*M* of **4**.

3. Results and discussion

In the first series of experiments the interaction of vancomycin and two small peptides, *N*-acetyl-D-Ala-D-Ala, **1**, and *N*-succinyl-D-Ala-D-Ala, **2**, was investigated. Vancomycin is a glycopeptide antibiotic that kills bacterial cells by inhibiting peptidoglycan biosynthesis. Vancomycin functions by binding to the terminal D-Ala-D-Ala dipeptide of bacterial cell wall precursors thereby impeding further processing of these intermediates into peptidoglycan [39]. In this technique a sample containing Van, MO and CAB were injected by pressure and electrophoresed in a solution of **1** for 2.0 min. The concentration of **1** was sequentially increased from 0 to 1150 μ*M* and the process repeated eight times until all concentrations of ligand were used.

Fig. 2 shows the electropherogram of Van generated using the multiple-step ligand injection ACE technique. Upon addition of increasing concentrations of **1** in the running buffer the Van peak shifts to the right for any given concentration of **1** with respect to the two non-interacting markers. MO and

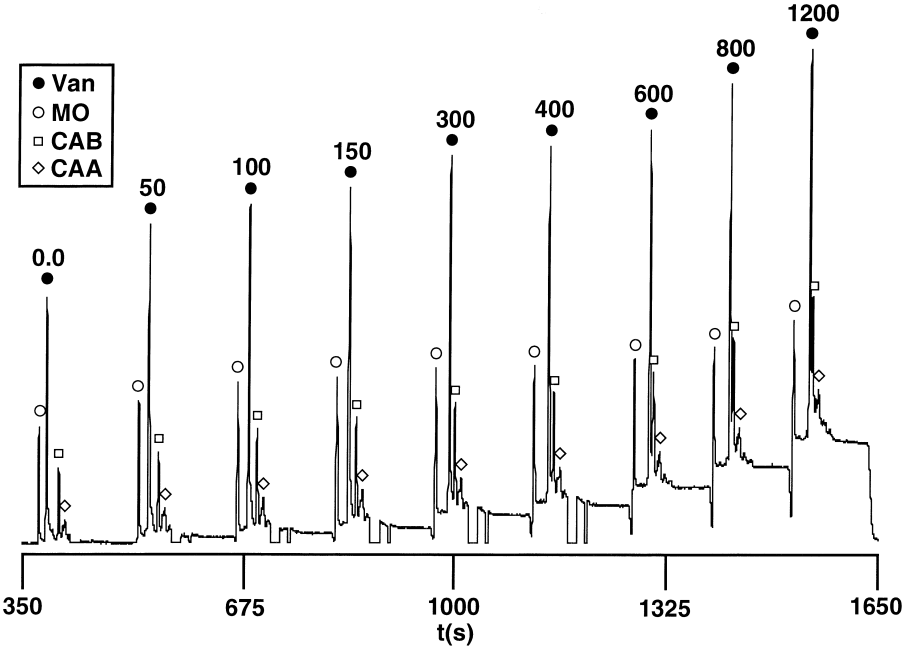


Fig. 2. A representative electropherogram of vancomycin (Van) in 0.192 *M* glycine–0.025 *M* Tris buffer (pH 8.3) containing various concentrations of **1** using the multiple-step ligand injection ACE technique. The total analysis time in each experiment was 27 min at 24 kV (current: 4.0 μA) using a 80.5 cm (inlet to detector)×50 μm I.D. open, uncoated quartz capillary. Mesityl oxide (MO) and carbonic anhydrase B (CAB, containing CAA and CAB isozymes) were used as internal standards. The number above each set of sample peaks refer to the concentration of **1** in μ*M*.

CAB are used as neutral and charged markers, respectively, in the analysis and do not interact with **1** in the running buffer under conditions of electrophoresis. Ligand **1** is a small negatively charged molecule and has a more negative electrophoretic mobility than both complexed and uncomplexed Van, hence, it elutes at a greater migration time than both Van and complexed Van. The complexation between **1** and Van resulted in an increasing negative charge and the peak for Van complexed to the ligand shifts to a longer migration time relative to the neutral marker MO increasing **1** in the running buffer. As can be seen in Fig. 2, a single electropherogram is generated using the multiple-plug ligand injection ACE technique. At any one time during the experiment only three different ligand concentrations and three sample plugs are contained in the capillary column. The instrument was programmed in order to ensure all plugs of sample were contained in one single electropherogram. The total time for the experiment was approximately 27 min. Traditional ACE techniques require in excess of 50 min depending on the number of repetitions of ligand run, voltage, capillary length and buffer conditions.

Fig. 3 is a Scatchard plot of the data for Van. In this form of analysis K_b is estimated using a dual-marker form of analysis which we term the RMTR of a receptor referenced to two non-interacting standards (Eq. (1)) [40]:

$$\mathrm{RMTR} = (t_r - t_s)/(t_{s'} - t_s) \quad (1)$$

Here, t_r, t_s, and $t_{s'}$ are the measured migration times of the receptor peak, and the two non-interacting standard peaks, respectively. In the present experiments, t_s and $t_{s'}$ are the migration times of MO and HHM, respectively. A Scatchard plot can be obtained via Eq. (2):

$$\Delta\mathrm{RMTR}_{R,L}/[L] = K_b\Delta\mathrm{RMTR}^{max}_{R,L} - K_b\Delta\mathrm{RMTR}_{R,L} \quad (2)$$

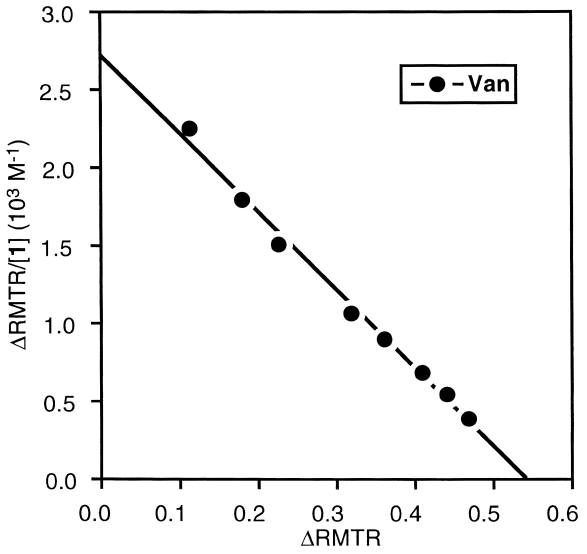


Fig. 3. Scatchard plot of the data for vancomycin according to Eq. (2).

Here, $\Delta RMTR_{R,L}$ is the magnitude of the change in the relative migration time ratio as a function of the concentration of ligand. Eq. (2) allows for the estimation of K_b on a relative time scale using two non-interacting standards and compensates for fluctuations in voltage in the capillary column. Table 1 summarizes the binding data for the two ligands and Van. These values agree well with previous ACE studies and with those obtained from other assays for structurally similar compounds [1,6,15,31,41–43].

There are a number of analyses that have been used in ACE to estimate K_b [44]. In cases where electroosmotic flow (EOF) is constant the use of electrophoretic mobilities (μ) and, in particular, changes in μ of the receptor referenced to a single non-interacting marker, is sufficient in estimating K_b. In the present experiments, it is not possible to relate changes in μ at different ligand concentrations, hence, another form of analysis (RMTR) that allows for simple correlation of changes in migration time of the receptor is warranted.

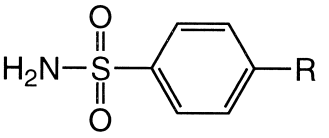
Of particular interest is the amount of ligand used in any given run. Unlike standard ACE techniques where the receptor is electrophoresed in a running buffer of increasing concentrations of ligand, the multiple-step ligand injection ACE technique uses much smaller quantities of material (1.9 nmol compared to approximately 9.9 nmol for standard ACE techniques) for any given ACE experiment since the capillary is not completely filled with ligand. In the present experiment we used a 87 cm long capillary in order to demonstrate the technique. We have used capillaries of smaller lengths and, hence, even smaller amounts of ligand can be used. In addition, the use of smaller capillaries afforded comparable binding constant information as that found with longer capillaries. The ability to obtain binding parameters using minute quantities of sample is integral in the development and design of new drugs and is the major advantage of the multiple-step ligand injection ACE technique over both traditional ACE techniques and other forms of binding assays.

A similar type of experiment was conducted with CAB and two charged arylsulfonamides (Fig. 4). CAB is an enzyme of the lyase class that catalyzes the equilibration of dissolved carbon dioxide and

Table 1
Experimental values of binding constants K_b ($10^3\ M^{-1}$) of ligands **1** and **2** and vancomycin measured by the multiple-step ligand injection ACE technique

Ligand	K_b [a]
1	5.0[b]
2	9.9[c]

[a] The reported binding constants are the average values from six experiments for each ligand.
[b] Previous estimates [15,31]: $K_b = 4.9–8.7 \cdot 10^3\ M^{-1}$.
[c] Previous estimate [1,6,15,31,41–43]: $K_b = 9.3 \cdot 10^3\ M^{-1}$.



3 R = CO_2^-

4 R = $CH_2NHC(O)(CH_2)_4CO_2^-$

5 R = CH_3

6 R = H

Fig. 4. Structures of compounds **3**–**6**.

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. Fig. 5 shows the electropherogram of CAB generated using the multiple-step ligand injection ACE technique with increasing concentrations (0 to 80 μ*M*) of **3**. The complexation between **3** and CAB resulted in an increasing negative charge and the complex is detected later than the uncomplexed form. Binding constants were obtained for two charged arylsulfonamides. Fig. 6 is a Scatchard plot of the data for CAB. Table 2 shows the binding constants obtained by Eq. (2). The values for the binding constants agree well with previous estimates by ACE and other binding techniques [1,10,15,31].

Although many ligands for receptors are charged, many are neutral at the pH of interest. In order to fully appreciate the versatility of the multiple-step ligand injection ACE technique we examined the

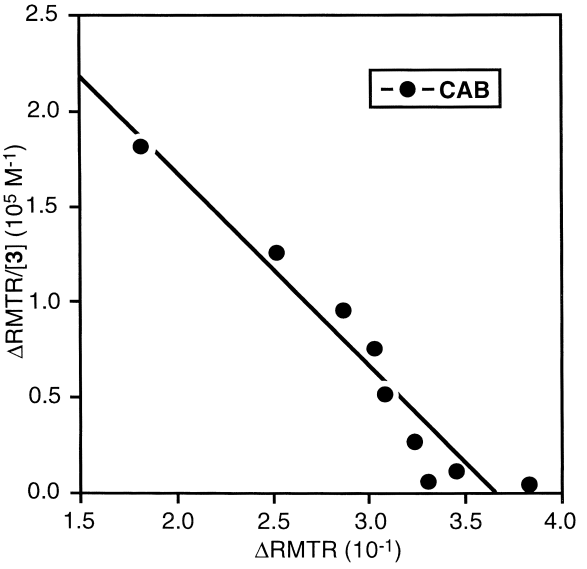


Fig. 6. Scatchard plot of the data for carbonic anhydrase B according to Eq. (2).

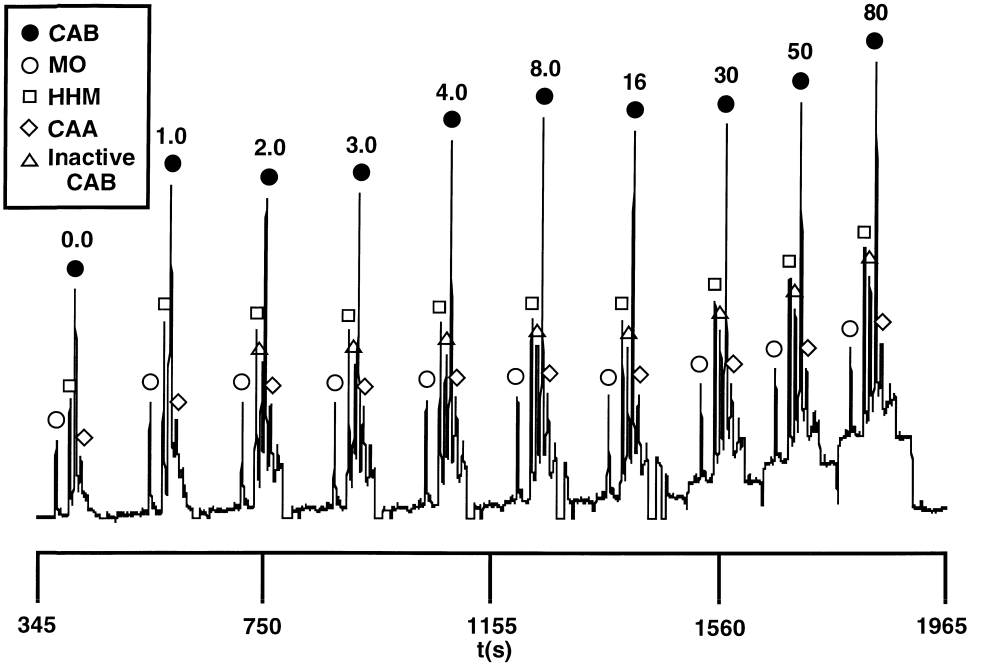


Fig. 5. A representative electropherogram of carbonic anhydrase B (CAB, containing CAA and CAB isozymes) in 0.192 *M* glycine–0.025 *M* Tris buffer (pH 8.3) containing various concentrations of **3** using the multiple-step ligand injection ACE technique. The total analysis time in each experiment was 27 min at 24 kV (current: 4.0 μA) using a 80.5 cm (inlet to detector)×50 μm I.D. open, uncoated quartz capillary. Mesityl oxide (MO) and horseheart myoglobin (HHM) were used as internal standards. The numbers above each set of sample peaks refer to the concentration of **3** in μ*M*.

Table 2
Experimental values of binding constants K_b (10^6 M^{-1}) of ligands **3**–**6** and carbonic anhydrase B measured by the multiple-step ligand injection ACE technique

Ligand	K_b [a]
3	1.0 [b]
4	0.91 [c]
5	2.5 [d]
6	0.91 [e]

[a] The reported binding constants are the average values from three experiments for ligands **3**–**6**.
[b] Previous estimate [1,10,15,31]: $K_b = 0.72–2.0 \cdot 10^6$ M^{-1}.
[c] Previous estimate [1,10,15,24,31]: $K_b = 0.45–0.92 \cdot 10^6$ M^{-1}.
[d] Previous estimate [24]: $K_b = 2.0 \cdot 10^6$ M^{-1}.
[e] Previous estimate [24]: $K_b = 1.1 \cdot 10^6$ M^{-1}.

binding of neutral arylsulfonamides to CAB using a competitive binding assay. In these series of experiments plugs of CAB and non-interacting standards were injected by pressure and electrophoresed in a buffer containing increasing concentrations of **5** and 20 μ*M* **3**. Ligand **3** is a weaker inhibitor of CAB than **5** and, hence, is appropriate to use as a competitive inhibitor in this study. Fig. 7 shows the electropherogram of CAB generated using the multiple-step ligand injection ACE technique with various concentrations of **5**. Upon addition of increasing concentrations of **5** the CAB peak shifts to the left for any given concentration of ligand. MO and HHM are used as neutral and charged markers respectively in the analysis. The complexation between **5** and CAB resulted in a decreasing negative charge on the protein and the complex is detected earlier than when complexed to ligand **1**. Fig. 8 is a Scatchard plot of the data for CAB. Table 2 shows the binding constants obtained for neutral ligands **5** and **6** by Eq. (2).

For the technique to be generally used several requirements must first be satisfied. One, knowledge about the electrophoretic mobilities of the receptor, non-interacting markers, and ligand is necessary in

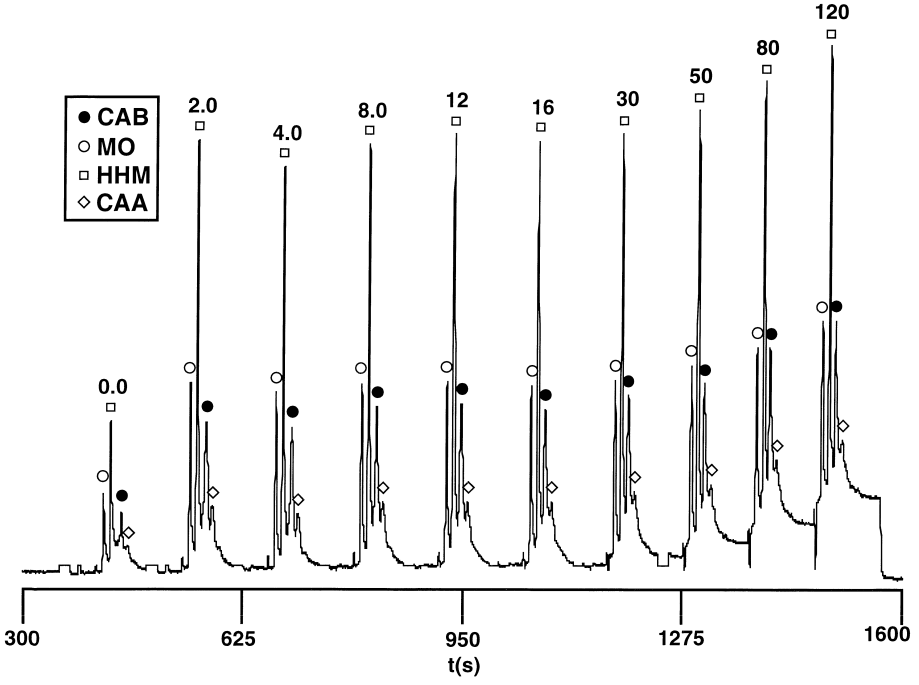


Fig. 7. A representative electropherogram of carbonic anhydrase B (CAB, containing CAA and CAB isozymes) in 0.192 *M* glycine–0.025 *M* Tris buffer (pH 8.3) containing various concentrations of **5** using the multiple-step ligand injection ACE technique. The total analysis time in each experiment was 27 min at 24 kV (current: 4.0 μA) using a 80.5 cm (inlet to detector)×50 μm I.D. open, uncoated quartz capillary. Mesityl oxide (MO) and horseheart myoglobin (HHM) were used as internal standards. The numbers above each set of sample peaks refer to the concentration of **5** in μ*M*.

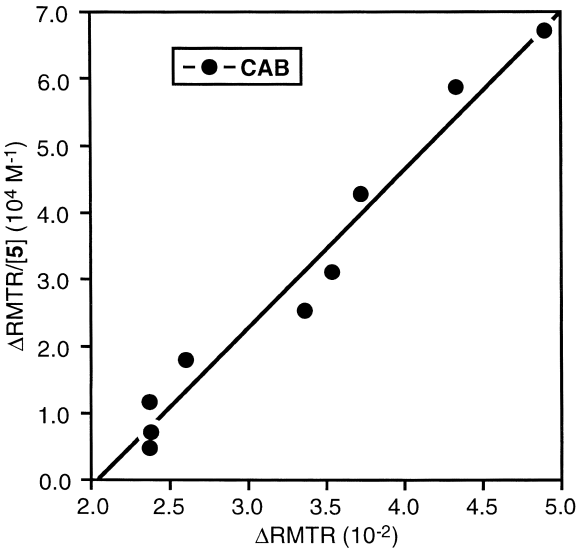


Fig. 8. Scatchard plot of the data for carbonic anhydrase B according to Eq. (2).

order to determine if overlap is sufficient at the point of detection. Two, a dynamic equilibrium must be satisfied between receptor and ligand prior to detection. Three, receptor, non-interacting markers, and ligand must not absorb onto the walls of the capillary in any measureable quantities. Four, the length of the ligand plug must retain a uniform concentration during the time of electrophoresis. Five, ligand injection must be reproducible.

4. Conclusion

Multiple-step ligand injection ACE can effectively be used to estimate binding constants of charged and uncharged ligands to receptors. We have shown this by the use of two model systems: vancomycin and D-Ala-D-Ala peptides and CAB and arylsulfonamides. In these experiments the capillary is sequentially filled with sample and electrophoresed in a buffer containing increasing concentrations of ligand. Binding constants are estimated using the relative migration time ratio. The binding constants obtained by this form of ACE agree well with those obtained by other assay methods and by other ACE techniques. Multiple-step ligand injection ACE has several advantages as a method for measuring biomolecular noncovalent interactions. First, it requires smaller quantities of receptor and ligand than in traditional ACE techniques. Second, the time required for analysis of a given receptor–ligand interaction is greatly reduced as compared to other ACE techniques. Third, the commercial availability of automated instrumentation, and the high reproducibility of data, make it experimentally convenient. Further work to demonstrate the versatility of this technique is in progress.

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