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Comparison of five methods for the study of drug–protein binding in affinity capillary electrophoresis

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Abstract

The qualitative and quantitative aspects of capillary electrophoretic methods used to study drug–protein interactions, viz. the affinity capillary electrophoresis (ACE), Hummel–Dreyer (HD), frontal analysis (FA), vacancy peak (VP) and vacancy affinity capillary electrophoresis (VACE) methods have been investigated. In the ACE and the VACE methods the binding parameters can be calculated from the change in the electrophoretic mobility of the drug on complexation with a protein. In the frontal analysis and the vacancy peak method the free drug concentration is measured with UV detection. In the Hummel–Dreyer method the amount of drug bound is measured with UV detection. For the comparison of these five methods the warfarin–bovine serum albumin (BSA) system was used. Several factors that might influence the determination of association parameters were examined. With the FA, VP, HD and VACE methods the absolute numbers of the different binding sites involved in the complex formation can be determined, a major advantage in drug-binding studies. © 1997 Elsevier Science B.V.

Keywords: Affinity capillary electrophoresis; Vacancy affinity capillary electrophoresis; Binding studies; Hummel–Dreyer method; Association constants; Frontal analysis method; Vacancy peak method; Warfarin; Albumin

1. Introduction

Binding studies involve the determination of association constants and the number of drug molecules that bind to the different classes of binding sites present on the protein molecule. In most cases, the drug–protein interactions are analysed assuming that the drug is bound to m classes of identical, independent binding sites. The fraction r of bound drug molecules per protein molecule is given by:

$$r = \frac{[D_b]}{[P_{total}]} = \sum_{i=1}^{m} n_i \frac{K_i[D_f]}{1 + K_i[D_f]} \quad (1)$$

where: $[D_f]$, $[D_b]$ and $[P_{total}]$ are, respectively, the concentrations of free drug, bound drug and total protein; n_i is the number of sites of class i and K_i is the corresponding association constant. The drug–protein data analysis often assumes two types of binding sites on the protein, and the binding parameters characterizing the interaction [1] are then n_1, K_1, n_2, K_2. Methods for studying binding phenomena measure either the concentration of the unbound (free) drug $[D_f]$ or the concentration of the bound

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drug [D_b]. Evidently both types of measurement should not disturb the equilibrium [1,2].

CE already proved to be an attractive method to study drug–protein binding, but so far little attention has been given to compare the performance of available CE methods. At present there are five CZE methods available to measure association constants. With the exception of the vacancy affinity capillary electrophoresis (VACE) method these methods have been developed in HPLC [1] before they were implemented in CZE. The present study aims to make such a comparison of these five methods: viz. the Hummel–Dreyer (HD) method, the affinity capillary electrophoresis method (ACE), the frontal analysis (FA) method, the vacancy peak (VP) method, and the vacancy affinity capillary electrophoresis (VACE) method. The comparison was made with respect to systematic errors of the measurements, the accuracy of the association parameters and the ability to obtain values for n_i with these methods.

This study will be focused on an important drug transport protein in plasma, i.e., albumin. Two model systems were selected: bovine serum albumin (BSA)–warfarin and human serum albumin (HSA)–warfarin. The HSA–warfarin system was chosen to evaluate the results of the FA method because the binding parameters are well documented in literature and the BSA–warfarin system to compare the available CZE methods. Usually the discussion will apply to the 1:1 case, while in some cases the consequences of more complicated reactions such as $n_i \neq 1$ and the occurrence of two K values will be considered. Interaction of other substances than drugs and proteins suitable for electrophoresis can be studied of course in quite a similar way.

2. Theoretical section

The various methods can be subdivided according the way the binding parameters are extracted viz. from the peak area (HD, VP) or plateau height (FA) of the elution profile or from the change of the mobility of the species (ACE, VACE). The experimental set-up of the ACE and the HD method is identical. Similarly the experimental set-up of the VACE and the VP method is also identical. The frontal analyses method uses a different experimental set-up. First the principles of these methods will be discussed in general to point out the methodological difference between them.

In the following text we will, for the sake of simplicity, refer to the substances denoted as protein (P) and drug (D), forming a complex C.

2.1. The Hummel–Dreyer method

In this type of experiment the capillary is filled with buffer containing the drug (D) to be studied at varying concentrations [3]. When a small amount of protein (P) is injected a typical elution profile appears as shown in Fig. 1 (line 2). The positive peak corresponds to the drug–protein complex (C) and the free protein, indicated by (●), and the negative peak emerges at the migration time of the

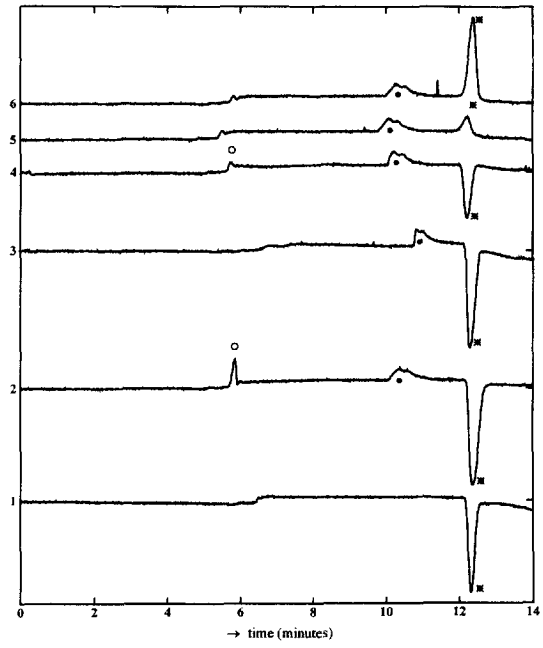

Fig. 1. Set of representative electropherograms obtained with the HD method for warfarin–BSA. Peak identification: (●) BSA–warfarin complex + free BSA, (*) warfarin trough peak, (o) artefact. Experimental conditions: hydrodynamic injection, 1.5 s, 169 mbar; separation voltage, 10 kV; $\lambda = 315$ nm. Separation buffer, phosphate buffer, 0.067 M (pH 7.4) + 309 μmol/l warfarin. Sample: line 1 = buffer, line 2 = 42 μmol/l BSA, line 3 = 42 μmol/l BSA + 259 μmol/l warfarin, line 4 = 42 μmol/l BSA + 388 μmol/l warfarin, line 5 = 42 μmol/l BSA + 518 μmol/l warfarin, line 6 = 42 μmol/l BSA + 776 μmol/l warfarin.

drug, indicated by (*). The area of the negative peak is directly related [4] to the amount of drug bound to the protein, $[D_b]$. The number of bound drug molecules per protein molecule is fixed by the drug concentration in the buffer and therefore can be considered constant during elution. The amount of bound drug may be quantified from either internal or external calibration [4,5] preferably of the peak area.

In the internal calibration a series of samples with a fixed protein concentration and increasing drug concentrations are injected. When increasing drug concentrations are injected with the protein the trough area will decrease, and for sufficient large concentrations a positive peak may even appear, as can be seen in Fig. 1 (lines 3–6). A simple interpolation allows the determination of the drug concentration for which the negative peak vanishes, schematically presented in Fig. 2A. From this value the amount of bound drug can be calculated.

In the external calibration method, the amount of bound drug is calculated from the difference of the trough area obtained upon injecting the protein sample (line 2 in Fig. 1), and the trough area obtained when plane buffer is injected, (line 1 in Fig. 1). The external calibration procedure is schematically presented in Fig. 2B. The value of the free concentration, $[D_f]$, also needed for the evaluation of the equilibrium, can be set equal to the concentration of drug in the buffer [4]. The HD method offers the possibility to check the stoichiometric model, because the bound drug concentration, $[D_b]$, is measured with this method. The fraction, r, representing the number of drug molecules that will be bound to a protein molecule can be obtained, by dividing the bound drug concentration by the concentration of the protein in the sample.

2.2. The vacancy peak method

In a vacancy peak experiment (VP) experiment, the capillary is filled with buffer containing both the protein and the drug [6]. The concentration of one compound, for instance the protein, is fixed and the concentration of the other component is varied. Then a small amount of plain buffer is injected and typical elution profiles appear as shown in Fig. 3.

The first negative peak arises due to a vacancy in the drug–protein complex and the free protein, indicated by (●), and the second negative peak is due to a vacancy in the free drug concentration indicated by (*). The area of the second negative peak depends directly on the amount of free drug in the buffer, $[D_f]$. The amount of free drug in each buffer may be quantified from internal calibration [5,6]. A series of samples (neat buffer) with increasing drug concentrations are injected. For large drug concentrations in the sample, the area of the trough peak changes from negative to positive. A simple interpolation allows the determination of the drug concentration for which the negative peak vanishes. This is schematically presented in Fig. 2C.

From this value the amount of free drug in the buffer can be found. It is assumed that the protein and the complex have approximately the same mobility [7]. The VP method offers the possibility to check the stoichiometric model. Using the measured value for $[D_f]$, the known total drug concentration in the buffer, $[D_{tot}]$, and the known protein concentration $[P_{tot}]$, the amount of drug bound by the protein can be calculated.

2.3. The frontal analysis (FA) method

In the frontal analysis method [5,8–11], the capillary is filled with buffer and subsequently a large sample plug is injected. This sample plug consists of drug and protein in equilibrium. This means that the sample plug will contain drug, protein and drug–protein complex. It is again assumed that the protein and the complex have approximately the same mobility [7,12]. Another requirement for the application of the method is that the mobility of the drug differs sufficiently from the mobility of the complex. Due to the difference in mobility, the free drug leaks out of the plug in a concentration equal to the free drug concentration in the injected sample. Representative electropherograms of the FA method are shown in Fig. 4. In the electropherogram two plateaus will be visible, one of these plateaus is related to the free protein and the complex, indicated by (●), the other plateau is related to the free drug, indicated by (*). The height of the latter plateau represents the free drug concentration $[D_f]$.

The free drug concentration can be calculated by comparison with a common calibration curve, obtained by injecting a sample containing only the

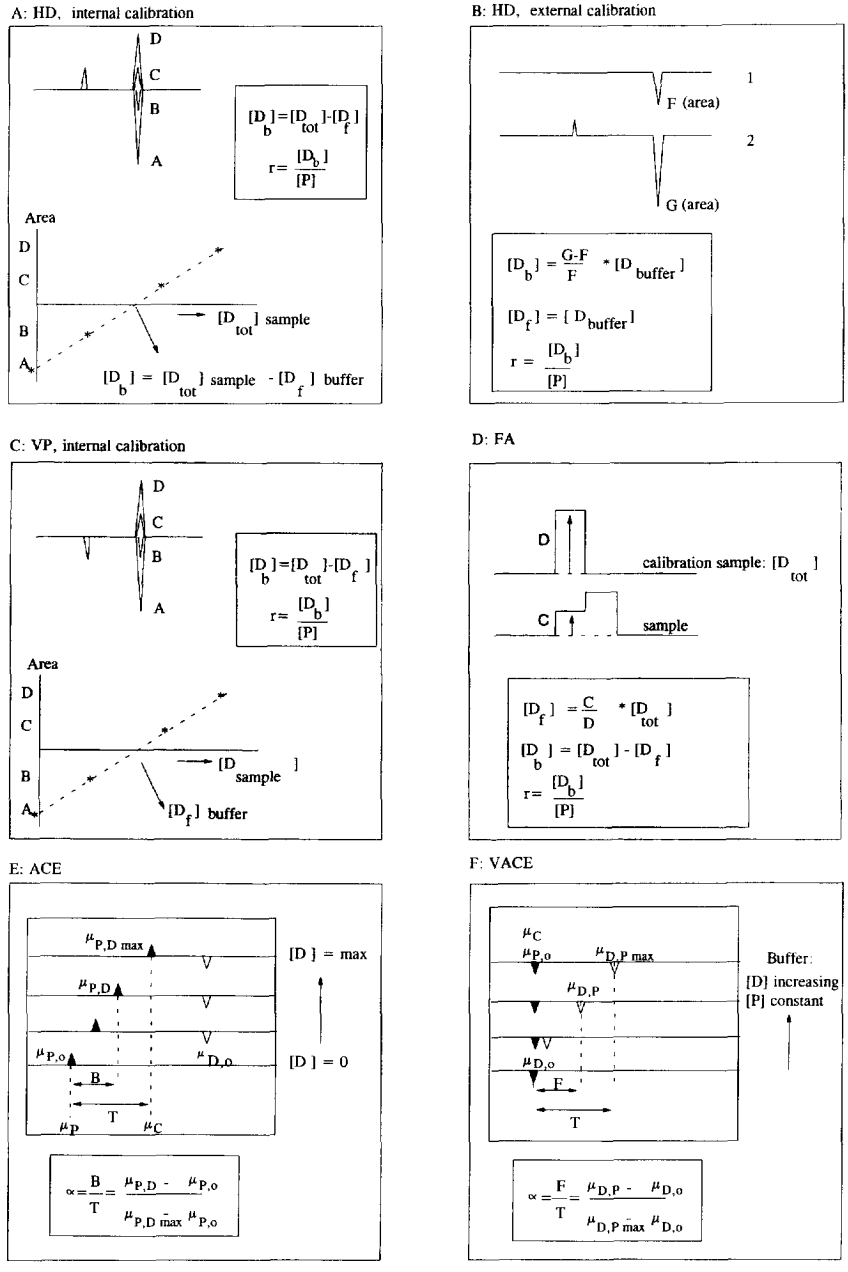

Fig. 2. Illustration of the calibration procedures for the methods: (A) HD internal calibration, (B) HD external calibration, (C) VP internal calibration, (D) FA, (E) ACE, (F) VACE method.

drug. From the known total and the measured free drug concentration, the association parameters can be calculated. The calibration procedure is schematically presented in Fig. 2D.

2.4. The affinity capillary electrophoresis (ACE) method

The ACE method uses an identical experimental

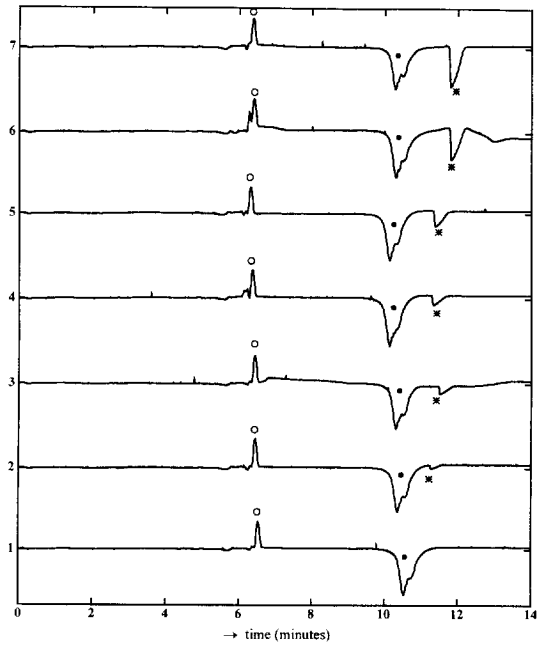

Fig. 3. Set of representative electropherograms obtained with the VP method for warfarin–BSA. Peak identification: (○) MO, (●) BSA+complex trough peak and (*) free warfarin trough peak. Experimental conditions, see Fig. 1, λ=215 nm. Sample, buffer+MO. Separation buffer, buffer+40 μmol/l BSA+varying concentrations of warfarin. Line 1=+102 μmol/l, line 2=+136 μmol/l, line 3=+204 μmol/l, line 4=+272 μmol/l, line 5=+509 μmol/l, line 6=+611 μmol/l, line 7=+815 μmol/l warfarin.

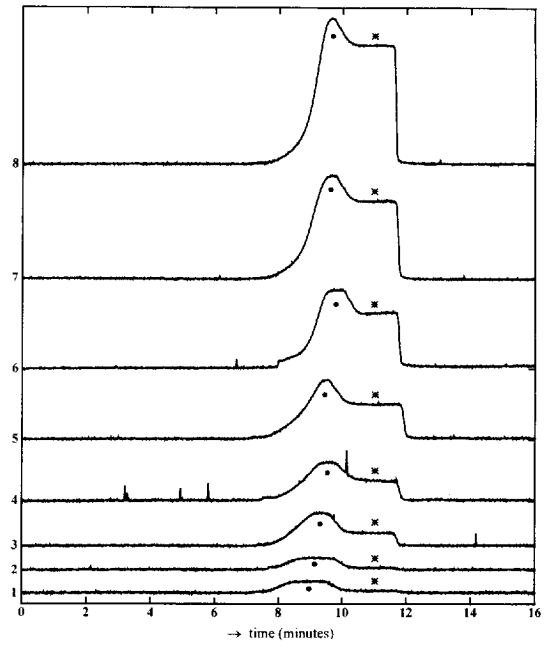

Fig. 4. Set of representative electropherograms obtained with the FA method for warfarin–BSA. Peak identification: (●) BSA–warfarin complex+free BSA, (*) free warfarin. Experimental conditions: hydrodynamic injection, 20 s, 169 mbar; separation voltage, 10 kV; λ=315 nm. Sample, 42 μmol/l BSA+varying concentrations of warfarin. Line 1=45 μmol/l, line 2=91 μmol/l, line 3=135 μmol/l, line 4=180 μmol/l, line 5=271 μmol/l, line 6=406 μmol/l, line 7=542 μmol/l, line 8=813 μmol/l warfarin.

set-up as the HD method. In ACE experiments the mobility of the injected component is monitored, in this example the protein [13–15]. A scheme of the ACE method is presented in Fig. 2E. Fig. 5 shows representative electropherograms when the drug is added in varying concentrations to the buffer and a fixed amount of the protein is injected.

The average mobility of the protein peak, $\mu_{P,D}$, will shift between two limiting values viz. $\mu_{P,o}$, the own mobility of the protein when no drug is present in the buffer and μ_C, the mobility of the complex and can be expressed as:

$$\mu_{P,D} = (1 - \alpha)\mu_C + \alpha\mu_{P,0} \quad (2)$$

where α reflects the ratio: $[P_b]/[P_{total}]$ of the injected sample. Often μ_C is not known and is replaced by $\mu_{P,D\ max}$, the maximum observed mobility shift when the drug concentration in the buffer is high. The fraction, α, of the (injected) protein having formed a complex with the drug, can be calculated in the following way:

$$\alpha = \frac{B}{T} = \frac{\mu_{P,D} - \mu_{P,0}}{\mu_{P,D\ max} - \mu_{P,0}} = \frac{[P_b]}{[P_{total}]} \quad (3)$$

where B reflects the fraction of the protein that is bound, and T reflects the total protein.

The ACE method, relates the change in the electrophoretic mobility $\mu_{P,D}$ of the protein, present in the sample, to the drug concentration, present in the buffer. This enables the determination of the association constant K_{ass}. If the mobility of the free protein is equal to the mobility of the complex, $\mu_{P,o}=\mu_C$, as is the case in Fig. 5, then no shift in mobility will be observed and the method cannot be used, while the HD method is still applicable.

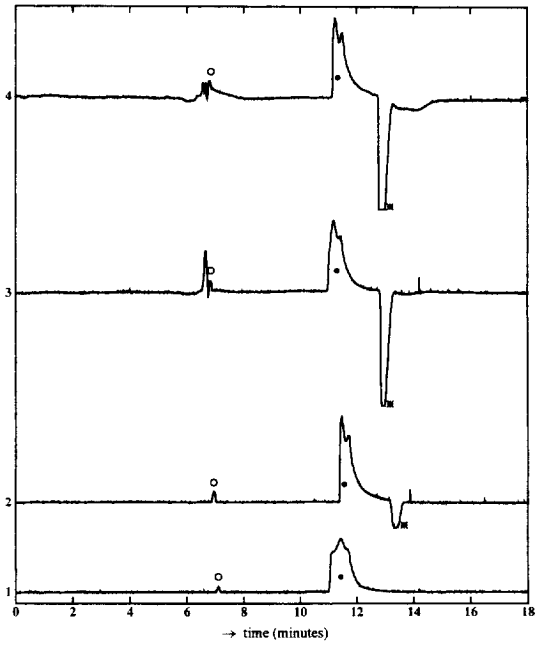

Fig. 5. Set of representative electropherograms obtained with the ACE method for warfarin–BSA. Peak identification: (○) MO, (*) Warfarin trough peak and (●) free BSA+BSA–warfarin complex. Experimental conditions, see Fig. 1. λ=215 nm; sample, 40 μmol/l BSA+MO. Separation buffer: line 1=buffer, line 2= buffer+200 μmol/l warfarin, line 3=buffer+500 μmol/l warfarin, line 4=buffer+1000 μmol/l warfarin.

The mobility of the negative peak in Fig. 5 (*), reflecting the amount of drug that is bound by the injected protein, migrates with the mobility of the free drug, $\mu_{D,o}$. The mobility of the free drug does not depend on the drug concentration in the buffer, therefore it won't shift upon increasing the drug concentration in the buffer.

In many cases binding cannot be described by a simple 1:1 stoichiometry. Complications involve two aspects: different values of successive binding constants, K_i, and bonding of more than one, n_i, molecules on sites having the same K_i [16].

With the ACE method the occurrence of multiple K-values already leads to difficulties. The point is that there is in general no linear mapping of the observed average mobility, of, e.g., P, on the r-value. This could only apply when the change in mobility with increasing complexation ($P \rightarrow PD \rightarrow PD_2$) is in proportion to the number of ligands. That may be the case, but is by no means certain. If this is not the case an appropriate model relating the mobilities of all the interacting species involved, should be incorporated in the data processing procedure. Also, one has to know whether r goes from 0 to 1 or from 0 to 2 (or even to higher values), as the only thing one observes is the conversion from one form ($\mu_{P,o}$) to another ($\mu_{P,D\ max}$) [15,17].

The second complication, multiple binding sites of one K_i ($n_i > 1$), turns up when studying literature on drug binding [1,2]. When there is no advance information on the magnitude of n_1 and n_2 available, it is impossible to find absolute values for r, even when the limiting mobility (for full conversion to PD_2) would be known [17].

With the ACE method one can arrive at estimates for K_1 and K_2, but the value of n_1 and n_2 have to be obtained form other types of measurement than the ACE method.

Another drawback of ACE experiments is the fact that the free drug concentration [D_f] in the migrating zone of P, is not directly measured, nor can this concentration be derived from other measurements in ACE. The free drug concentration, [D_f], in this zone is needed to construct the correct binding isotherm [1,2]. Perforce one has, as an approximation, to set the [D_f] in this zone to the concentration of the drug in the buffer [D_{buffer}]. The concentration of the drug in the buffer, [D_{buffer}], is plotted on the X-axis to estimate the association constant K_{ass}. This procedure may result in a systematic error in K_{ass}. This error is larger when, e.g., because of detection sensitivity, relatively high protein concentrations have to be used. Especially when the association constants are large, one may encounter this problem because the drug concentrations have to be small. It is recommended to keep the concentration of the injected species as low as possible in order to minimize this error [12,18].

2.5. The vacancy affinity capillary electrophoresis (VACE) method

The VACE method uses an identical experimental set-up as the VP method. Here the shift in the migration time of the negative peaks is used as a measure for the amount of binding [17]. Upon injecting a small amount of neat buffer, two negative

peaks will arise as already discussed with the VP method. When the concentration of the drug in the buffer is low, only a small fraction, $(1-\beta)$, of the protein is migrating with the mobility of the complex. If sufficient drug is added to the buffer to saturate the protein with the drug, the mobility of the protein peak will reach its maximum value, $\mu_{P,D\ max}$. The average mobility of the (negative) protein peak, $\mu_{P,D}$ can be expressed analogous to the ACE method:

$$\mu_{P,D} = (1-\alpha)\mu_C + \alpha\mu_{P,0} \quad (4)$$

where μ_C is the mobility of the complex, $\mu_{P,o}$ is the mobility of the protein when no drug is present and α reflects the ratio of $[P_f]/[P_{total}]$ in the buffer. If the mobility of the free protein, $\mu_{P,0}$, is equal to the mobility of the complex, μ_C, as assumed in Fig. 2F and shown in Fig. 3, then of course no shift in the mobility of the protein peak will be observed upon increasing the concentration of the drug in the buffer.

The situation is quite different for the drug peak (the second negative peak). Upon increasing the drug concentration in the mobility of the free drug will shift, as can be seen in Fig. 3. The average mobility of the drug peak depends on the fraction free drug and is schematically reflected in Fig. 2F. In this figure we assumed that the mobility of P, $\mu_{P,0}$ was equal to the mobility of the complex, μ_C, which is smaller than the mobility of the free drug, $\mu_{D,0}$. The average mobility of the drug peak, $\mu_{D,P}$ can be expressed as:

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

where β reflects the ratio of $[D_f]/[D_{total}]$ in the buffer.

The fraction, β, of the total drug added to the buffer which is unbound (free) can be expressed as:

$$\beta = \frac{F}{T} = \frac{\mu_{D,P} - \mu_{D,0}}{\mu_{DP,\ max} - \mu_{D,0}} = \frac{[D_f]}{[D_{total}]} \quad (6)$$

where F reflects the fraction drug that is free and T reflects the total drug (Fig. 2E).

With the VACE method, the change in the electrophoretic mobility, $\mu_{D,P}$, of the drug peak can be used to obtain the successive association constants and the values for the corresponding numbers of the binding sites, n_i as discussed in Ref. [17]. Furthermore, the change in the mobility of the protein peak, $\mu_{P,D}$, can be used to obtain only the association constants as discussed in Ref. [17]. The ability to obtain values for the number of the binding sites present on the protein can be considered as an advantageous feature of the VACE method when compared to the ACE method.

However, as discussed with the ACE method when PD_2 or higher forms occur, values for K_i and n_i can only be obtained from the equations presented in this work assuming that the changes in mobility with increasing complexation ($P \rightarrow PD \rightarrow PD_2$) is in proportion to the number of ligands. If this is not the case, an appropriate model relating the mobilities of all the interacting species involved, should be incorporated in the data processing procedure, inevitably complicating this even further. With the VACE method the free drug concentration in the zone is not known and as discussed with the ACE method, this may result to a systematic error in K_{ass}.

It is recommended to keep the concentration of the species in the buffer as low as possible in order to minimize this error [12].

3. Experimental section

3.1. Equipment

The capillary electrophoresis (CZE) system used in the study was an Applied Biosystems, Model 270A-HT (ABI, Foster City, CA, USA). The fused-silica capillary tubing (50 μm ID) was delivered by Polymicro Technologies (Phoenix, AZ, USA). The total length of the capillary was 50 cm, and the length from the inlet to the detector was 30 cm. A run voltage of 10 kV, 200 V/cm was used, or indicated in the figures. UV detection was performed at two different wavelengths: 215 nm or 315 nm, and will be specified in the figures. 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. Human serum albumin (HSA, essentially fatty acid free), bovine serum albumin (BSA, essentially fatty acid free), hydrochloric acid, sodium hydroxide, warfarin, and mesityl oxide (MO) were obtained from Sigma (St. Louis, MO, USA). Dipotassium hydrogenphosphate, potassium dihydrogenphosphate and ethanol were from Merck (Darmstadt, Germany). Distilled water was used to prepare the buffers.

3.3. Procedures

Before each measurement the capillary was flushed consecutively for 2 min each with ethanol, 0.03 mol/l HCl, 1 mol/l NaOH and buffer. Samples were introduced into the capillary using vacuum (169 mbar) injection for 1.5 s or 20 s, or using an electrokinetic injection for 60 s at 10 kV. The injection mode will be indicated in the figures and tables. In all the electrophoresis experiments a 0.067 mol/l potassium phosphate buffer was used, of pH 7.4. The buffer was prepared by combining 0.067 mol/l potassium dihydrogenphosphate with 0.067 mol/l dipotassium hydrogenphosphate such that a pH of 7.4 was measured. All samples were dissolved in the phosphate buffer.

3.4. Data processing

A general requirement in order to obtain valid binding parameters such as association constants and the number of binding sites is reaching a saturation maximum, reflected by the parameter that is used with a specific method [11,34]. Firstly the data processing used with the ACE method will be presented, after which the data processing used with the VACE method will be discussed. Finally the data processing used in combination with the FA, the HD and the VP method will be presented.

3.4.1. The ACE experiments

In these experiments the maximum mobility shift, $\mu_{P,D\ max}$, must be measured in order to use Eq. (3) directly to estimate binding constants. However, for many systems, $\mu_{P,D\ max}$ often cannot be measured accurately due to experimental limitations. Using the measured maximum $\mu_{P,D}$ as $\mu_{P,D\ max}$ will lead to a systematic error in the association constant.

Since the use of a Scatchard plot [19] for the estimation of binding parameters has some disadvantages [2,11], the direct more straight forward $\mu_{P,D} - [D_{buffer}]$ plot has the preference for the estimation of the association constant, K_{ass}, from ACE experiments [20].

By using a non-linear regression procedure [21,22], the $\mu_{P,D} - [D_{buffer}]$ plot enables the estimation of the value of K_{ass} without knowing $\mu_{P,D\ max}$, that is, K_{ass} as well as $\mu_{P,D\ max}$ are estimated simultaneously. The relationship between $\mu_{P,D}$ and the various parameters is given by:

$$\mu_{P,D} = \mu_{P,0} + (\mu_{P,D\ max} - \mu_{P,0}) \cdot K_{ass} \cdot \frac{[D_{buffer}]}{1 + K_{ass}[D_{buffer}]} \quad (7)$$

In this expression the concentration of D in the buffer, $[D_{buffer}]$ is known, $\mu_{P,D}$ and $\mu_{P,0}$ are measured and both K_{ass} and $\mu_{P,D\ max}$ are parameters that can be estimated with non-linear regression. Eq. (7) is only valid for a protein having one type of binding sites (only one association constant can be determined).

As described in Ref. [11] we used Monte Carlo simulations [23] to determine the confidence intervals of the K_{ass} and $(\mu_{P,D\ max} - \mu_{P,0})$.

3.4.2. The VACE experiments

As discussed with the VACE method the $\mu_{D,P} - [D_{total}]$ plot enables the estimation of the value of K_{ass} using a non-linear regression procedure. The relationship between $\mu_{D,P}$ and the various parameters is given by [17]:

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

where $\mu_{D,P}$, $\mu_{D,o}$, $\mu_{D,P\ max}$ are measured. The concentration of $[D_f]$ in the buffer in Eq. (8) can be calculated according to Eq. (9):

$$[D_f] = \frac{[D_{total}]}{3} - \frac{1}{(3K_1)} - \frac{1}{(3K_2)} - \frac{n_1[P_{total}]}{3} - \frac{n_2[P_{total}]}{3} - \frac{2^{1/3}I}{(3K_1K_2M)} + \frac{M}{(3\,2^{1/3}K_1K_2)} \quad (9)$$

where:

$$G = K_1 + K_2 + K_1K_2([P_{total}](n_1 + n_2) - [D_{total}])$$

$$H = 1 - [D_{total}](K_1 + K_2) + [P_{total}](K_1n_1 + K_2n_2)$$

$$I = (3K_1K_2H - G^2)$$

$$K = 27[D_{total}]K_1^2K_2^2 + 9K_1K_2HG - 2G^3$$

$$L = 4I^3 + K^2$$

$$M = (K + \sqrt{L})^{1/3}$$

and the total concentration of P respectively D in the buffer, $[P_{total}]$ respectively $[D_{total}]$ are known and K_1, K_2, n_1 and n_2 are estimated simultaneously from Eqs. (8) and (9) using non-linear regression [21,22].

3.4.3. The HD, VP and the FA experiments

With the HD, the VP and the FA method, the $r-[D_f]$ plot, i.e., Eq. (10) was used to estimate the binding parameters, using a non-linear regression procedure [21,22]:

$$r = n_1\frac{k_1[D_f]}{1 + k_1[D_f]} + n_2\frac{k_2[D_f]}{1 + k_2[D_f]} \quad (10)$$

In Eq. (10) (respectively Eq. (1)) $[P_{total}]$ is known, $[D_f]$ is measured and n_1, K_1, n_2, K_2 are parameters that can be estimated simultaneously with non-linear regression. This equation is valid for a complex having two types of binding sites (two association constants can be determined).

Monte Carlo simulations [23] were used to determine the confidence intervals of these parameters as described in Ref. [17]. Furthermore Eq. (9) can be used to extract the binding parameters from a $[D_f]-[D_{tot}]$ plot as was shown in Ref. [11] for a protein having two types of binding sites. The $[D_f]-[D_{tot}]$ relationship has the advantage that measurement errors do not affect the x-values of the plot, which gives a clearer statistical interpretation.

4. Results and discussion

The warfarin–BSA system was selected to evaluate five different CZE methods, viz the FA, the HD, the VP, the ACE and the VACE method. The association parameters for the warfarin–HSA system were measured to validate the results obtained with the FA method with conventional experimental methods.

Protein adsorption on the wall may influence the determination of the binding parameters and therefore the measurements should preferably be performed with coated capillaries. However, the long term stability of the coatings available to us appeared to be too small to do all the measurements under the same conditions. For that reason we decided to use an uncoated capillary. The extent of BSA adsorbed on this capillary was investigated by measuring the retention factor and the peak area under pressure driven conditions. It appeared that the retention factor is <0.01 and the peak area of the first injection was slightly (about 1%) smaller than the next injections. This means that indeed adsorption occurs but the amount of adsorption of BSA is very small and has a negligibly small effect on the calculation of the binding parameters. The deformed protein peaks reflected in the electropherograms cannot be attributed to adsorption and arise due to the slow kinetics of the complexation as was verified by us by computer simulations [12] and recently pointed out by Whiteside et al. [18].

It can be noticed that protein adsorption is also a problem with other techniques, such as equilibrium dialysis, ultrafiltration and HPLC [1,2,34]. In that respect the favourable surface to volume in CZE can be considered as an advantage.

4.1. The FA method

Fig. 6 shows a typical binding curve for warfarin–BSA obtained using hydrodynamic injection and a separation voltage of 10 kV. The experimental data fits adequately the theoretical curve when assuming two independent classes of binding sites.

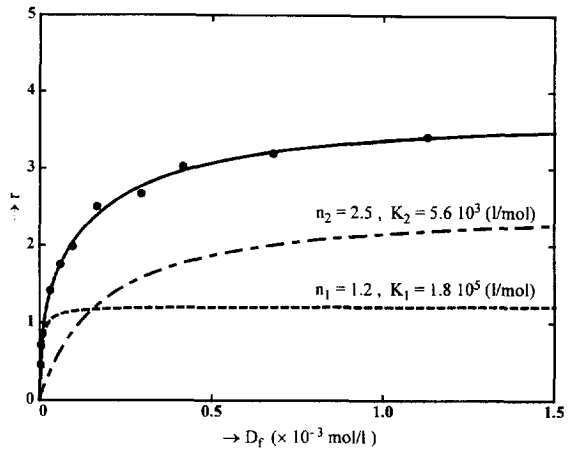

Fig. 6. Typical binding curve obtained for warfarin–BSA with the FA method. Experimental conditions, see Fig. 4. Samples, 42 μmol/l BSA + varying concentrations warfarin (0–1200 μmol/l).

This means that the curve is characterized by four parameters, n_1, K_1, n_2, and K_2; being the numbers of the different binding sites and their respective association constants. Both affinity sites on the BSA for warfarin can be distinguished with the FA method. The separate binding curves are included in Fig. 6. The values obtained from the $[D_f]-[D_{total}]$ plot as presented in Ref. [11] using Eq. (9) are also included in Table 1. They agree very well with the values obtained from the $r-[D_f]$ plot using Eq. (10).

4.1.1. Effect of the separation voltage and type of injection

In order to determine the effect of the separation voltage and type of injection on the association parameters, isotherms were measured at different voltages, using electrokinetic and hydrodynamic

Table 1
Results of the CZE measurements for warfarin–BSA

	[BSA] μmol/l	Injection[a]	[Warfarin] μmol/l	Run voltage	n_1	K_1 l/mol	n_2	K_2 l/mol
Frontal analyses								
	40	E	0–1200	5 kV	1.7	$0.8\cdot10^5$	2.6	$2.1\cdot10^3$
	40	E	0–1200	10 kV	1.3	$1.3\cdot10^5$	2.5	$3.9\cdot10^3$
	40	E	0–1200	15 kV	1.7	$0.7\cdot10^5$	2.8	$1.8\cdot10^3$
	40	H_2	0–1200	10 kV	1.2	$1.8\cdot10^5$	2.5	$5.6\cdot10^3$
	80	H_2	0–1200	10 kV	1.1	$1.2\cdot10^5$	2.6	$4.4\cdot10^3$
	500	H_2	0–1200	10 kV	1.8	$0.6\cdot10^5$	3.0	$1.1\cdot10^3$
	40	H_2	0–1200	10 kV	1.2	$2.0\cdot10^5$	2.6	$5.7\cdot10^3$ $[D_f]-[D_{tot}]$ plot
Hummel–Dreyer							n_{total}	K_{total} l/mol
	40	H	0–1200	10 kV				
EC[b]	peak height						1.0	$0.8\cdot10^4$
	peak area						3.6	$1.5\cdot10^4$
IC[b]	peak height						3.8	$1.2\cdot10^4$
	peak area						3.7	$1.4\cdot10^4$
ACE								K_{total} l/mol
	0–70	H	10	10 kV				$5.5\cdot10^4$
	0–100	H	50	10 kV				$2.1\cdot10^4$
	0–400	H	260	10 kV				$0.6\cdot10^4$
	0–400	H	500	10 kV				$0.2\cdot10^4$
Vacancy peak					n_1	K_1 l/mol	n_2	K_2 l/mol
	40	H	0–1200	10 kV	1.9	$0.7\cdot10^5$	2.1	$1.5\cdot10^3$
VACE					n_1	K_1 l/mol	n_2	K_2 l/mol
	40	H	0–1200	10 kV	1.0	$1.3\cdot10^5$	2.3	$1.9\cdot10^3$

[a] E, electrokinetic, 60 s, 10 kV; H, hydrodynamic, 1.5 s, 169 mbar; H_2, 20 s, 169 mbar.
[b] EC, external calibration; IC, internal calibration.

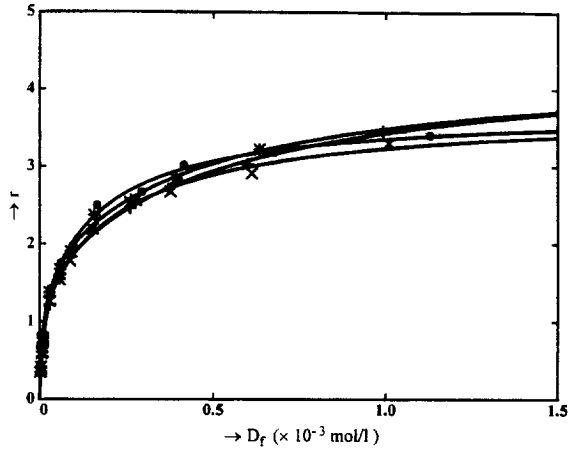

Fig. 7. Influence of the separation voltage and type of injection on the binding curves of warfarin–BSA obtained with the FA method. Experimental conditions, see Fig. 4. Electrokinetic injection, 60 s, 10 kV; separation voltage: 5 kV (*), 10 kV (×), 15 kV (+). Hydrodynamic injection, 20 s, 169 mbar; separation voltage, 10 kV (●).

injection. The effect of the separation voltage on the binding curve was investigated by varying the voltage, 5, 10, 15 kV, while keeping the injection voltage the same. The results of these measurements are given in Fig. 7 and Table 1. As can be seen the isotherms coincide reasonably. The values of the estimated binding parameters differ somewhat but the confidence intervals, as indicated in Table 3, are overlapping, except for K_2. The effect of the type of injection was investigated by measuring the binding curve at a separation voltage of 10 kV with hydrodynamic and electrokinetic injection. The association constants obtained with the hydrodynamic injection are somewhat larger and may indicate that there is a slight but not striking effect of the type of injection. From Fig. 7 and Table 1, it can be concluded that the separation voltage has no systematic effect on the measurement of the binding parameters. The difference can probably be attributed to migration discrimination as occurring with electrokinetic injection. In that respect in all CZE methods the use of hydrodynamic injection is strongly recommended.

The accuracy (here used as indicating absence of systematic errors) of the binding parameters of the warfarin–BSA system as obtained with the FA method cannot be checked because of the lack of reference values. In order to be able to judge the accuracy of the FA method also the binding of the warfarin–HSA system was studied. The warfarin–HSA system has great similarities with the warfarin–BSA system and has been extensively studied. The results of these measurements, including the values found in literature, are given in Table 2. The results found with the FA method seem to agree well with the results obtained with the conventional methods.

4.1.2. *Effect of the protein concentration*

So far the isotherms were measured using 40 μmol/l of BSA. In order to determine whether the protein concentration influences the FA measurements, the warfarin–BSA isotherm was also measured with 80 μmol/l and 500 μmol/l BSA. The latter albumin concentration is close to the physio-

Table 2
Comparison of the FA results for warfarin–HSA with several other experimental methods

Method	([Protein], °C)	n_1	K_1 $\cdot 10^5$ l/mol	n_2	K_2 $\cdot 10^3$ l/mol	$\Sigma n_i \cdot Ki$ $\cdot 10^5$	Reference
Ultrafiltration	(2 g/l, 37°C)	1.9	0.9			1.7	[28]
Chromatography							
FA	(3 g/l, 37°C)	1.4	2.0	3.7	2.2		[29]
HD (IC)	(2 g/l, 37°C)	1.0	2.5	4.0	3.9		[30]
VP (IC)	(0.01 g/l, 37°C)	1.3	2.2	3.8	4.2		[6]
Retention analysis (ACE in HPLC)	(0.01–1 g/l, 37°C)					3.2	[13]
Equilibrium dialysis	(2 g/l, 37°C)	1.0	2.3	3.7	5.9		[31]
	(4 g/l, 4°C)	1.0	14	2.0	1.8		[32]
CZE							
FA (20 s, 169 mbar/10 kV)	(3 g/l, 27°C)	1.0	3.0	2.8	7.4	3.2	this work

logical state of plasma (in vivo). The samples were injected hydrodynamically, the separation voltage was again set to 10 kV. The established binding parameters are included in Table 1. It appears that upon increasing the BSA concentration the association constants are decreasing. Several explanations for the dependence of binding constants upon albumin concentration have been offered. These include inhibition at high albumin concentrations by highly bound contaminants of the albumin preparation [24,25] and the displacement of bound ligand through molecular aggregation of albumin at high protein concentrations [26,27]. The fact that the binding parameters can be determined at various protein concentrations is an attractive feature of the FA method.

4.2. The HD method

The HD method can be performed in two ways: the warfarin can be added to the buffer and the BSA is injected, or the BSA is added to the buffer and the warfarin is injected. In the first set-up the intensity of the trough peak (peak height or area) is linearly related to the amount of warfarin that is bound by the injected protein. In the second set-up the intensity of the trough peak is related to the amount of bound BSA. However, in the latter set-up the measurement of the amount of BSA that is bound is hardly possible because the difference in mobility of the warfarin peak (complex) and of the BSA peak (trough) is very small. The difference becomes even less at high degree of binding. This is the reason why the latter experimental set-up was not useful in the HD mode. Therefore, only the first set-up in which the warfarin is added to the buffer in appropriate amounts, 0–1200 μmol/l, and BSA (40 μmol/l) is injected, was selected. In the study special attention was given to the effects of the external and internal calibration using the peak height and peak area. Since the measurement with the HD method is very laborious we decided to restrict the number of data points to eight. A consequence of this is that only an overall association constant can be determined instead of two separate association constants, as we demonstrated before [5]. However, since we aim to observe possible pitfalls with the HD method, the measurement of a complete isotherm is not necessary.

4.2.1. External and internal calibration

Fig. 8 shows the data points (isotherms) constructed via external and internal calibration by using the height and the area of the trough peak. The binding curve obtained with the FA method is included as a reference. Hydrodynamic and electrokinetic sample introduction were used. The data points obtained with electrokinetic injection are surrounded by a circle. The estimated association constants obtained with the HD method are included in Table 1. In Fig. 8 it can be seen that, with hydrodynamic injection, the data points scatter somewhat but approximately the same curves are obtained with the internal calibration procedure using either peak height or peak area, and for the external calibration procedure using the peak area. However, the binding curve obtained with the external calibration procedure using the peak height, is significantly shifted compared to the three other curves. The reason has to be attributed to the different shape of the trough peak obtained when injecting the protein sample (broader peak) and that obtained

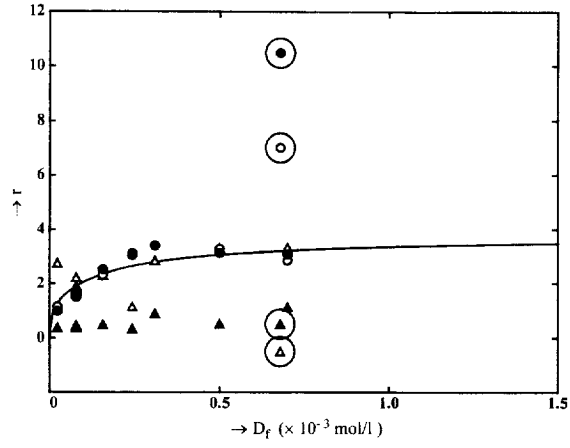

Fig. 8. Binding curve of warfarin–BSA obtained with the HD method. Experimental conditions, see Fig. 1. Separation buffer, buffer + warfarin (0–1200 μmol/l). Sample, 42 μmol/l BSA. (Solid line) Frontal analysis, (▲) external calibration using the peak height, (△) external calibration using the peak area, (●) internal calibration using the peak height, (○) internal calibration using the peak area. Data points marked with a circle were obtained with electrokinetic injection, 2 s, 10 kV.

when injecting plain buffer (see also Fig. 1, lines 1 and 2). This can be explained as follows: the amount of bound drug, $[D_b]$, is represented by the area of the negative peak [4,5]. The peak height can be used provided that the peak shape is constant. The difference in shape therefore prohibits the use of the peak height in the external calibration procedure and therefore with this procedure the peak area has to be used. In the internal calibration procedure both the peak area and the peak height can be used, because all calibration samples contain BSA and warfarin, resulting in similar peak shapes of the trough peak. The results obtained with electrokinetic injection are striking in that part of the curve, as can be seen in Fig. 8. This effect can most probably be attributed to sample-stacking.

4.3. The ACE method

The ACE method can be used when the migration time of one or both solutes change due to binding. Since the electro-osmotic flow changes when adding a solute to the buffer, the mobilities have to be corrected for the variation in the electro-osmotic flow and sometimes also for changes in the viscosity of the buffer solution [14]. A correction for the variation of the electro-osmotic flow upon adding solutes to the buffer was implemented by injecting a neutral solute (mesityl oxide) added to the samples. ACE experiments can in principle be performed in two different set-ups: (A) the protein is added to the buffer and the drug is injected, or (B) the drug is added to the buffer and the protein is injected, as mentioned above. However, when the protein and the complex have the same mobility the ACE experiments can only be performed with set-up A. In the case of a 1:1 complex, (DP), and when the mobility of all species differ, both set-ups can be performed. For a 2:1 complex (D_2P), the results with both set-ups will differ. In set-up A, large amounts of the drug have to be injected to first saturate the binding site with the highest affinity constant on the protein, in order to be able to measure the binding site with the lowest affinity, as will be shown in the experimental section. But on the other hand the concentration of the injected species, in this case the drug, should be kept as low as possible, as was already pointed out. So, if the protein has more than one binding site that is participating in the equilibrium, the drug should be added to the buffer and the protein has to be injected.

The mobility of the BSA peak, $\mu_{P,D}$, does not show a significant shift upon binding warfarin, as can be seen in Fig. 9. Therefore only the second experimental set-up could be used for the warfarin–BSA system. Representative electropherograms are shown in Fig. 10. In the ACE method the injected concentration should be as low as possible. In order to check on errors introduced by the injected concentration, the experiments were also performed with different warfarin concentrations, viz. 10, 50, 260 and 520 μmol/l. The BSA concentration in the buffer was varied in the range from 0–450 μmol/l. The binding curves of these measurements can be seen in Fig. 11. They were obtained by plotting the difference in the average mobility of the warfarin peak, $\mu_{D,P}$, when protein was present in the buffer, and the mobility of the warfarin peak when no protein was present in the buffer, $\mu_{D,0}$, as a function of the BSA concentration in the buffer. These $\mu_{D,P} - [BSA_{buffer}]$ plots were used to extract the association constants using Eq. (7).

The estimated association constants are included in Table 1. As can be seen the shapes of the curves differ significantly and so do the values of the association constants, they decrease with increasing

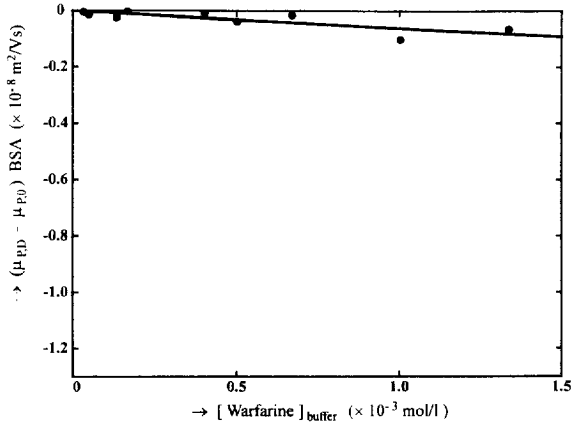

Fig. 9. The mobility of the BSA peak as function of the warfarin concentration present in the separation buffer as measured with the ACE method. Experimental conditions, see Fig. 5. Sample: 40 μmol/l BSA+MO, Separation buffer, buffer+warfarin (0–1300 μmol/l).

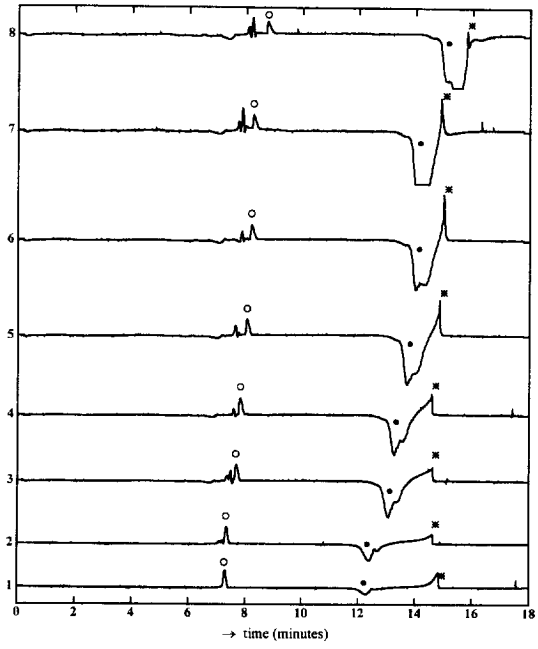

Fig. 10. Set of representative electropherograms obtained with the ACE method for warfarin–BSA. Peak identification: (○) MO, (●) BSA trough peak and (*) free warfarin+BSA–warfarin complex. Experimental conditions, see Fig. 5. Sample, 267 μmol/l warfarin+MO. Separation buffer, buffer+varying concentrations BSA. Line 1=plain buffer, line 2=+6.5 μmol/l, line 3=+15.6 μmol/l, line 4=+41.5 μmol/l, line 5=+52.1 μmol/l, line 6=+73.0 μmol/l, line 7=+91.1 μmol/l, line 8=+116.9 μmol/l, BSA.

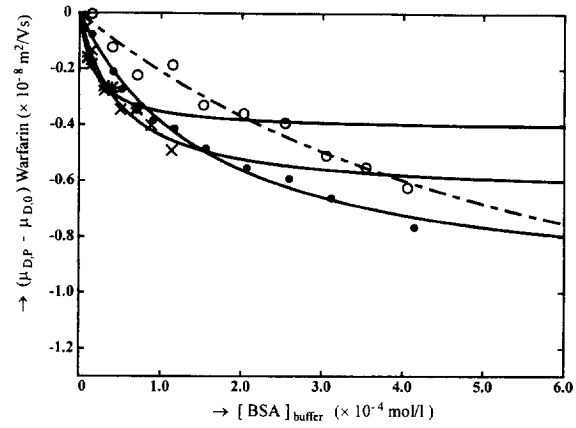

Fig. 11. Binding curve of warfarin–BSA obtained with the ACE method, in which the BSA was added to the separation buffer. Experimental conditions, see Fig. 10. Separation buffer, buffer+varying BSA concentrations (0–400 μmol/l). Sample warfarin+MO: (○) 500 μmol/l warfarin, (●) 260 μmol/l warfarin, (x) 50 μmol/l warfarin, (*) 10 μmol/l warfarin.

warfarin concentration. This effect can probably be attributed to the fact that with increasing warfarin concentrations, sufficient warfarin is injected to saturate the affinity site with the highest association constant in that (protein) zone; as a result there will be an increasing amount of warfarin available to occupy also the second binding site. The estimated overall association constant can be considered as an average of both association constants. The value of the overall K_{ass} obtained with the ACE method is in between the values of K_1 and K_2 obtained with the FA method.

Furthermore, as mentioned in the theoretical section, it is not correct to plot the BSA concentration in the buffer on the X-axis, because this concentration need not be equal to the free BSA concentration in the migrating zone of warfarin. This effect will be the greatest in the first part of the curve, where the BSA concentration is low. The K_{ass} values obtained with the ACE method may therefore differ from data obtained with the frontal analysis.

4.4. The VP method

Representative electropherograms of the VP method are shown in Fig. 3. As explained in the theory section, the intensity of the (second negative) drug peak is linearly related to the amount of free drug in the buffer. The warfarin was added to the buffer in appropriate amounts, 0–1200 μmol/l, together with a fixed amount of BSA (40 μmol/l) and neat buffer was injected. Fig. 12 shows the binding curve obtained with the VP method for the warfarin–BSA system, using the internal calibration procedure. The binding parameters are listed in Table 1. The association parameters obtained with the VP method agree reasonably with those obtained with the FA method. The FA and the VP method have similarities with respect to sample and buffer composition. The samples in the frontal analysis method and the buffers in the vacancy peak method contain a fixed amount of the BSA (40 μmol/l) and varying warfarin concentrations (0–1000 μmol/l). In fact, the samples that are prepared according to the frontal analysis method can be used as buffers in the

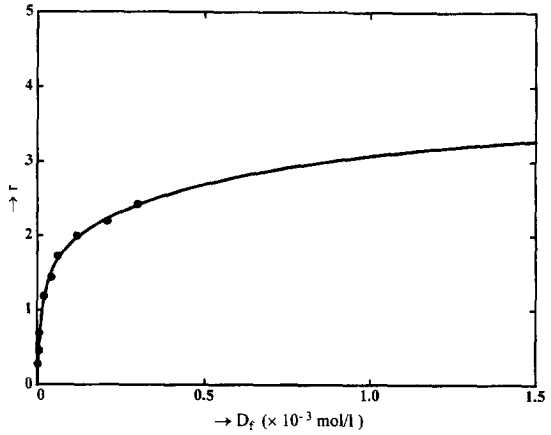

Fig. 12. Binding curve of warfarin–BSA obtained with the VP method, in which the BSA and the warfarin were added to the separation buffer. Experimental conditions, see Fig. 3. Separation buffer, buffer + varying warfarin concentrations (0–500 μmol/l), Sample, buffer + MO. The internal calibration procedure was used here.

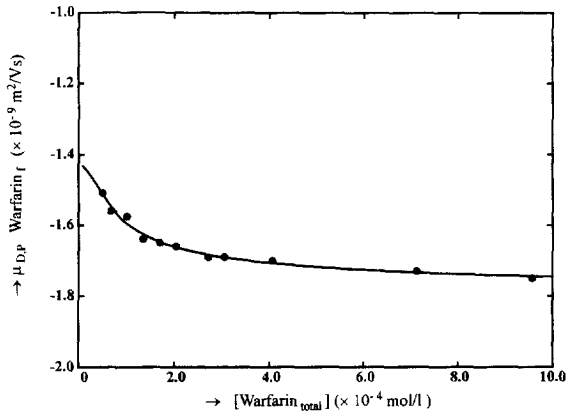

Fig. 13. Binding curve of warfarin–BSA obtained with the VACE method, in which the BSA and the warfarin were added to the separation buffer. Experimental conditions, see Fig. 3. Separation buffer, buffer + 40 μmol/l BSA + varying warfarin concentrations (0–1000 μmol/l). Sample, buffer + MO.

vacancy peak method. With the VP method it is possible to obtain the numbers of the different binding sites.

4.5. The VACE method

Analogous to the ACE method also VACE experiments can in principle be performed in two different set-ups: (A) the concentration of the protein is kept constant and the concentration of the drug is varied in the buffer, or (B) the concentration of the drug is kept constant and the concentration of the protein is varied in the buffer. Similar to the ACE method also the VACE method can not be performed in the reversed way, that is varying the concentration of the protein and keeping the concentration of the drug constant, when we are dealing with a 2:1 complex (D_2P).

Fig. 13 shows the binding curve obtained with the VACE method using set-up A. Contrary to the ACE method the VACE method allows to perform the experiments in which the BSA concentration is kept constant and the warfarin concentration is increased in the buffer. This experimental set-up could not be used with the ACE method because the free BSA and the complex had the same mobility.

Representative electropherograms of the VACE method are shown in Fig. 3. The first (negative) peak reflects the free BSA and the complex; it will not shift upon increasing the warfarin concentration because $\mu_{P,o} = \mu_C$. The situation is different for the second negative peak, representing the free warfarin. The average mobility of the warfarin, $\mu_{D,P}$, will shift upon increasing the warfarin concentration in the buffer. This shift was used to construct the binding curve presented in Fig. 13. The results obtained with the VACE method using Eqs. (8) and (9) are included in Table 1. The values for n_1, K_1, n_2 and K_2 agree well with the results obtained with the FA method. The fact that with the VACE values for n_1 and n_2 can be obtained is an advantage of this method when compared with the ACE method.

4.6. Comparison of the performance of the methods

As can be seen in Table 1, the results obtained with the FA, the VP, the HD, the VACE and the ACE method clearly show that for drug–protein binding studies the FA method seems to be the most favourable one. The FA method allows the determination of the numbers of the different binding sites, and is far less laborious than the HD and the VP method. With the same effort and less sample it clearly yields more respectively equal information about the binding process studied when compared to the ACE

Table 3
Repeatability of the CZE measurements and confidence intervals of the association parameters for warfarin–BSA

Frontal analyses

Inj.[a]	Sample [BSA] μmol/l	Sample [warf] μmol/l	Sep. volt. kV	$[D_f]$ S.D.[b] μmol/l	r S.D.[b] $\cdot 10^{-3}$	r_{max}	Related confidence intervals of n_1, K_1, n_2, K_2				
							n_1	K_1 $\cdot 10^5$ l/mol	n_2	K_2 $\cdot 10^3$ l/mol	$r_{max}/S.D._r$
E	42	42	5	0.4	9.47	3.23	1.3–1.9	0.7–1.1	2.5–2.8	1.6–3.0	341
E	42	40	10	0.3	8.35	3.31	1.2–1.4	1.0–1.7	2.4–2.6	3.4–4.4	391
E	42	40	15	0.1	2.18	3.46	1.6–1.7	0.6–0.7	2.7–2.8	1.7–1.9	1587
H	42	45	10	0.4	8.37	3.41	1.0–1.4	1.4–3.2	2.4–2.7	4.9–7.0	407

ACE

Inj.[a]	Buffer: [BSA] μmol/l	Sample: [warfarin] μmol/l	Warfarin S.D.[b] $\mu_{D,P}$ $m^2/Vs \cdot 10^{10}$	$(\mu_{D,P} - \mu_{D,0})$ $m^2/Vs \cdot 10^{-8}$	Related confidence intervals of K_{ass} and $(\mu_{D,P\,max} - \mu_{D,0})$		
					K_{ass} total l/mol $\cdot 10^4$	$(\mu_{D,P\,max} - \mu_{D,0})$ $m^2/Vs \cdot 10^{-8}$	$(\mu_{D,P\,max} - \mu_{D,0})/S.D._{\mu D,P}$
H_2	80	50	2.39	0.65	1.5–2.8	0.57–0.76	27
H_2	90	260	2.21	1.0	0.5–0.7	0.94–1.1	45

[a] E, Electrokinetic, 60 s, 10 kV; H, hydrodynamic, 20 s, 169 mbar; H_2, 1.5 s, 169 mbar.
[b] S.D., standard deviation for the individual result, $n = 5$.

respectively the VACE method. As discussed the results obtained with the FA method are accurate when the mobility of the protein equals the mobility of the complex.

The performances of the FA and the ACE method were compared because the ACE method is most often used in literature while we consider the FA method as the most favourable one.

In order to compare the performance of the FA and the ACE method, the repeatability of the measurements and the confidence intervals of the association parameters were determined. The confidence intervals were determined by Monte Carlo simulations [23] using the results of the repeatability measurements as was shown before [11]. The results of the repeatability measurements for the FA method with varying separation voltages are presented in Table 3. Except for the experiments performed at 15 kV, the standard deviations (S.D.) for $[D_f]$, respectively r, can be considered being the same for the experimental conditions studied. As can be seen the repeatability of both the FA and the ACE method can be considered good.

For a more general comparison of the performance of the ACE method and the FA method, the following measure for the 'signal-to-noise' ratio was used: the maximum value of the variable which is plotted on the Y-axis, respectively $(\mu_{D,Pmax} - \mu_{D,o})$ in the ACE method and r_{max} in the FA method, divided by the repeatability of this variable; $(\mu_{D,Pmax} - \mu_{D,o})/\text{S.D.}_{\mu_{D,P}}$ respectively $r_{max}/\text{S.D.}_r$. This value is informing us about the number of data points that can be distinguished. The results of these calculations are also collected in Table 3.

As can be seen form this value, with the FA method more data points can be distinguished compared with the ACE method. On basis of this finding and more importantly, the fact that with the FA method both association constants and the absolute number of the different binding sites can be measured, it can be concluded that for the warfarin–BSA system the performance of the FA method is superior to the ACE method.

The disadvantage of the ACE method, i.e. that the number of the different binding sites n_i cannot be found, can be circumvented by determining n_i from one experiment in the saturated part of the curve with either the HD [33] or the FA method.

With the FA method only two measurements (sample + calibration sample) are enough to construct a datapoint, while with the HD method it is necessary to perform at least four measurements (internal calibration procedure) to construct one data point. Therefore we recommend the FA method to measure n_{total}.

However, with the same effort and less sample the FA method can be performed instead of the ACE method combined with the FA or the HD method offering more information: K_1, K_2 and n_1, n_2.

5. Conclusions

The investigated five CZE methods viz.: the frontal analysis, the Hummel–Dreyer (HD), the vacancy peak (VP), the vacancy affinity capillary electrophoresis (VACE) and the affinity capillary electrophoresis (ACE) method are all suitable to study drug–protein interactions. The methods can be considered as complementary rather then competitive techniques, because each method exhibits specific ranges of applicability, advantages and disadvantages. For instance with the HD, the VP, the FA and the VACE method, both association constants and the number of the different binding sites can be determined, while with the ACE method only the binding constant can be determined and no information about the number of binding sites can be obtained. The FA method requires that $\mu_P = \mu_C$ (or $\mu_D = \mu_C$) while in the ACE method the contrary holds [12]: there should be a difference in the mobilities of the free and complexed species.

The ability to obtain values for the number of the binding sites present on the protein can be considered as an advantageous feature of the VACE method when compared to the ACE method. However, these values can only be obtained from the equations presented in this work when the change in mobility with increasing complexation ($P \rightarrow PD \rightarrow PD_2$) is in proportion to the number of ligands. If this is not the case, an appropriate model relating the mobilities of all the interacting species involved should be incorporated in the data processing procedure, inevitably complicating this even further. Moreover, with the ACE and the VACE method a systematic deviation in the value of the association constant

may be introduced when using the concentration free drug in the buffer, instead of the required free drug concentration, $[D_f]$ in the migrating zones. On the other hand, an advantage of the ACE and the VACE method is the simplicity of the measurements, provided corrections are made for changes in the electro-osmotic flow and possibly the viscosity, which often occurs when changing the composition of the buffer.

The HD and the VP methods are rather laborious because of the extensive calibration procedure. Moreover, the scatter in the data points appears to be considerably larger than with the other techniques.

When the mobility of the protein is equal to the mobility of the complex, by far the most favourable technique to study drug–protein binding is the FA method. The FA method is simple, robust, easy to implement, can deal with multiple equilibria and requires less material (nl) than all the other methods. The results of the FA method agree very well with those obtained with conventional methods. Moreover, the FA method is suitable to determine the binding parameters at protein concentrations similar to the plasma concentrations in vivo. In principle the FA method is suitable for the study of competitive drug–protein binding but for such an application one of the other methods has is preferable.

A main limitation in all CZE methods is the limited detector sensitivity due to the short optical path length. In that respect the use of a cell with an extended path length, such as the bubble-cell [11] can often be quite useful for binding studies.

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