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# Vacancy affinity capillary electrophoresis to study competitive protein–drug binding

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**Abstract**

The suitability of the vacancy affinity capillary electrophoretic method (VACE) for study of displacement of a target drug from a protein by simultaneously administered drugs was investigated. As test system, the displacement of warfarin from bovine serum albumin (BSA) by furosemide and phenylbutazone was selected. It appears that the displacement can be observed well from the shift of the actual mobility of warfarin when a displacer drug is added. Also, the competitive action of the displacer drugs (affinity for BSA) is clearly visible. The VACE method seems to be attractive for rapid assesssment of information about the competitive properties of coadministered compounds. © 1998 Elsevier Science B.V. All rights reserved.

*Keywords:* Vacancy affinity capillary electrophoresis; Protein–drug binding

## 1. Introduction

The pharmacological activity of a drug is closely related to the free drug concentration in the blood. Since drugs tend to bind reversibly to blood constituents, particularly to the plasma proteins, the free drug concentration will be considerably lower than the overall concentration. In order to adjust the optimum therapeutic dose of a drug in man, the extent of drug binding has to be known. Also, the effect of displacement of the drug by simultaneously administered exogenous compounds should be known to avoid unwanted side effects for the patient. Therefore there is a need for analytical methods to study protein–drug binding.

Various analytical techniques have been developed of which the size-exclusion chromatographic (SEC) techniques are the most frequently used nowadays [1]. A promising new technique is capillary zone electrophoresis (CZE) [2]. The technique is very simple and its potential for studies of drug binding is now well recognized. In analogy to the SEC methods, several CZE methods have been applied, viz. frontal analysis, vacancy peak method (VP), Hummel–Dreyer method, affinity capillary electrophoresis (ACE) and vacancy affinity capillary electrophoresis (VACE) [3–7]. With the first three techniques either the free or bound drug concentration is directly measured from the elution profile [3]. With the ACE and VACE method the binding parameters are estimated from the migration behaviour of the drug [4,6]. The ACE method has been used frequent-

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ly to study ligand–protein binding [8–10] and to establish the effect of competitive ligands [11–13]. The VACE method has been developed recently and its applicability to protein–drug binding studies has been demonstrated [6].

In this paper, the VACE method was tested for its suitability to study displacement in protein–drug binding. The displacement of warfarin from bovine serum albumin (BSA) by furosemide and phenylbutazone was selected as a model system.

## 2. General aspects of the VACE method

The principle of the VACE method has been extensively described in Ref. [6]. Briefly, the method is as follows: the capillary is filled with a solution containing buffer, protein and drug. The presence of these solutes causes a large detector signal. Then, a small volume of plain buffer is injected and the voltage is applied. When the mobility of the protein and drug differ, two negative peaks will appear in the electropherogram. The negative peaks (vacancies) arise due to a local deficiency of free protein and free drug, respectively. The area of the negative peak is related to the concentration, while the position of the peak (the mobility) contains information about the degree of complexation. Both the area and the mobility of the negative peaks can be used to estimate the binding parameters [7]. The corresponding two methods have been named the VP and VACE method, respectively.

For the determination of the binding parameters with the VACE method the concentration of one of the compounds is kept constant, the concentration of the other compound varied and the actual mobility of the drug is measured. Usually, the protein concentration is kept constant and that of the drug varied. The binding parameters can be obtained from the plot of $r$ versus the free drug concentration according to [14]:

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

where: $[D_b]$ and $[D_f]$ are the bound and free drug concentration and $[P_{tot}]$ the total protein concentration in the buffer; $m$ is the number of identical independent binding sites on the protein and $n_i$ is the number of sites of class $i$.

The free and bound drug concentration can be calculated from the actual mobility, $\mu_{D,P}$ of the drug. The actual mobility of the drug, $\mu_{D,P}$, ranges between the mobility of the free drug, $\mu_{Do}$, and the mobility of the complex, $\mu_C$ and can be expressed as:

$$\mu_{D,P} = \frac{[D_f]}{[D_{tot}]} \cdot \mu_{Do} + \frac{[D_b]}{[D_{tot}]} \cdot \mu_C \quad (2)$$

where: $[D_{tot}]$ is the total drug concentration in the buffer

Since $[D_{tot}]$ and $[P_{tot}]$ are known, $\mu_{Do}$ and $\mu_C (= \mu_{BSA})$ can be determined, the free and bound drug concentration can be calculated from the actual measured mobility of the drug and thus the plot of $r$ versus $[D_f]$ can be constructed. In principle the assumption that $\mu_C = \mu_{BSA}$ is only valid for neutral drugs. However, for small charged drugs it is a good approximation since the mobility of the protein will be hardly affected by the drugs bound to the large protein molecule.

The association constants and the number of binding sites can be estimated graphically by the Scatchard method [15] or by other, often more mathematical treatment of the data [6].

When a second drug, called in sequel the ''displacer'' is added to a protein–drug system, the original drug may be displaced from the protein by competitive interaction. The displacement occurs when both drugs show affinity for the same primary binding sites on the protein. In that case the extent of displacement depends on the individual concentration of the drugs in the buffer and on the value of their association constants. The occurrence of displacement of the bound drug from the protein by a displacer can be recognized from the shift of the actual mobility of the drug. The release of the bound drug from the protein by the displacer will increase the free drug concentration and as a result the actual mobility of the drug is shifted according to Eq. (2). The use of the shift of the actual mobility is a simple way to quickly establish the effect of displacement of the target drug by simultaneously administered compounds. The only requirement for the use of the VACE method as a technique to recognize displace-

ment is that either the mobilities of the target drug and displacer drug differ or the displacer drug does not respond to the detector. When both drugs respond to the detector and have a different mobility three negative peaks will appear in the electropherogram. The first negative peak arises due to a deficiency of free protein and the second and third due to a deficiency of the free concentrations of the drugs.

## 3. Experimental

### 3.1. Apparatus

The VACE experiments were performed with a commercial CE system (Model 270A-HT, Applied Biosystems, Foster City, CA, USA). The data processing was carried out with the Caesar software programme (Prince, Emmen, The Netherlands). For measurements of the displacement of warfarin by furosemide the wavelength was set to 308 nm and for measurements with phenylbutazone the wavelength was set to 280 nm. The temperature of the column was maintained at 25°C. An untreated capillary (Polymicro Technology, Phoenix, AZ, USA), 52 cm×50 μm I.D. with an effective length of 29 cm was used. The buffer plug was introduced into the capillary by vacuum injection ($0.17 \cdot 10^5$ Pa for 0.8 s). The running voltage was 10 kV and the current ranged between 100 and 130 μA.

### 3.2. Chemicals

Fatty acid free BSA and warfarin were purchased from Sigma (St. Louis, MO, USA). Furosemide and phenylbutazone were obtained from Aldrich (Axel, The Netherlands). The running buffer consisted of BSA and drugs dissolved in 0.067 mol/l sodium phosphate buffer, pH 7.4. The sodium phosphate buffer was previously purified by passage through a PSC filter assembly (Barnstead, Boston, MA, USA). The injection solution consisted of 0.067 mol/l sodium phosphate, pH 7.4 and mesityl oxide (MO) as a marker for the electroosmotic flow. All solutions were prepared daily. Before each measurement the capillary was flushed with the running buffer solution.

## 4. Results and discussion

The test solute warfarin consists of a mixture of enantiomers and a stereoselective binding to BSA might occur. In a previous paper a separation of the enantiomers of warfarin was obtained by adding BSA to the running buffer which contained 10% (v/v) *n*-propanol [16]. No separation of the two enantiomers of warfarin occurs in the absence of *n*-propanol indicating that no stereoselective binding occurs under the selected conditions.

Fig. 1 shows a typical binding curve obtained for BSA–warfarin. It is generally assumed that BSA has two types of binding sites on its molecule. This means that the binding of warfarin is characterized by two association constants ($K_1$, $n_1$, $K_2$, $n_2$).

The solid line in Fig. 1 represents the estimated curve by nonlinear regression of the data using Eq. (1) [6]. As can be seen, the experimental points at lower free warfarin concentrations fit well to the estimated curve. However, at larger free warfarin concentrations, the data points scatter significantly. This scattering can be attributed to the imprecision of measurements of small shifts in the actual mobility which introduces an uncertainty in the estimation of $K_2$. The estimated association constants were $K_1 = 1.2 \cdot 10^5$, $n_1 = 1.1$ and $K_2 = 2.3 \cdot 10^3$, $n_2 = 1.9$, respectively. These values agree well with the values obtained with other techniques [7].

The suitability of the VACE method for monitoring displacement was investigated using BSA–warfarin

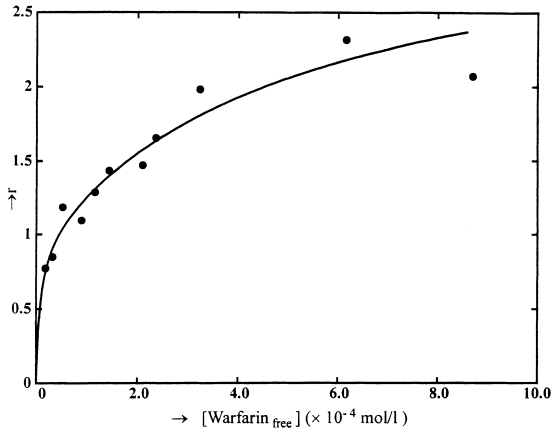


Fig. 1. Typical binding curve for warfarin–BSA obtained with the VACE method.

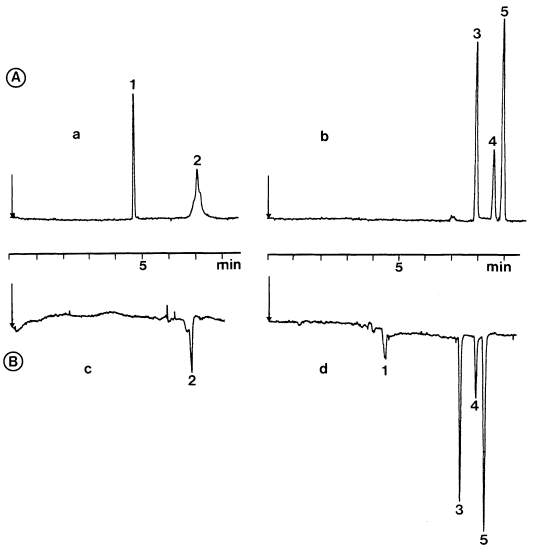


Fig. 2. Electropherograms to demonstrate the mobility order of the investigated test solutes and the occurance of the vacancy peaks with the VACE method. (A) Running buffer: 0.069 mol/l sodium phosphate. Injection solution: (a) BSA+MO dissolved in buffer; (b) drugs dissolved in buffer; peaks: (1) MO; (2) BSA; (3) phenylbutazone; (4) warfarin; (5) furosemide. (B) Running buffer: (c) BSA dissolved in buffer; (d) drugs+MO dissolved in buffer. Injection solution: 0.069 mol/l sodium phosphate.

as a binding system and two drugs, furosemide and phenylbutazone, as displacer drugs. Fig. 2A shows the electropherogram of BSA and the separate drugs. The mobility of BSA is larger than that of the drugs. Although the drugs are charged species it can be assumed that the mobilities of the large BSA molecule and that of the BSA–drug complex are the same. Fig. 2B shows the electropherogram obtained when BSA and the drugs are dissolved in the running buffer and plain buffer containing MO is injected. The negative peaks (the vacancies) correspond to the mobilities of the drugs.

In these experiments the BSA and warfarin concentrations were kept constant and the concentration of the displacer drug was varied. For the experiments with furosemide, the BSA concentration was set to 80 μmol/l and the warfarin concentration to 100 μmol/l. The furosemide and warfarin can be well detected at 308 nm. However, for phenylbutazone, a wavelength of 280 nm had to be used and a lower BSA concentration (50 μmol/l) had to be used in

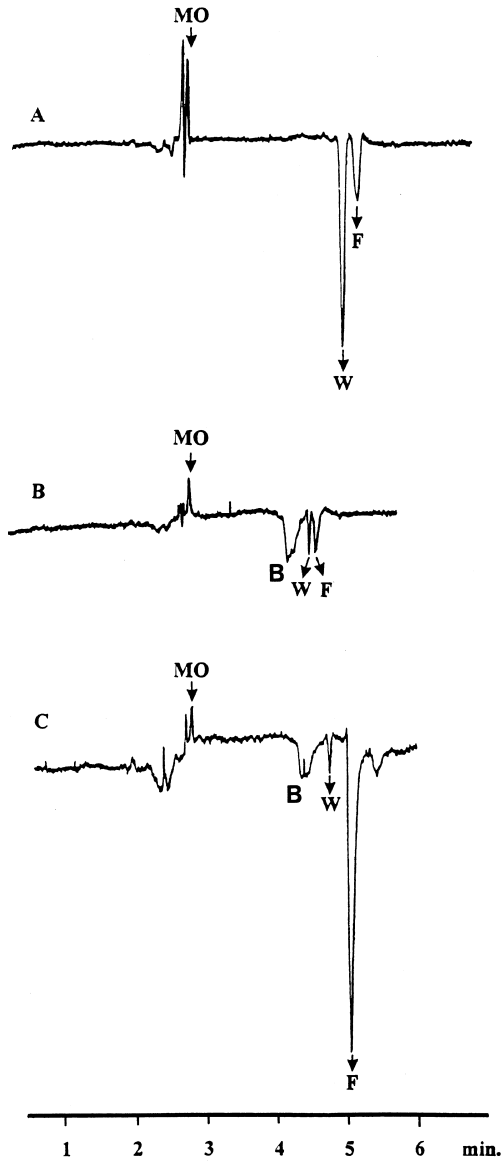


Fig. 3. Some representative electropherograms obtained with the VACE method. (A) Capillary filled with buffer+100 μmol/l warfarin+100 μmol/l furosemide. Injection solution: 0.067 mol/l sodium phosphate pH 7.4+MO. MO=mesityl oxide; B=BSA; W=warfarin; F=furosemide. (B) Capillary filled with buffer+100 μmol/l warfarin+100 μmol/l furosemide+80 μmol/l BSA. Injection solution: 0.067 mol/l sodium phosphate pH 7.4+MO. (C) Capillary filled with buffer+100 μmol/l warfarin+1000 μmol/l furosemide+80 μmol/l BSA. Injection solution: 0.067 mol/l sodium phosphate pH 7.4+MO.

order to decrease the background signal of BSA. The neutral electroosmotic marker was always added (MO) to the injection buffer in order to correct the mobilities for changes in the viscosity of the electrolyte solution when varying the displacer drug concentration.

Fig. 3 shows some typical electropherograms of the BSA–warfarin–furosemide system. In the absence of BSA and injecting buffer and MO, two negative peaks appear, reflecting the mobility of the free solutes and a positive peak of MO (Fig. 3A). The positive peak in front of MO appears due to refractive index differences. In the presence of BSA, a third negative peak is visible, reflecting the free and complexed BSA (Fig. 3B). Furthermore, the peak heights of the drugs decrease considerably due to binding to BSA and the positions of the peaks shift towards the protein peak as predicted by Eq. (2).

It can be seen that BSA migrates as a broad peak. This is due to the presence of a dimer and trimer in the BSA sample and to the fact that BSA is adsorbed slightly on the untreated capillary. The slight adsorption of BSA has no effect on the equilibria and mobilities but increases considerably the peak width and shape of BSA. However, the protein peak is not used since the mobility of the drug is monitored.

When increasing the concentration of furosemide, the free warfarin concentration increases and the warfarin peak is shifted again towards the mobility of the free warfarin (Fig. 3C). The negative peak migrating after the furosemide peak can be attributed to an impurity.

With the BSA–warfarin–phenylbutazone system, the same behaviour was observed with the difference that phenylbutazone migrated in front of warfarin.

Fig. 4 shows the effect of the addition of furosemide (Fig. 4A) and phenylbutazone (Fig. 4B) on the bound fraction of warfarin. The displacement of warfarin is clearly visible. In the presence of a tentimes larger displacer concentration, the bound fraction of warfarin dropped by about a factor of two with furosemide and a factor of three with phenylbutazone. It is known that phenylbutazone has a significantly larger affinity for BSA than furosemide [17], and thus will act as a stronger displacer. The results in Fig. 4 show that the VACE method can quickly provide qualitative information about the competitive properties of simultaneously administered drugs.

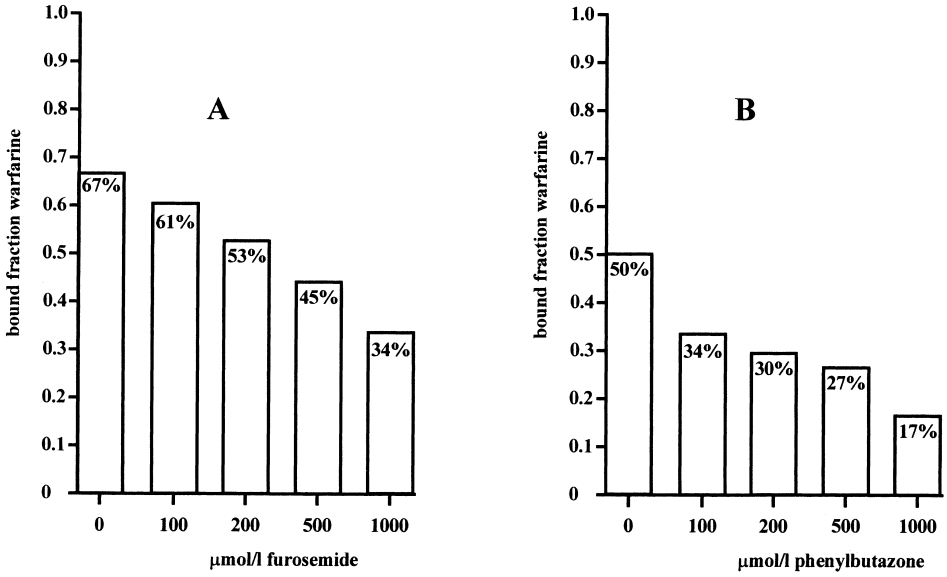


Fig. 4. Effect of the furosemide concentration (A) and phenylbutazone concentration (B) on the bound fraction warfarin. (A) Capillary filled with 100 μmol/l warfarin + 80 μmol/l BSA + (0–1000) μmol/l furosemide dissolved in buffer. (B) Capillary filled with 100 μmol/l warfarin + 50 μmol/l BSA + (0–1000) μmol/l phenylbutazone dissolved in buffer.

## 5. Conclusion

The VACE method is suitable for rapid screening of the competitive properties of simultaneously administered compounds towards a given protein–drug system. The displacement becomes visible from the shift of actual mobility of the parent drug in the absence and presence of the drug. The method is applicable when the mobility of the coadministered drug differs from that of the parent drug or has no response to the detector.

The VACE method to determine displacement is simple and it should be interesting to investigate whether drugs can be classified according to their ability to displace a reference drug from BSA or human serum albumin (HSA) under standard conditions. Since BSA (and HSA) has two main binding regions, e.g., the warfarin site (site 1) and the indole site (site 2) [18], the displacement should be studied with two reference drugs, for instance, warfarin (site 1) and naproxen (site 2). A study in that direction is now undertaken by us.

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