

Multiple-injection affinity capillary electrophoresis to examine binding constants between glycopeptide antibiotics and peptides

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Abstract

Multiple-injection affinity capillary electrophoresis (MIACE) was used to determine binding constants (K_b) between vancomycin, ristocetin, and teicoplanin from *Streptomyces orientalis*, *Nocardia lurida*, and *Actinoplanes teichomyceticus*, respectively, and fluorenylmethoxycarbonyl (Fmoc)-(Gly, Ala, Val, and Phe)-D-Ala-D-Ala peptides. In this technique, separate plugs of sample containing non-interacting standards, peptide one, buffer, and peptide two, were injected into the capillary column and electrophoresed. Peptides migrate through the column at similar electrophoretic mobilities but remain as distinct zones due to the buffer plug between peptides. The electrophoresis is then carried out in an increasing concentration of antibiotic in the running buffer. Continued electrophoresis results in a shift in the migration time of the peptides upon binding to the antibiotic. Analysis of the change in the relative migration time ratio (RMTR) of the resultant complexes relative to the non-interacting standards, as a function of the concentration of antibiotic yields a value for K_b . MIACE is a versatile technique that can be used to measure affinity constants between ligands of similar relative molecular mass and charge without the need of separate binding experiments. The findings described, herein, demonstrate the advantages of using MIACE to estimate binding parameters between ligands and receptors.

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1. Introduction

Vancomycin (Van)-group antibiotics are glycopeptides that kill bacterial cells by inhibiting peptidoglycan biosynthesis [1–8]. They function by binding to the terminal D-Ala-D-Ala dipeptide of bacterial cell wall precursors, thereby, impeding further processing of these intermediates into peptidoglycan. Van, from *Streptomyces orientalis*, has been called the antibiotic of last resort because of its effectiveness in treating infections caused by bacteria resistant to other antibiotics such as *Staphylococcus aureus*. Bacteria have also conferred resistance to Van through the substitution of the D-Ala-D-Ala terminus of the peptidoglycan precursor by D-Ala-D-Lac (Lac = lactic acid). The great number of mutations that have evolved in bacteria have made it increasingly important to develop new Van-group antibiotics, study their physicochemical parameters, and to examine their activity against Van-resistant enterococci (VRE).

At present there is a major need to develop analytical techniques that can easily assess and evaluate one or more biological

parameters expeditiously and accurately. Such information is critical in developing cures to some of mankind's most serious afflictions including Parkinson's and Alzheimer's disease, AIDS-HIV, and cancer. During the past 10 years advances in molecular biology have yielded great insights into the many molecular interactions involved in human disease. These interactions are critical in understanding the functions and molecular mechanisms of biological systems and the roles they play in health and human disease. Similarly, combinatorial chemistry techniques have generated many millions of potential drugs and drug precursors. In order to assess and examine the large array of receptors and ligands that have been found, it is paramount that new analytical techniques be developed that can easily estimate the level and extent of interaction between biological species.

During the past decade affinity capillary electrophoresis (ACE) has emerged as a useful and sensitive technique for studying bimolecular non-covalent interactions and for determining binding and dissociation constants of formed complexes. In 1992 the first reports documenting the use of ACE to study receptor–ligand interactions were published [9–13]. Since then a myriad of interactions including protein–ligand [14–25], peptide–metal [26,27], peptide–peptide [28–38], protein–peptide [39], protein–antibody [40], polymer–peptide

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[41], antibody–antigen [24], enzyme–drug [43], and polymer–cyclodextrin [44] have been successfully examined using ACE [14–44]. For example, Varenne et al. [14] used ACE to examine the binding of fucoidan, an anticoagulant polysaccharide of marine origin, to antithrombin [14]. Kaddis et al. [15] recently determined binding constants for the activator fructose-6-phosphate (F6P) and substrate ATP to the recombinant wild-type (WT) *Rhodobacter sphaeroides* adenosine 5'-diphosphate-(ADP)-glucose pyrophosphorylase (ADPGlc PPase) using ACE [15]. Finally, Castagnola et al. [26] studied the binding of metal ions (Ni^{2+} , Cu^{2+} , and Zn^{2+}) to the dodecapeptide bacitracin by ACE. In ACE the mobility of a receptor (or ligand) changes upon binding to a ligand (or receptor) that is present in the electrophoresis buffer. The change in migration time can be correlated to a binding constant via Scatchard analysis or other form of analysis.

Although CE has been shown to be a powerful technique to separate mixtures of materials a criteria for separation is that the molecules in question have either a differing charge or mass or both. In the case of charge even a partial charge difference can result in separation of compounds with similar mass; for mass, a larger difference is required if there is no difference in charge. When analyzing small molecules with the same charge and only a small difference in mass (for example, the amino acid glycine replaced with alanine in a peptide) it is frequently problematic to separate them by CE the results being a single peak or, at best, two peaks overlapped with little to no baseline resolution. Hence, CE techniques that can separate similar species are highly warranted.

We recently developed the use of a new technique called multiple-injection affinity capillary electrophoresis (MIACE) [37]. In this technique, multiple samples of receptor are injected into the capillary column and electrophoresed in an increasing concentration of ligand. Multiple binding constants for the same interaction are subsequently obtained, thereby, shortening the ACE experiment.

Herein, we expand on the use of MIACE using as model systems vancomycin, ristocetin, and teicoplanin (Fig. 1). In this work multiple injections of peptides with similar charge and mass, buffer, and standards are injected into the capillary column and electrophoresed. Zones of peptides of similar mass and charge remain separate throughout the ACE experiment and their binding affinities to antibiotics readily obtained.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were analytical grade. Vancomycin, D-Ala-D-Ala, nicotinamide adenine dinucleotide (NAD), and nicotinamide adenine dinucleotide, reduced form (NADH) were purchased from Sigma (St. Louis, MO, USA) and were used without further purification. Ristocetin was obtained from Bio-Data (Horsham, PA, USA) and was used without further purification. Teicoplanin-HCl was purchased from Advance Separation Technologies (Whippany, NJ, USA) and was used without further purification. Fmoc-Gly-NHS, Fmoc-Ala-NHS, Fmoc-Val-

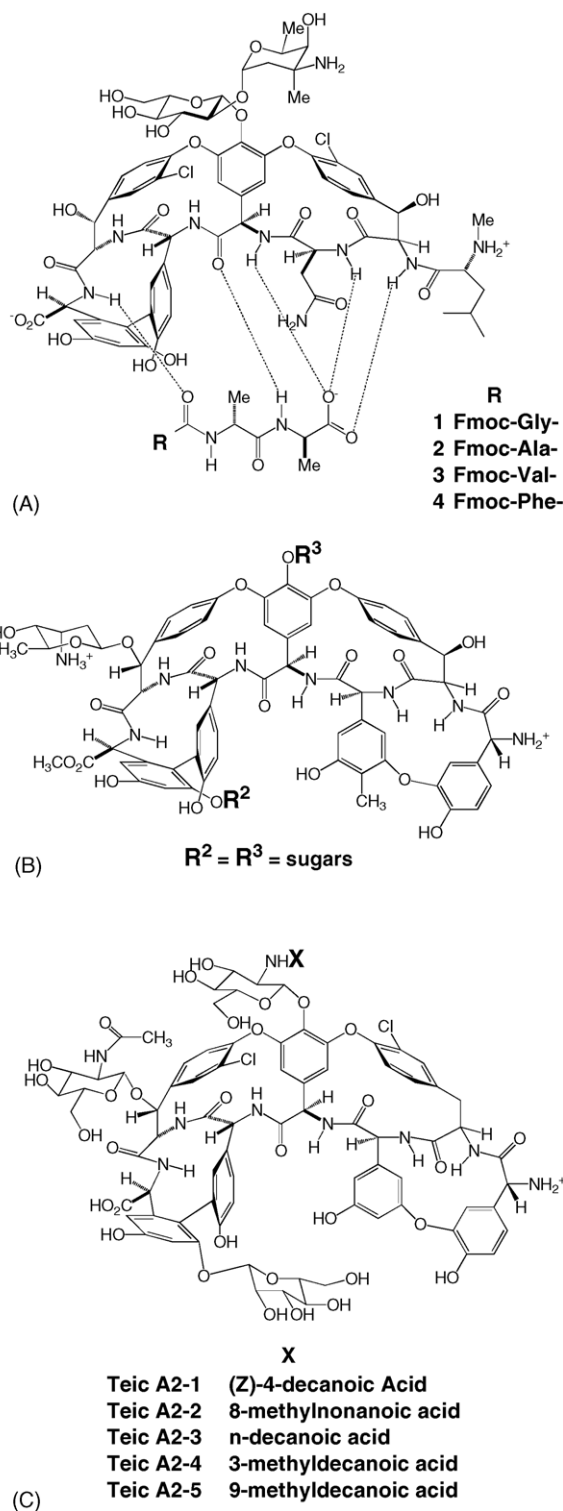


Fig. 1. The glycopeptide antibiotics (A) vancomycin, (B) ristocetin, and (C) teicoplanin and ligands 1–4 used in this study.

NHS, and Fmoc-Phe-NHS were purchased from Bachem California (Torrance, CA, USA). Mesityl oxide (MO) was obtained from Calbiochem (San Diego, CA, USA). Stock solutions of Van (0.2 mg/mL), Teic (1.0 mg/mL), NAD (0.5 mg/mL), and NADH (0.5 mg/mL), were each prepared by dissolving in buffer

(192 mM glycine–25 mM Tris; pH 8.3). Stock solutions of the N-protected amino acids (4 mM) were prepared by dissolving the compounds in buffer. Fmoc-Gly-D-Ala-D-Ala (**1**), Fmoc-Ala-D-Ala-D-Ala (**2**), Fmoc-Val-D-Ala-D-Ala (**3**), and Fmoc-Phe-D-Ala-D-Ala (**4**) were prepared based on literature procedures [45].

2.2. Apparatus

The capillary electrophoresis (CE) system used in this study was a BeckmanCoulter MDQ (Fullerton, CA, USA). The capillary tubing (Polymicro Technology, Inc., Phoenix, AZ, USA) used for the experiment was uncoated fused silica with an internal diameter of 50 μm , length from inlet to detector of 40.5 cm (49 cm for Teic and compounds **1** and **2**), and a length from detector to outlet of 6.5 cm (11 cm for Teic and compounds **1** and **2**). The conditions used in CE were as follows: for Van, Rist, and Teic, voltage, 25 kV; current, 6.8 μA for Van, 7.9 μA for Rist and Teic; detection, 200 nm; temperature, 23.0 ± 0.1 °C. Data were collected and analyzed with BeckmanCoulter 32 Carot software.

2.3. Procedures

For analysis of **1** and **2**, the capillary was first equilibrated with buffer (192 mM glycine–25 mM Tris; pH 8.3) containing increasing concentrations of Van (0–230 μM). Separate plugs of sample solution (3.6 nL) containing the marker (MO), second marker (NAD), **2**, buffer (18.0 nL), and **1** were then introduced by pressure injection. The electrophoresis was carried out using Tris–glycine buffer with increasing concentrations of Van at 25 kV for 7.0 min to complete the detection of all species. For Rist the markers NAD and NADH were used for the studies.

3. Results and discussion

In the first series of experiments we attempted to separate ligands **1** and **2** using CE. At pH 8.3 both ligands have the same charge and vary in molecular weight by only 12 g/mol. Initially, we injected by pressure into the capillary a sample containing an equimolar mixture of **1** and **2**. Upon electrophoresis no separation between the two species was observed (Fig. 2, bottom). This result was not unexpected since the only difference between **1** and **2** is in the amino acid alpha to the Fmoc protecting group. The use of a longer capillary column did not allow for separation of the two species.

We then attempted to separate the two peptides by use of the MIACE technique. Here, separate plugs of sample containing MO (plug one), NAD (plug two), **1** (plug three), buffer (plug four), and **2** (plug five) were injected onto the capillary column. All of the samples, excluding the buffer plug (vacuum injection for 18 s at low pressure), were injected at a pressure of 6.894 MPa for 1 s and electrophoresed for 7.0 min. Fig. 3 shows the schematic of the plug injection used to examine peptides of similar mass and the same charge. Upon electrophoresis all species migrate through the capillary at their respective electrophoretic mobilities. Because of the buffer plug inserted between peptides **1** and **2** both peptides remain as separate zones of sample during electrophoresis and do not mix during the

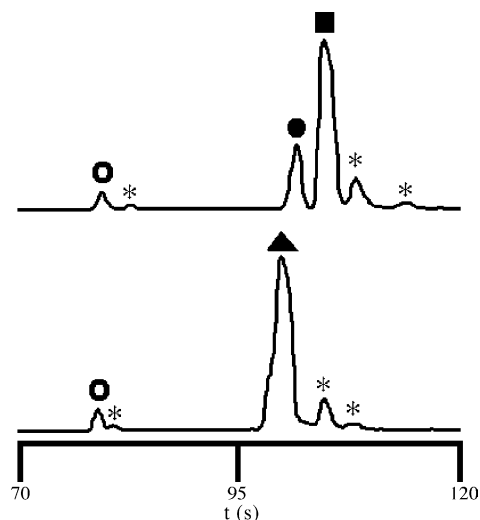


Fig. 2. Electropherograms of ligands **1** (closed square) and **2** (closed circle) in 192 mM glycine–25 mM Tris buffer (pH 8.3) (top) using the MIACE technique and (bottom) same sample injected together. The total analysis time was 7.0 min at 24 kV (current 34–35 μA) using a 49-cm (inlet to detector), 50- μm I.D. open, uncoated quartz capillary. MO (open square) was used as an internal standard. The asterisks (*) are impurities in the samples.

experiment. At the point of detection they are observed as distinct peaks and are baseline resolved (Fig. 2, top).

Upon achieving separation of **1** and **2** we then conducted an ACE experiment. Using a similar sequence of plug injections the capillary is equilibrated with an increasing concentration of Van and electrophoresed. Fig. 4 shows a representative series of electropherograms of ligands **1** and **2** in a capillary filled with increasing concentrations of Van at 200 nm. The peaks for both **1** and **2** are baseline resolved and can easily be differentiated from each other. At $[\text{Van}] = 0$ μM both **1** and **2** elute after the non-interacting standard NAD (open square). At $[\text{Van}] = 30$ and 60 μM the peaks for **2** and **1**, respectively, migrate with the same electrophoretic mobility as the marker NAD and are not observed in the electropherograms. As the concentration of

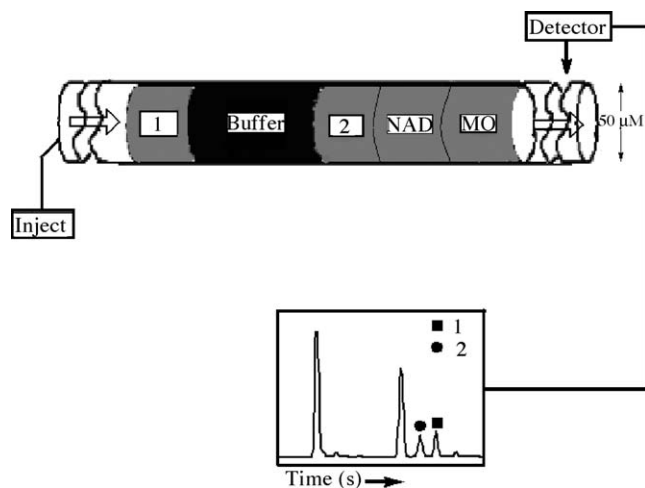


Fig. 3. Schematic of the plug-sequence used to separate peptides of similar charge and mass using the multiple-injection affinity capillary electrophoresis (MIACE) technique.

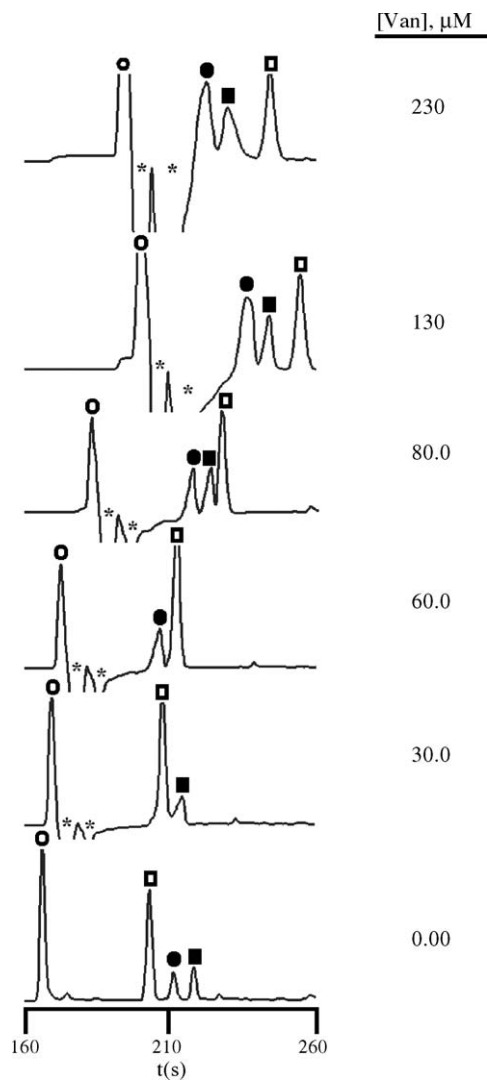


Fig. 4. A representative set of electropherograms of ligands **1** (darkened square) and **2** (darkened circle) in 192 mM glycine–25 mM Tris buffer (pH 8.3) containing various concentrations of Van using the multiple-injection affinity capillary electrophoresis (MIACE) technique. The total analysis time in each experiment was 7.0 min at 25 kV (current 34–35 μ A) using a 49-cm (inlet to detector), 50- μ m I.D. open, uncoated quartz capillary. MO (open circle) and NAD (open square) were used as internal standards. The negative peaks are explained in the text.

Van was increased (0–230 μ M) in the running buffer the peaks for **1** and **2** shift to a shorter migration time on formation of the **1**-Van and **2**-Van complexes and are clearly observed in the electropherogram. The complexes are of greater relative molecular mass than the uncomplexed peptides reason for the shift in migration time. In order to provide clarity to Fig. 4 only six representative electropherograms are presented although 12 concentrations of Van were used in the binding assay. The choice of standard is based on several criteria: (1) it must not interact with either receptor or ligand; (2) its migration time must not overlap with the peak in question; (3) it must be chromophoric at the wavelength of analysis; (4) it must not adsorb onto the capillary column, and; (5) it must elute as a symmetrical peak and reproducibly.

At [Van] = 80 μ M the peaks for **1**-Van and **2**-Van are both observed left of NAD and are baseline resolved. The inverted peaks to the right of MO are due to the dilution of Van in the running buffer upon complexation to **1** and **2**. These negative peaks are commonly observed in ACE studies and are particularly pronounced when the receptor or ligand in the running buffer is chromophoric and/or when high concentrations of the receptor/ligand are used for the binding assay. As can be seen in Fig. 4 the greater the concentration of Van the more pronounced the negative peaks are. Due to the higher mass of the newly formed complex that results on increasing the concentration of Van in the running buffer, the height of the peaks for the peptides increase.

To estimate K_b we used a dual marker form of analysis. Here, K_b is estimated using two non-interacting standards, which we term the relative migration time ratio (RMTR) Eq. (1) [17].

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

Here, t_p , t_s , and $t_{s'}$ are the measured migration times of the peptide peak (for example, **1**), and two non-interacting standard peaks (MO and NAD), respectively. A Scatchard plot can be obtained via Eq. (2).

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

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

Fig. 5 details the Scatchard plots of the data for ligands **1** and **2** upon binding to Van using Eq. (2). As can be seen straight lines result from the Scatchard analysis. Ligands **1** and **2** were found to bind of similar affinities. In our previous work, we found **2** to bind Van four times stronger than **1** to Van but this result was from ACE experiments conducted at pH 7.5. It is well known that pH effects the value of K_b obtained by both ACE and other assay techniques. Ligand **3** was found to bind Van seven times greater than **4**. We can provide no explanation as to the variance in these ligands to Van as previous studies note that derivatization at the *N*-terminus of D-Ala-D-Ala peptides does not significantly affect the binding of these peptides to glycopeptide antibiotics. Binding constant information using the MIACE technique is found in Table 1. It must also be emphasized that the binding constants obtained using any ACE technique are related to the ionic strength of the electrophoresis buffer and the temperature of the assay vis-à-vis binding constants are apparent and not thermodynamic association constants of the complexes studied.

We then examined the binding of ligands **1–4** to Rist. Rist, like Van, also inhibits cell wall peptidoglycan biosynthesis in susceptible bacteria by binding to key peptidoglycan intermediates. It was clinically employed to treat bacterial infections into the late 1950s, but due to undesirable side effects, was discontinued. Recently, the increasing incidence of Van-resistant strains

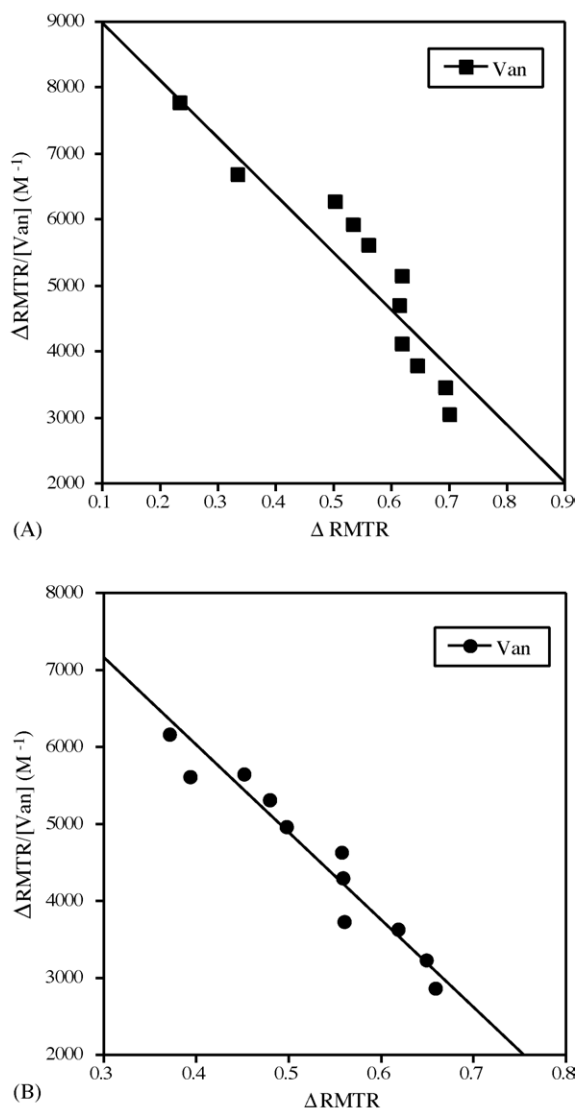


Fig. 5. Scatchard plots of the data for (A) **1** and (B) **2** according to Eq. (2).

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

Ligand	Van	K_b Rist	Teic
1	9.8 (0.931) ^a	11.7 (0.936) ^b	237.5 (0.930) ^c
2	11.4 (0.936) ^d	17.1 (0.864) ^e	153.2 (0.913) ^f
3	171.4 (0.987)	13.0 (0.845) ^g	21.5 (0.808)
4	24.3 (0.808)	25.5 (0.964) ^h	15.5 (0.877)

^a Previous estimates [31]: $K_b = 41.6 \times 10^3 \text{ M}^{-1}$ (pH 7.5).

^b Previous estimates [36]: $K_b = 41.4 \times 10^3 \text{ M}^{-1}$ (pH 8.3); [31]: $K_b = 8.2 \times 10^3 \text{ M}^{-1}$ (pH 7.5).

^c Previous estimates [31]: $K_b = 21.8 \times 10^3 \text{ M}^{-1}$ (pH 7.5).

^d Previous estimates [31]: $K_b = 174.5 \times 10^3 \text{ M}^{-1}$ (pH 7.5).

^e Previous estimates [36]: $K_b = 9.1 \times 10^3 \text{ M}^{-1}$ (pH 8.3); [31]: $K_b = 52.4 \times 10^3 \text{ M}^{-1}$ (pH 7.5).

^f Previous estimates [31]: $K_b = 185.1 \times 10^3 \text{ M}^{-1}$ (pH 7.5).

^g Previous estimates [36]: $K_b = 25.3 \times 10^3 \text{ M}^{-1}$ (pH 8.3).

^h Previous estimates [36]: $K_b = 16.4 \times 10^3 \text{ M}^{-1}$ (pH 8.3).

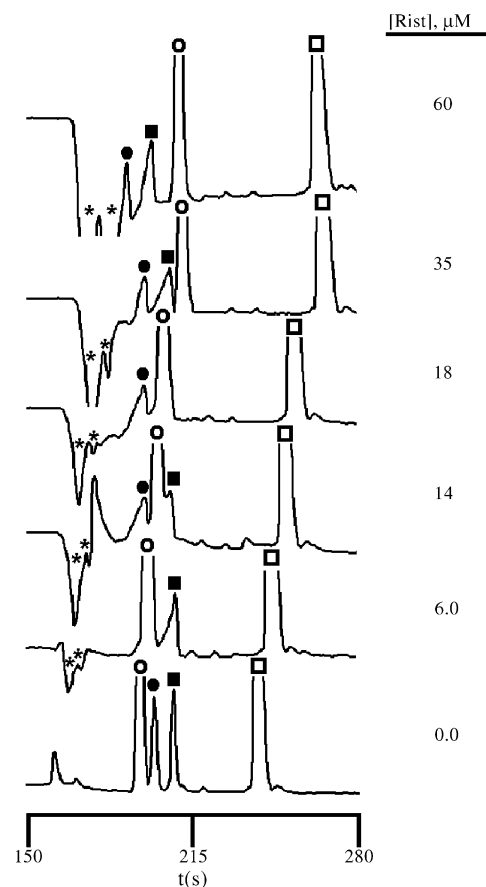


Fig. 6. A representative set of electropherograms of ligands **3** (darkened square) and **4** (darkened circle) in 192 mM glycine–25 mM Tris buffer (pH 8.3) containing various concentrations of Rist using the multiple-injection affinity capillary electrophoresis (MIACE) technique. The total analysis time in each experiment was 7.0 min at 25 kV (current 34–35 μA) using a 49-cm (inlet to detector), 50- μm I.D. open, uncoated quartz capillary. NAD (open circle) and NADH (open square) were used as internal standards. The negative peaks are explained in the text.

of infectious bacteria has sparked interest in the synthesis of these new Rist derivatives.

Fig. 6 is a representative series of electropherograms of peptides **3** and **4** in capillaries filled with Rist. For clarity, only six electropherograms are shown. As can be seen ligands **3** and **4** show a similar peak pattern as that found for ligands **1** and **2**. At $[\text{Rist}] = 6$ and $18 \mu\text{M}$ ligands **3** and **4** migrate at similar electrophoretic mobilities as the marker NAD, respectively, and are not observed in the electropherograms. In an earlier experiment injection of a sample of **3** and **4** did not realize separation of the peptides as both compounds have almost identical electrophoretic mobilities and elute nearly at the same migration time. The negative peaks are due to the dilution of Rist in the electrophoresis buffer. Fig. 7 are Scatchard plots of the data for ligands **3** and **4** binding to Rist using Eq. (2). For Fig. 7A there is a gap in the data for **3** overlaps with NAD at the intermediate concentrations of Rist. A similar effect is only nominally observed for ligand **4** in the series of electropherograms. There is some variation in the values for K_b between the four ligands and Rist. Structural studies have shown that the amino acid alpha

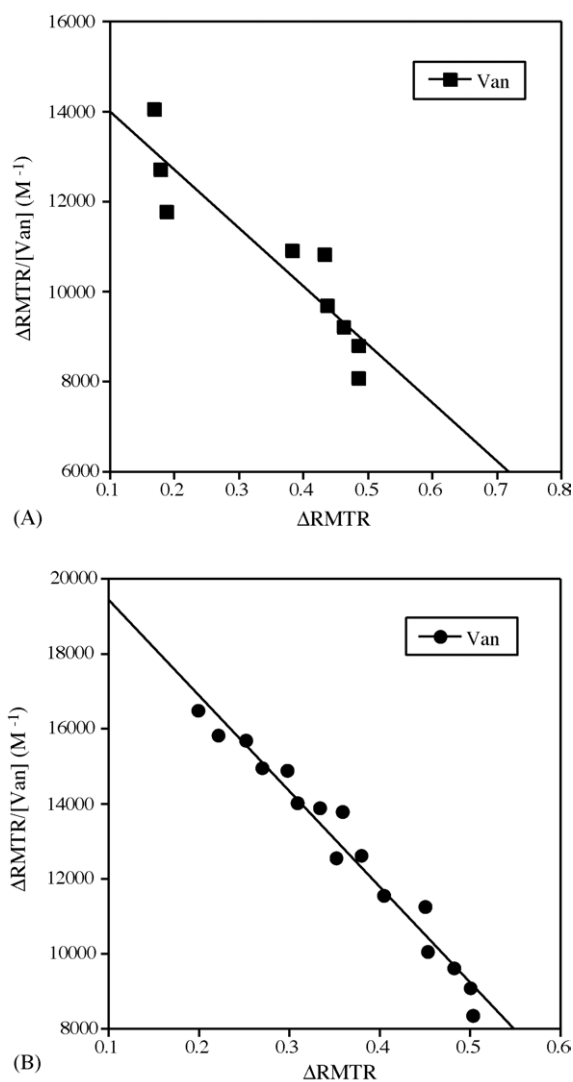


Fig. 7. Scatchard plots of the data for (A) **3** and (B) **4** according to Eq. (2).

to the D-Ala-D-Ala fragment has little effect on the binding to some glycopeptide antibiotics.

We also examined the binding of ligands **1–4** to Teic. Like Van, Teic is a linear heptapeptide, cross-linked between residues 1 and 3, 2 and 4, and 4 and 6 by diphenyl ether bridges and between residues 5 and 7 by a biphenyl bridge. Teic is a mixture of five closely related analogues, designated T-A₂₋₁ through T-A₂₋₅ [6,18]. They differ by approximately 20 molecular mass units because of the variation of the carbon length and substituent groups of the hydrophobic acyl side chain (hydrophobic tail) that is attached to a 2-amino-2-deoxy- β -D-glucopyranosyl moiety. This hydrophobic tail gives Teic its unique characteristics. Table 1 lists the binding data for Teic and ligands **1–4**.

The advantages of MIACE are several fold: one, smaller quantities of receptor and ligand are needed to conduct the studies in comparison to other assay techniques. Two, purification of the sample prior to injection is not necessary as long as the component to be analyzed can be separated from other species. Three, binding constants for different species can be obtained, thereby, shortening the amount of time required to conduct the

assay. Four, compounds with similar charge and mass can be analyzed for binding using MIACE. Finally, the commercial availability of automated instrumentation, and high repeatability of data, make it experimentally convenient.

4. Conclusion

The present study demonstrates the ease of using multiple-injection ACE (MIACE) to estimate binding constants between peptides of similar charge and mass and the glycopeptide antibiotics vancomycin, ristocetin, and teicoplanin. In this technique separate plugs of peptides and standards are injected onto the capillary column and are subjected to an increasing concentration of antibiotic in the running buffer. The separate injections of peptide allow for the separation of like charge and mass peptides. Changes in migration time of the peptides upon complexation to antibiotic was used in the Scatchard analysis and realized multiple binding constants comparable to those obtained using other ACE techniques and traditional assay methods. The work detailed here serves as a foundation for using MIACE to examine the binding of other receptor-ligand combinations. Future work will focus on expanding the versatility of the technique by modifying the plug–plug injection sequence to other systems.

Acknowledgments

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