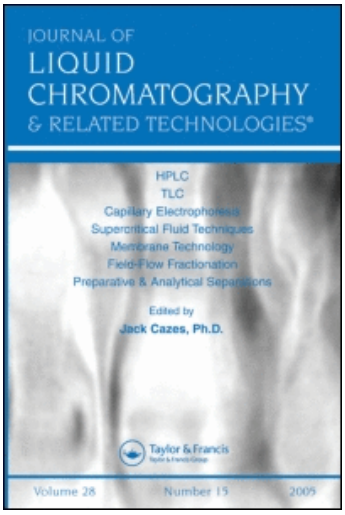

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DETERMINATION OF BINDING CONSTANTS BETWEEN TEICOPLANIN AND D-ALA-D-ALA TERMINUS PEPTIDES BY AFFINITY CAPILLARY ELECTROPHORESIS

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DETERMINATION OF BINDING CONSTANTS BETWEEN TEICOPLANIN AND D-ALA-D-ALA TERMINUS PEPTIDES BY AFFINITY CAPILLARY ELECTROPHORESIS

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ABSTRACT

Binding constants between the glycopeptide antibiotic, Teicoplanin (Teic), and D-Ala-D-Ala terminus peptides were determined by affinity capillary electrophoresis (ACE) and by on-column ligand synthesis coupled to ACE. In the first technique, a plug of Teic and two non-interacting standards are injected and electrophoresed. Analysis of the change in the relative migration time ratio (RMTR) of Teic, relative to the non-interacting standards, as a function of the concentration of D-Ala-D-Ala peptide, yields a value for the binding constant. In the second technique, 9-fluorenylmethoxycarbonyl (Fmoc)-amino acid-D-Ala-D-Ala species are first synthesized on-column. The initial sample plug contains a D-Ala-D-Ala terminus peptide and two non-interacting standards. Plugs two and three contain solutions

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of Fmoc-amino acid-*N*-hydroxysuccinimide (NHS) ester and buffer, respectively.

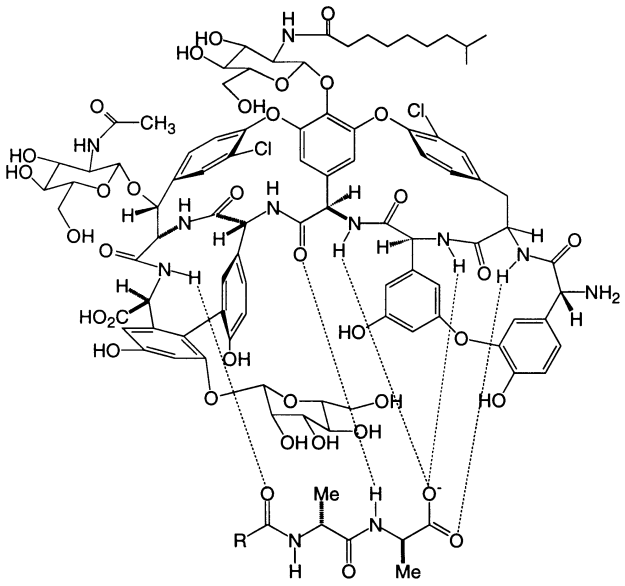
Upon electrophoresis, the initial D-Ala-D-Ala peptide reacts with the Fmoc-amino acid NHS ester, yielding the Fmoc-amino acid D-Ala-D-Ala peptide. Continued electrophoresis results in the overlap of the Teic in the running buffer and the plug of Fmoc-amino acid-D-Ala-D-Ala peptide, and non-interacting markers. Subsequent analysis of the change in RMTR of the peptide relative to the non-interacting standards, as a function of the concentration of Teic, yields a value for the binding constant. Binding constants were determined for five ligands and Teic by ACE and by on-column ligand synthesis coupled to ACE. The findings described here demonstrate the advantage of using ACE and on-column ligand synthesis techniques coupled to ACE for estimating binding parameters between antibiotics and ligands.

Key Words: Teicoplanin; Affinity capillary electrophoresis; Binding constant

INTRODUCTION

Teicoplanin (Teic), a glycopeptide antibiotic from *Actinoplanes teichomyceticus*, disrupts the growth of methicillin-resistant *Staphylococcus aureus* and other Gram-positive organisms, by inhibiting cell wall biosynthesis (Fig. 1).[1] It binds to the D-Ala-D-Ala terminus of bacterial cell wall precursors, thereby, impeding further processing of these intermediates into peptidoglycan.[2] Unlike the closely related antibiotic, vancomycin, there is limited binding constant information for Teic and D-Ala-D-Ala peptides. These studies, though, have shown the affinity between Teic and D-Ala-D-Ala peptides to be stronger than that between vancomycin and these peptides.[3,4] Due to the lack of efficient and accurate analytical techniques to characterize the extent of interaction between Teic and its derivatives with target molecules, the development of new techniques to measure binding parameters is warranted.

The ability to accurately determine the extent of interaction between antibiotics and their target molecules is basic with respect to determining the efficacy of these drugs for treatment of disease. Affinity capillary electrophoresis (ACE) is one such technique that has emerged as a useful and sensitive method for studying bimolecular noncovalent interactions, and for determining binding and dissociation constants of formed complexes.[5–32] This technique uses the resolving power of CE to distinguish between the free and bound forms of a



R
1 *N*-acetyl-
2 N_α,N_ε-diacetyl-lys-
3 *N*-succinyl-
4 Fmoc-Gly-
5 Fmoc-Ala-

Figure 1. Teicoplanin A2-2 and D-Ala-D-Ala ligands **1**–**5** used in this study.

receptor as a function of the concentration of free ligand.[5] 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 Scatchard analysis yields a value for the binding constant, K_b.

A number of interactions have been examined by ACE, affording information on binding parameters. For example, ACE was used by Qian et al. to investigate an epitope on human immunodeficiency virus by a monoclonal antibody.[6] Mito et al. introduced a form of analysis termed relative migration time ratio (RMTR) to obtain binding constants for ligands of carbonic anhydrase and vancomycin.[7] Finally, Kiessig et al. have used ACE to examine the interaction of the enzyme cyclophilin with the immunosuppressive drug cyclosporin A.[8]

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We recently described a two-step procedure whereby on-ligand synthesis and partial-filling affinity capillary electrophoresis (PFACE) were coupled to each other to determine binding constants of *N*-protected amino acid-D-Ala-D-Ala species to vancomycin. In these studies, differential electrophoretic mobility is utilized to merge zones of analyte and reagent(s) under the influence of an electric field. Upon electrophoresis, zones of sample overlap yielding product(s), which is then transported to the detector.

In this paper, we determine binding constants between Teic and D-Ala-D-Ala peptides using ACE. In the first technique, an increasing concentration of peptide in the running buffer is used to create a shift in migration time of the formed Teic-peptide complex. Subsequent Scatchard analysis yields a value for the binding constant. In the second technique, Fmoc-amino acid peptides are synthesized on-column, and are subsequently evaluated for their binding to Teic using ACE. This data demonstrates the advantages of using these CE methods for estimating affinity parameters between antibiotics and their ligands.

EXPERIMENTAL

Chemicals and Reagents

All chemicals were analytical grade. Teic·HCl was purchased from Advanced Separation Technologies Inc. (Whippany, NJ, USA) and was used without further purification. Nicotinamide adenine dinucleotide (NAD), D-Ala-D-Ala, *N*-acetyl-D-Ala-D-Ala, N_{α}, N_{ε}-diacetyl-Lys-D-Ala-D-Ala, carbonic anhydrase B (CAB, EC 4.2.1.1, containing CAA and CAB isozymes from bovine erythrocytes, carboxybenzene sulfonamide (CBSA), and horse heart myoglobin (HHM) were purchased from Sigma Chemical Company (St. Louis, MO, USA)) and were used without further purification. Mesityl oxide (MO) was purchased from Calbiochem (San Diego, CA, USA). Benzoic acid (BA) was purchased from Fisher Scientific Company (Fair Lawn, NJ, USA). Fmoc-Gly-D-Ala-D-Ala and *N*-succinyl-D-Ala-D-Ala were synthesized off-column, based on literature procedures.[32] Stock solutions of bovine carbonic anhydrase B (1 mg mL^{-1}), Teic (0.5 mg mL^{-1}), and horse heart myoglobin (0.5 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 BA (1.0 mg/mL), NAD (1 mM), CBSA (2 mM), and D-Ala-D-Ala (10 mM) were each prepared by dissolving in buffer (20 mM phosphate buffer; pH 8.3). Stock solutions of Fmoc-Gly-NHS ester (10 mM) and Fmoc-Ala-NHS ester (10 mM) were prepared by dissolving the compounds in acetonitrile.

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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, Inc., Phoenix, AZ, USA) used for the ACE experiments was of uncoated fused silica with an internal diameter of 50 μm, length from inlet to detector of 30.5 cm, and a length from detector to outlet of 6.5 cm.

The conditions used in CE were as follows: voltage, 25 kV current, 7.6–8.0 μA; detection, 200 and 205 nm; temperature, 25.0 + 0.1°C. The capillary tubing (Polymicro Technologies, Inc., Phoenix, AZ, USA) used for the on-column synthesis coupled with ACE experiments was of uncoated fused silica with an internal diameter of 50 μm, length from inlet to detector of 50.5 cm, and a length from detector to outlet of 6.5 cm. The conditions used in CE were as follows: voltage, 18 kV; current, 3.5–4.0 μA; detection, 205 and 214 nm; temperature, 25.0 + 0.1°C. Data were collected and analyzed with Beckman System Gold software.

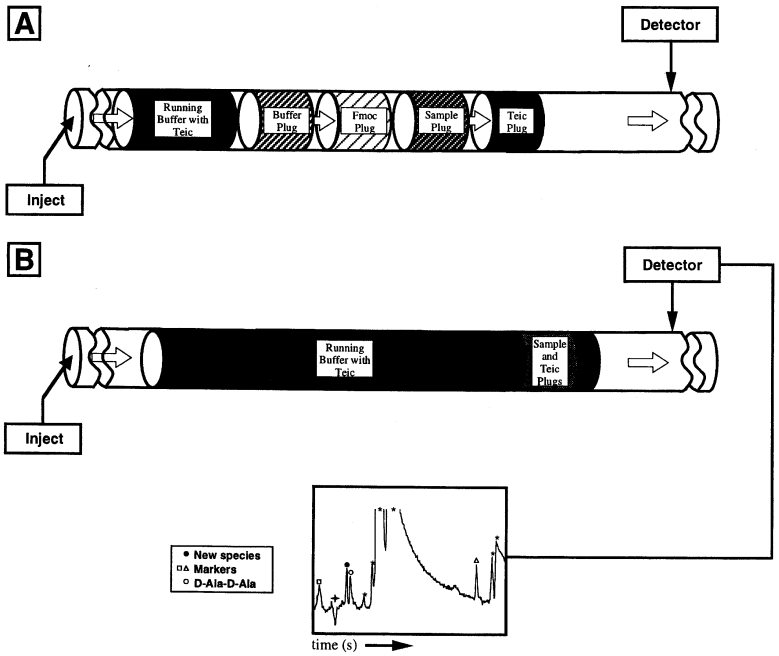

Figure 2. Schematic of an on-column synthesis ACE experiment.

Procedures

In the general ACE technique, a sample (3.6 nL) of Teic (0.02 mg mL^{-1}), CAB (0.04 mg mL^{-1}), HHM (0.07 mg mL^{-1}), and MO (0.08 mg mL^{-1}) in buffer was vacuum injected into the capillary for 3 sec at high pressure. The electrophoresis was carried out using a 192 mM Tris-25 mM Gly buffer (pH 8.3) and repeated at increasing concentrations of the ligand (0.5–200 uM) for 2.0 min. In the on-column ligand synthesis ACE technique (Fig. 2), the capillary was first equilibrated with buffer (192 mM Tris-25 mM Gly; pH 8.3) containing increasing concentrations of Teic (0–25 μM). Separate plugs (3.6 nL) (a 1-s time of injection equates to 1.2 nl of volume of liquid) of buffer (20 mM phosphate; pH 8.3), sample solution (1.2 nL) containing D-Ala-D-Ala terminus peptides, and the non-interacting markers (NAD and CBSA or CAB and BA), Fmoc-amino acid-NHS ester in acetonitrile (2.4 nL), and buffer (3.6 nL) (20 mM phosphate; pH 8.3) were then introduced by pressure injection. The electrophoresis was carried out using the Tris-Gly buffer with increasing concentrations of Teic (0–25 μM) and run at 18 kv for 6.5 min. to complete the detection of all species.

RESULTS AND DISCUSSION

The sample of Teic used in this study was comprised of five closely related compounds differing at the *N*-acyl aliphatic chain at the *β*-D-glucosamine (Fig. 1).[33–35] Using this mixture, we focused on obtaining binding constants between Teic A2-2, the main component in the complex, and D-Ala-D-Ala terminus peptides.

In the first series of experiments, we examined the interaction between Teic A2-2 and *N*-acetyl-D-Ala-D-Ala, **1**. Previous studies have shown that **1** has a greater affinity for Teic than to vancomycin. Here, a plug of sample containing Teic, HHM, CAB, and MO was vacuum injected into the capillary at high pressure for 3 sec. The sample was then electrophoresed in a running buffer containing increasing concentrations of **1**. HHM, CAB, and MO do not interfere in the binding event and, hence, were used as standards in the data analysis.

Figure 3 shows a representative series of electropherograms of Teic in buffer containing increasing concentrations (0 to 200 μM) of **1**. At increasing concentrations of **1** in the running buffer, the Teic peak shifts to the right relative to its initial position at $[\mathbf{1}] = 0\,\mu M$. The complexation between **1** and Teic resulted in an increasing negative charge and the complex is detected later than the uncomplexed form. Some peak broadening was observed at intermediate concentrations of **1**. This is caused by the retardation of migrating molecules due to their frequent interactions with the ligand in the region of intermediate status. The inverted peak (*) is observed above $[\mathbf{1}] = 20\,\mu m$ and is

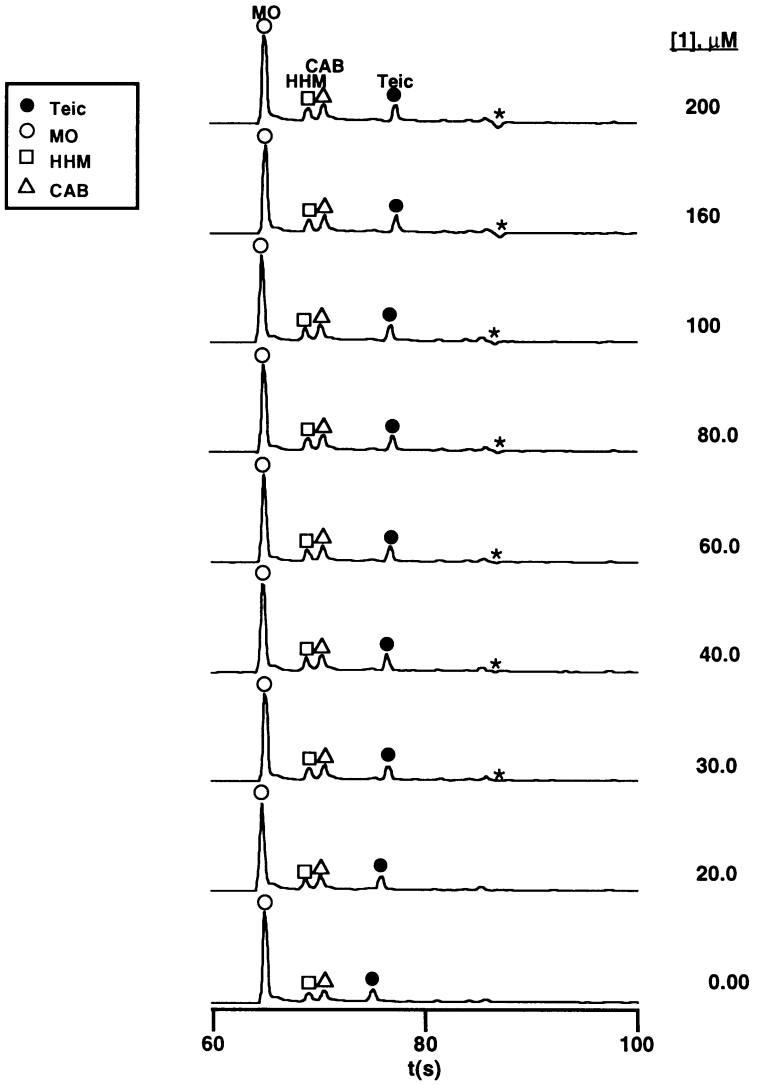

Figure 3. A representative set of electropherograms of Teic in 0.192 M glycine-0.025 M Tris buffer (pH 8.3) containing various concentrations of **1** using affinity capillary electrophoresis. The total analysis time in each experiment was 2.0 min at 25 kV (current: 7.6–8.0 μA) using a 30.5 cm (inlet to detector), 50 μm I.D. open, uncoated quartz capillary. Mesityl oxide (MO), carbonic anhydrase B (CAB), and horse heart myoglobin (HHM) were used as internal standards. The symbol (*) is explained in the text.

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due to the dilution of **1** upon complexation to Teic. The other four compounds in the Teic complex were present in the sample in small quantities, and are observed as small peaks eluting after Teic A2-2. These peaks were not reproducible and, hence, binding constants could not be estimated for their interaction with **1**.

Figure 4 is a Scatchard plot of the data for Teic. In this form of analysis K_b is estimated using a dual marker form of analysis, which we term the relative migration time ratio (RMTR) Eq. (1).

$$\text{RMTR} = (t_r - t_s')/(t_s' - t_s) \tag{1}$$

Here, t_r, t_s, and t_s' are the measured migration times of the receptor peak, and 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).

$$\Delta\text{RMTR}_{R,L}/[L] = K_b\Delta\text{RMTR}_{R,L}{}^{\max} - K_b\Delta\text{RMTR}_{R,L} \tag{2}$$

Here, $\Delta R_{R,L}$ is the magnitude of the change in the RMTR as a function of the concentration of ligand. Equation 2 allows for the estimation of smaller K_b on a relative time scale using two non-interacting standards and compensates

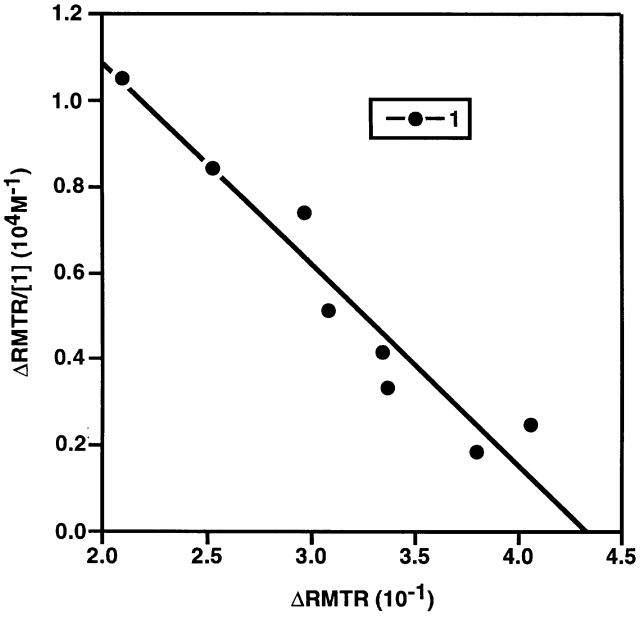

Figure 4. Scatchard plot of the data for Teic and compound **1** according to Eq. (2).

for fluctuations in voltage and electroosmotic flow (EOF) in the capillary column.

Table 1 summarizes the binding data for the peptide ligands examined in this study obtained by Eq. (2). The experiments were conducted at pH 8.3, and values for K_b are expected to be an order of magnitude smaller than previously published values obtained at pH 5 and 7.[2,4] At higher pH, it is proposed that the 1,2- and 3,4-peptide linkages of glycopeptide antibiotics are in the enolic form.[4,35] Therefore, the contribution of NH-protons from Teic residues 2 and 4 to hydrogen bonding with D-Ala-D-Ala terminus peptides is weaker than at more acidic pH. As expected, the binding constant for **1** is lower than the reported value obtained under more acidic conditions using other techniques.[3] The binding constant obtained for **2** is consistent with the value obtained at basic pH.[2,4] We also examined the interaction between Teic and two other peptides, *N*-succinyl-D-Ala-D-Ala, **3**, and Fmoc-Gly-D-Ala-D-Ala, **4**, for which data had not yet been obtained. Table 1 summarizes the binding data of Teic to ligands **3** and **4** using Eq. (2).

In a second series of experiments, we examined the binding of a D-Ala-D-Ala terminus peptide to Teic using an on-column ligand synthesis ACE technique. Here, a multi-plug injection technique was utilized. After equilibrating the capillary with an increasing concentration of Teic in Tris-Gly buffer, a plug of phosphate buffer is injected followed by a solution of sample containing D-Ala-D-Ala terminus peptide and non-interacting standards. A sample of Fmoc-amino acid-NHS ester in acetonitrile followed by a plug of phosphate buffer, are then

Table 1. Experimental Values of Binding Constants K_b ($10^5\,M^{-1}$) of Teicoplanin and Ligands 1–5 Obtained by Eq. (2)

Ligand	K_b (Correlation Coefficient)
1	0.44 (0.99)[a,c]
2	2.6 (0.99)[a,d]
3	0.34 (0.97)[b]
4	2.2 (0.98)[b]
5	4.2 (0.97)

[a]The reported binding constants are the average values from two experiments of four repetitions for each ligand concentration.
[b]The reported binding constants are the average values from three experiments of three repetitions for each ligand concentration.
[c]Previous estimate [3]: $K_b = 1.7 \times 10^5\,M^{-1}$.
[d]Previous estimate [2, 4]: $K_b = (2.1 - 26) \times 10^5\,M^{-1}$.

injected and the sample plugs are electrophoresed in increasing concentrations of Teic. The plugs of phosphate buffer are used to shield the Fmoc reagent and the D-Ala-D-Ala peptides from coming in contact with the Tris-Gly buffer prior to their reaction. It also ensures the maximum yield of product. Overlap of the separate zones of species yields the new Fmoc-Ala-D-Ala-D-Ala (**5**) species. To prevent hydrolysis, the Fmoc reagent is dissolved in acetonitrile. Continued electrophoresis allows for the zone of Teic to migrate into the zone of **5**, thereby, forming a dynamic equilibrium between Teic and **5**.

Figure 5 shows a representative series of electropherograms of **5** in a capillary filled with increasing concentrations of Teic at 205 nm. With the addition of increasing concentrations of Teic in the running buffer, **5** shifts to the left (shorter migration time). A change in the height of the peptide peak is observed and is due to the increasing mass of the newly formed complex upon increasing concentrations of Teic. Based on the peak height of D-Ala-D-Ala we estimate the yield of **5** to be approximately 15%. Since we observed peaks of constant heights and areas from repetition to repetition within a given concentration of Teic, we assumed that similar quantities of peptide were synthesized throughout the duration of the experiment. Although the D-Ala-D-Ala shifted to shorter migration times upon increasing the concentration of Teic, we were unable to determine a binding constant of it to teicoplanin. The inverted peak (+) is due to the dilution of Teic upon binding to **5**. Unreacted peptide and reaction side products (*) are also observed in the series of electropherograms and do not interfere in the binding experiment.

Figure 6 is a Scatchard plot of data for **5** according to Eq. (2). CAB and BA were used as markers in the analysis, and do not interact with any of the species in the running buffer under conditions of electrophoresis. Due to the change in electroosmotic flow (EOF) caused by the increasing viscosity of the running buffer, all peaks shift to the left with increasing concentrations of Teic. The use of RMTR in the Scatchard analysis compensates for the change in EOF during the course of the experiment. We confirmed the use of the on-column ligand synthesis ACE technique by synthesizing **4** off-column. A K_b for Teice; and **4** was obtained by both the on-column ACE technique and by a traditional ACE technique, whereby a shift in **4** is obtained upon increasing the concentration of Teic in the running buffer.

We obtained binding constants for five D-Ala-D-Ala terminus peptides (Table 1). The average correlation coefficients (R) are 0.98 for the ACE experiments and 0.97 for the on-column ligand synthesis ACE technique. This demonstrates that ACE is a viable method to measure binding constants. To our knowledge, binding constants for ligands **3–5** have yet to be reported.

ACE has several advantages as a method for measuring biomolecular noncovalent interactions. First, it requires smaller quantities of receptor and ligand than in traditional binding techniques. Second, purification of the sample

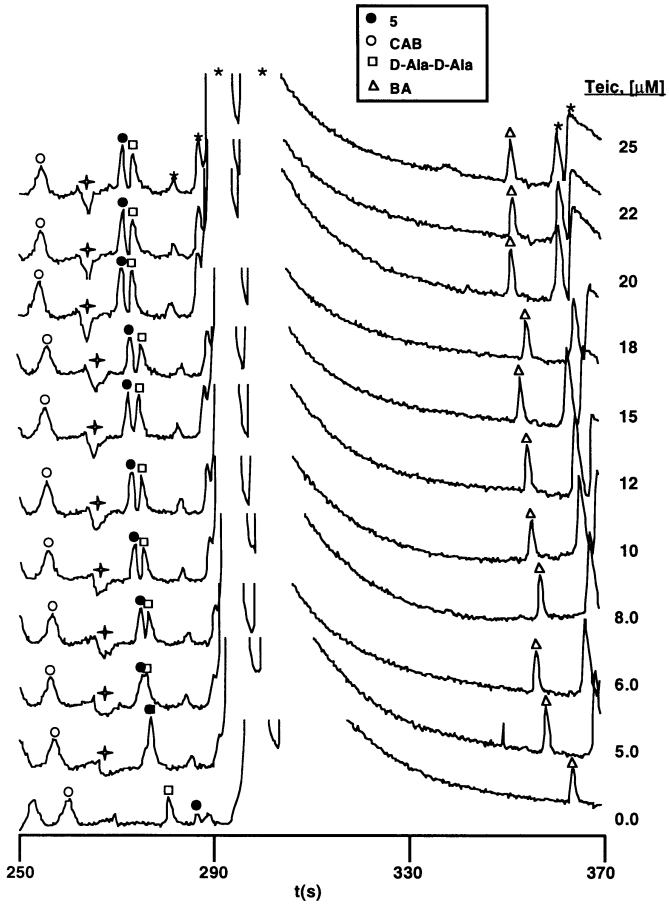

Figure 5. A representative set of electropherograms of Fmoc-Ala-D-Ala-D-Ala, **5**, in 0.192 M glycine-0.025 M Tris buffer (pH 8.3) containing various concentrations of Teic using the on-column synthesis ACE technique. The total analysis time in each experiment was 6.5 min at 18 kv (current: 3.5–4.0 μA) using a 50.5 cm (inlet to detector) 50 μm I.D. open, uncoated quartz capillary. Carbonic anhydrase (CAB) and benzoic acid (BA) were used as internal standards. The symbols * and + are explained in the text.

prior to injection is not necessary as long as the component to be analyzed can be separated from other species. Third, it does not require radio labelled ligands. Fourth, the commercial availability of automated instrumentation, and the high reproducibility of data, makes it experimentally convenient. Further work to characterize Teic and its derivatives using CE is in progress.

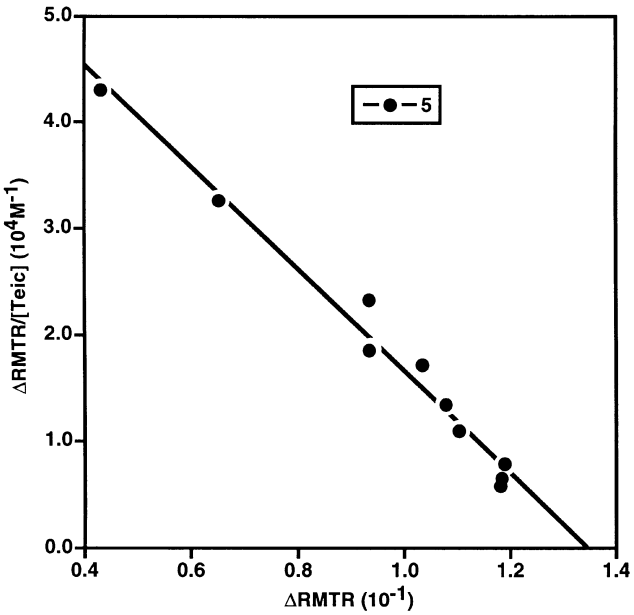

Figure 6. Scatchard plot of the data for Teic and compound **5** according to Eq. (2).

CONCLUSION

Traditional ACE and on-column ligand synthesis coupled to ACE can effectively be used to determine binding constants of Teic to D-Ala-D-Ala terminus peptides. We have verified the applicability of ACE in assessing the affinity of Teic to D-Ala-D-Ala terminus peptides by measuring binding constants for several ligands. In the traditional ACE technique, a plug of Teic and two interacting standards are injected and electrophoresed. Analysis of the shift in Teic, relative to the non-interacting standards as a function of peptide, yields a value for K_b. In the on-column technique, plugs of buffer, sample, and Fmoc reagents are injected and electrophoresed in increasing concentrations of teicoplanin in the running buffer. The migration time of the readily formed peptide is then examined and a K_b is obtained.

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