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Study of Competitive Protein-Drug Binding by Capillary Electrophoresis

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The competitive affinity of two drugs (the anticoagulant warfarin and the diuretic furosemide) to bovine serum albumin (BSA) is studied by capillary electrophoresis. The competition of these two drugs for the same site on BSA is shown. The binding constant between the displacer drug (furosemide) and BSA is estimated relative to the known warfarin-BSA binding constant.

Keywords: Capillary electrophoresis, protein, drug, binding constant.

1. Introduction

After introduction into the circulation most drugs are bound to the different blood constituents. In order to adjust the optimum therapeutic dose of a drug, the extent of drug binding has to be known. Therefore there is a need for analytical methods to study protein-drug binding.

Various analytical techniques have been developed to measure binding constants. Most of them are based on the use of separation techniques under equilibrium conditions. Among these separation techniques, a promising new technique is capillary electrophoresis (CE). CE differentiates charged species on the basis of mobility under the influence of an electric field. The technique is very simple and its potential for studies of drug binding is now well recognized [1]. Several CE methods are applicable to measure binding constants, *e.g.*, affinity capillary electrophoresis (ACE), Hummel-Dreyer method (HD), vacancy peak method (VP), frontal analysis (FA), and vacancy affinity capillary electrophoresis (VACE) [2-4].

We have recently reported a qualitative way to see the competitive affinity of two drugs (the anticoagulant warfarin and the diuretic furosemide) to bovine serum albumin (BSA) using the VACE method [5]. Within the plasma proteins, serum albumin is undoubtedly the most important carrier for drugs and other small molecules. It is now generally accepted that in certain but not all cases the albumin binding of a drug is a significant factor for the pharmacokinetics. Furthermore, the displacement of one drug by another from albumin binding sites can at least contribute to drug interaction phenomena [6]. The binding interaction of warfarin and serum albumin has been extensively studied with various methods. The present paper reports a fast and simple quantitative study to estimate the binding constant of the competitive drug by detecting the displacement of warfarin by another drug from albumin binding sites using VACE measurements.

2. Method and materials

2.1. Principle of VACE

Theoretical aspects of the method can be found in reference [2]. In this part, after a brief description of VACE, its application to the competitive binding equilibrium will be given.

In the vacancy experiments, the capillary is filled with buffer, protein, and ligand. Then, a small volume of buffer is injected and a voltage is applied. Three deficiency peaks can be observed on the electropherogram, which represent the deficiency of complex, free protein, and free ligand respectively. It is possible to construct a correlation between the mobility of vacancy peaks and binding parameters. This approach is the basis of the VACE method.

Keeping protein concentration in buffer constant and increasing drug concentration, or following the reversed procedure, *i.e.* keeping the drug concentration constant and increasing protein concentration, the position of drug and/or protein vacancy peaks is measured.

Experimentally, in CE, the electrophoretic mobility of a solute is determined according to the following equation,

$$\mu = L_t \mathrm{L}_d / \mathrm{V}(\mathrm{t\text{-}t}_{eo}), \quad (1)$$

where L_d is the length of the capillary from the

injection end to the detector, L_t is the total length of the capillary, V is the voltage that is applied across the capillary, t and t_{eo} are the migration times of the solute and a neutral marker respectively.

Overall mobility of a drug peak is given as

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

where $\mu_{D,P}$ is the overall mobility of the drug, μ_D is the free mobility of the drug, μ_C is the mobility of the complex at which the active sites of the protein is completely saturated by drug, and α is the free fraction of drug.

In the same manner, the overall mobility of the protein peak, $\mu_{P,D}$,is equal to

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

where μ_P is the free mobility of the protein and β free fraction of the protein.

As can be deduced from the equations, the overall mobility of the protein vacancy peak will shift to the limit value of free protein mobility with the increasing concentration of free protein in the equilibrium, or shift to the limit value of saturated complex mobility with the increasing concentration of complex, *e.g.*, decreasing concentration of free protein in the equilibrium. The same behaviour is valid for the drug vacancy peak. The mobility of the vacancy drug peak changes between the two limit values, *e.g.*, free drug mobility and saturated complex mobility depending on the equilibrium concentrations. Therefore, in the application of VACE method, it should be an acceptable difference between the complex mobility and the free protein mobility or between the complex mobility and the free drug mobility to observe differences in the mobility shift of vacancy peaks. However, even one of the free mobilities of solutes is equal to the complex mobility, the measurements of the mobility shift of the second solute give enough information for binding association. For small charged drugs, it is a good approximation that μ_C is similar to the mobility of free protein since the mobility of the protein will hardly be affected by the drugs bound to the large protein molecule. Once free and actual mobilities of the drug are obtained from the mobility measurements, the correlation between equilibrium concentrations and the association constant can easily be formed.

From Eq.2, the following equations are obtained for free and bound concentrations of drug.

$$[D] = \alpha C_D =$$

$$C_D \frac{[D]}{[D]+[DP]} = C_D \frac{(\mu_{D,P}-\mu_c)}{(\mu_D-\mu_c)}, \quad (4)$$

$$[DP] = (1-\alpha)\, C_D =$$

$$C_D \frac{[DP]}{[D]+[DP]} = C_D \frac{(\mu_D-\mu_{DP})}{(\mu_D-\mu_c)}, \quad (5)$$

where [D] and [DP] are the free and bound drug concentrations, C_D is the total drug concentration.

The reversible binding of a ligand to a macromolecule is expressed by the following equation [7,8].

$$r = \frac{[DP]}{[C_P]} = \sum_{i=1}^{m} n_i \frac{K_i\,[D]}{1+K_i\,[D]}, \quad (6)$$

where r represents the mean number of moles of ligand bound per mole of macromolecule and C_P is the total protein concentration in the buffer, m is the number of identical independent binding sites on the protein such that each class i has n_i sites with binding affinity K_i.

It is possible to perform the VACE experiments when the protein interacts with a mixture of drugs. In that case the shift in mobility values is affected from the competition between two drugs for the same binding sites of the protein. When the buffer contains the second drug, in addition to vacancy peaks described above, the new vacancy peak reflecting second the drug will appear in the electropherogram. Simultaneous binding of two drugs can be measured in two types of experiments. The concentrations of protein and one of the drugs are kept constant and the concentration of the other drug is increased, or concentrations of the both drugs are kept constant and the concentration of the protein is increased. The first series of experiments allow observing the decrease in the affinity of one drug to the protein-binding site with the increasing concentration of the other drug. The release of the bound drug from the protein by the displacer drug will increase the free drug concentration and as a result the actual mobility of the first drug is shifted to the free drug mobility according to Eq. 2.

Both measurements allow the calculation of the binding constant of one drug from the known constant of the other. Since it is the case in our model system, in the following theoretical part, the protein mobility is assumed as equal to the complex mobility. In that case, the procedure involves only the determination of the shifts in the mobility of vacancy peaks of the two drugs relative to free drug mobilities. For the different equilibrium conditions according to the followed experiments, mobilities of two drug peaks are measured. The mobilities of free drugs are determined at the same system in the absence of protein in the buffer.

In the situation under consideration, two drugs are competing for the same binding class of the protein. If the two drugs compete for the same primary binding sites of the protein and electrostatic interactions between the anionic species are neglected as proposed by Klotz et al.[7], equilibrium between protein and each individual drug can be treated separately. If represents the moles of bound drug per mole of total protein, we can write this ratio for drug A and B as,

$$r_A = \frac{(C_A - [A])}{([P] + [PA] + [PB])} = \frac{nK_A[A]}{(1 + K_A[A] + K_B[B])}. \quad (7)$$

$$r_B = \frac{(C_B - [B])}{([P] + [PA] + [PB])} = \frac{nK_B[B]}{(1 + K_A[A] + K_B[B])}. \quad (8)$$

Taking the $r_{A/}r_B$ ratio, Eq.9 is obtained:

$$\frac{(C_A - [A])[B]}{(C_B - [B])[A]} = \frac{K_A}{K_B}. \quad (9)$$

If two drugs are both bound at the same primary binding site on the protein, the left site of the Eq.9 will be constant for the equilibrium concentrations of the experimental sets.

Equation 9 provides the calculation of the association constant of one drug using the known value of that for the other drug and VACE experimental data, *i.e.*, mobilities of vacancy peaks.

From the equations 4, 5, and 9, Eq. 10 can be formed to estimate the binding constants:

$$\frac{(\mu_A - \mu_{A,P})}{(\mu_{A,P} - \mu_C)} \frac{(\mu_{B,P} - \mu_C)}{(\mu_B - \mu_{B,P})} = \frac{K_A}{K_B}. \quad (10)$$

2.2. Apparatus

All VACE experiments were performed with an Applied Biosystems Model 270A-HT CE system (Foster City, CA, USA). Data processing was carried out with the Caesar software (Prince, Emmen, the Netherlands). The wavelength was set at 308nm. The temperature of the column was maintained at 25 °C. An untreated capillary (Polymicro Technology, Phoenix, AZ, USA), 52.3cmx50μm I.D. with an effective length of 29.2 cm. was used. The buffer plug was introduced into the capillary by vacuum injection($0.17.10^5$ Pa for 0.8s). The applied voltage was 15 kV.

2.3. Chemicals

BSA and warfarin were purchased from Sigma Chemical Co. (St.Louis, MO, USA). Furosemide is from Aldrich (Axel, The Netherlands). All solutes are dissolved in the 0.067mol/L phosphate buffer at pH 7.4. The injection solution consisted of 0.067 mol/L sodium phosphate, pH 7.4 and mesityl oxide (MO) as a marker for electroosmotic flow. All solutions were prepared fresh daily.

3. Results and discussion

The application of VACE method to the protein and two drug systems was performed with using bovine serum albumin (BSA) as protein, warfarin (anticoagulant) and furosemide (diuretic) as drugs.

Location of the binding sites and binding constants of serum albumin has been extensively investigated due to its pharmacological activity. It is now obvious that, one of the most important binding sites of serum albumin is warfarin binding site. Actually this site of albumin is the a big area and the drug binding one site of this area always displace ligands bound to the other part of this area to a certain degree [6]. Since it is proved that the drugs used in our model bind to the same primary site of albumin, *e.g.*, the warfarin site [9-11], and both are small negatively charged molecules, our model is based on the assumption that two individual competitive equilibrium for n possible sites of one classified area on the albumin.

Fig. 1. shows a typical electropherogram of the BSA-warfarin-furosemide system.

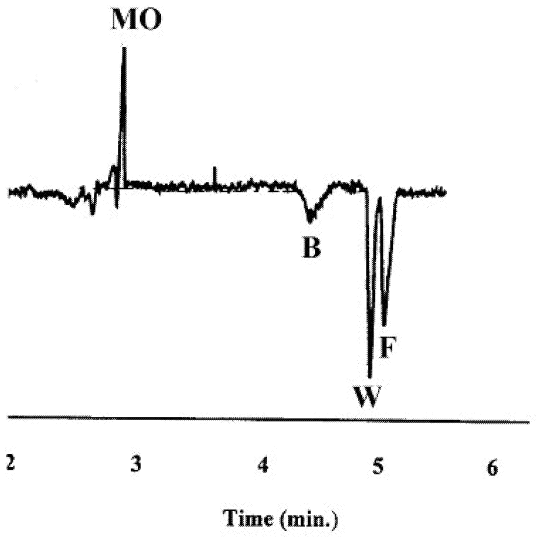

Figure 1. A representative electropherogram obtained with the VACE method. Capillary filled with buffer+20μmol/l BSA+100μmol/l warfarin+100 μmol/l furosemide. Injection solution: 0.067mol/l sodium phosphate pH 7.4+mesityl oxide. MO: mesityl oxide (neutral marker); B=BSA; W=warfarin; F=furosemide.

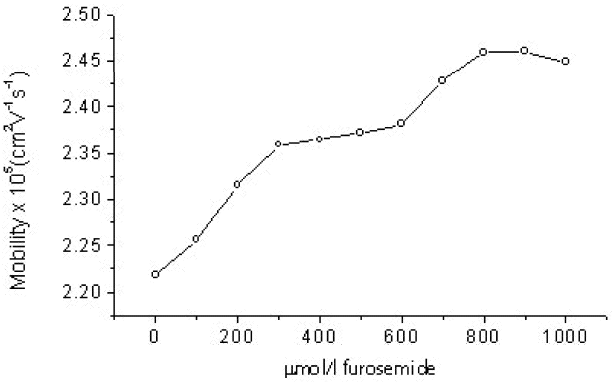

Figure 2. Mobility of warfarin *vs.* furosemide concentration. W= 100 μmol/l; BSA=100 μmol/l.

Simultaneous binding of drugs to BSA was measured with two sets of experiments. In one of them, the concentrations of both warfarin and BSA are kept constant in the running buffer at 100 μmol/l and 80μol/l respectively; furosemide concentration was increased between 0-1000 μmol/l. A small volume of buffer containing mesityloxide as an electroosmotic marker is injected. From the migration times of neutral marker and vacancy peaks of drugs, the electrophoretic mobilities of drugs are found according to Eq.1. In that way, the changes in the mobilities due to the changes in the buffer viscosity are prevented.

A shift in both mobility of warfarin and furosemide to the direction of free drug mobilities are observed. This shift shows the increase in the free concentrations of both drug in equilibrium. The addition of furosemide in equilibrium media cause an increase in the free furosemide concentration, but the increase in the warfarin concentration indicates a decrease in the affinity of warfarin for BSA with increasing concentration of furosemide. In Fig.2, the electrophoretic mobilities of warfarin *vs.* increasing concentration of furosemide is given. The displacement of warfarin is clearly visible.

In the second series of experiments, the concentrations of both drugs are kept constant at 100 μM and the BSA concentration is increased between 0-100 μM in the running buffer. In that case a shift in the mobilities of two drugs to the direction of saturated complex mobility are observed with the increasing concentration of the protein. This shift reflects the simultaneous binding of the two drugs to BSA (Fig. 3).

The corrected mobilities of vacancy peaks according to neutral marker in the absence of protein were used as the free mobilities of the drugs in the calculations. Using the mobilities of free drugs and saturated complex (in that case $\mu_C = \mu_{BSA}$), as explained in the theoretical part, the ratio of primary binding site constants can be calculated from the measured mobilities of drug vacancy peaks. Since the binding association of warfarin and serum albumin was extensively studied, we used the literature values of warfarin binding constant and calculated the furosemide constant writing the Eq. 10.

Table 1 gives the mean value of binding constant for furosemide and BSA, K_F, for two separate sets of experiments (I and II). The first col-

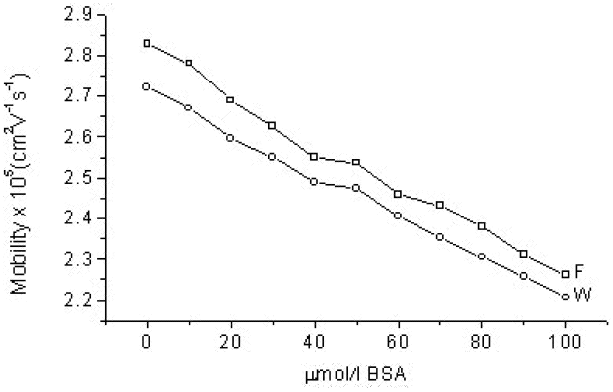

Figure 3. Mobilities of warfarin and furosemide vs. BSA concentration. W= F=100 μmol/l.

Table 1
Values of the binding constant for furosemide-BSA system obtained with VACE method.

	I	II
K_F 10^{-5}	1.57 ($\pm$0.18)	1.62 ($\pm$0.25)

umn shows the K_F value obtained from the first series of experiments (I), *e.g.*, the concentrations of warfarin and protein constant, the concentration of furosemide is increasing. The K_F value in the second column (II) was obtained from the experiments that the concentrations of the drugs are constant and the concentration of the protein is increasing. We used Sebille's binding constant value for warfarin as $2.1.10^5$ in the calculations[11]. The association constant value for furosemide found by same researcher is $1.68.10^5$. The values in brackets show standard deviations of constants for 10 data point for each experiment set. The result suggests that the two drugs are both bond at the same primary bindind site on BSA.

4. Conclusion

The results obtained from the experiments shows that the VACE method can be used for the simultaneous competition of two drugs for the binding sites of a protein. The procedure is rapid and only small quantities of protein and drug are required. The results from experiments also give considerable information about the affinity of one drug to the location of the binding site on the protein molecule, by using a drug with a known binding site location. If a competition is observed, this is the indication of two-drug use of the same binding site on protein. VACE provides an easy qualitative application in that respect. Furthermore, simply measuring the mobility shifts of the drugs, the method allows estimation of the binding constant of the competitive drug to the protein using the known parameter of other drug.

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