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Vacancy affinity capillary electrophoresis, a new method for measuring association constants

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Abstract

A new method to determine association constants, the vacancy affinity capillary electrophoretic (VACE) method is described. The capillary is filled with a mixture of the interacting components and neat buffer is injected. The concentration of one of the components is fixed, and the concentration of the other one is varied during a series of experiments. The presence of both species in the capillary causes a large background signal. Two negative peaks will appear in the electropherogram. The migration times of the negative peaks reflect the fractions of the free and bound components in the mixture, and depends on the concentration in the buffer. This shift can be used to construct a binding curve from which the association parameters can be estimated. Nearly the whole system is saturated with the interacting components, so that losses due to wall adsorption are less detrimental. © 1997 Elsevier Science B.V.

Keywords: Vacancy affinity capillary electrophoresis; Association constants; Affinity capillary electrophoresis; Binding studies; Vancomycin; Peptides; Acetylalanylalanine

1. Introduction

The characterization of specific interactions is the focus of much biochemical research [1]. Several methods have been developed to measure binding parameters for non-covalent interactions. Many of these methods are based on the use of a separation method under equilibrium conditions [2,3]. Capillary electrophoresis is one, relatively new, separation technique that offers advantageous features for studying such interactions.

At present there are several capillary zone electrophoresis (CZE) methods available to study affinity interactions. Recently, the frontal analysis method [4–6], the Hummel and Dreyer method [5], the vacancy peak method [5] and affinity capillary electrophoresis (ACE) [7–9], have been used to characterize the binding of ligands to proteins. Affinity interactions between protein–protein [9,10], protein–DNA [1,11], protein–drug [5,12–17], protein–carbohydrate [18,19], peptide–peptide [20–24], peptide–carbohydrate [25], carbohydrate–drug [26] and antibody–antigen [6,27] have been demonstrated by using CZE.

A new binding assay for obtaining association constants called the vacancy affinity capillary electrophoresis method (VACE), is described in this paper. In the VACE method both interacting species are added to the buffer. Changes in the electro-

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phoretic mobility of the interacting components can be correlated to the association constant.

This method is illustrated by analysis of the binding between the glycopeptide, vancomycin and the dipeptide, N-Ac-D-alanyl-D-alanine.

2. Theoretical section

The association of two species denoted as D and P, e.g. a drug and a protein, forming a complex, C, is used to discuss and compare the ACE and the VACE methods in general. Furthermore it is assumed that a (1:1) complex is formed, that the complexation is fast and that the detector responds to all species. First the principles of the ACE method will be discussed in order to point out the methodological difference between the two methods.

2.1. *Affinity capillary electrophoresis (ACE) method*

In the ACE method one component is added in varying concentrations to the buffer and the other one is injected. The concentration of the injected component is fixed. For instance D is added to the buffer and P is injected. In that case the mobility of P as a function of the concentration of D in the buffer is monitored. A scheme of the ACE method is presented in Fig. 1. The peak of P shifts upon increasing the concentration of D in the buffer. When no D is present in the buffer, P will migrate with its own mobility ($\mu_{P,0}$). Upon adding D to the buffer, the injected P will form a complex with D and this will cause a shift in the mobility of P. The average mobility of the peak of P will shift between the mobility of P when no D is added to the buffer, $\mu_{P,0}$, and the mobility when the injected P is completely bound to D, $\mu_{P,D\ max}$. At the intermediate concentrations of D, the average mobility is determined by the fraction P free, α, and P bound $(1-\alpha)$ of the (injected) amount of P. The fraction of bound P will migrate with the mobility of the complex, μ_C, and the fraction of P free will migrate with the mobility of P free, $\mu_{P,0}$. The average mobility of the peak of P, $\mu_{P,D}$, can be expressed in the following way:

$$\mu_{P,D} = (1-\alpha)\mu_C + \alpha\mu_{P,0} \tag{1}$$

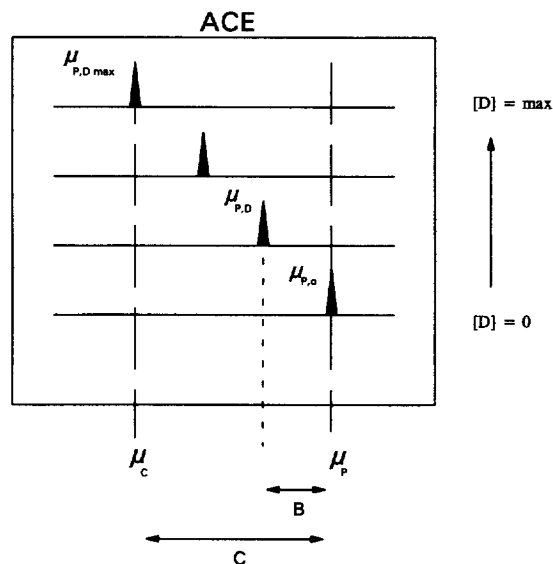

Fig. 1. Illustration of the ACE method. In this example D was added to the buffer and P was injected. Assumption: $\mu_P > \mu_C$

So, in an ACE experiment the fraction, $(1-\alpha)$, of the (injected) P that will form a complex with D, is actually being monitored as a shift in the mobility of the peak of P, and can be calculated in the following way:

$$(1-\alpha) = \frac{B}{C} = \frac{\mu_{P,D} - \mu_{P,0}}{\mu_{P,D\ max} - \mu_{P,0}} = \frac{[P_b]}{[P_{total}]} \tag{2}$$

where B in Fig. 1 reflects the fraction of P that has been bound, and C reflects the total amount of P.

The reversed experiment can also be performed i.e. adding P to the buffer and injecting D.

The ACE method relates the change in the average electrophoretic mobility $\mu_{P,D}$ of P present in the sample to the concentration of D present in the buffer, $[D_{buffer}]$. This enables the determination of the association constant K_{ass} [7].

2.2. *Vacancy affinity capillary electrophoresis (VACE) method*

In the VACE method, the capillary is filled with buffer containing both P and D. The concentration of one of the components is fixed and the concentration of the other component is varied. The situation will

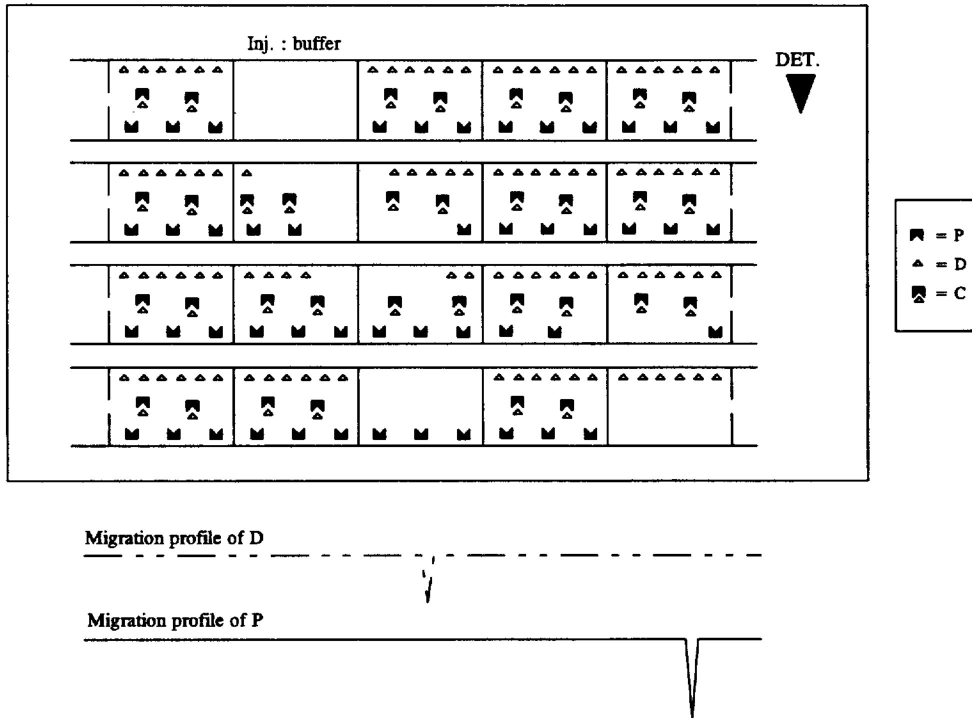

Fig. 2. Illustration of the migration process in a typical VACE experiment. Assumption: $\mu_P > \mu_D$

be discussed in which the concentration of P is kept constant. Fig. 2 shows a scheme of the principle of the VACE method. The presence of D and P causes a large background detector response. A small volume of neat buffer is injected and the power supply is switched on. Two negative peaks appear in the electropherogram. The occurrence of the two negative peaks can be explained in the following manner: As there are only two independent concentrations (for which the concentrations of D and P in the buffer can be chosen) there are only two zones possible [28,29]. We assume that P, irrespective of its form (PD=C or P) always migrates faster than D irrespective of its form (PD=C or D). At the rear edge of the plug, P migrates faster in the injection zone than D, moving ahead of the D boundary and finally catching up with D at the front edge of the injection zone. Because D at the rear edge of the injection zone has the same mobility as D at the front edge of the zone, a 'vacancy' of D occurs. This 'vacancy' is actually a zone with a deficiency of D. A similar reasoning can be applied to the P molecules, a zone with a deficiency in P (P-'vacancy') migrates with $\mu_{P,D}$. After a certain time, the zone lacking in P and the zone lacking in D are separated by a zone in which the equilibrium is attained again. From that point on a steady state is reached, resulting in two negative peaks in the electropherogram. The first (negative) peak is detected because in this zone P is lacking. The second (negative) peak is detected because in this zone D is lacking. The area of the negative peaks contains information about the degree of complexation. The area of the first peak reflects the concentration of free P in the buffer and the second peak reflects the concentration of free D in the buffer. Sebille et al. [30] have given evidence that for liquid chromatography the area of the negative peaks indeed reflects the concentration of the species in the buffer and a similar reasoning can be applied to CZE [5]. The method in which the area of the negative peak is used to construct a binding isotherm is called the vacancy peak method [30].

Apart from using the area of the negative peak, information on the association constant can also be

attained from the shift of the migration times of the (negative) peaks. This new method is the VACE method explored in this paper. The mobilities of the two negative peaks depend on the concentrations of the components in the buffer. This means that like the ACE method the VACE method can also be performed in two ways: the concentration of D in the buffer is varied and the concentration of P is kept constant in the buffer, or the concentration of P is varied and the concentration of D is kept constant. The procedure in which the concentration of D is varied will be discussed.

Upon increasing the concentration of D the mobility of the P-'vacancy' shifts. This can be explained in the following manner: the average mobility of the peak of P (first negative peak) is determined by the fraction of free P, α, and bound P, $(1-\alpha)$, present in the buffer. Fig. 3a shows the dependence of the average mobility of the peak of P, reflecting the fraction of P that becomes bound, in relation to the total concentration of D in the buffer. When the concentration of D in the buffer is small, only a small fraction, $(1-\alpha)$, of P migrates with the mobility of the complex, μ_C. The larger part of P, α, migrates with the mobility of the free (unbound) P, $\mu_{P,0}$. Therefore, the average mobility of the peak of P is dominated by the mobility of the free P. Upon increasing the concentration of D, the average mobility of the peak of P changes, because the fraction of free P molecules, α, decreases. If enough D is added to the buffer to saturate P with D, the mobility of the peak of P reaches its maximum value, $\mu_{P,D\ max}$. In this situation most of P in the buffer is bound and the average mobility of the peak reflecting P approaches the mobility of the complex, μ_C. The average mobility of the peak of P, $\mu_{P,D}$, can be expressed as:

$$\mu_{P,D} = (1-\alpha)\mu_C + \alpha\mu_{P,0} \quad (3)$$

The fraction of P that has been bound, $(1-\alpha)$, can be calculated with the following equation:

$$(1-\alpha) = \frac{E}{F} = \frac{\mu_{P,D} - \mu_{P,0}}{\mu_{P,D\ max} - \mu_{P,0}} = \frac{[P_b]}{[P_{total}]} \quad (4)$$

where E in Fig. 3a reflects the fraction of P that has been bound, and F reflects the total amount of P.

The VACE method relates the change in the mobility, $\mu_{P,D}$, observed for P as a function of the total concentration D in the buffer, $[D_{total}]$. This relationship enables the determination of the association constant K_{ass}.

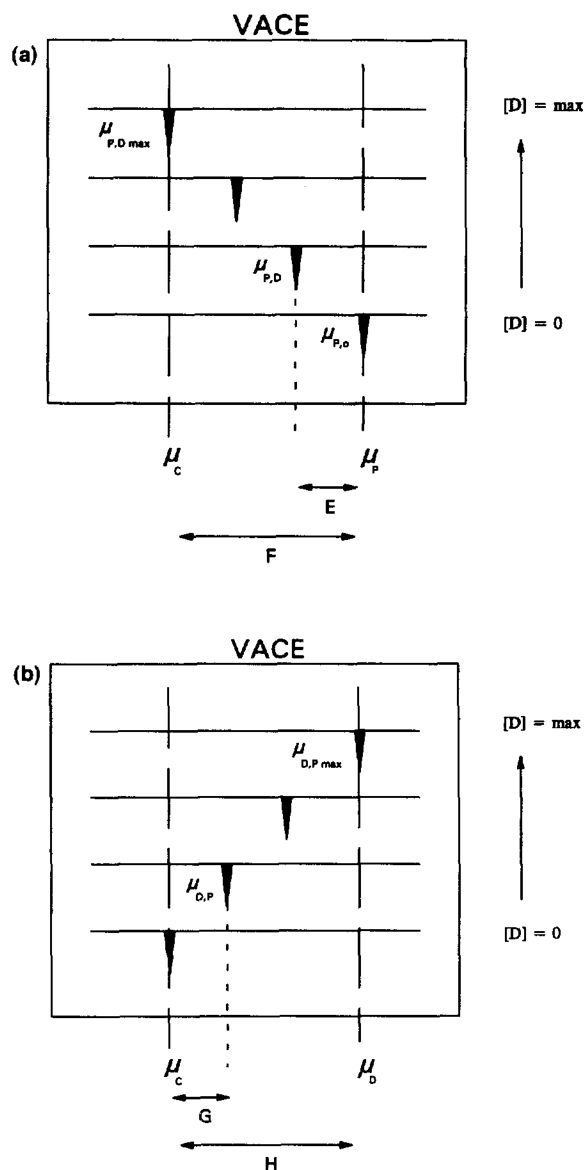

Fig. 3. (a) Illustration of the VACE method. In this example the concentration of D is increasing in the buffer. Assumption: $\mu_P > \mu_C$. (b) Illustration of the VACE method. In this example the concentration of P is increasing in the buffer. Assumption: $\mu_D > \mu_C$

In addition to using the change in the mobility of

the P-'vacancy' with the VACE method the change in the mobility of the second negative peak representing a deficiency in D (D-'vacancy') can also be used to obtain the association constant.

The behaviour of the mobility of the D-'vacancy' is quite similar. The average mobility of the peak of D, $\mu_{D,P}$, also changes with increasing concentrations of D in the buffer. Fig. 3b shows the dependence of the average mobility of the (negative) peak of D, $\mu_{D,P}$, in relation to the total concentration of D in the buffer. When the concentration of D in the buffer is small, most of D, $(1-\beta)$, migrates with the mobility of the complex, μ_C. As a result, the average mobility of the peak of D is dominated mainly by the mobility of the complex. When the concentration of D in the buffer is large, most of D, (β), migrates with the mobility of the free D, $\mu_{D,o}$. A shift in the average mobility of the peak of D, $\mu_{D,P}$, occurs according to Eq. (5):

$$\mu_{D,P} = (1-\beta)\mu_C + \beta\mu_{D,0} \quad (5)$$

where the fraction of free D in the buffer is reflected by β, and the fraction of bound D is reflected by $(1-\beta)$. Therefore the fraction, β, of the total D added to the buffer that is free can be calculated with the following equation:

$$\beta = \frac{G}{H} = \frac{\mu_{D,P} - \mu_{D,0}}{\mu_{D,Pmax} - \mu_{D,0}} = \frac{[D_f]}{[D_{total}]} \quad (6)$$

where G in Fig. 3B reflects the fraction of D that will remain free, and H reflects the total amount of D. Also this shift can be related to the association constant.

The reversed experiment can also be performed with the VACE method; i.e. varying the concentration of P and keeping the concentration of D constant.

3. Experimental

3.1. Equipment

The (CZE) system used in this study was an Applied Biosystems Model 270A-HT (ABI, Foster City, CA, USA). The 50 μm I.D. fused-silica capillary tubing was obtained from Polymicro Technologies (Phoenix, AZ, USA). The total length of the capillary was 76.3 cm, and the length from the inlet to the detector was 49.6 cm. A run voltage of 13 kV, 173 V cm^{-1} was applied. UV detection was performed at 215 nm. The temperature was set to 27±1°C. To record the electropherograms (ASCII files) we used the CAESAR programme (Prince Technologies, Emmen, Netherlands). For convenience the ASCII files were exported to Matlab (The MathWorks, MA, USA). With this program data processing was done and figures were made.

3.2. Materials

All chemicals used were of analytical grade. The glycopeptide vancomycin was purchased from Aldrich (Milwaukee, WI, USA). Dipotassium hydrogenphosphate, potassium dihydrogenphosphate were from Merck (Darmstadt, Germany). Hydrochloric acid, sodium hydroxide and N-Ac-D-alanyl-D-alanine, mesityl oxide (MO) were obtained from Sigma (St. Louis, MO, USA). Distilled water was used to prepare the buffers.

3.3. Procedures

Before each measurement the capillary was flushed consecutively for 2 min each with 0.03 *M* HCl, 1 *M* NaOH and buffer. Samples were introduced into the capillary using vacuum (169 mbar) injection for 1.5 s. In all the electrophoresis experiments a 0.05 *M* potassium phosphate buffer (pH 6.0) was used. The buffer was prepared from stock solutions of 50 m*M* dipotassium hydrogenphosphate and potassium dihydrogenphosphate. With the ACE method appropriate amounts of N-Ac-D-alanyl-D-alanine were added to the buffer. In the VACE experiments both the vancomycin and the N-Ac-D-alanyl-D-alanine were added in appropriate amounts to the buffer. MO was added to the sample to determine the variation in the electro-osmotic flow. The electrophoretic mobility of a solute, $\mu_{e,i}$, used for the construction of the curves, was calculated by subtracting the mobility of MO, μ_{MO}, from the effective mobility of the solute, $\mu_{eff,i}$ [8]:

$$\mu_{e,i} = \mu_{eff,i} - \mu_{MO}$$

3.4. Data processing

3.4.1. ACE method

For the estimation of the association constant from the ACE experiments, the untransformed raw data was used: the $\mu_{P,D}$–$[D_f]$ plot. Using this plot if the shift is halfway, we know that half of P is bound to D, but whether this is PD, PD_2 or...PD_n remains unknown. This can be explained as follows; substituting $[P_{total}]-[P_b]$ for $[P_f]$, Eq. (1) can also be expressed as:

$$\mu_{P,D} = \frac{[P_b]}{[P_{total}]}\mu_{P,D\ max} + \frac{[P_f]}{[P_{total}]}\mu_{P,0} \quad (7)$$

Eq. (7) can be rewritten as:

$$\frac{[P_b]}{[P_{total}]} = \frac{\mu_{P,D} - \mu_{P,0}}{\mu_{P,D\ max} - \mu_{P,0}} \quad (8)$$

Furthermore it is known that [3]:

$$\frac{[D_b]}{[P_{total}]} = \frac{n_{bind}K_{ass}[D_f]}{1 + K_{ass}[D_f]} \quad (9)$$

Only when assuming e.g. that $[P_b]$ is equal to $[D_b]$, in the case of a (1:1) complex, Eq. (8) may be set equal to Eq. (9). Once this assumption is made the value for n_{bind} is set. In fact, the shift in the mobility of the peak of P is actually representing the part of P that will have bound D. In order to be able to obtain values for n_{bind}, representing the number of the binding sites present on P, the fraction of D that has been bound by P, should be known.

The relationship (Eq. (10)) between the mobility of the peak of P allows therefore only for the estimation of the association constant, K_{ass}, and not the estimation of the numbers of the binding sites present on P, n_{bind}.

The relationship between $\mu_{P,D}$ and the various parameters is given by:

$$\mu_{P,D} = \mu_{P,0} + (\mu_{P,D\ max} - \mu_{P,0})\cdot\frac{K_{ass}[D_f]}{1 + K_{ass}[D_f]} \quad (10)$$

where $\mu_{P,D}$ and $\mu_{P,o}$ are measured, whereas K_{ass}, $\mu_{P,D\ max}$ and if necessary $\mu_{P,0}$ are estimated simultaneously using non-linear regression [31].

Furthermore, with the ACE method the concentration of free D in the migrating zone of P, $[D_f]$, is unknown [32]. Therefore this has to be set equal to the concentration of D in the buffer $[D_{buffer}]$. Using $[D_{buffer}]$ instead of the local concentration of free D may lead to a systematic error in the value found for K_{ass}, because the local free concentration of D will differ from the concentration of D in the buffer. Unfortunately there is only one condition under which $[D_f]$ is equal to $[D_{buffer}]$, that is when $\mu_P = \mu_C$, exactly the case where this experiment is impossible to perform [32]. This effect can be minimized by lowering the concentration of P in the sample.

3.4.2. VACE method

With the VACE method, in principle, two possibilities exist to extract a value for the association constant, K_{ass}. The shift in the mobility of D, $\mu_{D,P}$, as well as the shift in the mobility of P, $\mu_{P,D}$, can be plotted against the total concentration of D in the buffer, $[D_{total}]$: the $\mu_{P,D}$–$[D_{total}]$ plot and the $\mu_{D,P}$–$[D_{total}]$ plot.

An advantage of the VACE method is the fact that the plot of $\mu_{D,P}$ vs. $[D_{total}]$ contains information on the absolute number of the different binding sites present on P, n_{bind}, because the shift in the average mobility of the peak of D, $\mu_{D,P}$, represents the fraction of D that is bound by P. Plotting $\mu_{D,P}$, vs. $[D_{total}]$ therefore enables one to obtain information about n_{bind}.

On the other hand, when the relationship between the shift in the mobility of P, $\mu_{P,D}$ and $[D_{total}]$ is used, it is only possible to obtain the association constant K_{ass}, but not the value for n_{bind}. A similar reasoning, as was discussed for the ACE method can be applied here. The shift in the mobility of the peak of P represents the part of P that is bound to D. Plotting $\mu_{P,D}$, vs. $[D_{total}]$ therefore does not enable one to obtain information about n_{bind}.

As discussed for the ACE method, correct results for the association parameters can only be obtained when the free D concentration, $[D_f]$, is used in the calculation of the association parameters. With the VACE method part of D added to the buffer will be bound by P, $[D_b]$, therefore the free concentration of D in the buffer will already be lower than the total concentration of D added to the buffer.

With the VACE method it is not possible to measure the free D concentration in the buffer, however it can be calculated, because $[D_{total}]$ and

$[P_{total}]$ in the buffer are known, in the following way, the mass balance for D is:

$$[D_{total}] = \frac{n_{bind}\, K_{ass}\, [D_f]}{1 + K_{ass}\, [D_f]} \cdot [P_{total}] + [D_f] \qquad (11)$$

Eq. (11) relates the known total concentration of D in the buffer to the free concentration of D in the buffer. Taking the inverse of this relationship (Eq. (11)) results in Eq. (12) with which the free concentration of D can be calculated when the total concentration of D in the buffer, and in principle, also the values for K_{ass} and n_{bind} are known:

$$[D_f] = (-1 + [D_{total}]K_{ass} - [P_{total}]K_{ass}n_{bind} + \sqrt{(4[D_{total}]K_{ass} + \frac{((1 - [D_{total}]K_{ass}) + [P_{total}]K_{ass}n_{bind})^2}{(2K_{ass})}} \qquad (12)$$

However, with a value for K_{ass} and n_{bind} assumed in an iteration step in the fitting procedure, Eq. (12) can be used to calculate $[D_f]$ values, with which then estimates of μ, to be compared with the observed values for μ, can be found. This procedure will be explained below.

(I) $\mu_{P,D}$–$[D_{total}]$ plot. The relationship between $\mu_{P,D}$ and the various parameters for this plot of the VACE method is also given by Eq. (10), whereby $\mu_{P,D}$ is measured and in the case of the VACE method Eq. (12) can be used to calculate $[D_f]$, provided the stoichiometry (n_{bind}) of the complex is known. The parameters K_{ass}, $\mu_{D,0}$, $\mu_{P,D\ max}$ can be estimated simultaneously from the data, with non-linear regression [31] using Eqs. (10) and (12). For this model system n_{bind} was set to 1 [7].

(II) $\mu_{D,P}$–$[D_{total}]$ plot. The relationship between $\mu_{D,P}$ and $[D_{total}]$, Eq. (5), can also be expressed as:

$$\mu_{D,P} = \mu_{D,P\ max} + \frac{[D_f]}{[D_{total}]} \cdot (\mu_{D,0} - \mu_{D,P\ max}) \qquad (13)$$

where $\mu_{D,P}$ is measured, the value for $[D_f]$ can be calculated with Eq. (12), $[P_{total}]$ and $[D_{total}]$ in the buffer are known. The value for K_{ass}, $\mu_{D,0}$, $\mu_{D,P\ max}$ and n_{bind} are estimated simultaneously using non-linear regression [31] from the data using Eqs. (13) and (12).

By using the $\mu_{D,P}$–$[D_{total}]$ plot, in principle the absolute number of the binding sites can be estimated with the VACE method [36]. For the selected model system, vancomycin and N-acetyl-D-alanyl-D-alanine, n_{bind} is known to be 1 [7].

4. Results and discussion

The vancomycin–N-Ac-D-alanyl-D-alanine system was selected to investigate the VACE method, because this complex has been described in literature and was already studied with the ACE method [7,20,21,24]. With the selected buffer, the effective mobilities of the complex (C) and free N-Ac-D-alanyl-D-alanine (denoted as D) appear to be the same but smaller than the effective mobility of the free vancomycin (denoted as P). So in the investigated system $\mu_P > \mu_D = \mu_C$; because $\mu_C = \mu_D$, the mobility of the free D cannot be used the estimate to association constant.

In this particular case the reversed experiment, i.e. varying the concentration of P in the buffer and injecting D, cannot be performed with the ACE method, but can with the VACE method.

4.1. ACE method

In order to evaluate the results obtained with the VACE method, as a reference the selected model system was also studied with the ACE method.

In this experiment D was added to the buffer in varying concentrations (0–500 μM) and P was injected (50 μM). Fig. 4 shows representative electropherograms obtained with the ACE method. The first peak is P, indicated by (*) and the second peak is MO, indicated by (○).

As can be seen the P peak shifts towards the migration time of the complex, upon increasing the concentration of D in the buffer (see lines 2–6 in Fig. 4). The mobility of the complex appears to be very small, its peak approaches that of MO in this buffer system.

Fig. 5 shows the plot of the shift in the mobility of the P, $(\mu_{P,D} - \mu_{P,0})$, versus the concentration of D

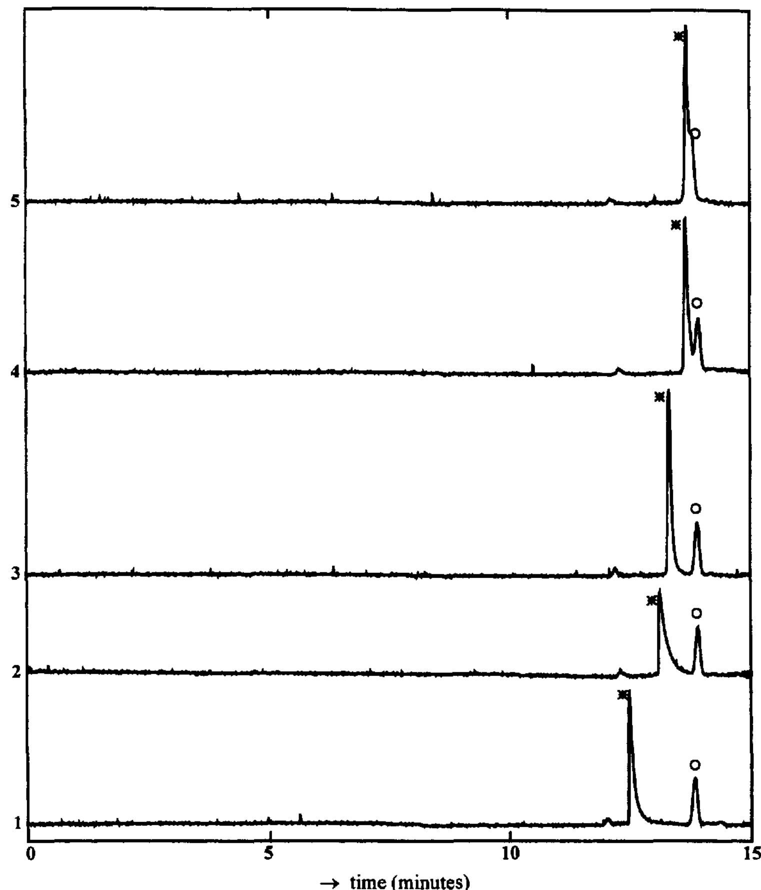

Fig. 4. Representative electropherograms of the ACE method for the vancomycin–N-Ac-D-alanyl-D-alanine system. MO (○) and vancomycin (*). Conditions: hydrodynamic injection, 1.5 s, 169 mbar; separation voltage, 13 kV; $\lambda=215$ nm, 0.01 AU; sample, 50 μM vancomycin+MO; separation buffer, 0.050 M phosphate buffer (pH 6.0)+varying concentrations of N-Ac-D-alanyl-D-alanine: trace 1=0, trace 2=8, trace 3=16, trace 4=42, trace 5=82 μM N-Ac-D-alanyl-D-alanine.

in the buffer. The association constant estimated from this curve, using Eq. (10) is given in Table 1.

4.2. VACE method

In the VACE method both P and D are dissolved in the buffer. Phosphate buffer containing the neutral marker MO is injected. In the VACE method either the P or the D concentration in the buffer can be varied. Since D is not very sensitive to UV detection, the shift in the mobility of P, $\mu_{P,D}$, has to be studied. The experiment in which P is constant is arbitrarily indicated as the normal VACE experiment and the measurements in which the concentration of P is varied as the reversed VACE experiment.

4.2.1. Normal VACE experiment

Fig. 6 shows representative electropherograms obtained with the normal VACE experiment. Two negative and one positive peak (MO) should appear in the electropherogram when injecting phosphate buffer containing MO. However, as can be seen from Fig. 6 only one negative peak (belonging to P) but two positive peaks are observed. The last positive peak was identified as MO. The mobility of the complex resembles the mobility of the MO, these peaks therefore coincide. As a result only one

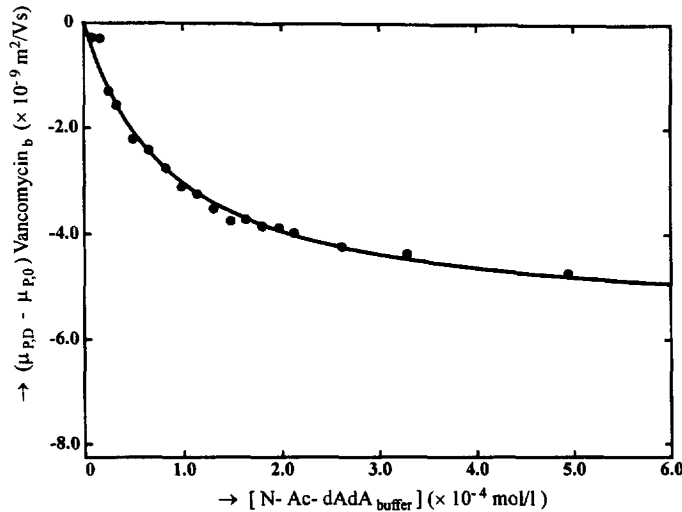

Fig. 5. Binding curve obtained with the ACE method for the vancomycin–N-Ac-D-alanine-D-alanine system, using Eq. (10). Experimental conditions as in Fig. 4.

negative peak should appear in the electropherogram. However, the absence of the second negative peak (reflecting the complex and D) is not serious because with the VACE method the shift in the mobility of the P peak can also be monitored. So far no clear explanation of the occurrence of the other positive peak can be given. The presence of the second positive peak was also noted in similar experiments using other complexing species and MO as a marker. In the absence of MO this second peak was not noticed in all our experiments.

From Fig. 6 it can be seen that with increasing D concentration, the negative P peak shifts towards the MO peak (which has the same mobility as the complex and the D). The shift in the migration time of the P peak reflects the fraction of bound P with

Table 1
Experimental values of the association constant obtained with the VACE method and comparison with other experimental methods

	Buffer		K_{ass}	Reference
Exp.	50 m*M* phosphate (pH 6.0)			
	(μ*M*)	(μ*M*)	(*10^{04} l/mol)	
VACE	[N-Ac-D-Ala-D-Ala]: 0–500	[Vancomycin] = 50	2.6	This work
VACE	[Vancomycin]: 0–500	[N-Ac-D-Ala-D-Ala] = 50	2.5	This work

Comparison with other experimental methods

Method	Buffer (μ*M*)	Sample (μ*M*)	K_{ass} (*10^{04} l/mol)	Reference
ACE[a]	50 m*M* phosphate (pH 6.0)		1.7	This work
	0–500 [N-Ac-D-Ala-D-Ala]	[vancomycin]:50		
Fluorescence-based assay				
	100 m*M* phosphate (pH 7.0)		1.6	[33]
UV-difference binding assay				
	20 m*M* citrate (pH 5.1)		2.0	[34]
UV-difference binding assay				
	20 m*M* citrate (pH 5.1)		1.8	[35]

[a] N-Ac-D-Ala-D-Ala added to the buffer and injection of vancomycin.

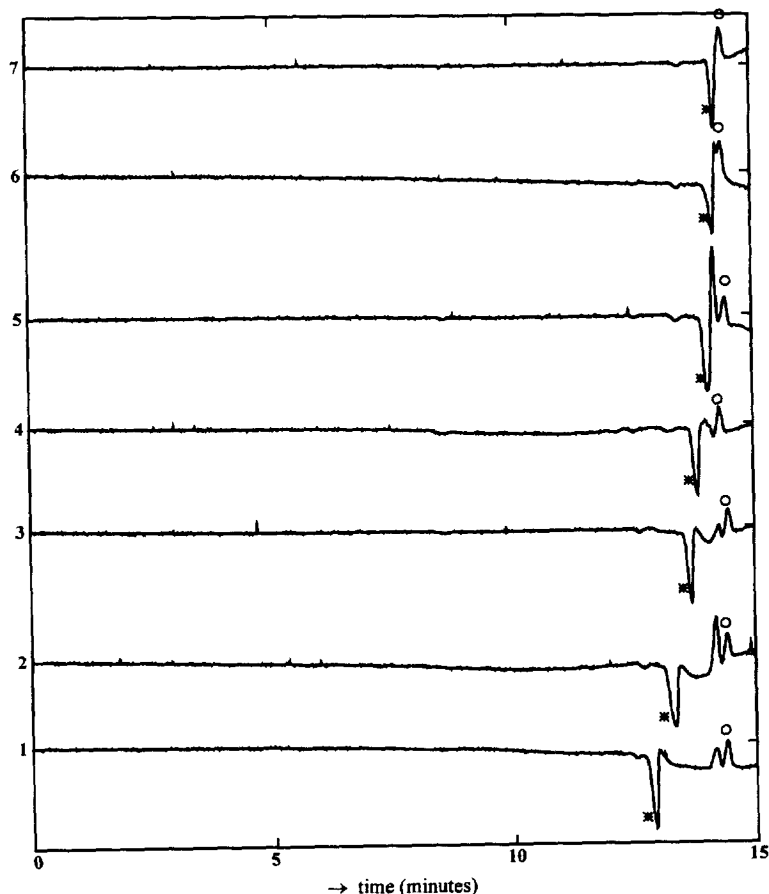

Fig. 6. Representative electropherograms of the VACE method for the vancomycin–N-Ac-D-alanyl-D-alanine system. MO (○) and the vancomycin trough peak (*). Experimental conditions as in Fig. 4. Sample: phosphate buffer+MO. Separation buffer: phosphate buffer 0.050 M (pH 6)+50 μM vancomycin+varying concentrations N-Ac-D-alanyl-D-alanine: trace 1=16, trace 2=33, trace3=82, trace 4=132, trace 5=148, trace 6=214, trace 7=248 μM N-Ac-D-alanyl-D-alanine.

increasing D concentration. Fig. 7 shows the mobility of P, $\mu_{P,D}$, vs. the D concentration in the buffer.

From this curve the association constant can be estimated using Eqs. (10) and (12); the value is included in Table 1.

4.2.2. Reversed VACE experiment

The experiment as described in Fig. 6 can be reversed by varying the P concentration and keeping the D concentration constant. In this experiment the shift in the mobility of P reflects the fraction of P that remains free. Fig. 8 shows representative electropherograms obtained under these conditions. At the most sensitive detector setting a baseline shift occurs, but after a few minutes the baseline will come back to its original position (see lines 5 and 6). At very small P concentrations, the negative P peak is very close to the MO peak (having the same mobility as the complex and D). This is expected since most of the P is bound, and therefore migrates with the mobility of the complex. When the P concentration is increased, the negative P peak shifts towards the mobility of the free P. Under these conditions the average mobility of P is largely dominated by the free P.

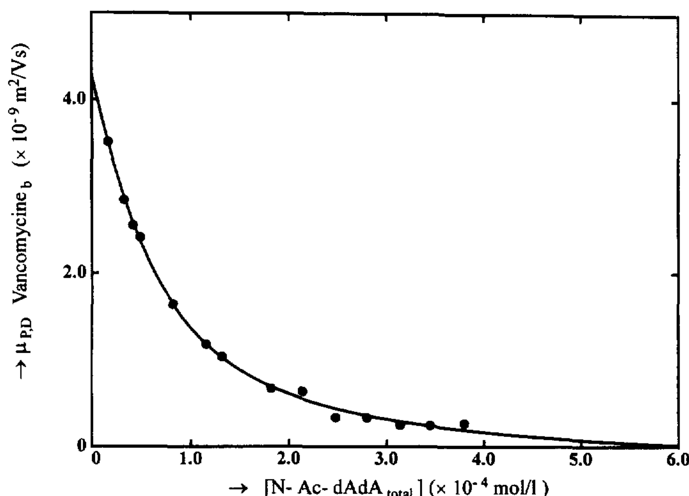

Fig. 7. Binding curve obtained with the VACE method for the vancomycin–N-Ac-D-alanyl-D-alanine system, using Eqs. (10) and (12). Experimental conditions as in Fig. 6.

Fig. 9 shows the plot of the mobility of vancomycin, $\mu_{D,P}$, vs. the concentration of P in the buffer. The value of the estimated association constant obtained from Eqs. (13) and (12), is included in Table 1.

5. Comparison of the obtained results and conclusions

In Table 1 the association constants obtained with the VACE and ACE method are gathered, including literature values obtained with other experimental methods. From this table it can be concluded that the values of the association constant obtained with both VACE procedures are in good agreement, although somewhat higher than the values found with other methods. This may be partly attributed to a difference in the selected pH. In a separate experiment using pH 8.2 instead of pH 6.0, we noticed that almost no shift in the mobility of vancomycin occurred. This indicates that complexation depends on pH, and might explain the smaller value as reported by e.g. [31].

On basis of the results obtained in this study it can be concluded that the developed VACE method, in which the mobility of negative peaks is used, appears to be suitable for the determination of association constants. The ACE and VACE methods should be regarded as complementary rather than competitive techniques for a (1:1) complex. A disadvantage of the VACE method is the fact that a larger quantity of the component which concentration is fixed is used, because it is present in all the buffers. For binding studies in which both interacting species respond to the detector and the mobilities of all species differ, both techniques can be applied equally well. When the mobility of one of the interacting species is equal to the mobility of the complex, the ACE method can only be applied in one way, while with the VACE method the measurements can be performed in both the normal and the reversed mode. Table 2 summarizes the conditions under which the ACE and VACE methods can be applied for a 1:1 complexation.

With the ACE as well as with the VACE method the found association constants have to be considered as estimates, because of the uncertainty in the [D_f] concentration in the migrating zone of P [32]. The difference between the local free concentration of D in the migrating zone of P and the concentration of D in the buffer is small at relatively large concentration of D in the buffer but at small concentrations of D, deviations occur. How large this the effect will be on

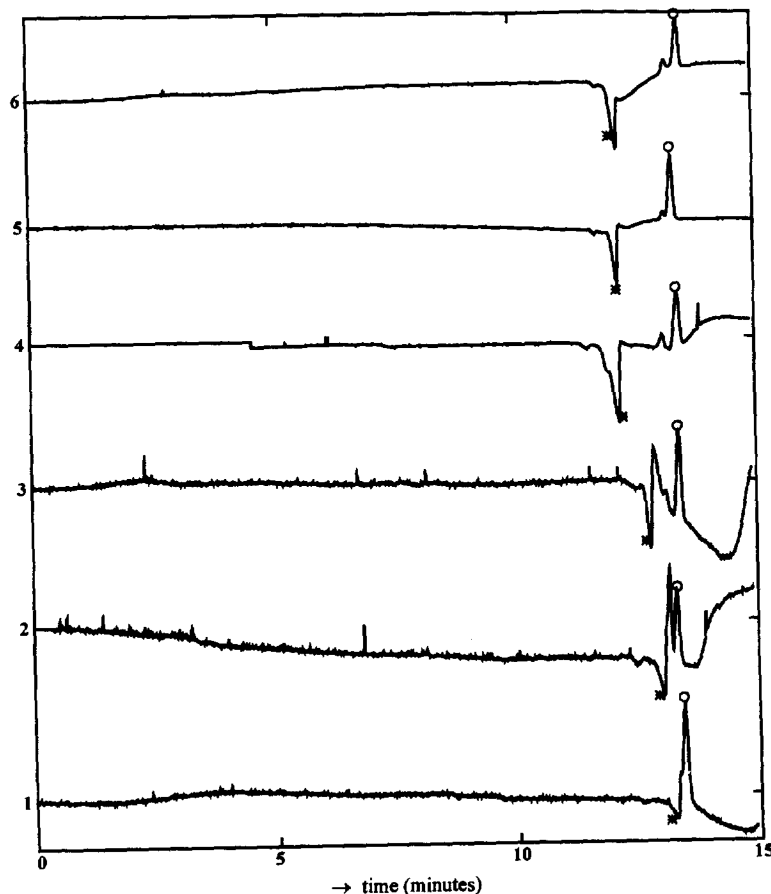

Fig. 8. Representative electropherograms of the VACE method for the vancomycin–N-Ac-D-alanyl-D-alanine system. MO (O) and the vancomycin trough peak (*). Conditions: hydrodynamic injection, 1.5 s, 169 mbar; separation voltage, 13 kV; λ=215 nm; traces 1–3: 0.005 AU; traces 4–6, 0.10 AU; sample, phosphate buffer+MO. Separation buffer, 0.050 M phosphate buffer (pH 6)+50 μM N-Ac-D-Alanyl-D-alanine+varying concentrations vancomycin: trace 1=14, trace 2=28, trace 3=35, trace 4=123, trace 5=178, trace 6=232 μM N-Ac-D-alanyl-D-alanine.

the value of the association constant is being investigated.

The VACE method may be especially convenient for ligands that as such are weakly soluble in water; the presence of both the protein and the ligand in the separation buffer increase the ligand's solubility because of binding interactions.

The VACE method could also be useful to study competitive binding of several ligands to the same protein site, provided that the different ligands can be separated [3].

With the VACE method in principle the absolute numbers of the different binding sites, n_{bind}, can be measured, [36]. This can be considered as an advantage when studying multiple equilibria.

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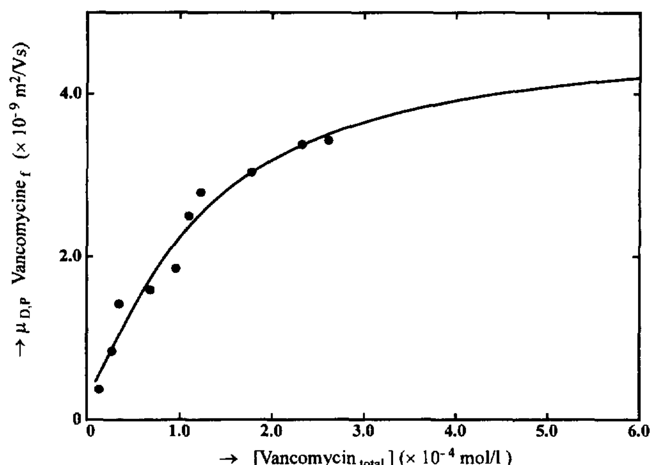

Fig. 9. Binding curve obtained with the VACE method for the vancomycin–N-Ac-D-alanyl-D-alanine system, using Eqs. (13) and (12). Experimental conditions as in Fig. 8.

Table 2
Comparison of the possibilities of the ACE and the VACE method, with respect to the mobilities, detection sensitivity and limited sample volume of the solutes for a 1:1 complex

	Experimental set-up	Method can be performed	Detector response		Sample volume	
			P 'insensitive'	D 'insensitive'	D limited	P limited
Mobility ratio: $\mu_P \neq \mu_C \neq \mu_D$						
ACE	Inj.: D, P in buffer	Y	Y	No	Y	No
	Inj.: P, D in buffer	Y	No	Y	No	Y
VACE	Increasing: D, P constant	Y	Y	Y	No	Y
	Increasing; P, D constant	Y	Y	Y	Y	No
Mobility ratio: $\mu_P \neq \mu_C = \mu_D$						
ACE	Inj.: D, P in buffer	No	No	No	Y	No
	Inj.: P, D in buffer	Y	No	Y	No	Y
VACE	Increasing: D, P constant	Y	No	Y	No	Y
	Increasing; P, D constant	Y	No	Y	Y	No
Mobility ratio: $\mu_P = \mu_C \neq \mu_D$						
ACE	Inj.: D, P in buffer	Y	Y	No	Y	No
	Inj.: P, D in buffer	No	No	Y	No	Y
VACE	Increasing: D, P constant	Y	Y	No	No	Y
	Increasing; P, D constant	Y	Y	No	Y	No

Method can be performed: according to the ratio of the mobilities of the solutes to be studied.
P 'insensitive': P is insensitive to the applied detection method.
D 'insensitive': D is insensitive to the applied detection method.
D limited: the quantity of D is very limited, or D is expensive.
P limited: The quantity of P is very limited, or P is expensive.

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