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7 Multiple-injection affinity capillary electrophoresis to estimate 8 binding constants of receptors to ligands

9 Received: 1 April 2005 / Revised: 18 July 2005 / Accepted: 21 July 2005
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11 **Abstract** Multiple-injection affinity capillary electropho-
12 resis (MIACE) is used to determine binding constants (K_b)
13 between receptors and ligands using as model systems
14 vancomycin and teicoplanin from *Streptomyces orientalis*
15 and *Actinoplanes teichomyceticus*, respectively, and their
16 binding to D-Ala-D-Ala peptides and carbonic anhydrase
17 B (CAB. EC 4.2.1.1) and the binding of the latter to
18 arylsulfonamides. A sample plug containing a non-inter-
19 acting standard is first injected followed by multiple plugs
20 of sample containing the receptor and then a final injection
21 of sample containing a second standard. Between each
22 injection of sample, a small plug of buffer is injected which
23 contains an increasing concentration of ligand to effect
24 separation between the multiple injections of sample.
25 Electrophoresis is then carried out in an increasing con-
26 centration of ligand in the running buffer. Continued elec-
27 trophoresis results in a shift in the migration time of the
28 receptor in the sample plugs upon binding to their re-
29 spective ligand. Analysis of the change in the relative mi-
30 gration time ratio (RMTR) or electrophoretic mobility (μ)
31 of the resultant receptor–ligand complex relative to the
32 non-interacting standards, as a function of the concentra-
33 tion of ligand yields a value for K_b . The MIACE technique
34 is a modification in the ACE method that allows for the
35 estimation of binding affinities between biological inter-
36 actions on a timescale faster than that found for standard
37 ACE. In addition sample volume requirements for the
38 technique are reduced compared to traditional ACE assays.
39 These findings demonstrate the advantage of using MIACE
40 to estimate binding parameters between receptors and
41 ligands.

Keywords Multiple-injection affinity capillary · 42
Electrophoresis · Vancomycin · Teicoplanin · 43
Carbonic anhydrase B · Binding constants 44

Abbreviations MIACE: Multiple-injection affinity 45
capillary electrophoresis · Van: Vancomycin · Teic: 46
teicoplanin · RMTR: relative migration time ratio 47

48 Introduction

49 Currently there is a major need to develop analytical tech- 49
50 nologies that can aid in the screening of drugs for many 50
51 health-related problems. For example, Alzheimer's disease, 51
52 AIDS-HIV, and cancer are but a few of the many human 52
53 diseases for which a rapid screening technique would ex- 53
54 pedite the development of a cure and the saving of human 54
55 lives. Over the past ten years advances in molecular bi- 55
56 ology have yielded great insights into the many molecular 56
57 interactions involved in human disease. Such interactions 57
58 are critical in understanding the functions and molecular 58
59 mechanisms of biological systems and the roles these in- 59
60 teractions play in health and human disease. Because of the 60
61 large array of receptors and ligands that have been found, 61
62 and those yet discovered, it is critical to develop analytical 62
63 techniques that can estimate the level and extent of in- 63
64 teraction between biological species. 64

65 During the past decade affinity capillary electrophoresis 65
66 (ACE) has emerged as a useful and sensitive technique for 66
67 studying bimolecular non-covalent interactions and for 67
68 determining binding and dissociation constants of formed 68
69 complexes [1–25]. Since the initial reports in 1992 docu- 69
70 menting the use of CE to study receptor–ligand inter- 70
71 actions, ACE has been successfully used to examine a wide 71
72 array of interactions including protein–drug, protein–DNA, 72
73 peptide–carbohydrate, peptide–peptide, DNA–dye, carbo- 73
74 hydrate–drug, and antigen–antibody [26–30]. For example, 74
75 Kaddis et al. recently used ACE to determine binding 75
76 constants for the activator fructose-6-phosphate (F6P) and 76
77 substrate ATP to the recombinant wild-type (WT) *Rhodo-* 77
78 *bacter sphaeroides* adenosine 5'-diphosphate-(ADP)-glu- 78

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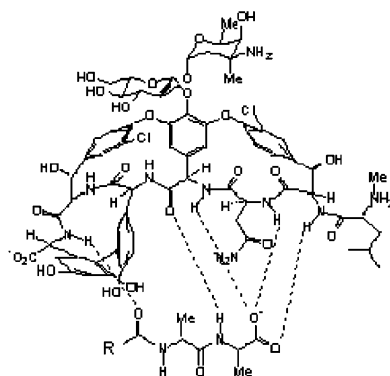
79 cose pyrophosphorylase (ADPGlc PPase) [1]. McKeon et
 80 al. used ACE to determine the binding between heparin-
 81 like glycosaminoglycans and the heparin-binding domain
 82 (96–110) of amyloid precursor protein (APP) [2]. Finally,
 83 Varenne et al. used ACE to examine the binding of
 84 fucoidan, an anticoagulant polysaccharide of marine ori-
 85 gin, to antithrombin [3]. The underlying principle of ACE
 86 is that the electrophoretic mobility of a receptor (μ_R)
 87 changes upon binding to a ligand that is present in the
 88 electrophoresis buffer. The change in μ (or other parameter
 89 if an alternate form of analysis is used) as a function of
 90 ligand concentrations allows binding constants to be de-
 91 termined via Scatchard analysis [15].

92 Although ACE has been shown to be effective in
 93 estimating binding parameters of ligands to receptors, in
 94 cases where only small quantities of material are available,
 95 analysis by traditional ACE techniques is made difficult. In
 96 addition, expeditious analysis of the interaction in question
 97 may also be desirable particularly when combinatorial
 98 approaches to drug design are utilized. In cases where both
 99 conditions are needed, modifications in the standard ACE
 100 technique are warranted.

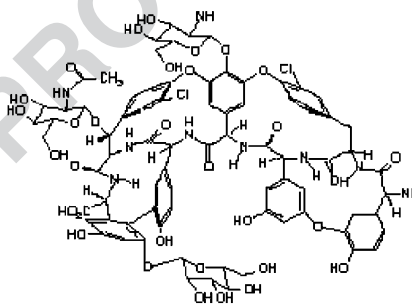
101 In two previous studies we described multiple-plug
 102 injection sequences that allowed for the estimation of
 103 binding constants between receptors and ligands [14, 22].
 104 In the first study, a series of four plugs containing receptor
 105 and standards were injected and exposed to an increasing
 106 concentration of ligand in the electrophoresis buffer [22].
 107 Four binding constants for the same receptor–ligand inter-
 108 action were subsequently obtained. A drawback to this
 109 technique was that the number of injections was limited as
 110 too short a delay between injections caused unwanted
 111 overlap of adjacent plugs of sample thereby making separa-
 112 tion and differentiation of peaks difficult. In the second
 113 study, a more elaborate plug sequence was demonstrated
 114 utilizing partial-filling techniques [14]. Here, a sample of
 115 receptor and standards were multiply injected each separa-
 116 ted by an increasing concentration of ligand. The whole
 117 sequence of ligand concentrations could be visualized in
 118 one electropherogram and a shift in the receptor migration
 119 time was observed thereby allowing for the estimation of
 120 a binding constant. This technique, too, had a drawback
 121 in that a long capillary was needed for the separation thus
 122 lengthening the time required for the experiment. In addi-
 123 tion, timing was critical since receptor and markers were
 124 required to migrate into the zone of ligand by the point
 125 of detection.

126 Herein we describe a new technique called multiple-
 127 injection affinity capillary electrophoresis (MIACE) using
 128 as model systems vancomycin (Van) and teicoplanin (Teic)
 129 (Fig. 1a) and carbonic anhydrase B. In this technique
 130 multiple injections of sample containing receptor, non-
 131 interacting standards, and buffer are injected. The sample
 132 of buffer and running buffer contain an increasing con-
 133 centration of ligand that, upon interaction with the sample,
 134 causes a shift in migration time of the receptor due to
 135 complexation with the ligand. Subsequent Scatchard anal-
 136 ysis yields a value for K_b . The present study requires only
 137 one set of standards and can accommodate multiple in-

A

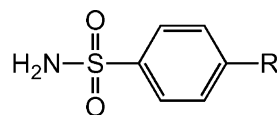
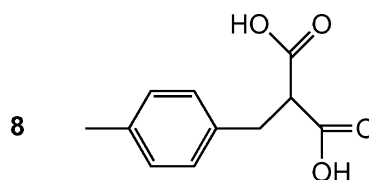


- R
- 1 Fmoc-Gly-
 - 2 Fmoc-Ala-
 - 3 Fmoc-Phe-
 - 4 Fmoc-Val-
 - 5 *N*-Acetyl-
 - 6 *N,N*-Diacetyl-Lys-



- R
- 1 Fmoc-Gly-
 - 2 Fmoc-Ala-
 - 5 *N*-Acetyl-

B

7 R = CO₂H

8

Fig. 1 a Structures of vancomycin (*top*), teicoplanin (*bottom*), and D-Ala-D-Ala ligands 1–6 used in this study. b Ligands 7 and 8 used in this study

jections of receptor. The number of receptor injections can
 be increased and is dependent on the capillary length and
 applied voltage.

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 139
 140

141 **Materials and methods**

142 Chemicals and reagents

143 All chemicals were of analytical grade. Vancomycin, D-
144 Ala-D-Ala, *N*-acetyl-D-Ala-D-Ala (**5**), *N,N'*-diacetyl-Lys-
145 D-Ala-D-Ala (**6**), nicotinamide adenine dinucleotide (NAD),
146 and 4-carboxybenzenesulfonamide (CBSA) (**7**) were pur-
147 chased from Sigma Chemical Company (St. Louis, MO,
148 USA) and were used without further purification. Teico-
149 planin-HCl was purchased from Advance Separation Tech-
150 nologies Inc. (Whippany, NJ, USA) and was used without
151 further purification. Fmoc-Gly-NHS, Fmoc-Ala-NHS, Fmoc-
152 Phe-NHS, and Fmoc-Val-NHS were purchased from Bachem
153 California Inc. (Torrance, CA, USA). Mesityl oxide (MO)
154 was purchased from Calbiochem (San Diego, CA, USA).
155 Stock solutions of Van (0.2 mg mL^{-1}), Teic (1.0 g L^{-1}), and
156 CBSA (0.5 g L^{-1}) were each prepared by dissolving in
157 buffer (20 mM phosphate buffer; pH 7.5). Stock solutions
158 of the *N*-protected amino acids (4 mM) were prepared by
159 dissolving the compounds in buffer. Fmoc-Gly-D-Ala-D-
160 Ala (**1**), Fmoc-Ala-D-Ala-D-Ala (**2**), Fmoc-Phe-D-Ala-D-
161 Ala (**3**), and Fmoc-Val-D-Ala-D-Ala (**4**) were prepared
162 based on literature procedures [31]. Compound **8** was a
163 gift of S. Mallik (North Dakota State University) [32].

164 Apparatus

165 The capillary electrophoresis (CE) system used in this
166 study was a Beckman Model P/ACE 5510 (Fullerton, CA,
167 USA). The capillary tubing (Polymicro Technology, Inc.,
168 Phoenix, AZ, USA) used for the experiment was uncoated
169 fused silica with an internal diameter of 50 μm , length from
170 inlet to detector of 40.5 cm (49 cm for Teic and compounds
171 **1** and **2**), and a length from detector to outlet of 6.5 cm (11
172 cm for Teic and compounds **1** and **2**). The conditions used
173 in CE were as follows: for Van and Teic, voltage, 25 kV;
174 current, 6.8 μA for Van, 7.9 μA for Teic; detection, 200
175 nm; temperature, $23.0 \pm 0.1^\circ\text{C}$; for CAB, voltage, 30 kV;
176 current, 7.5 μA ; detection, 200 nm; temperature, $23.0 \pm$
177 0.1°C . Data were collected and analyzed with Beckman
178 System Gold or 32 Carot software.

179 Procedures

180 For Van, the capillary was first equilibrated with buffer
181 (192 mM glycine/25 mM Tris; pH 8.3) containing in-
182 creasing concentrations of peptide (0–100 μM). Separate
183 plugs of sample solution (3.6 nL each) containing the
184 marker mesityl oxide (MO), five plugs of Van, and second
185 marker (CBSA) were then introduced by pressure injection
186 each separated by a plug of buffer (18-s injection). The
187 electrophoresis was carried out using Tris-glycine buffer
188 with increasing concentrations of ligand at 25 kV for
189 6.0 min to complete the detection of all species. Exper-
190 imental conditions for Teic and CAB were similar except
191 only three plugs of receptor were introduced and exper-

iments were run for 7.0 min to Teic. For CAB, horse heart
myoglobin (HHM) was used as a marker and was intro-
duced directly after introduction of the first marker MO.

Forms of analysis

Two forms of analysis, one using two markers and the other
using a single marker, were used to estimate K_b . In the
dual marker form of analysis, K_b is estimated using two
non-interacting standards, which we term the relative migra-
tion time ratio (RMTR) (Eq. (1)) [17].

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

Here, t_p , t_s , and $t_{s'}$ are the measured migration times of the
antibiotic peak (e.g., Van), and two non-interacting stan-
dard peaks (CBSA and MO), respectively. A Scatchard plot
can be obtained via Eq. (2).

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

Here, $\Delta \text{RMTR}_{P,A}$ is the magnitude of change in the RMTR
as a function of the concentration of peptide. Equation 2
allows for the estimation of K_b on a relative time scale
using two non-interacting standards and corrects for
changes in EOF that occur in the capillary column due to
variations in viscosity and Joule heating.

In the second form of analysis, K_b is estimated using
one non-interacting standard which relates changes in the
electrophoretic mobility ($\mu_{P,L}$) of, for example, Van on
complexation with the ligand (L) present in the buffer to
 K_b . Analysis of the magnitude of the change in mobility,
 $\Delta \mu_{P,L}$ as a function of the concentration [L] of ligand
yields K_b (Eq. (3)).

$$\begin{aligned} \Delta \mu_{P,L} &= \mu_{P,L} - \mu_P \\ &= l_c l_d / V [(1/t_{P,L} - 1/t_{m,L}) - (1/t_P - 1/t_m)] \quad (3) \end{aligned}$$

Here, $\Delta \mu_{P,L}$ is the change in mobility of Van as a function
of the concentration of peptide; $t_{P,L}$ and $t_{m,L}$ are the
measured migration times of Van and a non-interacting
standard (e.g., MO or CBSA) at the concentration of
peptide, respectively; l_c (cm) is the total length of the
capillary; l_d (cm) is the length of capillary from the inlet
end of the capillary to the detector; t_m (s) is the measured
migration time of the non-interacting standard; t_P is the
measured migration time of the Van; and V is the voltage
across the capillary. The values of $\Delta \mu_{P,A}$ obtained using
Eq. (3) over a range of concentrations of peptide are then
used for Scatchard analysis (Eq. (4)).

$$\Delta \mu_{P,A} / [A] = K_b \Delta \mu_{P,A}^{\max} - K_b \Delta \mu_{P,A} \quad (4)$$

In general, either Eq. (2) or (4) can be used for ACE
studies although we have found that the use of two markers

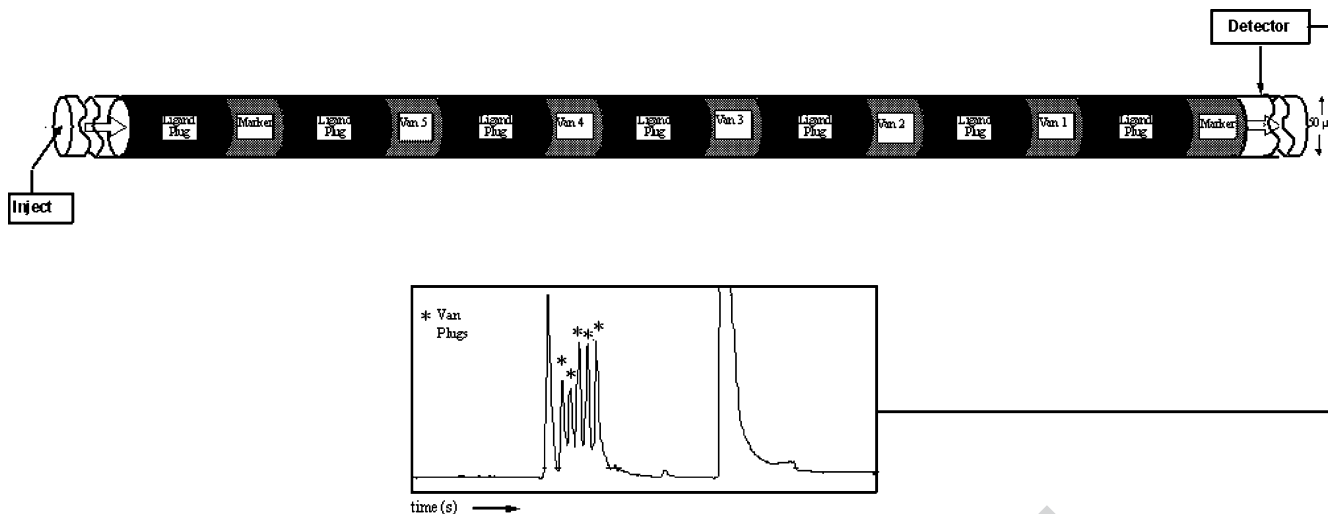


Fig. 2 Schematic of a multiple-injection affinity capillary electrophoresis (MIACE) experiment

240 (Eq. (2)) yields a more accurate value for K_b especially
241 when EOF varies from run to run.

242 Results and discussion

243 In the first series of experiments, we examined the binding
244 of D-Ala-D-Ala terminus peptides to Van by using the
245 MIACE technique. Van-group antibiotics are glycopep-
246 tides that kill bacterial cells by inhibiting peptidoglycan
247 biosynthesis [33–40]. They function by binding to the
248 terminal D-Ala-D-Ala dipeptide (binding also occurs to
249 D-Tyr-D-Ala and D-Tyr-D-Ser terminus peptides) of bac-
250 terial cell wall precursors, thereby impeding further pro-
251 cessing of these intermediates into peptidoglycan.

252 In the MIACE technique, a plug of sample (0.5 psi at 3 s)
253 containing the non-interacting standard mesityl oxide
254 (MO) was first injected into the capillary column followed
255 by five plugs (0.5 psi at 3 s) of sample containing Van
256 (Fig. 2). Between each injection of Van was placed a small
257 plug (0.5 psi at 18 s) of buffer to aid in the separation
258 of all Van peaks. A final plug (0.5 psi at 3 s) of the
259 second non-interacting standard, 4-carboxybenzenesulfon-
260 amide (CBSA), was then injected and electrophoresed.

261 Upon electrophoresis individual plugs of sample migrate
262 through the capillary column to afford seven peaks (five for
263 Van and two for the standards) at the point of detection.
264 Figure 3 shows a representative series of electrophero-
265 grams of Van in a capillary filled with increasing con-
266 centrations of 1 at 200 nm. The peaks for Van are not
267 baseline resolved but can easily be differentiated from each
268 other. As the concentration of 1 was increased (0–300 μ M)
269 in the running buffer the peaks for Van shifted to longer
270 migration times as the Van–1 complexes are more negative
271 than Van itself. The inverted peaks to the right of the Van
272 peaks are due to the dilution of ligand 1 in the running
273 buffer upon complexation to Van. These negative peaks are
274 commonly observed in ACE studies and are particularly

275 pronounced when the ligand or receptor in the running
276 buffer are chromophoric and/or when high concentrations
277 of the ligand/receptor are used for the binding assay. Due
278 to the higher mass of the newly formed complex upon
279 increasing the concentration of ligand 1, the height of the
280 peaks for Van increase in comparison to the MO marker.
281 Analysis of the change in the relative migration time ratio
282 (RMTR) or electrophoretic mobility (μ) of the resultant
283 complex relative to the non-interacting standards, as a

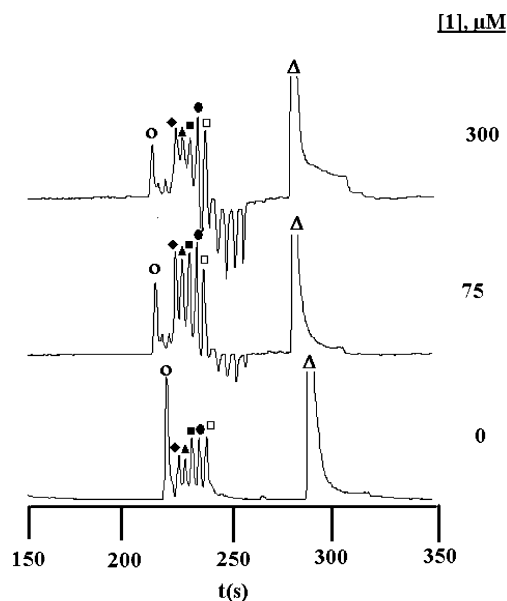


Fig. 3 Representative set of electropherograms of Van (filled diamonds, triangles, squares, and circles, and open squares) in 192 mM glycine/25 mM Tris buffer (pH 8.3) containing various concentrations of ligand 1 using the MIACE technique. The total analysis time in each experiment was 6.0 min at 25 kV (current 6.8 μ A) using a 40.5-cm (inlet to detector), 50- μ m I.D. open, uncoated quartz capillary. MO (open circles) and CBSA (open triangles) were used as internal standards

284 function of the concentration of ligand (or receptor), yields
 285 a value for K_b .

286 Figure 4 shows Scatchard plots of the data for the five
 287 Van peaks using Eq. (2); all five Van peaks result in similar

Scatchard plots. In this experiment three repetitions were
 run at each concentration of ligand and the third repetition
 was used for the analysis. In our previous work with ACE
 we have observed that the initial repetitions tend to yield

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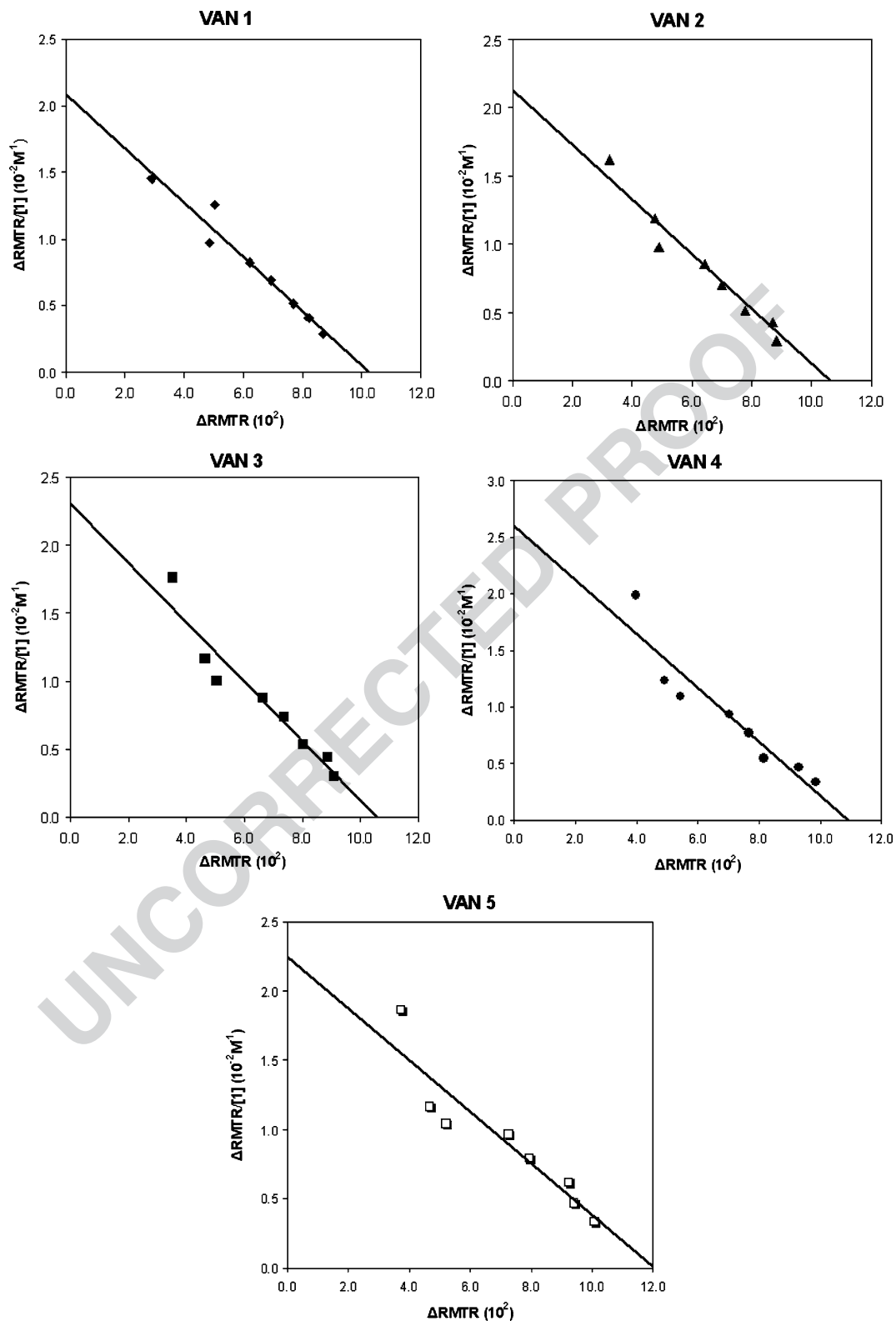


Fig. 4 Scatchard plots of the data for Van and ligand 1 according to Eq. (2)

Van peak	K_b	R^2
1	21.4	0.950
2	22.5	0.960
3	23.0	0.922
4	25.1	0.890
5	19.6	0.871

Table 1 Experimental values of binding constants K_b ($10^3 M^{-1}$) of ligand **1** to Van obtained by Eq. (2)

^aPrevious estimates [19]: $K_b=41.6 \times 10^3 M^{-1}$ (pH 7.5)

292 binding constants with smaller values for R^2 [1, 4, 6]. We
 293 attribute this to the capillary not having reached equilib-
 294 rium with the ligand in the running buffer. Table 1 sum-
 295 marizes the binding data obtained for Van and ligand 1.
 296 The average binding constant was determined to be $22.3 \times$
 297 $10^3 M^{-1}$. In our previous work we determined a value for
 298 K_b of $41.6 \times 10^3 M^{-1}$ [19]. This study, though, was con-
 299 ducted at a pH of 7.5 and in phosphate buffer. It should
 300 be noted that previous studies have shown that pH affects
 301 the value of K_b obtained by both ACE and other assay
 302 techniques.

303 We then determined the binding affinities of Van to
 304 ligands 2–6 by using a similar injection sequence and the
 305 results are summarized in Table 2. The binding constants
 306 obtained using MIACE were comparable to those deter-
 307 mined using standard ACE techniques [4, 17–19].

308 We also examined the binding of several D-Ala-D-
 309 Ala terminus peptides to Teic. Like Van, Teic is a linear
 310 heptapeptide, cross-linked between residues 1 and 3, 2 and
 311 4, and 4 and 6 by diphenyl ether bridges and between
 312 residues 5 and 7 by a biphenyl bridge. Teic is a mixture of
 313 five closely related analogues, designated T-A₁₋₁ through
 314 T-A₁₋₅ [6, 18]. They differ by approximately 20 molecu-
 315 lar mass units because of the variation of the carbon length and
 316 substituent groups of the hydrophobic acyl side chain
 317 (hydrophobic tail) that is attached to a 2-amino-2-deoxy- β -
 318 D-glucopyranosyl moiety. This hydrophobic tail gives Teic
 319 its unique characteristics. Table 2 lists the binding data for
 320 Teic and ligands 1, 2, and 5. In general, the values for K_b
 321 obtained by MIACE parallel the values found in previous
 322 ACE studies [4, 17–19].

Antibiotic	Ligand	K_b
Teic	1	144.0 ^a
Van	2	15.3 ^b
Teic	2	21.1 ^c
Van	3	40.2
Van	4	25.9
Van	5	3.7 ^d
Teic	5	20.7 ^e
Van	6	43.0

Table 2 Experimental values of binding constants K_b ($10^3 M^{-1}$) of ligands 1–6 to Van and Teic obtained by Eqs. (2) and (4)

^aPrevious estimates [4, 19]: $K_b=220 \times 10^3 M^{-1}$ (pH 8.3),
 $K_b=21.8 \times 10^3 M^{-1}$ (pH 7.5)

^bPrevious estimate [19]: $K_b=174.5 \times 10^3 M^{-1}$ (pH 7.5)

^cPrevious estimate [18]: $K_b=185.1 \times 10^3 M^{-1}$ (pH 7.5)

^dPrevious estimate [17, 19]: $K_b=3.6-5.0 \times 10^3 M^{-1}$ (pH 8.3)

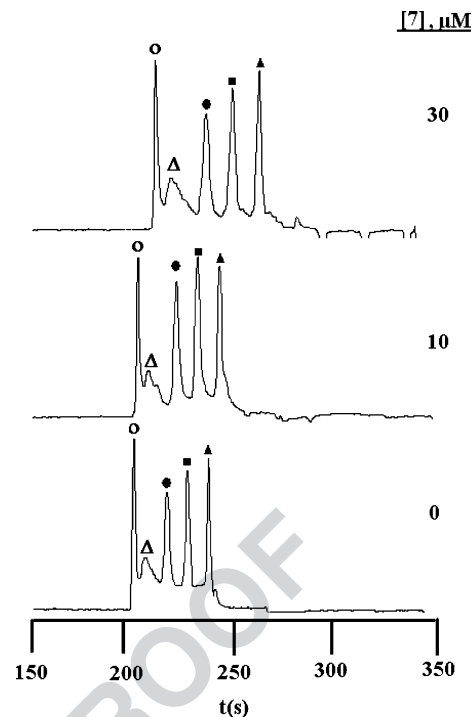


Fig. 5 Representative set of electropherograms of CAB (filled diamonds, squares, and triangles) in 192 mM glycine/25 mM Tris buffer (pH 8.3) containing various concentrations of ligand 7 using the MIACE technique. The total analysis time in each experiment was 6.0 min at 30 kV (current 7.5 μ A) using a 40.5-cm (inlet to detector), 50- μ m I.D. open, uncoated quartz capillary. MO (open circles) and HHM (open triangles) were used as internal standards

323 We then examined the binding of arylsulfonamides to
 324 the enzyme CAB. CAB is a zinc protein of the lyase class
 325 that catalyzes the equilibration of dissolved carbon dioxide
 326 and carbonic acid. It is strongly inhibited by sulfonamide-
 327 containing molecules. Using a similar series of injections
 328 as that used for the Van studies, three samples of CAB were
 329 injected onto the capillary column and their binding to
 330 ligands 7 and 8 (Fig. 1b) examined. Figure 5 shows a
 331 representative series of electropherograms of CAB in a
 332 capillary filled with increasing concentrations of 7 at 200
 333 nm. Compound 7 is negatively charged and when bound to
 334 CAB shifts the migration time of the CAB–7 complex to a
 335 greater migration time. Table 3 lists the values for K_b for
 336 ligands 7 and 8 binding to CAB. Negative peaks represent
 337 the dilution of ligand in the electrophoresis buffer upon
 338 binding to CAB. The voids are common in ACE techniques
 339 and are more pronounced when the ligand (or receptor) in
 340 the running buffer is highly chromophoric.

Table 3 Experimental values of binding constants K_b ($10^5 M^{-1}$) of ligands 7 and 8 to CAB obtained by Eqs. (2) and (4)

Ligand	K_b
7	4.84 ^a
8	4.47

^aPrevious estimates [14]: $K_b=0.72-2.0 \times 10^6 M^{-1}$ (pH 8.3)

341 **Conclusion**

342 The present study demonstrates the ease of using multiple-
 343 injection ACE (MIACE) to estimate binding constants
 344 between receptors and ligands. Vancomycin, teicoplanin,
 345 and carbonic anhydrase B were used as model systems and
 346 estimates of binding constants of these receptors to their
 347 respective ligands were derived. In this technique multiple
 348 injections of sample containing receptor were subjected to
 349 an increasing concentration of ligand in the running buffer.
 350 Scatchard analysis was applied to the change in migration
 351 time upon formation of complex to afford multiple binding
 352 constants comparable to those obtained using other ACE
 353 techniques and traditional assay methods. The advantages
 354 of MIACE are several. One, smaller quantities of ligand are
 355 needed to conduct the studies in comparison to other assay
 356 techniques. Two, purification of the sample prior to injection
 357 is not necessary as long as the component to be
 358 analyzed can be separated from other species. Three, multiple
 359 binding constants can be obtained in a series of ACE
 360 experiments thus shortening the amount of time required
 361 to conduct the assay. Finally, the commercial availability
 362 of automated instrumentation, and high reproducibility of
 363 data, make it experimentally convenient. This work serves
 364 as a foundation for using MIACE to probe the binding of
 365 other receptor–ligand combinations. Future work will focus
 366 on expanding the versatility of the technique by modifying
 367 the plug-plug injection sequence to other systems.

368 **Acknowledgements** The authors gratefully acknowledge financial
 369 support for this research by grants from the National Science
 370 Foundation (CHE-0136724 and DMR-0351848), and the National
 371 Institutes of Health (R15 AI055515–01). K.M. was a Partnership for
 372 Research and Education in Materials (PREM) High School Fellow.

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